Selective loss of *Grin2a* in adolescent dopamine neurons results in a phenotype relevant to psychosis

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List of Abbreviations

AAV - adeno-associated virus

ACSF - artificial cerebrospinal fluid

ANOVA - Analysis of Variance

cKO - conditional knockout

CRISPR - clustered regularly interspaced short palindromic repeats

CS - conditioned stimulus

DI - discrimination index

DLPFC- dorsal lateral prefrontal cortex

DS - dorsal striatum

EEG - electroencephalogram

EPM - elevated plus maze

FCL - flexible contingency learning

FR1 - Fixed Ratio 1

FR5 - Fixed Ratio 5

GABA - gamma-aminobutyric acid

GFP - green fluorescent protein

GWAS - genome wide association study

HA - hemagglutinin

IACUC - Institutional Animal Care and Use Committee

I_h - hyperpolarization-activated cation current

IP - intraperitoneal

ITI - inter-trial interval

kg - kilogram

LoF - loss of function

mg - milligram

MK801 - dizocilpine

NAc - Nucleus Accumbens

NMDA - *N*-methyl-D-aspartate

OF - open field

OHSU - Oregon Health & Science University

P# - postnatal day #

PAM - positive allosteric modulator

PBS - Phosphate buffered saline

PCP - phenylcyclohexyl piperidine

PET - positron emission tomography

PFA - paraformaldehyde

PH - Pearce Hall

PR - Progressive Ratio

PRP - post-reinforcement pause

RW - Rescorla Wagner

SN - substantia nigra

SPECT - single-photon emission computed tomography

TH - tyrosine hydroxylase

US - unconditioned stimulus

VTA - Ventral Tegmental Area

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Chapter 1

Introduction

Overview

Schizophrenia is a severe mental health disorder that affects around 1 in 300 people globally (Solmi et al., 2023). Despite the social, economic, and personal burden of this disorder, its complex nature has led to a lack of effective, well-tolerated treatment options. This is largely due to limitations in our understanding of disease etiology. It is necessary to develop accurate animal models of schizophrenia so we can better understand and, consequently, more precisely target its biological underpinnings.

The scientific perception of schizophrenia has been evolving for over a hundred years. From its antiquated diagnosis as a consequence of maternal neglect, to its current classification as the result of complex interactions between multiple risk factors, there have been many iterations of hypotheses surrounding the development of this disorder. Our understanding of the biological basis of schizophrenia has progressed with the advancement of investigative tools. Associations between diagnosis and certain genetic mutations, developmental conditions, and the environment have been established, but there remains much to be discovered about the mechanism of action of these risk factors and the complex interactions that occur between them.

Neuroscientists have not been able to—nor will they likely ever—generate a 'schizophrenic rat', yet animal models are a powerful tool when asking questions about the

neurobiology of psychiatric disorders. While it is unrealistic to expect a complete recapitulation of this uniquely human disorder, we can utilize models to determine how etiologically relevant manipulations affect specific aspects of the brain and behavior. Isolating individual risk factors is imperative to understanding their discrete contributions to the disorder. Driven by recent genome wide association studies (GWAS), the primary focus of this dissertation was to model an etiologically relevant genetic loss of function and investigate how this particular disruption contributes to a schizophrenic phenotype.

This dissertation is organized as follows: 1) an introduction reviewing literature relevant to previous rodent models of schizophrenia and the recent advances in science that have led to the development of novel, genetic models of this disorder, 2) the characterization of rodent adolescent GluN2A profiles in bran regions relevant to schizophrenia, 3) the characterization of a novel CRISPR-generated virus in brain tissue, 4) the characterization of the behavioral phenotype of adolescent rats with a dopamine neuron-specific loss of *Grin2a*, and 5) the characterization of effects of this loss of function on basic dopamine neurotransmission in vivo, using fiber photometry. I then provide a general discussion of the key findings of this work and how they can inform future research.

Clinical perspective of schizophrenia

Schizophrenia is a heterogeneous psychiatric disorder characterized by the presence of three symptom classifications: positive, negative, and cognitive. Positive symptoms, such as delusions and hallucinations, are commonly thought of as the hallmark of schizophrenia but negative symptoms (anxiety, amotivation, blunted affect) and cognitive symptoms (deficits in memory

and attention) are also core contributors to the burden of this disorder (Blanchard & Cohen, 2006; Carbon & Correll, 2014; Heinrichs & Zakzanis, 1998; Jauhar et al., 2022). While commonly diagnosed following the first psychotic episode in late adolescence or early adulthood, many individuals experience attenuated symptoms earlier on (Fusar-Poli et al., 2012; Piskulic et al., 2012). This time that precedes the manifestation of frank psychosis is referred to as the prodromal stage. During late adolescence or early adulthood, this stage is marked by subclinical disturbances in perception, cognition, motivation, language, and motor function. This period differs from late stages of schizophrenia in that symptoms are expressed with less intensity, frequency, and duration (Olsen & Rosenbaum, 2006). It is considered a crucial period for intervention as addressing symptoms in the prodrome may halt disease progression, rather than merely blunting symptom severity later in life with antipsychotics (Cornblatt et al., 2003; Goulding et al., 2013; Yung & McGorry, 1996).

Following its conception the early 20th century, schizophrenia was initially treated somatically. Popular methods included chemical induction of narcosis, seizures, or comas as well as electro-convulsive therapy and frontal lobotomy (Tueth, 1995). This changed in the 1950's with the development of neuroleptic medications, or what came to be known as 'typical' antipsychotics. Initially it was unclear how these drugs worked, but it was later determined that they blocked dopamine receptors (Seeman et al., 1975; Seeman et al., 1976). While dopamine receptor antagonism proved to assuage some schizophrenia symptoms, it also induced undesirable side effects, most prominently extrapyramidal effects resulting in involuntary movements (Harrison, 1999). These unwanted effects contribute to medication nonadherence and have driven the development of 'atypical' antipsychotics which are less likely to cause these

side effects. Atypical antipsychotics still block dopaminergic receptors, but have lower affinity for them than typical antipsychotics and also work as antagonists at serotoninergic receptors (Meltzer et al., 1989). Unfortunately, there remains a large proportion of people with schizophrenia who do not adequately respond to either of these drugs, highlighting the need to identify novel treatment options for people with this disorder (Potkin et al., 2020). To do this, it is necessary that we continue to explore the neurobiological basis of schizophrenia and in doing so we will be able to better treat the root cause of this disorder.

Neurochemical theories of schizophrenia

Dopamine is a neurotransmitter produced in the ventral tegmental area (VTA) and substantia nigra. Dopaminergic neurons in these regions send projections to the striatum and cortex via the nigrostriatal, mesolimbic, and mesocortical systems (Swanson, 1982). Within the synapse, dopamine plays a modulatory role and can have either an inhibitory or excitatory effect, depending on the receptor type it is bound to (Beaulieu & Gainetdinov, 2011). Dysfunctional dopaminergic signaling has been associated with schizophrenia for almost six decades. Support for its role was first established in 1963, when it was determined that typical antipsychotics increase levels of catecholamine metabolites (Carlsson & Lindqvist, 1963). This led to the development of the hypothesis that these drugs worked via dopamine receptor blockade (van Rossum, 1966), which was ultimately proven soon after with radioligand binding assays (Seeman et al., 1975; Seeman et al., 1976) (reviewed in (Seeman, 2021)). These findings contributed to the development of the 'original dopamine hypothesis' which posited that schizophrenia symptoms are a result of excess dopamine transmission. Subsequent studies furthered this idea

showing drugs that stimulate dopamine release can induce psychosis in healthy individuals and can worsen symptoms in individuals with schizophrenia (Angrist et al., 1980; Lieberman et al., 1987). More evidence for this hypothesis was discovered following the development of positron-emission tomography (PET), which revealed striatal hyperdopaminergia in affected individuals (Abi-Dargham et al., 1998; Howes et al., 2009; Howes et al., 2013; Laruelle & Abi-Dargham, 1999; Laruelle et al., 1996).

While the data associating schizophrenia with elevated dopamine are compelling, inconsistencies in experimental findings belie the original dopamine hypothesis. In cortical regions, individuals with schizophrenia show blunted amphetamine-induced dopamine release and reduced dopamine levels when performing cognitive tasks (Rao et al., 2019; Slifstein et al., 2015). These data prove that reducing dopamine receptor availability alone is an insufficient intervention. In fact, growing evidence suggests that the dopaminergic abnormalities in schizophrenia result from disruptions in presynaptic function—not post. Clinical imaging studies show there is a lack of evidence supporting abnormalities in dopamine transporter or receptor availability but instead identify abnormalities in dopamine synthesis capacity and release as the major contributors to dopaminergic dysfunction in schizophrenia (Howes, Kambeitz, et al., 2012). Growing evidence supports the theory that schizophrenia symptomology is not a result of an over-active dopaminergic system but is instead a product of a dysregulated one. This updated hypothesis provokes the investigation of systems providing afferent control over dopamine release when investigating pathology.

Given the profound behavioral impact of dopaminergic signaling, neurons that synthesize and release this neurotransmitter are under carefully balanced inhibitory and excitatory control. Glutamatergic and GABAergic afferents into the midbrain tightly regulate dopamine release by influencing neuronal firing patterns. GABA receptors on these neurons maintain inhibitory tone (Tepper & Lee, 2007) while excitation via NMDA receptors promotes a shift from tonic activity to phasic burst firing (Chergui et al., 1993; Zweifel et al., 2009) and subsequent dopamine release in regions targeted by efferent projections (Karreman et al., 1996; Overton & Clark, 1997). The dopaminergic dysregulation seen in schizophrenia could result from an imbalance in this inhibitory/excitatory control.

So, while the dopamine hypothesis of schizophrenia has been a prominent theory in the field, theories implicating glutamatergic signaling have also garnered attention over the years. Primarily focused on the dysfunction of the NMDA receptor, these theories posit that schizophrenia symptoms result from diminished NMDA receptor signaling (Moghaddam & Javitt, 2012; Olney & Farber, 1995). Indeed, antagonists that target this receptor such as phencyclidine (PCP) possess psychotomimetic properties in healthy individuals and can exacerbate symptoms in people with schizophrenia (Javitt, 1987; Javitt & Zukin, 1991; Luby et al., 1959). Adding to this are data showing NMDA receptor deficits in neural tissue of patients (Law & Deakin, 2001) and in single-photon emission computed tomography (SPECT) experiments (Pilowsky et al., 2006). With strong clinical evidence implicating both dopaminergic and glutamatergic systems in this disorder, these neurotransmitters have been the target of many manipulations when attempting to model the clinical condition within a laboratory setting.

Pharmacological models of schizophrenia and their limitations

Rodent models of schizophrenia have been an effective tool when investigating the biological basis of the disorder. Pharmacological models, where behavioral correlates of symptoms are induced by either chronic or acute drug exposure are commonly utilized in basic science experiments. Because amphetamine increases striatal dopamine in both humans and (Howes, Fusar-Poli, et al., 2012) rats (Sharp et al., 1987), it is used in animals to induce a hyperdopaminergic state. Administration of this drug results in hyperlocomotion and stereotyped behaviors, which have been related to the positive, psychotic symptoms of schizophrenia (Robinson & Becker, 1986; Segal & Mandell, 1974). This model is considered to have predictive validity because these behaviors can be normalized with some antipsychotics (Arnt, 1995; Jackson et al., 1994).

A major criticism of amphetamine models is that the drug fails to reproduce behaviors reflecting the full spectrum symptoms, so alternative pharmacological models have been developed. Models utilizing NMDA receptor antagonists like PCP have been more successful in replicating the positive, negative, and cognitive aspects of schizophrenia. Similar to amphetamine models, PCP induces hyperlocomotion and stereotyped behaviors that can be reversed with atypical antipsychotics (Castellani & Adams, 1981; Freed et al., 1984; Kitaichi et al., 1994; Murray & Horita, 1979; Sturgeon et al., 1982) but can also induce behaviors associated with negative symptomology by inducing social behavior deficits (Sams-Dodd, 1996; Steinpreis et al., 1994) and reducing motivation (Noda et al., 1995).

These pharmacological models have been successful in recapitulating aspects of schizophrenia in animals, and in doing so have provided valuable insight into the neurotransmitter systems involved in its biology. However, they are incomplete models focused on reproducing symptomology, not etiology. Pharmacological manipulations do not fully capture the developmental course of disease and fall short when modeling chronic disorders such as schizophrenia. To generate a more accurate model, it is important to mimic the biology that is driving the pathology.

Genetic models of NMDA receptor hypofunction

To address this, genetic animal models of NMDA receptor hypofunction have been developed to build upon the discoveries made in pharmacological models. While a complete loss of NMDA receptors is neonatally fatal (Forrest et al., 1994), mice with global reductions in or conditional knockouts of *Grin1* have proven to be useful animal models for schizophrenia. Like PCP models, NMDA receptor-deficient mice show increases in motor activity as well as disrupted sociability (Mohn et al., 1999). More specific targeting of the receptor on parvalbumin (PV) interneurons results in novelty induced hyperlocomotion, increased anxiety-like behavior in the open field, and deficits in rapid working memory (Belforte et al., 2010; Bygrave et al., 2016; Carlen et al., 2012; Korotkova et al., 2010). Deletion of this gene in specific excitatory neurons can also induce relevant behaviors like memory impairments, reduced sociability, and deficits in sensory gating (Niewoehner et al., 2007; Rompala et al., 2013; Tatard-Leitman et al., 2015). As well, knocking out NMDA receptors on dopaminergic neurons results in abnormalities in phasic dopamine release (Parker et al., 2010; Zweifel et al., 2009) and behaviors reflective of negative

schizophrenia symptoms (Jastrzebska et al., 2016). Genetic models such as these have helped us to gain insight into the effects of sustained NMDA receptor hypofunction, thus bringing us a step closer to a biologically relevant model.

Human genomic data inform more specific models

Schizophrenia is a highly heritable disorder (Cannon et al., 1998; Cardno et al., 1999; Hilker et al., 2018; Sullivan et al., 2003), so many studies have focused on determining the genetic factors that contribute to its pathogenesis. This has recently become possible due to advances in genotyping and sequencing technology allowing for large scale genomic studies. Highly powered experiments comparing the genome of affected and unaffected individuals have led to a rich data set identifying multiple schizophrenia risk genes (Ripke et al., 2013; Schizophrenia Working Group of the Psychiatric Genomics, 2014; Singh et al., 2022; Stefansson et al., 2009; Trubetskoy et al., 2022). Several genes involved with glutamatergic signaling have been implicated, but here we are prioritizing mutations that result in a loss of function (LoF) of the gene encoding the GluN2A subunit of the NMDA receptor (*GRIN2A*). This gene is of particular interest because both rare and common variants are associated with schizophrenia diagnosis. Moreover, there appears to be a natural dose-dependent effect of these variants where common variants influence disease risk modestly, but more damaging ones (such as protein-truncating variants) confer more risk (Hall & Bray, 2022).

These genomic findings have driven investigation into the specific role of *Grin2a* in schizophrenia pathophysiology. Recent studies in mice characterizing the effects of a genetically induced loss of *Grin2a* have proven to be promising. Mice with a heterozygous loss of *Grin2a*

show electroencephalogram (EEG) abnormalities that are also observed in humans with the disorder (Herzog et al., 2023) as well as a hypersensitivity to amphetamine, resulting in an increased locomotor response to the drug (Farsi et al., 2023). Moreover, adolescent mice with *Grin2a* deficits had upregulated expression of genes related to dopamine response and signaling in the striatum (Farsi et al., 2023). This finding bridges deficits in *Grin2a* expression with downstream effects on dopaminergic neurotransmission.

