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NMDA RECEPTOR DYSFUNCTION IN THE DEVELOPING PREFRONTAL CORTEX IN TWO ANIMAL MODELS FOR SCHIZOPHRENIA: EXPRESSION PROFILE, EPIGENETIC MECHANISMS, AND PHYSIOLOGY

BY

YELENA GULCHINA

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A Dissertation Presented to the Faculty of Drexel University College of Medicine

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Ramesh Raghupathi, Ph.D., Chair
Professor
Neurobiology and Anatomy

Wen-Jun Gao, Ph.D., Advisor
Professor
Neurobiology and Anatomy

Barry Waterhouse, Ph.D.
Professor and Chair
Cell Biology and Neuroscience
Rowan University

Felice Elefant, Ph.D.
Associate Professor
Biology
Drexel University

Mikhail Pletnikov, M.D., Ph.D.
Professor
Psychiatry and Behavioral Sciences
Johns Hopkins University

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DEDICATION

This work is dedicated to individuals coping with mental health disorders, whether undiagnosed or in treatment. During good and bad days, when struggling to put one foot in front of the other, perseverance in the face of these obstacles is a daily fight. This work is a reflection of that fight, and trying to use every day's successes and failures as motivation for tomorrow.

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TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xii

LIST OF ABBREVIATIONS xiii

ABSTRACT 1

CHAPTER 1