Other studies have targeted *Grin2a* in specific cell types to further dissect the role of this gene in schizophrenia. Loss of GluN2A in cortical and hippocampal excitatory neurons induced behaviors relevant to positive (hyperlocomotion, impaired sensorimotor gating) as well as negative symptoms (disrupted nest building, reduced social novelty seeking). On the other hand, loss of GluN2A in the inhibitory neurons of these regions only induced negative symptom-related behaviors (Lu et al., 2024). In addition to cell-type specific effects of the mutation, it was also shown that the timing of the manipulation plays a role in its effect on behavior. When a global knockout of *Grin2a* was induced in adult mice (P80+), the behavioral phenotype differed from when this manipulation was induced in younger animals (P49-56). Earlier knockouts resulted in behavioral abnormalities relevant to positive and negative symptoms as well as deficits in working memory. If this knockout was induced later, in adults, animals only exhibited negative symptom-related behaviors, suggesting an interaction between *Grin2a* and neurodevelopment and highlighting the importance of considering age as a factor when manipulating this gene (Lu et al., 2024).

The generation of animal models with human genetic validity is an important step towards understanding the biological processes that underly schizophrenia. Recent studies provide encouraging evidence that genetic manipulations resulting in a LoF of *Grin2a* are sufficient to induce phenotypes relevant to schizophrenia. They prompt further investigation into specific cell types and circuits where genetic variants of *Grin2a* contribute to this disorder.

Purpose of this dissertation

Animal models that accurately depict etiological aspects of schizophrenia are needed to target the root cause of the disorder more specifically. Advancements in our understanding of the contribution of genetic risk factors have allowed for the development of more specific animal models to utilize when investigating current treatment mechanisms and identifying new ones. Driven by insights from human literature, research into the specific role of *Grin2a* in schizophrenia has begun to emerge. With this dissertation, I aim to build upon the literature and investigate how genetic deficiencies of *Grin2a* in dopaminergic neurons contribute to behaviors and physiology associated with schizophrenia.

We examined the relationship between adolescent neurodevelopment and *Grin2a*. We found GluN2A protein levels fall specifically in the midbrain during adolescence (Chapter 2). We then augmented this process by inducing a loss of *Grin2a* in the dopaminergic neurons of this region during adolescence (Chapter 3). Reducing *Grin2a* in these cells resulted in behaviors (Chapter 4) and dysfunctional dopaminergic signaling (Chapter 5) analogous with positive, but not negative or cognitive, symptoms of schizophrenia. These findings suggest a novel mechanism

of reduced functionality of *GRIN2A* in dopaminergic neurons in the pathophysiology of schizophrenia.

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Chapter 2

Regional profiles of GluN2A during adolescent neurodevelopment

Introduction

NMDA receptors are comprised of four subunits: two obligatory GluN1 subunits accompanied by some combination of GluN2 (A-D) or GluN3 (A-B) subunits. The composition of the receptor dictates its permeability, gating, pharmacological properties, trafficking, and downstream cascades (Cull-Candy et al., 2001; Cull-Candy & Leszkiewicz, 2004; Paoletti et al., 2013). Interestingly, GluN1 is expressed in virtually all neurons, but GluN2 subunits display distinct spatiotemporal expression patterns. GluN2A does not express in the human or rodent brain before birth, but with experience and increases in neural activity, synapses mature and levels of GluN2A-containing NMDAs receptors increase (Bar-Shira et al., 2015; Barria & Malinow, 2002; Bellone & Nicoll, 2007; Monyer et al., 1994; Ohi et al., 2016; Philpot et al., 2001; Stocca & Vicini, 1998; Watanabe et al., 1992; Wenzel et al., 1995).

Genes with this form of developmental regulation are particularly interesting within the framework of schizophrenia risk because it is a neurodevelopmental disorder. It is hypothesized that its etiology is related to the disruption of typical ontogenetic processes because different symptoms emerge at different ages (Paus et al., 2008). Cognitive and negative symptoms such as memory and attention deficits, flattened affect, and social withdrawal typically start expressing

during childhood. It isn't until late adolescence that we begin to see the presentation of positive symptoms (Howes & Murray, 2014). These age-related changes in phenotype suggest that good candidate risk genes for schizophrenia will also have developmentally regulated expression. Previous studies have shown that cortical shifts in *Grin2a* expression occur early in development (Monyer et al., 1994; Watanabe et al., 1992; Wenzel et al., 1995), which coincides with the emergence of cognitive and negative symptoms. However, this does not help explain the delayed adolescent onset of positive symptoms. Because these symptoms are associated with dopaminergic abnormalities (Abi-Dargham et al., 2000; Abi-Dargham et al., 2009; Breier et al., 1997; Egerton et al., 2013; Howes et al., 2013), we hypothesized that there would be shifts in *Grin2a* expression in brain regions associated with this circuitry during adolescence.

The transition from adolescence to adulthood is marked by significant neurobiological and behavioral changes (Spear, 2013). Despite clear differences between the adolescent and adult brain, adolescent animals remain underutilized in basic science research. One concern with studying the adolescent period is that it remains to be precisely defined in humans, making distinguishing this period challenging in animal subjects. While generally described in humans as the period between 12-18 years old, it is also argued to last until age 25. This debate is reflected in literature describing rodent adolescence which is conservatively defined as the period between P28-42 but is also described within a broader range of P21-60 (Spear, 2000; Spear & Brake, 1983). Guided by these definitions, we chose to collect tissue at four different time points, spanning the age range of P21-P80.

Rodent studies focusing on early, perinatal neurodevelopment show both *Grin2a* mRNA expression and GluN2A protein levels are sensitive to age. Adolescence is another

period of significant neurological change that is strongly linked to the emergence of psychiatric disorders, so we wanted to determine regional and developmental profiles of GluN2A in adolescent animals. Our findings showed region-specific changes in GluN2A during adolescence, with a significant effect of age on protein levels specifically in regions containing dopamine cell bodies.

Methods

Subjects

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Oregon Health & Science University (OHSU). Naïve male (n=12) and female (n=12) Long-Evans rats were utilized in these experiments. All animals were bred in house and maintained on a reverse 12hr light/dark cycle with ad libitum access to food and water.

Tissue collection and protein extraction

Brain levels of GluN2A were examined in animals aged P21, P35, P45, and P80. Tissue collection methods were adapted from Wager-Miller (Wager-Miller et al., 2020). Briefly, at age of interest, brains were harvested, and flash frozen in isopentane that was pre-chilled with dry ice. Whole brains were stored in conical tubes at -80°C until dissection.

While maintaining a frozen condition, brains were sliced into 1mm sections using a pre-chilled brain matrix and razor blades. Bilateral micropunches of tissue were collected from regions of interest using a rat brain atlas as a reference (Paxinos, 1998). To extract protein, tissue samples were homogenized in a lysis buffer containing protease and phosphatase

inhibitors. Protein concentration was quantified with a Pierce BCA Protein Assay Kit (ThermoFisher). Homogenates were aliquoted and stored at -80°C until use.

Protein quantification

Automated immuno-quantification was run on a Wes instrument (Bio-Techne) according to the manufacturer's protocol (Luck et al., 2021). Briefly, each tissue sample was diluted to a concentration of 0.3 µg/µl protein and mixed with dithiothreitol and a fluorescent molecular weight marker at a ratio of 5:1. These samples were heated to 95°C for 5 min for protein denaturation and then loaded into the allocated wells of the Wes plate. Other wells were loaded with blocking reagent, primary antibody (Rabbit-anti-NMDAR2A (Sigma Aldrich)), horseradish peroxidase (HRP)-conjugated secondary antibody, and a mix of Luminol-S and Peroxide (Bio-Techne Anti-Rabbit Detection Module, #DM-001). Size-based separation electrophoresis, immobilization, and immunodetection were automatically run using the capillary array system of the Wes. All samples were normalized to total protein as a loading control (Bio-Techne Total Protein Detection Module for Chemiluminescence, #DM-TP01). Densitometric analysis was performed using the Compass software from ProteinSimple.

Statistical analysis

Statistical analyses were performed in GraphPad Prism (Version 10.0.1). Data were analyzed with an ordinary one-way ANOVA and followed up with Tukey's multiple comparisons test for post hoc analysis when main effect of age was significant ($p < 0.05$).

Data exclusion

Data were excluded if the Compass software determined a potential data issue during peak analysis of either the GluN2A or total protein signal for a sample.

Results

The GluN2A subunit of the NMDA receptor has a temporal expression profile that coincides with critical periods of neurodevelopment. Previous studies show dorsal brain regions such as the frontal cortex and hippocampus have drastic shifts in subunit expression during perinatal stages of development, with little change after that (Monyer et al., 1994; Watanabe et al., 1992; Wenzel et al., 1995). To expand our understanding of the developmental timeline of GluN2A, we chose to collect tissue beyond the perinatal period—with a closer focus on adolescence. To do so, we collected tissue punches from juvenile (P21), adolescent (P35, P45), and adult (P80) brains (Figure 2.1 A). This tissue was collected from brain regions closely associated with schizophrenia: the prefrontal cortex (PFC), hippocampus, dorsal striatum (DS), nucleus accumbens (NAc), ventral tegmental area (VTA), and the substantia nigra (SN). Consistent with previous studies, we found GluN2A levels in dorsal brain regions to have stabilized by the juvenile time point. Between ages P21 and P80, there was no significant effect of age on GluN2A levels in the PFC or hippocampus. A similar pattern was observed in the NAc whereas the DS showed a transient increase in GluN2A between P21 and P35, then returned to juvenile levels in adulthood (Figure 2.1 C). The most remarkable change during adolescence was observed in the VTA and SN. In both regions there was a significant loss of GluN2A after P21, throughout adolescence, which persisted into adulthood (Figure 2.1 D). Thus, in contrast to cortical regions where GluN2A levels remain stable after childhood, GluN2A levels were labile in dopamine-rich regions during adolescence.

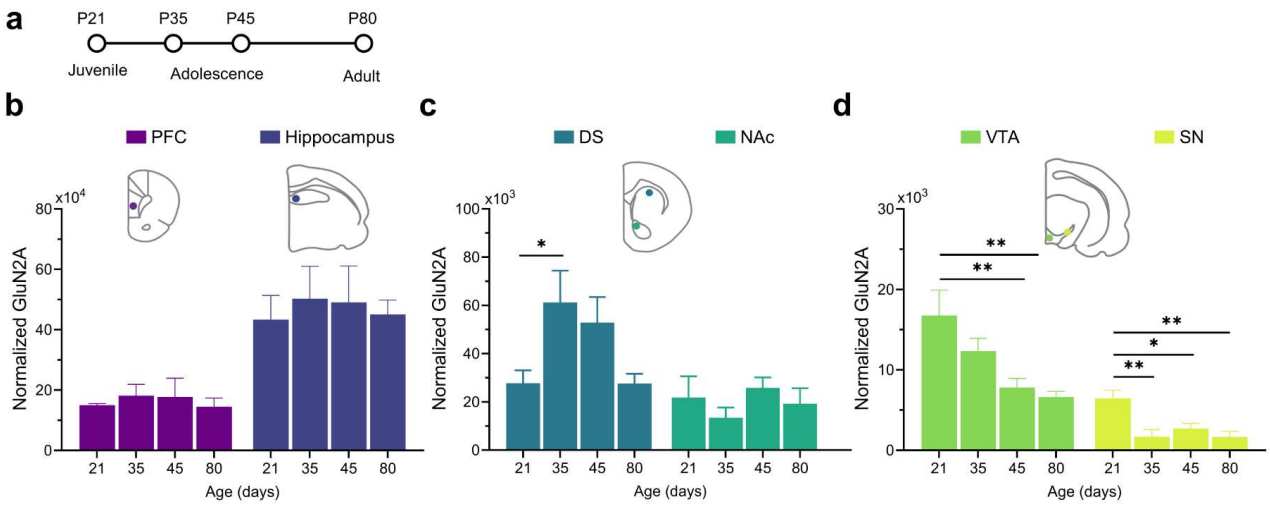

Figure 2.1 Regional differences in GluN2A protein levels during adolescence

A Timeline of tissue collection ages for juvenile (P21), adolescent (P35, P45), and adult (P80) samples. B There was no significant effect of age on normalized GluN2A levels in the prefrontal cortex (PFC) or hippocampus; *n*=5-6. C There was a significant effect of age on GluN2A levels in the dorsal striatum (DS); GluN2A increased from P21 vs P35 (*p=0.0491). Levels were not affected by age in the nucleus accumbens (NAc); *n*=4-6 brains. D There was a significant effect of adolescent age on normalized GluN2A levels in dopaminergic midbrain regions. The VTA showed reductions in GluN2A at P21 vs P45 (*p=0.0157) and vs P80 (**p=0.0058) and the substantia nigra (SN) from P21 vs P35 (**p=0.0036) vs P45 (*p=0.0318) and vs P80 (**p=0.0033); *n* = 5-6 brains. The data are represented as mean ± SEM with representative schematics of location of tissue micropunches displayed above corresponding graphs.

Discussion

While NMDA receptor hypofunction has long been associated with schizophrenia pathophysiology (Moghaddam & Javitt, 2012; Olney & Farber, 1995), deeper investigation into the role of specific NMDA receptor subtypes in this disorder has been a more recent development. Genome-wide association analysis has identified variants resulting in a LoF of *GRIN2A* as schizophrenia risk genes, necessitating investigation into how this specific receptor subtype contributes to pathophysiology (Ripke et al., 2013; Singh et al., 2022; Trubetskoy et al., 2022). Because this gene has spatiotemporal regulation and has been implicated in a disorder that has distinct temporal patterns of symptom expression, it is important to understand how

age affects its expression within different brain regions to determine how a LoF of this gene could have variable effects on different neural systems.

Levels of GluN2A-containing receptors are influenced by critical neurodevelopmental periods. Childhood onset of cognitive and negative schizophrenia symptoms coincides with the rise of cortical *GRIN2A* expression. So, while a loss of function of *GRIN2A* could explain the childhood onset of cognitive and negative symptoms, how these mutations contribute to delayed expression of positive symptoms during adolescence is less clear. These late emerging, psychotic symptoms are attributed to abnormalities in dopaminergic signaling (Creese et al., 1976; Seeman et al., 1976; Snyder, 1976). Our findings identify NMDA-receptor related maturation in dopaminergic regions during adolescence as a potential mechanism contributing to the late-stage phenotype.

We determined adolescent maturation in the midbrain involves significant reductions in GluN2A. This was a critical finding as it connected adolescent developmental alterations of GluN2A with the well-established involvement of the dopamine system in schizophrenia. With typical development, there are decreases in midbrain GluN2A. If an individual expresses a genetic variant resulting in insufficient GluN2A production, that loss would be exaggerated and may contribute to the adolescent emergence of behavioral and physiological deficits. In future chapters we will explore how augmenting this adolescent loss of GluN2A in dopaminergic regions affects the brain and behavior.

Chapter 3

Modeling loss of function of *Grin2a* in dopamine neurons of the midbrain

Introduction

Early descriptions of schizophrenia implicated neurodegeneration in its etiology because it has a delayed onset. The idea that this disorder stems from the deterioration of the brain led to Emil Kraepelin's initial classification of the disorder as *dementia praecox* or 'early onset dementia'. Since then, evidence has shifted this hypothesis away from that of accelerated neurological decline to one of altered development, with pathology manifesting even before birth (Cannon & Murray, 1998; Lewis & Levitt, 2002). The idea that schizophrenia has developmental origins is supported by associations between diagnosis and risk factors that are present in utero. Complications of pregnancy or during delivery have significant associations with schizophrenia diagnosis (Cannon et al., 2002; Cannon & Murray, 1998; Geddes et al., 1999; Lewis & Murray, 1987). As well, it has been shown that aberrations in the maternal environment due to age (Fountoulakis et al., 2018; Lopez-Castroman et al., 2010), stress (Huttunen & Niskanen, 1978; Khashan et al., 2008; van Os & Selten, 1998), metal exposure (Modabbernia et al., 2016; Opler et al., 2004; Opler et al., 2008), or infection (Brown et al., 2004; Brown et al., 2005; Buka et al., 2008; Mortensen, Norgaard-Pedersen, Waltoft, Sorensen, Hougaard, et al., 2007; Mortensen, Norgaard-Pedersen, Waltoft, Sorensen, Hougaard, & Yolken, 2007) are associated with increased incidence in progeny.

The neurodevelopmental theory of schizophrenia was proposed to explain how pathologies present in early life interact with typical brain maturation to uncover symptoms over time (Weinberger, 1987). It offers an explanation as to why adolescence is the peak age range of onset for most psychiatric disorders, including schizophrenia (Paus et al., 2008; Solmi et al., 2022). Because there are many neurobiological processes that undergo reconstruction during adolescence, it is hypothesized that some aspects of schizophrenia result from an exaggeration of typical adolescent development. Genetic variants can offset developmental trajectories, resulting in age-dependent symptom expression.

Adolescent maturation of the human brain is marked by many events, including reductions in delta-wave sleep periods (Campbell & Feinberg, 2009), gray matter volume loss in the prefrontal cortex (Gogtay et al., 2004), and synapse elimination (Huttenlocher & Dabholkar, 1997; Petanjek et al., 2011). In support of the neurodevelopmental hypothesis, these processes have been observed to be amplified in people with schizophrenia. People with this disorder have even more pronounced reductions in delta-wave sleep (Feinberg, 1982; Keshavan et al., 1994), cortical gray matter loss (Sporn et al., 2003), and 'over' pruning of synapses (Moyer et al., 2015). For this reason, we wanted to identify an adolescent process that had the potential to be augmented by a *Grin2a* LoF mutation. Guided by our previous findings (Chapter 2) that midbrain GluN2A levels decline in adolescents, our goal was to enhance this process with region- and cell type-specificity. To do so, we employed the use of a Cre-dependent CRISPR/Cas9 virus to knockout *Grin2a* in dopaminergic neurons of the midbrain.

This tool was initially developed and validated in mice (Hunker et al., 2020), so we ran a battery of experiments to determine the efficacy of this tool in the rat brain. Through

immunohistochemistry, slice electrophysiology, and protein quantification we determined that virally mediated delivery of CRISPR/SaCas9 generated a conditional *Grin2a* knockout (cKO) model that would serve as a useful tool in future experiments to better understand how a LoF of *Grin2a* contributes to the adolescent phenotype of schizophrenia.

Methods

Subjects

All animal procedures were approved by the IACUC at OHSU. Rodents utilized in this study were transgenic Long Evan's rats expressing Cre recombinase under the control of the tyrosine hydroxylase (TH) promoter (Witten et al., 2011). All animals were maintained on a reverse 12hr light/dark cycle with ad libitum access to food and water.

Viral production

The Cre-inducible recombinant adeno-associated virus (AAV) vector constructs utilized in this study were produced and gifted by Larry Zweifel (Hunker et al., 2020). Viruses in these experiments were: AAV1-FLEX-Cas9-U6-sgGrin2a (*Grin2a* cKO) and AAV1-FLEX-Cas9-U6-sgRosa26 (control) (Addgene # 159914). These viruses were co-infused with AAV1-hSyn-DIO-EGFP (Addgene # 50457) to aid with visualization in subsequent experiments.

Stereotaxic surgery

Juvenile rats (P21±1) were anesthetized with isoflurane and placed in a stereotaxic apparatus and body temperature was maintained using a water heating circulation pump (E-Z Systems). The scalp was incised and two bilateral craniotomies over the VTA were performed. Using a syringe pump (World Instruments) and micro infusion syringe (Hamilton), we delivered four 250

nl infusions at the bilateral VTA sites (AP= -4.9 mm, ML= ±0.7 mm, DV= -6.5 and -6.0 mm from dura) at a rate of 100 nl/min. To prevent backflow, the syringe was not removed until 5 min after infusions. The incision was then closed with surgical sutures and treated with a triple antibiotic. Animals remained on heat and were administered oxygen until consciousness was regained, then returned to their home cage. There was a minimum of a two-week incubation period before experiments were run.

Protein extraction and quantification

Tissue dissection, micropunching, protein extraction, and protein quantification were run as described previously, in Chapter 2. Punches were collected from the VTA of control and *Grin2a* cKO animals.

Immunohistochemistry (IHC)

Animals were euthanized with an intraperitoneal (IP) injection of 400 mg/kg chloral hydrate and perfused via the vascular system with 0.1 M phosphate buffer solution (PBS) followed by 4% paraformaldehyde (PFA). Brains were removed and stored in 4% PFA at 4°C overnight, then transferred to a 30% sucrose solution and stored at 4°C until sectioning.

Coronal sections (35 µm) of each brain were collected using a cryostat (Lecia) and stored in 0.1 M PBS with 0.05% sodium azide. Free-floating sections were blocked and permeabilized at room temperature for 2 hrs in 0.1 M PBS, 0.25% Triton X, 10% normal donkey serum. Sections were then incubated overnight at 4˚ C with the following primary antibodies: chicken anti-TH (1:500, #76442, abcam), rabbit anti-GFP (1: 500, #290, abcam), or mouse anti-HA (1:500, MMS-101P, Biolegend). Sections were washed, then incubated in secondary antibodies (AlexaFluor-

594; 488, 1:1000) for 2 hrs at room temperature. Sections were washed again and mounted onto Super Frost+ slides. Coverslips were applied using Vectashield HardSet antifade mounting media. Fluorescence was imaged using a Ziess Axiovert 200 microscope and cell counting was done in Zen 2 software (Zeiss).

Slice electrophysiology

Two to three weeks following virus infusion (P35-42), rats were anesthetized with isoflurane and decapitated. The brain was collected and deposited in artificial cerebrospinal fluid (ACSF) at 32–35°C containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 25 $NaHCO_3$, and 11 Dextrose. The ACSF used for brain extraction, slicing, and recovery also contained 2 mM kynurenic acid (Sigma) to prevent NMDA-mediated excitotoxic damage. Horizontal slices (220 µm) containing the midbrain were cut using a vibratome (Leica) in warm ACSF bubbled with 95% O_2/5% CO_2. Slices were allowed to recover in the same buffer at 30°C for at least 30 min prior to recording. Hemisected slices were transferred to the recording chamber, which was continuously perfused at 2 to 3 mL/min with bubbled ACSF at 34°C. In most experiments the recording buffer was identical to the collection buffer except it did not contain any $MgCl_2$, to allow for NMDA receptor activation by glutamate iontophoresis. In a subset of recordings, experiments were started in Mg-containing ACSF and Mg-free ACSF was perfused later. All experiments were completed within 7 h of slicing.

VTA dopamine neurons were identified by their morphology and the presence of green fluorescence protein (GFP). Recordings were made using glass pipettes (1.0 – 2.5 MΩ resistance) filled with an internal solution containing (in mM): 100 K-gluconate, 20 NaCl, 1.5 $MgCl_2$, 10 HEPES (K), 2 ATP, 0.2 GTP, 10 phosphocreatine, and 10 BAPTA (4K). Recordings were made in

whole-cell configuration. After break-in, cells were voltage-clamped at −55 mV and resistance and capacitance were monitored; cells with a series resistance ≥12 MΩ were discarded. The dopaminergic identity of the patched cells was further confirmed by the presence of a hyperpolarization-induced depolarizing current (I_h). Cells with an I_h < 200 pA were discarded. Another glass pipette was filled with a 1 M solution of monosodium glutamate (Sigma Aldrich). This pipette was lowered into the slice and placed in proximity to the patched cell, while a continuous backing current of +1.0 nA was applied. Glutamate was iontophoretically delivered by the application of 20 ms negative current pulses (-40.0 to -80.0 nA). Location and current intensity were adjusted to produce a stable depolarizing current in the patched cell.

Data were acquired with AxoGraph software (Berkeley, CA) and recordings were monitored with LabChart (AD instruments, Colorado Springs, CO). Current responses in Mg-free ACSF were recorded until at least 10 stable responses were obtained. For experiments where Mg-free ACSF was perfused after patching this took up to 25 min. The slice was then perfused with Mg-free ACSF containing the GluN2A-specific antagonist TCN201 (30 µM; Sigma Aldrich SML0416). Responses to glutamate puffs were then recorded until a stable state was obtained or for a maximum of 20 minutes. The slice was then perfused with Mg-free ACSF containing ifenprodil (10 µM; Hello Bio HB0339), a GluN2B-specific antagonist. Responses were measured until a stable state was reached, or a maximum of 20 min. Lastly, we bath applied AP5 (50 µM; Sigma Aldrich A8054), a non-selective NMDA blocker, to confirm the identity of the measured currents. Because both TCN201 and ifenprodil do not readily wash out, each slice was only used for one attempted recording. Recordings were analyzed post-hoc using AxoGraph by averaging

the last 5 stable pulses for each condition and measuring the peak amplitude of the resulting trace. Data are expressed as a fraction of the Mg-free baseline.

Statistical analysis

Statistical analyses were performed in GraphPad Prism (Version 10.0.1). Unpaired t-tests were run in instances where two groups were compared. Otherwise, the data were analyzed with a one-way ANOVA with Tukey post hoc corrections if significance was reached ($p<0.05$).

Results

Dopamine neuron-specific expression of *Grin2a* cKO virus

We induced a dopamine neuron-selective knockout of *Grin2a* by infusing a Cre-inducible CRISPR/Cas9 virus targeting Grin2a into the VTA of TH-Cre^{+} rats. Viral surgeries were done after weaning (P21) to allow for viral expression during adolescence. To aid in visualization, the *Grin2a* cKO or control virus were co-infused with a Cre-dependent virus that would express GFP (Figure 3.1 A). Immunohistochemistry was used to confirm dopamine neuron-specific virus expression. Because the *Grin2a* cKO virus had a hemagglutinin (HA)-tag, we looked for co-expression between HA and GFP. We found that there was significant overlap between HA+ and GFP+ cells (Figure 3.1 B, C). To determine that this expression was specific to dopamine neurons, we also looked for co-expression between GFP and TH. We determined significant overlap between TH+ and GFP+ cells (Figure 3.1 D, E) (for 10x magnification tiled image, see Supplementary Figure A.1)

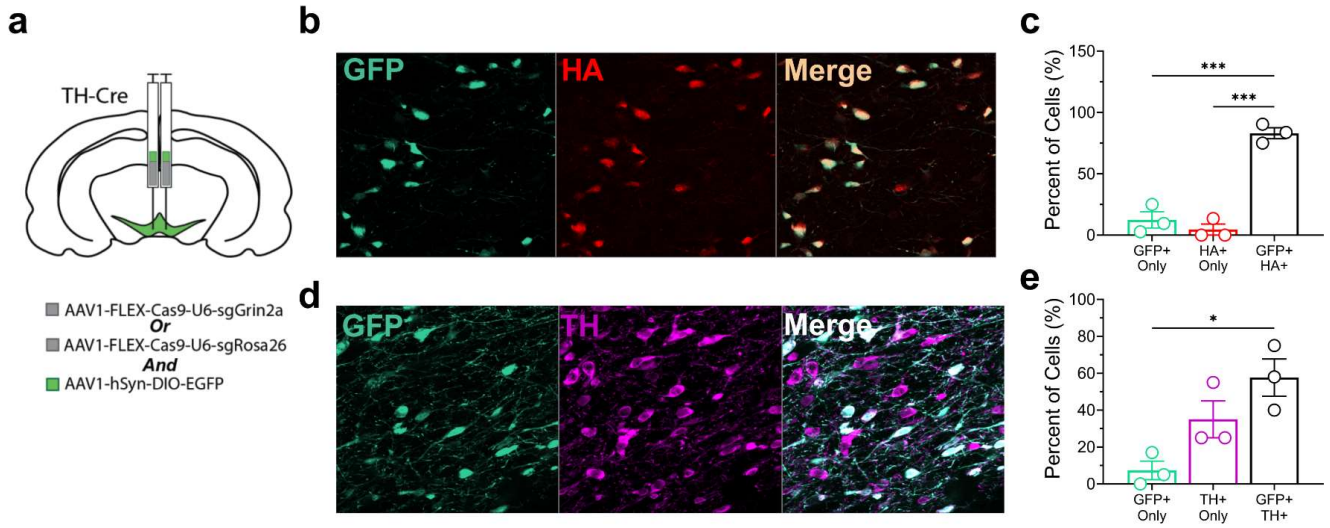

Figure 3.1 Expression of *Grin2a* cKO virus is specific to dopamine neurons

A Schematic representation showing infusion of the *Grin2a* cKO or control virus mixed with a GFP virus at a 3:1 ratio. **B-C** Immunohistochemistry experiments were run to determine the level of co-expression between the virus resulting in GFP expression and the hemagglutinin (HA)-tagged *Grin2a* cKO virus. There was significant overlap between cells expressing HA and GFP with significantly more cells expressing both GFP and HA than only GFP (***p=0.0002) or with only HA (***p=0.0001); *n* = 3 brains. **D-E** Immunohistochemistry experiments were run to determine dopamine neuron-specific expression of GFP. There was significant co-expression of GFP and tyrosine hydroxylase (TH) with more cells expressing both GFP and TH than only GFO (*p=0.0152); *n* = 3 brains. Images were collected at 20x magnification, the data are represented as mean ± SEM.

Grin2a cKO virus infusion results in functional loss of GluN2A

Once dopamine neuron-specific expression of the virus was confirmed, functional reduction of GluN2A in GFP+ established with recordings in an acute slice preparation (Figure 3.2 A). Iontophoretic delivery of glutamate proximal to patched cells resulted in large inward currents that were collected as a baseline. Bath application of a selective antagonist to block GluN2A-containing NMDA receptors (TCN201, 30 µM), followed by application of a GluN2B-specific antagonist (ifenprodil, 10 µM) (Figure 3.2 B, C) allowed us to compare cellular response to drug between treatment groups. TCN201 application resulted in a reduction of the glutamate-induced

current in controls, but cells from *Grin2a* cKO animals showed a significantly blunted response to the antagonist (Figure 3.2 E), confirming that our viral construct worked as expected. This blunting was specific to GluN2A-containing NMDA receptors as ifenprodil produced similar effects in both control and *Grin2a* cKO animals (Figure 3.2 F). Finally, the extent of this regional loss of GluN2A was determined through Western blot analysis. We observed an approximate 50% reduction of GluN2A in the VTA of animals administered the *Grin2a* cKO virus compared to control (Figure 3.2 G).

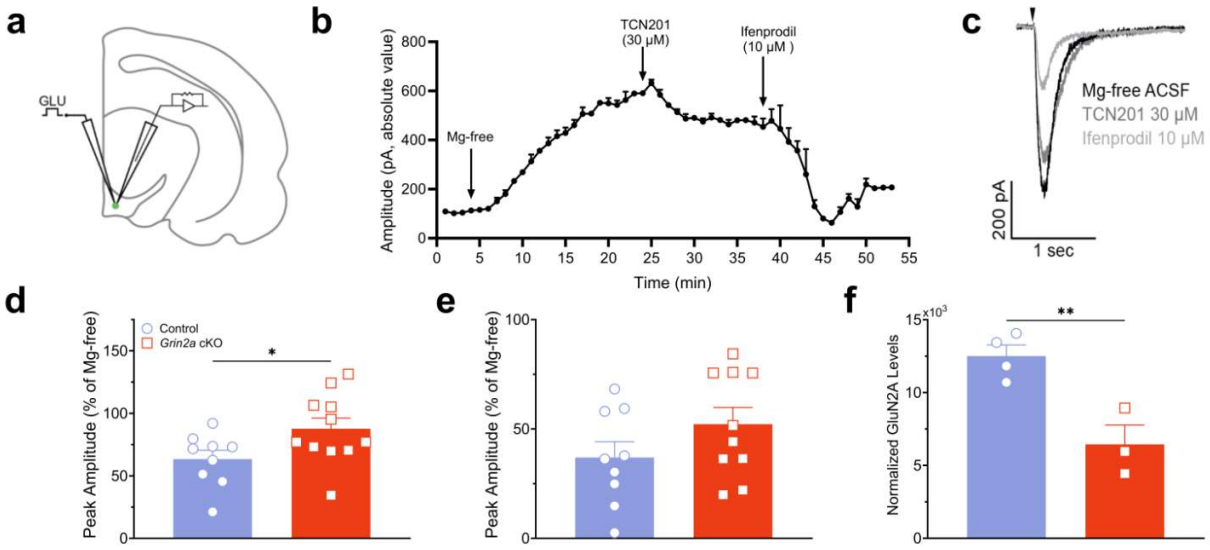

Figure 3.2 *Grin2a* cKO virus results in significant loss of functional GluN2A

A Schematic representation of slice electrophysiology experiments. GFP+ neurons were patched onto and recorded from following the application of glutamate iontophoresis. **B** Experimental design: iontophoretic delivery of glutamate proximal to patched cells resulted in currents collected as a baseline, antagonists were then bath applied. **C** Example traces of recording from control slice following iontophoresis an antagonist application. **D** Quantification of peak amplitudes show that dopamine cells with *Grin2a* cKO virus were significantly less effected by TCN201 than controls (*p=0.049); *control n* = 9, *Grin2a* cKO *n* = 11 cells. **E** Quantification of peak amplitudes show that dopamine cells with *Grin2a* cKO virus were similarly affected by ifenprodil as controls, *control n* = 9, *Grin2a* cKO *n* = 10 cells. **F** To determine the extent of the viral KO, Western blot analysis was used to determine a significant reduction in GluN2A in the VTA of animals infused with the *Grin2a* cKO. There was a significant loss in *Grin2a* cKO animals compared to controls (**p=0.0082); *control n* = 4, *Grin2a* cKO *n* = 3 brains. The data are represented as mean ± SEM.

Discussion

We previously found that during adolescence, GluN2A levels naturally decline in dopamine cell-containing brain regions (Chapter 2). LoF variants of the gene encoding this protein have been associated with schizophrenia diagnosis, so we wanted to augment this process to determine how excessive loss of this protein could contribute to symptomology. To do so, we employed viral gene silencing techniques to conditionally express SaCas9 along with single guide RNA to directly target *Grin2a* in dopamine neurons. We confirmed that infusion of the *Grin2a* cKO virus resulted in cell-specific expression and a functional loss of GluN2A. Combining immunohistochemistry, slice electrophysiology, and protein quantification methods, we confirmed a significant loss of functional GluN2A in midbrain dopamine neurons. These experiments established the efficacy of our strategy, and confirmed the virus was a viable tool to use in future studies.

Chapter 4

Behavioral phenotype of adolescents with *Grin2a* cKO

Introduction

Schizophrenia symptoms are often categorized into 3 types: cognitive, negative, and positive. Cognitive symptoms include working memory and attentional deficits; negative symptoms include low motivation, flattened affect, and anxiety; positive symptoms include hallucinations and delusions which can be exacerbated by psychomimetic drugs. These characteristic symptoms may seem impossible to identify in a subject without the capacity to self-report, however many rodent behaviors have been identified as relevant to the human condition. Establishing rodent behavioral correlates of symptoms allows us to take advantage of the high level of control allowed for by studies in animal models. Moreover, these models are crucial for in depth investigation into the biology driving symptom expression.

Working memory in rodents can be assessed by observing their spontaneous alternation when placed in a multi-armed maze. Mice or rats with intact working memory are expected to alternate between arms of the maze, tending to enter novel arms over previously visited ones (Kraeuter et al., 2019). Tests of anxiety-like behaviors in rodents are commonly based in the innate aversion rodents have to open spaces. The Open Field (OF) and Elevated Plus Maze (EPM) are frequently used to assess levels of avoidance of portions of a maze that are exposed, with the idea that animals with more anxiety spend less time in the open (Broadhurst, 1969; Montgomery, 1955). Amotivation can be assessed in animals with the use of progressive ratio

schedules of responding. In these tasks, experimental animals are trained to take an action to receive a reward, and once behavior is stabilized, the number of actions needed to receive the reward increases progressively. In this task, animals must maintain their operant responding under increasing demand. The behavioral readout for this task is breakpoint—or when the subject ceases responding. Theoretically, if an animal has motivational deficits, they will have low breakpoints (Hodos, 1961). Additional behavioral readouts such as response rate during the cue or latency to respond to the cue also reveal information about reinforcement learning in this task (Bradshaw & Killeen, 2012).

The simplest way behavioral correlates of positive schizophrenia symptoms are studied in rodents is through observation of basic locomotor function. Baseline hyperactivity as well as hyperreactivity to the locomotor effects of psychotomimetic drugs are thought to be correlates of positive symptoms (Powell & Miyakawa, 2006). While these studies are useful for initial observations, more clinically relevant behaviors should be applied to better study specific aspects of positive schizophrenia symptoms.

Psychosis can be difficult to model behaviorally, but if broken down, components of this complex symptom can be studied in a rodent. The aberrant salience hypothesis of schizophrenia explains hallucinations and delusions as a result of misattributed significance (Howes & Nour, 2016). Typically, our brains begin to ignore predictable sensory input to conserve the overwhelming amount of energy that would be required to attended to everything in our surroundings. If an individual is experiencing abnormalities in perception, they will attend to stimuli that would generally be ignored. Over-assigning importance to irrelevant information could manifest as the abnormal beliefs and perceptions which are characteristic of emerging

positive symptoms in schizophrenia (Fletcher & Frith, 2009). In rodent models, we can look at salience attribution when animals are conditioned to pair cues with outcomes of varying significance. Fortunately, association learning in rodents is a well-studied behavior and can be investigated in both Pavlovian and operant conditioning paradigms.

With the following experiments, we tested relevant behaviors that spanned the spectrum of schizophrenia symptomology to characterize the behavioral phenotype of adolescent animals with the *Grin2a* cKO. Reducing *Grin2a* in dopamine neurons resulted in adolescent behaviors analogous with positive—but not negative or cognitive—symptoms of schizophrenia. These findings suggest a novel mechanism through which reduced functionality of *GRIN2A* in dopaminergic neurons contribute to positive prodromal symptoms in schizophrenia.

Methods

Subjects

Rodents utilized in this study were transgenic TH-Cre Long Evan's rats bred in house. Animals were maintained on a reverse 12hr light/dark cycle with ad libitum access to food and water (unless otherwise specified). All behavioral experiments were run during the adolescent period (P35-P60).

Stereotaxic surgery and virus infusion

Grin2a cKO and control virus was infused into the VTA of juvenile rats at weaning (P21±1) as described in the previous chapter. All animals were allowed a two-week recovery and incubation period before behavioral experiments were run.

Operant and Pavlovian conditioning

All operant box behavior was performed on animals aged P35-P45. Rats were mildly food restricted and habituated to operant boxes (Coulbourn Instruments) two days prior to experimentation. All behavioral tasks were run in red light and behavior recorded with Graphic State software (Coulbourn Instruments). Each animal received one of the following tasks:

Progressive ratio of reinforcement

Operant chambers contained one wall with a nose poke hole that could be illuminated and an opposing wall with a food trough where sucrose pellets were dispensed. Animals were initially trained on a fixed ratio one (FR1) schedule where one nose poke in response to a light cue resulted in the delivery of one sucrose pellet. After five days of training, animals advanced to an FR5 schedule for two days. For the final four days, animals were tested with a progressive ratio schedule, where the response ratio increased according to the formula $5e^{(0.2n)-5}$, where n = trial number. This resulted in an exponential increase in response requirement (1, 2, 4, 6, 9, 12 etc.) when rounded to the nearest whole number. Sessions were terminated if there was a 5 min cessation in responding, if a trial took longer than 45 min to complete, or after 3 hr total. Latencies to respond to the cue, to retrieve a reward, and total rewards received were calculated during FR1 and FR5 trials. We determined the breakpoint for each animal across the four test dates. Running response rate and post reinforcement pause (PRP) lengths were determined from the average of the testing days. Data were excluded if animals did not reach a response ratio at least two times.

Flexible contingency learning (FCL)

Operant chambers contained one wall with a light cue and speaker and an opposing wall with a food trough where sucrose pellets were dispensed. We utilized a modified version of a previously published task (Kim et al., 2012; Kim et al., 2010), abbreviated to better fit within the adolescent time window. Briefly, animals were presented with two conditioned stimuli (CS): a 10 sec light or tone. In a counterbalanced fashion, animals were conditioned to pair one CS with the delivery of a sucrose pellet and the other with a mild foot shock (180 ms, 0.2 mA). These unconditioned stimuli (US) immediately followed the termination of either CS. After five days of conditioning, the initial associations were reversed for Sessions 6-10. In each session, animals underwent 100 stimulus pairings (50 trials of each contingency) delivered pseudo-randomly with a 20 sec inter-trial interval (ITI). CS_A represents the cue that was initially appetitive and then became aversive, CS_B represents the cue that was initially aversive and then switched to appetitive.

Behavior was analyzed by normalizing the amount of nose pokes into the food trough during CS to baseline food trough entries during the ITI. The following equation was used to calculate this discrimination index (DI):

$$\left(\frac{\text{CS entries} - \text{last 10s ITI entries}}{\text{CS entries} + \text{last 10s ITI entries}}\right)$$

With this formula, a positive DI suggests conditioned approach, a null DI suggests no conditioning has occurred, and a negative DI suggests conditioned suppression. Average

DIs across Sessions 1-5 were considered initial learning, while average DIs across Sessions 6-10 were considered contingency reversal learning.

Maze behaviors

All maze testing was administered at P45-P55. Animals were habituated to handling and the testing environment two days preceding behavior trials. All behaviors were run under dim, white light. On the day of testing, animals were habituated to the room for 2 hrs before trials were run. The assays were run as follows:

Elevated plus maze (EPM)

The EPM consisted of two opposing open arms and two opposing arms bordered with 48 cm walls. Animals were placed in the center of the maze and allowed to explore for 10 min and their movement tracked with PanLab SMART v3.0 software (Harvard Apparatus). Percent time spent in open and closed arms was evaluated.

Spontaneous alternation test

A four-arm, plus maze was used to assess spontaneous alternation as a measure of spatial working memory. In this task, distinct visual cues were positioned on all four walls of the behavioral testing room. Animals were placed in the center of the maze and allowed to explore for 10 min. The order of arm entries was recorded manually during the task. Percent alternation and total number of arm entries were evaluated. An alternation consisted of four distinct arm choices out of five consecutive arm entries. A 4/5 alternation score was determined by dividing the total number of alternations in overlapping quintuplets by the number of possible alternations and multiplying by 100.

Open field (OF) and pharmacological challenge

Animals were placed in an opaque, square arena and allowed to explore for 20 min. Movement was tracked with PanLab SMART v3.0 software. During this time, baseline locomotion and percent time spent in center (defined as a 5 cm distance from the walls of the arena) was calculated. After OF habituation, animals received IP injections of 0.3 mg/kg dizocilpine (MK801) and placed back into the arena for 1 hr while video recordings were collected. Behavior was analyzed off-line by an experimenter blind to treatment. Ataxia severity was rated with previously published criteria (Wu et al., 2005). Briefly, scores ranged from 0-5 with lower scores representing less severe ataxia and 5 representing extreme ataxia. Scores were given during the first 30 sec of each 5 min block, then summed for a total behavioral score.

Statistical analysis

Statistical analyses were performed in GraphPad Prism (Version 10.0.1). Unpaired t-tests were run in instances where two groups were compared. Otherwise, the data were analyzed with a one-way ANOVA with Tukey post hoc corrections if significance was reached ($p<0.05$). In cases where two variables were compared (i.e., when data were separated by sex), a two-way ANOVA was run to determine if there were significant main effects or an interaction between variables. If the interaction was significant, the data were analyzed with Sidak post hoc corrections.

Computational modeling

We utilized computational modeling to further dissect data collected during the FCL task using DI (see above).

Models

We utilized two reinforcement learning models that explain learning as a function of immediate prediction error (Rescorla-Wagner, RW) (Rescorla, 1972) or prior stimulus associability (Pearce-Hall, PH) (Pearce & Hall, 1980). For each value we predicted the DI as our value for the level of conditioned strength (V) on each trial.

RW – Learning in the RW model depends on prediction error (δ). V develops for each trial according to the difference between actual (λ_n) and expected outcome (V_n) for a stimulus on a given trial (n). This prediction error was multiplied by a free parameter deemed learning rate (α, constrained between 0-1) which controls the rate of learning. This can be formulated as:

$$\delta = (\lambda_n - V_n)$$

$$V_n = V_{n-1} + \alpha\delta$$

PH - The PH model accounts for learning as a function of the associability (α) of a stimulus. V develops for each trial through prediction error (δ) given by the absolute value of the difference between actual (λ_{n-1}) and expected outcomes for a stimulus on the prior trial (V_{n-1}). We used an extended formulation of the PH model with gradual learning (Roesch et al., 2012) where α was dictated by prediction error and a weighted exponential average of prior value of associability (α_{n-1}) controlled by a free weighting parameter (γ, constrained between 0-1).Thus this model is formulated as:

$$\delta = |\lambda_{n-1} - V_{n-1}|$$

$$\alpha_n = \alpha_{n-1} + \gamma(\delta * \alpha_{n-1})$$

$$V_n = V_{n-1} + \alpha * \lambda_n$$

Model fitting

To address the high trial-by-trial response variability, we smoothed responses using a 5-trial moving average and fit each of the RW and PH models to the DI values for the pre- (Sessions 4-5) and post-reversal (Sessions 6-10) epochs for each subject. Expected value was determined for each subject by pre-reversal means (i.e., Sessions 4-5) and then shifted at reversal (Sessions 6-10) to the mean DI observed in the last session. This effectively normalized the minimum and maximum 'strength' of the cue across subjects, leaving the rate of learning controlled by α and γ for RW and PH as free parameters. Best-fitting model parameters were estimated through grid search across a range of 1000 equally spaced values and selected via least-squares approach.

Model comparison

We compared models based on the total sum of squares (SS) for each group and selected the model parameters with the lowest total SS for each group. We also quantified the difference in SS between RW and PH fits for each subject and compared groups using a two-tailed t-test.

Data simulations

We validated parameter estimates for α or γ by simulating group differences in the learning process using group averaged values for parameters from the superior model.

Results

Grin2a cKO results in pharmacological sensitivity to NMDA receptor antagonism in adolescents

NMDA receptor antagonism can induce positive symptoms in unaffected individuals and exacerbate those symptoms in people with schizophrenia (Javitt, 1987; Javitt & Zukin, 1991). To determine if this phenotype was present in the *Grin2a* cKO animals, we administered the NMDA receptor antagonist MK801 (0.3mg/kg) and determined the severity of locomotor impairment by scoring drug-induced ataxia levels (Figure 4.1 A). Our initial analysis showed both treatment groups had locomotor impairment following the injection (Figure 4.1 B). Because our data appeared to have a bimodal distribution and it has been established that females have enhanced sensitivity to MK801 (McDougall et al., 2020), we then separated our data by sex. This analysis reproduced previous findings: females in both treatment groups were significantly more sensitive to MK801 than males. This analysis also revealed a significant overall effect of treatment, suggesting a hypersensitivity to the locomotor effects of MK801 in both male and female *Grin2a* cKO animals (Figure 4.1 C; for time binned ataxia scores see Supplementary Figure A.2). This finding prompted a deeper investigation into other positive-symptom related behaviors in our model.

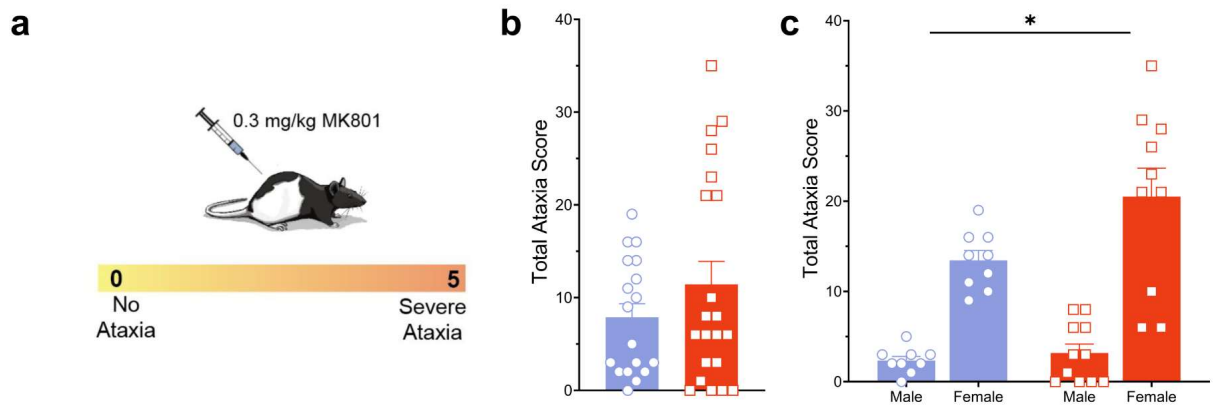

Figure 4.1 *Grin2a* cKO adolescents show hypersensitivity to locomotor effects of MK801

A Schematic of experimental design; MK801 (0.3 mg/kg) was injected intraperitoneally, and ataxia levels scored at 5 min intervals on a scale from 0-5. **B** MK801 results in ataxia in both treatment groups. **C** When separated by sex, *Grin2a* cKO animals showed elevated total ataxia scores (*p=0.0362) compared to controls; *control n* = 18, *Grin2a* cKO *n* = 21 rats. The data are represented as mean ± SEM.

Psychosis-relevant behavioral phenotyping and behavioral modeling of *Grin2a* cKO adolescents

Positive symptoms of schizophrenia include delusions, hallucinations, and perceptual disorganization. While quantifying the entire spectrum of these symptoms is not feasible in laboratory animals, behavioral constructs associated with selective symptoms can be quantified. This includes—but is not limited to—aberrant salience attribution (Fletcher & Frith, 2009; Howes & Nour, 2016). Given the time constraints of the rodent adolescent period, we chose two tasks that could be completed within two weeks and provide measures of association learning and salience attribution. These included the progressive ratio of reinforcement task and the FCL task.

In the progressive ratio task, response requirements to receive a constant reward increase over consecutive sessions. This task allows for measures of (1) association learning (2) motivational state through breakpoint and (3) measures of effort optimization and salience attribution through running response rate and length of post reinforcement pause. In our design,

animals first learned to execute a nose poke in response to a light cue to receive a sucrose pellet reward (fixed ratio 1 or FR1) for 5 sessions. They then progressed to 2 sessions of executing 5 actions to receive a sugar pellet (FR5) before being tested with a progressive ratio for 4 consecutive test sessions. During the progressive ratio phase, rats began at an FR1 but with each subsequent trial, the response requirement increased exponentially (Figure 4.2 A). Control and *Grin2a* cKO animals performed similarly in the FR1 and FR5 sessions. Their cue-action latencies, action-reward latencies, and completed trials were not significantly different during the 7 days of training (Figure 4.2 B-D). The *Grin2a* cKO adolescents also did not differ in their breakpoint or running response rates during the component of the task (Figure 4.2 E, F). These findings suggested that reduction in GluN2A function in adolescent dopamine neurons did not produce deficits in general learning or motivation for reward guided actions. Robust differences were, however, observed in post reinforcement pause, which was the delay between cue onset and trial initiation after a successful trial. This latency to respond to the cue typically increases as a function of an increasing response requirement (Bradshaw & Killeen, 2012). In control animals, we observed the expected rise of post reinforcement pause as the task became more effortful, whereas *Grin2a* cKO animals did not flexibly change this response time (Figure 4.2 G). Thus, while general motivational responding was normal in the *Grin2a* cKO animals, their abnormal post reinforcement behavior suggests impaired salience attribution and an inability to adjust stimulus-related actions as the task became more effortful.

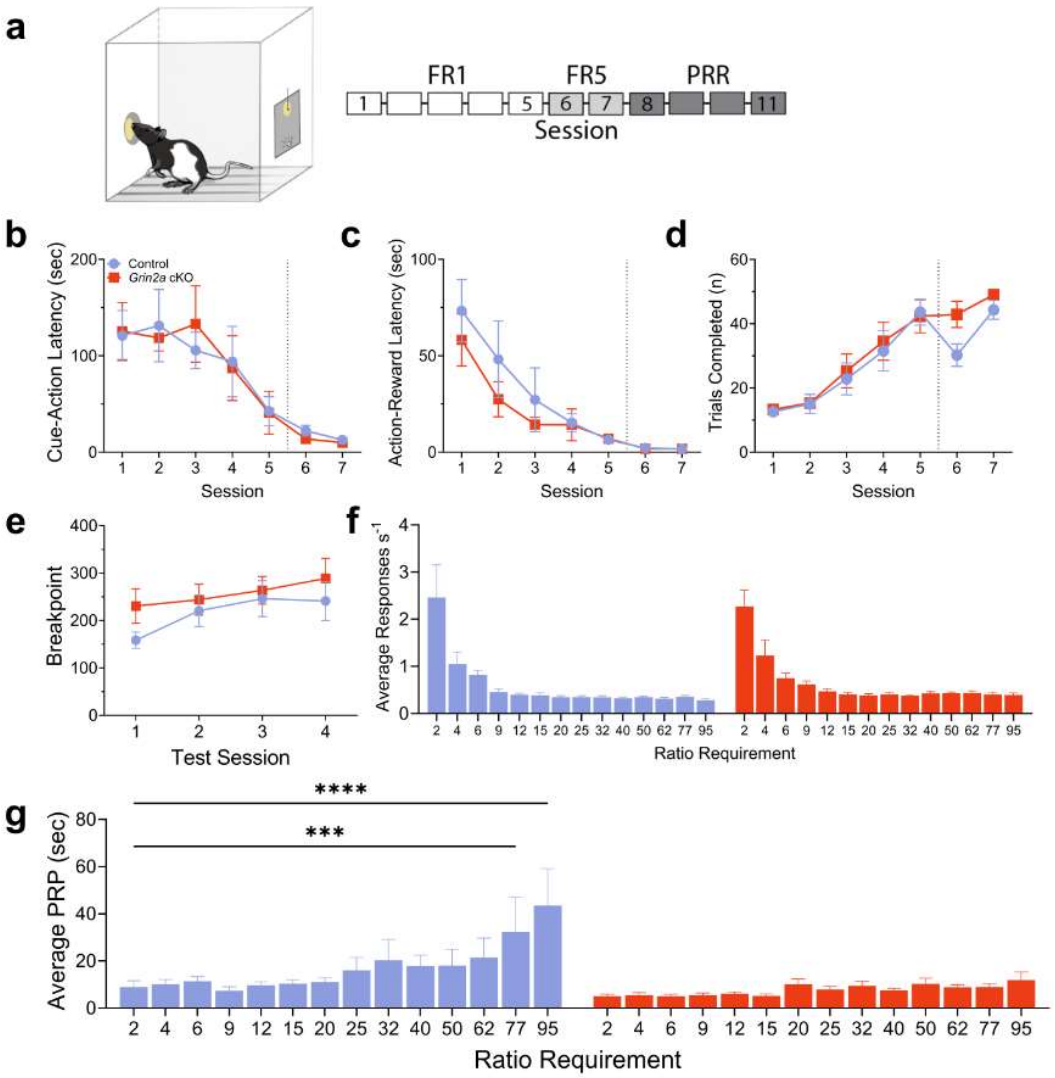

Figure 4.2 *Grin2a* cKO adolescents show no motivation deficits but aberrant salience attribution in progressive ratio task

A Representative schematic of the operant box and experimental design; animals were initially trained on an FR1 for five sessions, then two sessions of FR5, and finally tested on a progressive ratio for the final four sessions. **B-D** Data collected during the FR1 and FR5 sessions show no group differences in initial operant performance. Cue-action latency (B), action-reward latency (C), and total trials completed (D) were similar between groups. **E** There were no differences in breakpoint between control and *Grin2a* cKO animals across the four testing days. **F** Response rate was determined by dividing total nose pokes by the time each trial took after the initial nose poke. Response rates averaged across testing sessions were similar between control and *Grin2a* cKO animals. **G** Post reinforcement pause is the delay between cue onset and trial initiation. There was a significant interaction between treatment and ratio requirement (**p=0.0065), post hoc analysis revealed control animals increased average post-reinforcement pause when the response requirement increased FR2 vs FR77 (***p=0.0005) and vs FR95 (****p<0.0001). This was not seen in the *Grin2a* cKO animals; *control n* = 10, *Grin2a* cKO *n* = 10 rats. The data are represented as mean ± SEM.

In the FCL task, animals were exposed to two novel conditioned stimuli (CS), which appeared in random order during the behavior session, each of which predicted either a reward or shock unconditioned stimulus (US). After 5 sessions, during which CS-US associations were learned (Kim et al., 2010), the contingencies were switched so that the CS that was associated with reward (referred to as CS_A) now predicted shock and the CS that predicted shock (referred to as CS_B) was followed by reward delivery (Figure 4.3 A). A behavioral readout for the FCL task was discrimination index (DI), which was the number of reward trough nose pokes an animal made during each CS normalized to entries made during the intertrial interval (ITI). We found *Grin2a* cKO and control animals learned the initial association between CS_A and reward and its later association with shock similarly (Figure 4.3 B). However, when the shock-predicting CS switched to reward-predicting *Grin2a* cKO animals more rapidly formed an updated positive association with the CS, approaching the food trough during CS_B presentation in reversal sessions more than controls (Figure 4.3 C).

To gain a deeper understanding of the behavioral difference observed between control and *Grin2a* cKO rats in this task, we applied computational models rooted in Pavlovian learning theory to their data. We employed two distinct models: the Rescorla-Wagner model and the Pearce-Hall model (Figure 4.3 D). These models both work to explain how organisms learn associations between stimuli and outcome; however, they differ in their ability to account for the influence of earlier stimuli presentations on information updating and related phenomena such as latent inhibition (Lubow, 1989; Lubow & Moore, 1959). Because Pearce-Hall incorporates more factors like attention and surprise, it accounts for interactions between prior exposure and new learning. Both models were first fit to data collected during the initial 5 positive association

learning sessions and fit both control and *Grin2a* cKO animal data with similar accuracy (Figure 4.3 E). Data simulated with these models using group-averaged parameter estimates successfully recapitulated the observed data (Figure 4.3 F). These similarities were expected because with no earlier exposure to the reward-predicting cue, both models rely on similar principles of association learning.

When the models were fit to data from reward association learning *after* the contingency switch, we observed that the Pearce-Hall model better explained the control group's behavior, while the *Grin2a* cKO animal's behavior was better explained by Rescorla-Wagner mechanisms (Figure 4.3 G). Importantly, data simulated with group-averaged parameter estimates were able to reproduce the observed data (Figure 4.3 H). These results indicated that control animals were slower to update their response to the reward-predicting CS when that CS had previously been associated with punishment. Alternatively, *Grin2a* cKO animals expressed an inability to incorporate previous learning with new and this led to disrupted behavioral switching when outcome contingencies changed.

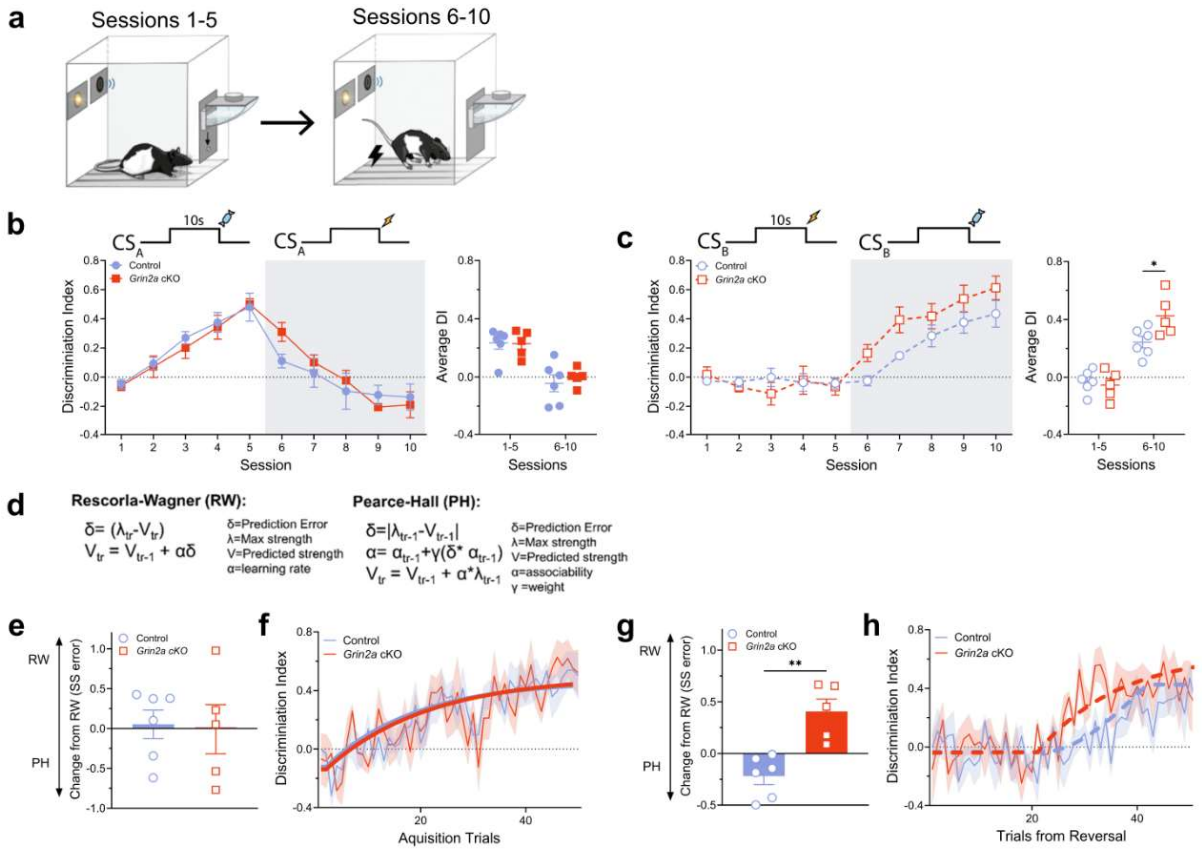

Figure 4.3 *Grin2a* cKO adolescents exhibit abnormally rapid conditioning to reward-prediction cue following contingency switch

A Representative schematic of the operant box. **B** Discrimination index (DI) over sessions and averaged across sessions were used a behavioral readout for the FCL task. Both treatment groups responded to CS_A similarly, increasing food trough entries relative to baseline during CS presentation as a positive association formed, then decreasing once the value of CS_A was switched on Session 6. **C** DI over session and averages across session in response to CS_B. There were no significant differences between treatment groups in response to CS_B over all sessions, but when DIs were pooled across initial learning (Session 1-5) and reversal (Session 6-10) *Grin2a* cKO animals had significantly higher DIs than controls after the contingency switch (*p=0.0204). **D** Summary Pavlovian learning models applied to FCL data. **E** Models were first fit to data from the initial learning (Sessions 1-5) of the positive association (CS_A). Average and individual values (symbols) for change in model fit between RW and PH. Values <0 indicate a better fit from PH mechanisms. These models fit the inital learning data with similar accuracy. **F** Actual (thin lines) and predicted (bold lines) DIs for the best fit model for each group recovered no differences in behavioral trajectories over initial learning between groups. **G** Models were then fit to data from contingency reversal learning (Session 6-10) of the positive association (CS_B). Average and individual values (symbols) for change in model fit between RW and PH. RW model better fit data from *Grin2a* cKO animals while PH better fit control data (**p=0.0016). **H** Actual (solid lines) and predicted (dashed lines) DIs for the best fit model for each group recovered differences in behavioral trajectories over learning between groups; *control* n = 6, *Grin2a* cKO n = 5 rats. The data are represented as mean ± SEM.

Adolescent *Grin2a* cKO behavioral phenotype does not generalize to anxiety-related symptoms or cognitive deficits

Finally, given the highly specific behavioral deficits observed above, we sought to determine if adolescents with the *Grin2a* cKO expressed psychosis-unrelated behaviors consistent with negative or cognitive schizophrenia symptoms. We conducted a battery of behavioral tasks aimed to assess spontaneous locomotor activity, innate anxiety, and spatial working memory. These included open field, elevated plus maze, and plus-maze to measure spontaneous alternation. In the open field, avoidance of the center portion of the maze is related to anxiety in rodents (Broadhurst, 1969). With this measure, we determined that control and *Grin2a* cKO rats showed no significant difference in anxiety-like behavior as they spent similar percent time in the center of the OF (Figure 4.4 A). Moreover, this was not a result of any impairment in locomotor ability, as both treatment groups traveled similar distances (Figure 4.4 B). As in the open field, avoidance of the open arms of the elevated plus maze and preference for the walled-in, closed arms can be used to ascertain anxiety-like behavior in rodents (Montgomery, 1955). Here we further established no change in anxiety-like behavior in the *Grin2a* cKO adolescents as they spent similar percent time in the open and closed arms of the elevated plus maze compared to controls (Figure 4.4 C, D). To compare spatial working memory between treatment groups, we measured spontaneous alternation in a plus maze. Because rats tend to explore novel arms as opposed to previously visited ones, impairments in working memory would emerge as reduced alternation in this task (Kraeuter et al., 2019). There were no significant between-group differences in spontaneous alternation in the plus maze, suggesting no effect of the *Grin2a* cKO on working memory in adolescents (Figure 4.4 E). This was not due to differences in overall

exploration of the maze as total arm entries were also similar between control and *Grin2a* cKO animals (Figure 4.4 F).

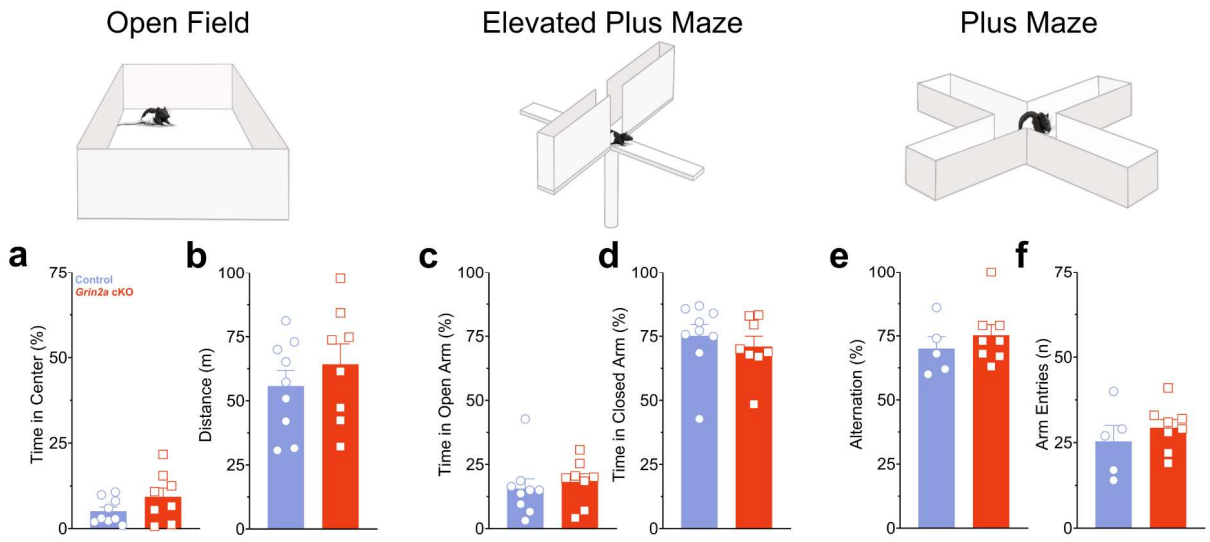

Figure 4.4 *Grin2a* cKO behavioral phenotype does not generalize to cognitive deficits or anxiety-like symptoms

A-B *Grin2a* cKO animals showed no significant differences from control animals in time spent in the center of the Open Field or in general locomotor activity; *control n* = 9, *Grin2a* cKO *n* = 8 rats. **C-D** Control and *Grin2a* cKO animals showed similar percent time spent in either the open or closed arms of the Elevated Plus Maze; *control n* = 9, *Grin2a* cKO *n* = 8 rats. **E-F** *Grin2a* cKO animals had no significant differences from controls in percent alternation or total arm entries in the Plus Maze; *control n* = 5, *Grin2a* cKO *n* = 8 rats. The data are represented as mean ± SEM.

Discussion

Clinically, schizophrenia is defined as a heterogeneous psychiatric disorder characterized by the presence positive, negative, and cognitive symptoms. To determine the extent of the effect of our manipulation, we included a variety of schizophrenia-related behavioral tasks in our battery. We found a specific behavioral phenotype in which adolescent animals with the *Grin2a* cKO showed behavioral abnormalities in line with early emerging positive schizophrenia symptoms.

Consistent with clinical studies showing the exacerbation of positive symptoms in people with schizophrenia by NMDA receptor antagonists (Javitt, 1987; Javitt & Zukin, 1991), we determined *Grin2a* cKO animals had increased sensitivity to the locomotor effects of MK801. This hyperreactivity to psychostimulants indicated the presence of positive symptom-like behaviors, so we chose to investigate this further in our association learning paradigms.

The key characteristics of positive schizophrenia symptoms are delusions, hallucinations, and disorganized thoughts and perceptions. The aberrant salience hypothesis of schizophrenia explains some of these symptoms as a result of misattributed significance (Howes & Nour, 2016). If an individual is experiencing abnormalities in perception, they attend to stimuli that would typically be ignored. Over-assigning importance to irrelevant information could manifest as the abnormal beliefs and perceptions which are characteristic of emerging positive symptoms in schizophrenia (Fletcher & Frith, 2009).

While psychosis can be difficult to model behaviorally, components of this complex collection of symptoms can be broken down and studied within a rodent. Elegant studies in mice have developed methods of quantifying hallucination-like precepts through an auditory detection task where animals with induced striatal hyperdopaminergia reported hearing signals that were not actually presented (Schmack et al., 2021). Inspired by these findings but restricted by prioritizing studying behavior within the limited time scale of rodent adolescence, we chose to look for perceptual abnormalities within simpler behavioral paradigms.

In both operant and classical conditioning tasks, we observed evidence of disrupted salience attribution wherein *Grin2a* cKO animals attributed importance to stimuli that were

being ignored by controls. During the progressive ratio, the onset of the nose poke light is expected to have reduced salience in late, high demand trials and, therefore, produce longer pauses in operant responding (Felton & Lyon, 1966; Powell, 1968). Control rats displayed this suppression of stimulus-driven responding, while *Grin2a* cKO animals did not. In the FCL task, we saw that the original negative association formed with a stimulus inhibited responding in control animals when its contingency had been reversed. This was not surprising, as attention to stimuli with previously learned associations typically interfere with the acquisition of new associations—a phenomenon described as latent inhibition (Lubow, 1989; Lubow & Moore, 1959). This effect was not observed in the *Grin2a* cKO rats who more readily developed conditioned approach in early reversal trials. Their abnormally rapid conditioning may be related to a reduced capacity to update learned associations, something commonly related to positive symptoms of schizophrenia (Lubow, 2005; Lubow & Gewirtz, 1995). This was further supported by the modeling data as the Pavlovian learning model that fails to adjust attention in response to prior stimulus exposure (RW) better fit the *Grin2a* cKO animal data while the PH model—which does account for this—better fit control data.

Our observations that *Grin2a* cKO adolescents were more likely to attend to irrelevant stimuli are in line with clinical findings. Latent inhibition is observed in healthy individuals but is absent in individuals at high risk for developing the disorder or suffering from acute schizophrenia (Baruch et al., 1988; Kraus et al., 2016). In addition to latent inhibition deficits, studies have also provided behavioral and physiological evidence suggesting people with this disorder display intense attentional fixation, regardless of if it aids task performance (Hahn et al., 2022; Luck et al., 2019). In schizophrenia, irrelevant information captures attention and is

inappropriately assigned significance, and this was a phenotype observed in our treatment group. These studies implicate reduced *Grin2a* expression in adolescent dopamine neurons with positive symptoms of schizophrenia.

Alternatively, our manipulation showed no effect on anxiety-like behaviors or working memory. These findings complement previous research implicating NMDA receptors on parvalbumin (PV)-positive interneurons in these behaviors (Belforte et al., 2010; Carlen et al., 2012; Korotkova et al., 2010) and provide evidence that the behavioral abnormalities induced by a loss of function of *Grin2a* on dopamine neurons were specific to analogs of positive symptoms.

Here, we showed that deficits in *Grin2a* expression in dopamine neurons caused behavioral dysfunction resulting in over-attendance to stimuli that control animals ignored. These studies implicate reduced *Grin2a* expression in dopamine neurons with deficits in salience attribution and, more broadly, positive symptoms of schizophrenia.

Chapter 5

Effects of *Grin2a* cKO on synaptic dopamine levels

Introduction

Our understanding of the role of dopaminergic signaling is one that is constantly evolving. Classic experiments in nonhuman primates initially observed increases in dopamine neuron activity in response to stimuli that triggered immediate movements (Schultz, 1986). Thus, one of the first roles attributed to dopamine cells is encoding 'behavioral activation'. Building on this theory, subsequent studies aimed to uncover how dopamine signaling motivated actions that result in reward. The role of phasic dopamine activity in encoding reward prediction errors (RPEs) is well-studied. Initial experiments in nonhuman primates showed dopamine neurons had phasic 'bursts' of activity during rewarding experiences, however it was not the consumption of the reward itself that resulted in activity, but instead, the signal was a prediction error calculation (Schultz et al., 1997). When there is a difference between what is expected and what occurs, dopamine neuron activity is modulated. When a reward is unexpected or larger than expected, positive prediction error occurs, resulting in strong dopamine cell excitation. Negative prediction error occurs when an expected reward is omitted or smaller than anticipated, and results in the suppression of dopamine cell firing. No prediction error occurs when a reward is cued—thus expected—so the cells have little to no response to the reward. Similarly, when a sensory cue provides information about a future reward, dopamine neurons react. They can increase activity in response to a reward-predicting cue, inhibit activity in response to a cue predicting reductions

in future rewards, and generally have little response to cues conveying no information about a future reward (Montague et al., 1996; Schultz, 1998). These early studies focused on the role of dopamine in encoding motivational *value* but since then, there has been increasing evidence that in addition to this role, dopamine neurons can encode motivational *salience*.

Salient events are important and worth attentional orienting. These events can have positive or negative value: a cue informing you to approach an upcoming reward is as important as a cue informing you to avoid future punishment. Therefore, if dopamine neurons fire in response to salience, they would increase firing regardless of value. Studies consistent with this theory show aversive events and the cues that predict those events can result in high levels of dopamine cell activation (Cohen et al., 2012; Matsumoto & Hikosaka, 2009; Schultz, 2013; Zweifel et al., 2011). These findings suggest dopamine neurons are not a homogenous population—some encode value, others encode saliency.

Further evidence for diversity in what dopamine cells encode come from studies showing that some of these neurons respond to neither rewarding nor aversive events but to novel sensory cues with no predictive value. They are considered to encode novelty as these responses reduce as a stimulus becomes familiar (Horvitz, 2000; Horvitz et al., 1997; Ljungberg et al., 1992). So, while historically dopamine neurons were determined to uniformly signal motivation during rewarding events, new evidence suggests a more complex role for dopaminergic signaling. In addition to rewarding events, they can also transmit information about salient, nonrewarding events with neutral or negative valence (Bromberg-Martin et al., 2010; Lammel et al., 2014).

In the following studies, we investigated how our dopamine-neuron specific loss of *Grin2a* influenced phasic dopamine release in response to novel, rewarding, and aversive stimuli. We found a specific disruption of dopamine release during value encoding. Cues predictive of either positive or negative outcomes resulted in abnormal dopamine release in *Grin2a* cKO animals. Interestingly, dopaminergic response to salient and novel events remained intact in these animals.

Methods

Subjects

All animal procedures were approved by the IACUC at OHSU. Rodents utilized in this study were transgenic Long Evan's rats expressing Cre recombinase under the control of the TH promoter (Witten et al., 2011). All animals were maintained on a reverse 12hr light/dark cycle with ad libitum access to food and water until otherwise noted.

Stereotaxic surgery

For fiber photometry experiments, juvenile (P21±1) animals were infused with either the control or *Grin2a* cKO virus mixed with AAV9-hsyn-GRAB_DA2m ($GRAB_{DA}$) (addgene # 140553) at a 1:1 ratio. These were infused bilaterally into the VTA as described previously. Once animals reached adulthood (P60+), they were implanted with an optical fiber (Doric Lenses Inc) into the nucleus accumbens core (AP= +1.6 mm, ML= 1.5 mm, DV= -7.0 mm). Animals were given at least one week to recover from the implant surgery before behavior was run.

Behavior

Two days preceding behavior trials, implanted animals were mildly food restricted and habituated to handling, being attached to the recording patch cord, and to the testing environment. Animals were then run on the FCL task as previously described (Chapter 5). Behavior was assessed by calculating the likelihood of food trough entry during the presentation of either CS_A or CS_B.

Recording

Fiber photometry experiments were run using an RZ10X fiber photometry system (Tucker-Davis Technologies). The Quick-Release Interconnect (Thorlabs) was used to mate the implanted fiber optic cannula and the patch chord. Excitation light was passed through a 400 µm patch cord from 465 and 405 nm LEDs (Doric Lenses Inc), sinusoidally modulated at 220 and 310-Hz, respectively. Digital signals were demodulated at 1kHz with a 6Hz low pass filter in real-time using Synapse software (Tucker-Davis Technologies). Time locked behavioral events from the operant box were recorded through the RZ10X digital input ports. Recording was performed on each day of the behavioral task.

Data analysis

Fiber photometry data were analyzed in Python 3 using the TDT Python package. Raw 465 nm ($GRAB_{DA}$) and isosbestic 405 nm data were split into trials (each trial began 10 s prior to CS onset and ended 10 s following US onset, a 30 s period). This was done to fit the $GRAB_{DA}$ signal to the isosbestic signal on a trial-by-trial basis. The fitted signals were then used to calculate change in fluorescence ($\Delta F/F$) for each trial to correct for motion artifacts and photobleaching.

To assess changes in synaptic dopamine around specific events (CS onset and US onset), peri-event z-scores were computed by comparing the ΔF/F to the 10 s baseline ΔF/F prior to CS onset, in the ITI. To quantify differences in synaptic dopamine between the treatment groups, we determined the average z-score across three, 2 s periods (P1, P2, P3). The CS onset time bin consisted of the first 2 s following the cue turning on (P1), the mid CS 2 s time bin was taken 5 s following the onset of the cue (P2), and the US onset time bin consisted of the first 2 s following the onset of the reward/shock (P3).

Histology

Animals were euthanized, transcardially perfused, and brain tissue preserved as described previously (Chapter 3). Brain sections containing the fiber tract were mounted onto Super Frost+ slides. Coverslips were applied using Vectashield HardSet antifade mounting media containing DAPI and imaged using a Ziess Axiovert 200 microscope. To confirm fiber placement in the nucleus accumbens core, images were superimposed with a brain atlas (Paxinos, 1998) (See hit map, Supplementary Figure A.3).

Excluded data

Behavioral and fiber photometry data were excluded from analysis if there was no signal because of a misplaced fiber tip.

Statistical analysis

Statistical analyses were performed in GraphPad Prism (Version 10.0.1). Average z-scores were analyzed with a two-way ANOVA with Sidak post hoc corrections if there was a significant interaction ($p<0.05$).

Results

Grin2a cKO adults show disrupted signaling in response to predictive stimuli during association learning

Aberrant dopamine neurotransmission has been theoretically linked to disrupted prediction-error signaling in schizophrenia (Katthagen et al., 2020; Millard et al., 2022). To explore causal mechanisms linking *Grin2a* cKO with aberrant dopamine release during prediction error, we measured synaptic dopamine levels using fiber photometry during the FCL task. This event-rich task allowed us to assess phasic dopamine responses to novel stimuli, their transition to predictive stimuli, and expected and unexpected reward and shock outcomes during association learning and under shifting contingencies. Dopamine transients were measured using the fluorescent dopamine reporter, $GRAB_{DA}$. This virus was co-infused with control or *Grin2a* cKO virus into the VTA and a fiber was implanted into the nucleus accumbens core (NAc core) (Figure 5.1 A, B) (see hit map Supplementary Figure A.3). This allowed for the assessment of synaptic dopamine levels in the midbrain terminal field within the NAc core during the FCL task (Figure 5.1 C).

To compare novelty responsivity between treatment groups, were first compared dopamine responses to CS_A and CS_B, in early trials of Session 1 before association learning occurred. We binned data from the first 25 presentations of either stimulus into trials of 5 to look at novelty response across trials. We determined the dopaminergic response to both CS_A and CS_B fell during trial progression similarly between treatment groups, suggesting novelty signaling was not disrupted by our manipulation (Supplementary Figure A.4). Because there was

a novelty response in the first 25 presentation of either stimulus, we only included the final 25 trials of each CS in subsequent analyses of Session 1 data.

To determine how dopamine response changed with positive association learning, we compared the average fluorescence response during the final 25 CS_A presentations of Session 1 to all 50 CS_A presentations of Session 5 (Figure 5.1 D). Quantitative analyses of this dopamine signal were focused to 3 periods (P1, P2, P3; Figure 5.1 E, top): 2 s after CS presentation, 2 s in the middle of the CS presentation, and 2 s following US presentation. Rationale for inclusion of P2 was to determine if there was sustained dopamine release throughout the presentation of the CS, beyond its onset.

Behaviorally, initial association learning progressed similarly in both groups (Supplementary Figure A.5) but there were pronounced differences in dopaminergic signaling during this stage of the task. By Session 5, control animals exhibited the expected increase in phasic dopamine response to the reward-predicting stimulus. This increase was absent in *Grin2a* cKO animals, which exhibited comparable dopamine responses to CS_A onset in Session 1 and 5 (Figure 5.1 E, left). During P2, control animals showed higher average z-scores than *Grin2a* cKO animals in Session 1 which remained present through Session 5, suggesting *Grin2a* cKO animals had deficits in sustaining dopamine release throughout the duration of the reward-predicting cue (Figure 5.1 E, middle) These deficits were specific to the CS, as the phasic response to the US (reward) was similar in both groups (Figure 5.1 E, right). Control animals began to exhibit a suppression of dopamine in response to CS_B during the final trials of Session 1, and this response remained through Session 5. This negative dopaminergic response to the shock-predicting cue was not observed in the *Grin2a* cKO animals (Figure 5.1 F, G left). There were no group

differences in response during P2 or to the shock itself (Figure 5.1 G middle, right). Like CS_A trials, response to the US was intact in *Grin2a* cKO animals, but their response to the punishment-predicting cue was elevated compared to controls. Because control animals had a negative response to CS_B during this task, this further suggested dysfunctional prediction error signaling in the *Grin2a* cKO animals.

We next wanted to determine if there were disruptions in reinforcement learning after the contingency reversal. We first compared changes in behavior and fluorescence recording from the final 25 CS_A trials of Session 6 to all 50 CS_A trials of Session 10. In these trials, control and *Grin2a* cKO animals appeared to have similar behavior and dopaminergic response to the CS and US (Figure 5.1 H, I; Supplementary Figure A.5). Alternatively, robust differences in response to the newly rewarding stimulus (CS_B) emerged after the contingency reversal (Figure 5.1 J, Supplementary Figure A.5). Early after the contingency switch, both treatment groups did not show a response to the onset of the reward-predicting cue. With learning, a response to CS_B onset developed in both groups, but this response was elevated in control animals (Figure 5.1 K, left). As well, between Session 6 and Session 10, control animals showed increased dopaminergic signaling during P2 compared to *Grin2a* cKO animals (Figure 5.1 K, middle). Finally, both treatment groups showed a dopaminergic response to the reward in late trials of Session 6, but control animals had significantly higher responses than *Grin2a* cKO animals. This increased response to the reward fell with learning in control animals but remained significantly higher than *Grin2a* cKO animals by Session 10 (Figure 5.1 K, right).

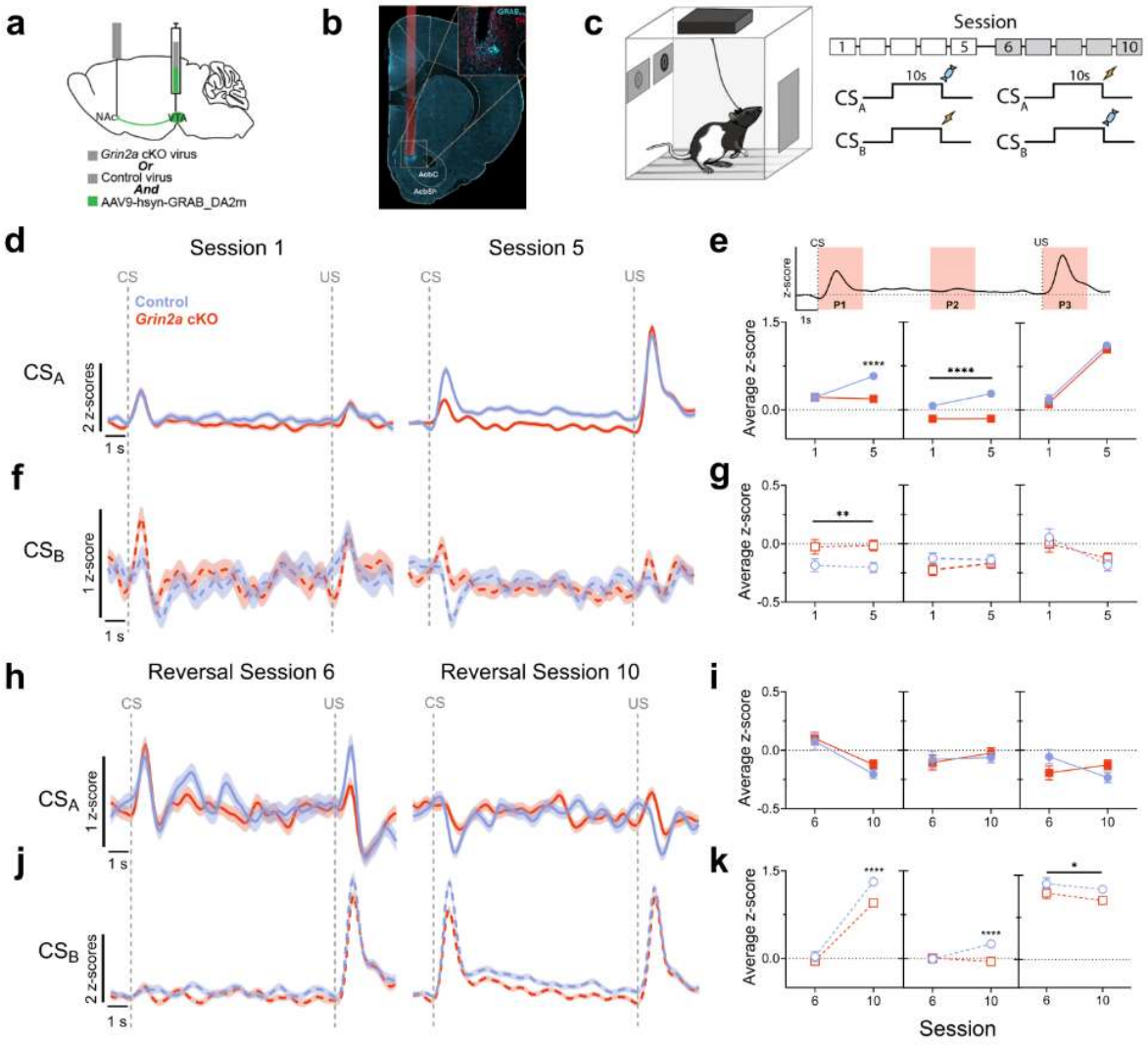

Figure 5.1 *Grin2a* cKO adults showed altered dopaminergic responses to predictive stimuli during association learning

A Schematic representation showing infusion of a mixture of *Grin2a* cKO/control virus and $GRAB_{DA}$ at a 1:1 ratio into the VTA and fiber implantation into the NAc. B Representative IHC image showing fiber placement in the NAc core. C Schematic of photometry setup and FCL task. D Average fluorescence response during the final 25 CS_A presentations of Session 1 and all 50 CS_A presentations of Session 5. E Representative schematic showing time bins used to take average z-score over 2 s periods: CS onset (P1), mid CS (P2), and US onset (P3) (top). Quantification of average z-scores during P1, P2, and P3 during initial learning of CS_A (bottom). Control animals showed an increase in average P1 z-score with learning that was not present in Grin2a cKO animals (****p<0.0001) (left). During both sessions, control animals had higher mid CS responses than Grin2a cKO animals (****p<0.0001) (middle). There was no difference between groups during P3 (right) F Average fluorescent response during the final 25 CS_B presentations of Session 1 and all 50 CS_B presentations of Session 5.

(legend continued on next page)

Figure 5.1 (continued)

G Quantification of average z-score during P1, P2, and P3. Control animals showed a negative average z-score during P1 across sessions, while *Grin2a* cKO animals did not (**p=0.0015) (left). There were no group differences in z-scores during P2 and P3 (middle, right); Session 1: control n = 125 CS_A trials, 125 CS_B trials (N = 5 rats), *Grin2a* cKO n = 150 CS_A trials, 150 CS_B trials (N = 6 rats). Session 5: control n = 250 CS_A trials, 250 CS_B trials (N = 5 rats), *Grin2a* cKO n = 300 CS_A trials, 300 CS_B trials (N = 6 rats). H Average fluorescence response during the final 25 CS_A presentations of Session 6 and all 50 CS_A presentations of Session 10. I Quantification of average z-score during P1, P2, and P3. There were no differences in z-scores during P1 (left) or P2 (middle). There was a significant interaction between session and treatment in response to US onset (p=0.0199), but post hoc analysis resulted in no significant p values. J Average fluorescence response during the final 25 CS_B presentations of Session 6 and all 50 CS_B presentations of Session 10. K Quantification of average z-score during P1, P2, and 3. With learning, control animals showed increases in average z-score during P1 (left) and P2 (middle) that were higher than *Grin2a* cKO animals (****p<0.0001). There also was a main effect of treatment such that control animals' response during P2 was higher than *Grin2a* cKO animals during both sessions (*p=0.0164)); Session 6: control n = 125 CS_A trials, 125 CS_B trials (N = 5 rats), *Grin2a* cKO n = 150 CS_A trials, 150 CS_B trials (N = 6 rats). Session 10: control n = 250 CS_A trials, 250 CS_B trials (N = 5 rats), *Grin2a* cKO n = 300 CS_A trials, 300 CS_B trials (N = 6 rats). The data are represented as mean ± SEM.

Grin2a cKO adults showed deficits in dopaminergic signaling during unexpected events

We next analyzed Session 6—the first day the contingencies were reversed— in more detail by dividing the session into 'Early' (first 25 trials of each CS) and 'Late' (final 25 trials of each CS) sections. In Early CS_A trials, control animals showed heightened responses during all three periods, but these decreased by the Late trials of Session 6. This elevation in dopamine in Early trials of Session 6 was significantly muted in the *Grin2a* cKO rats (Figure 5.2 A, B). Similarly, we compared fluorescent response to CS_B, which was initially associated with shock but switched to be rewarding across Early and Late trials of Session 6 (Figure 5.2 C). While neither treatment group showed a phasic response to CS_B onset in Session 6 (Figure 5.2 D, left), control animals had a small increase during P2 compared to *Grin2a* cKO animals (Figure 5.2 D, middle). As well, the response of control animals to the unexpected reward was high in the Early CS_B trials but fell by the Late trials. This was not observed in the *Grin2a* cKO animals (Figure 5.2 D, right).

Collectively, these data suggest that *Grin2a* cKO produces impairments in dopamine prediction error signaling. Our control animals showed the expected phasic dopaminergic responses during association learning and after the reward-contingency switch, and adapted their responding as they learned new contingencies. The *Grin2a* cKO animals showed disruptions during initial associative learning and after contingency switching consistent with deficits in prediction error encoding.

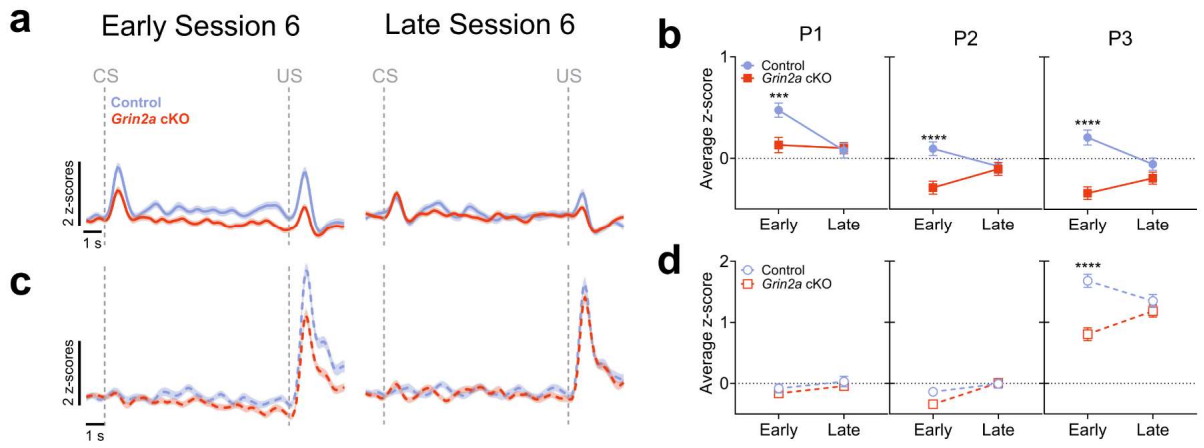

Figure 5.2 *Grin2a* cKO adults show dysfunctional dopaminergic response to unexpected outcome

A Average fluorescence response during the first 25 (Early) and final 25 (Late) CS_A trials. **B** Quantification of average z-score across 2 s time periods at CS onset (P1), mid CS (P2), and US onset (P3). Control animals had significantly higher responses than *Grin2a* cKOs in Early trials during P1 that fell by Late trials (left) (***p=0.0009). This difference was sustained through P2 (middle) (****p<0.0001) and P3 (right) (****p<0.0001). **C** Average fluorescence response during the first 25 (Early) and final 25 (Late) CS_B trials. **D** Quantification of average z-score during P1, P2, and P3. There were no differences during P1 (left) or P2 (middle). Control animals had significantly higher responses to the unexpected shock during Early trials that normalized by Late trials (****p<0.0001) (right); Early Session 6: control n = 125 CS_A trials, 125 CS_B trials (N = 5 rats), *Grin2a* cKO n = 150 CS_A trials, 150 CS_B trials (N = 6 rats). Late Session 6: control n = 125 CS_A trials, 125 CS_B trials (N = 5 rats), *Grin2a* cKO n = 150 CS_A trials, 150 CS_B trials (N = 6 rats). The data are represented as mean ± SEM.

Discussion

Our fiber photometry data propose a role for GluN2A-containing NMDARs on dopamine neurons in prediction error signaling. During association learning, dopaminergic response to reward and punishment was intact in the *Grin2a* cKO adults, however, controls developed a learned response to the predictive stimuli that they did not. An abnormal response was observed regardless of stimulus valance: response to the reward-predicting stimulus was reduced in *Grin2a* cKO animals while response to the punishment-predicting cue was elevated.

As well, we looked for changes in dopamine signaling with learning after the contingency reversal. Immediately following the reversal, we observed deficits in *Grin2a* cKO animals' ability to increase dopaminergic signaling in response to unexpected events. In later sessions, we also observed disruptions in dopaminergic response to reward following the contingency switch. Our data show that during reversal sessions, both control and *Grin2a* cKO animals developed a phasic dopamine response to the reward-predicting cue, but this was blunted in *Grin2a* cKO animals and they showed an overall reduction their response to reward delivery. These data suggest the *Grin2a* cKO adults were not updating reward-related signaling as efficiently as control animals.

Due to the well-established role of dopamine in prediction error, abnormalities in dopamine neurotransmission in schizophrenia are thought to disrupt this process. Evidence for faulty prediction error signaling in schizophrenia comes from fMRI studies showing abnormalities in regional activity during reinforcement learning. Specifically, it has been shown that midbrain and striatal activity levels in response to reward predicting stimuli are significantly lower than controls and, conversely, higher in response to neutral information (Ermakova et al., 2018; Kirschner et al., 2016; Murray et al., 2008; Radua et al., 2015). Additionally, people with

schizophrenia have reduced activity in the midbrain and ventral striatum when presented with an unexpected outcome while maintaining a typical level of activity in response to expected outcomes (Corlett et al., 2007; Morris et al., 2012). Our data provide a potential mechanism through which a genetic variant of *GRIN2A* could contribute to the deficits in prediction error encoding observed in people with schizophrenia.

Chapter 6

General Discussion

Main Findings

With this dissertation, I set out to develop an animal model relevant to schizophrenia with the hopes of elucidating some of the neural mechanisms driving the complex symptoms observed in people with this disorder. Clinical studies have highlighted the role of hypofunctional *GRIN2A*. We, therefore, sought to better understand the relationship between this gene, neurodevelopment, and the pathophysiology of schizophrenia. Because adolescence and the emergence of positive symptoms in schizophrenia coincide, we began by identifying an adolescent neurodevelopmental process that would interact with a deficit in *GRIN2A* expression. We determined the midbrain as a potential region of interest because there was significant loss of GluN2A in the VTA and substantia nigra during this developmental period. This led us to hypothesize that the *GRIN2A* LoF variants observed in the clinical population may interact with this developmental process and contribute to the emergence of positive schizophrenia symptoms in adolescents. To generate an animal model with construct validity, we utilized a Cre-driven CRISPR/Cas9 virus to induce a genetically driven deficit of *Grin2a* in the dopamine neurons of the midbrain region of rats.

When we characterized the behavioral phenotype of adolescent animals with this manipulation, we revealed a specific effect of the virus on behavioral correlates of positive

symptoms of schizophrenia while correlates of cognitive and negative symptoms remained unchanged. We did not see abnormalities in working memory, anxiety, or motivation in the *Grin2a* cKO adolescents. What we did observe was a hypersensitivity to MK801 and specific disruptions in salience attribution. In Pavlovian and operant conditioning paradigms, *Grin2a* cKO animals responded more to cues that had lost salience to control animals. Their inability to distinguish between relevant and irrelevant information is reflective of the abnormal thoughts and perceptions that emerge during the schizophrenia prodrome, lending face validity to our model.

With fiber photometry experiments, we identified disruptions in prediction error signaling in adult *Grin2a* cKO animals. While some aspects of dopaminergic signaling were intact in the experimental group, they failed to properly react to sensory cues that were contingent on outcome. Interestingly, this disruption in neurotransmission was evident regardless of the valence of the outcome. Compared to controls, striata; dopamine levels were reduced in *Grin2a* cKO animals in response to a cue with positive value and elevated in response to a cue with negative value. These animals also failed to respond with heightened dopamine signaling during unexpected events, further providing evidence for disruptions in prediction error encoding in these animals. These studies add to the face validity of our model as there are many clinical studies linking dopaminergic dysfunction in schizophrenia to abnormalities in prediction error.

Limitations and Future Directions

First, while we set out to model the human condition of schizophrenia, we took a specific approach with our manipulation. To tease apart how different cell-types and neural systems are

affected by *Grin2a* mutations, we knocked out its expression in a subset of cells of the brain. To this end, we did not produce a full model of schizophrenia, but generated a piece to a much larger puzzle. In the human condition, it is unlikely the mutation is confined to a single region, so it is likely that *GRIN2A* LoF in other regions or cell types result in different symptoms. It will be important for future studies to expand to other regions of interest to investigate the effects of this manipulation throughout the brain.

Second, due to the technical limitations of fiber photometry measurements and the short window of rodent adolescence, we chose to record dopamine activity in adult animals. While this approach is limited in that it does not provide neural correlates for the behavioral differences observed in adolescents, schizophrenia is a chronic disorder, making understanding the long-term consequences of the *Grin2a* cKO of critical interest. Moreover, because our experiments utilized both adults and adolescents, they have the potential to inform how the illness progresses.

We observed behavior abnormalities in both adolescent and adult animals, but the directionality of this abnormality was different. When the FCL was assessed in adolescents, our manipulation exaggerated the development of appetitive conditioning when contingencies were reversed. When assessed in adults, the *Grin2a* cKO animals had reduced appetitive conditioning during contingency reversal sessions. These age-dependent differences are not entirely surprising, as conditioning behaviors can be subserved by different underlying dopaminergic mechanisms when comparing adults and adolescents (McCane et al., 2021). It is possible, then, that the behavioral deficits we observed in the *Grin2a* cKO adolescents were driven by different physiological abnormalities than those driving behavioral differences in *Grin2a* cKO adults.

Similar opposing behavioral phenotypes have been reported with pharmacological approaches. In learning in paradigms where stimulus contingencies reverse, amphetamine administration facilitates reversal learning (Weiner & Feldon, 1986) while NMDA receptor blockade impairs it (Jentsch & Taylor, 2001; Li et al., 2016). Thus, the behavior we observed during the FCL task in adolescents may reflect a hyperdopaminergic state, whereas behavior in adults may reflect that of NMDA receptor hypofunction. Our fiber photometry experiments, performed in adults, revealed dysregulated dopamine signaling—not a single directional shift. So, it is possible that the *Grin2a* cKO is influencing behavior through separate mechanisms in adolescents and adults. Future work would benefit from continued characterization of dopamine release between *Grin2a* cKO adults and adolescents across different neural systems and timescales.

Third, while our model has construct and face validity, we did not investigate its predictive validity. It will be important for future studies to attempt to reverse some of the abnormalities induced by the *Grin2a* cKO with antipsychotics or novel treatment modalities directed at reducing schizophrenia symptoms. Of particular interest, GluN2A-selective positive allosteric modulators (PAMs) have been proposed as a potential therapeutic avenue in schizophrenia (Hanson et al., 2023). This model will be a useful tool for future work investigating the benefits of NMDA receptor modulation in schizophrenia. Moreover, because our model allows for intervention during adolescence, it will be possible to compare adolescent vs adult treatment to help determine if early intervention can halt disease progression.

Conclusions

With this work, we add to the body of literature focused on identifying the neurobiological underpinnings of schizophrenia. This work was driven by our initial finding that, unlike forebrain regions where GluN2A expression stabilizes in childhood, levels in dopamine neuron-containing brain regions declined throughout adolescence. This suggested that a LoF in *GRIN2A* may be most detrimental to dopamine neurotransmission during adolescence and contribute to the emergence of psychosis at this age (Figure 6.1). To investigate this potential link between *GRIN2A* LoF and post-adolescent expression of psychosis, we selectively manipulated *Grin2a* in adolescent dopamine neurons and found that it conferred a behavioral phenotype and dopaminergic abnormities that are consistent with psychosis.

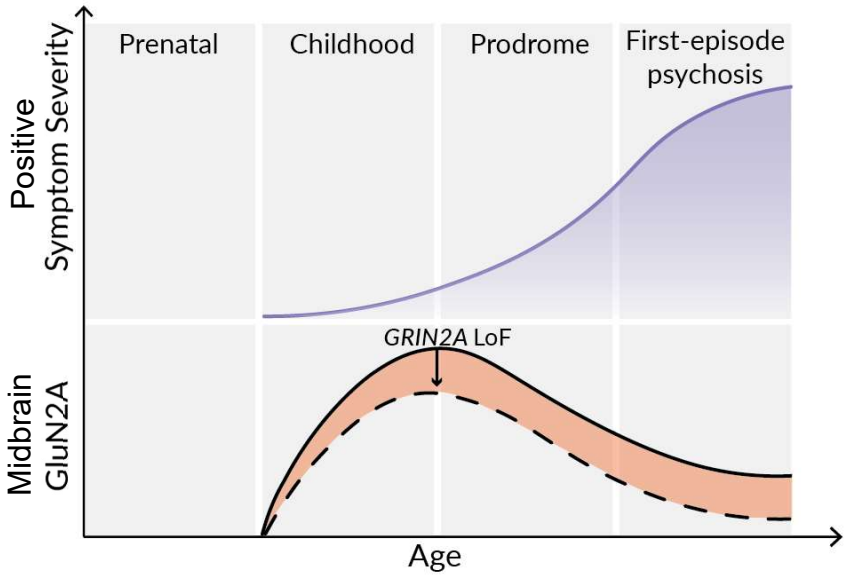

Figure 6.1 Developmental trajectory of positive schizophrenia symptoms and midbrain GluN2A levels

Adaptation of figure from (McCutcheon et al., 2020)

Supplementary Figures

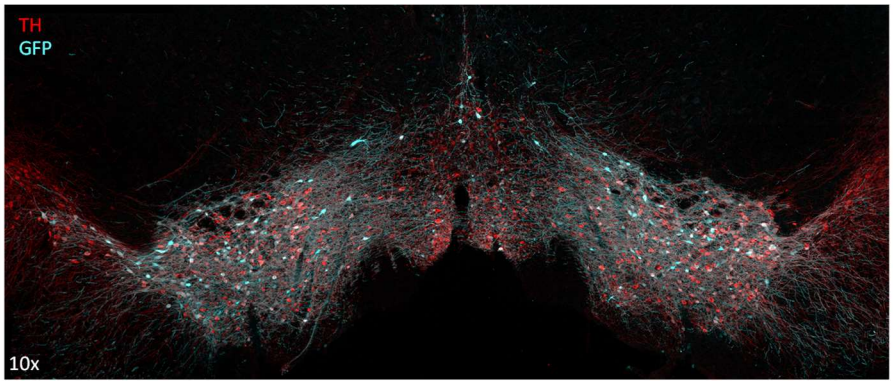

Figure A.1 Representative IHC of GFP+ neurons in the midbrain

TH+ cells are in red, GPF+ cells are in cyan.

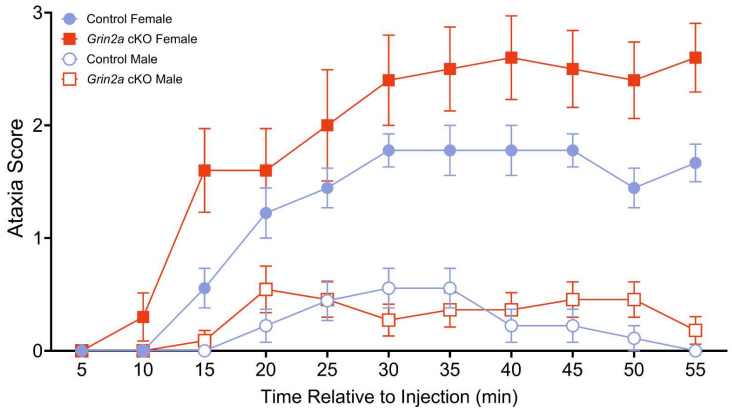

Figure A.2 MK801 ataxia scores over time

Significant effect of treatment on ataxia levels following MK801 (0.3 mg/kg) administration with Grin2a cKO animals showing increased ataxia scores compared to controls over time (*p=0.0428)

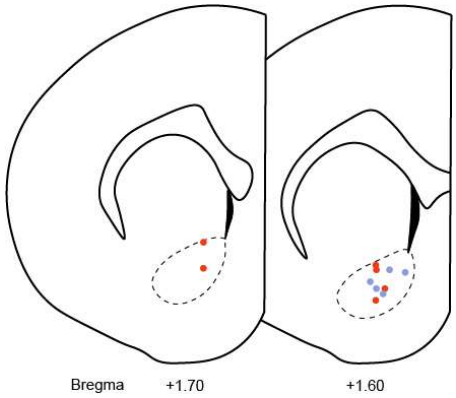

Figure A.3 Hit map of fiber tips in nucleus accumbens core

Location of fiber placement for fiber photometry experiments. Orange dots reflect *Grin2a* cKO animals, blue dots reflect control animals.

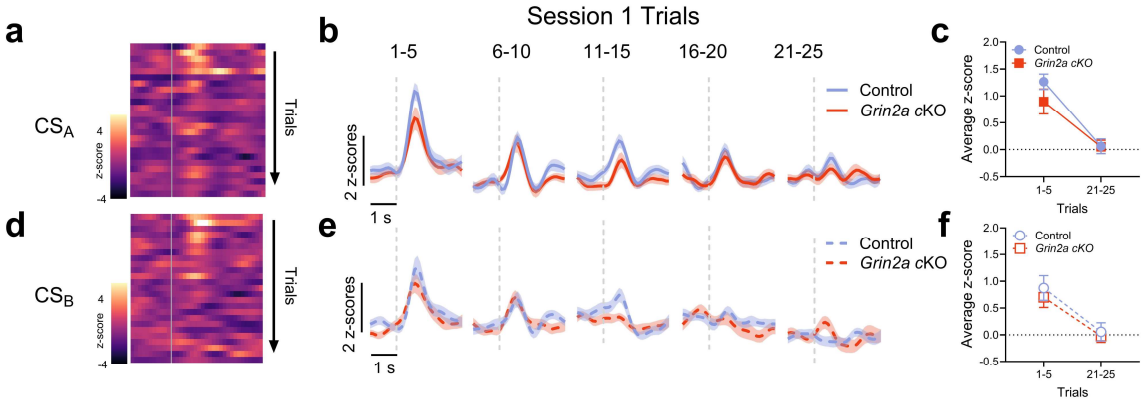

Figure A.4 Dopaminergic response to novelty is similar between treatment groups

A Heatmap of first 25 presentations of CS_A during Session 1. **B** Average fluorescent signal in first 25 presentations of CS_A during Session 1, binned into 5 trial sections. **C** Quantification of average z-score across 2 s following CS_A onset shows reduction in response from first 5 presentations to final 25 presentations. Both control and Grin2a cKO animals reduce response similarly. **D** Heatmap of first 25 presentations of CS_B during Session 1. **E** Average fluorescent signal in first 25 presentations of CS_B during Session 1, binned into 5 trial sections. **F** Quantification of average z-score across 2 s following CS_B onset shows reduction in response from first 5 presentations to final 25 presentations. Both control and Grin2a cKO animals reduce response similarly.

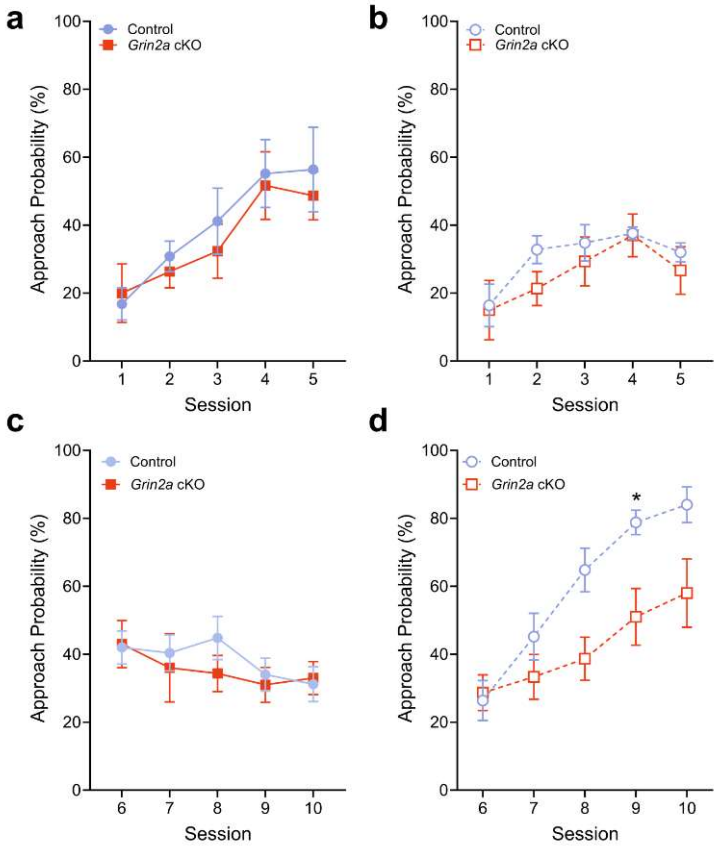

Figure A.5 Approach probability during fiber photometry experiments

A Approach probability during CS_A during initial learning sessions was similar between treatment groups. **B** Approach probability during CS_B during initial learning sessions was similar between treatment groups. **C** Approach probability during CS_A during contingency reversal sessions was similar between treatment groups. **D** Control animals were significantly more likely to approach the food trough during CS_B presentation than *Grin2a* cKO animals on Session 9 of the task (*p=0.0336).

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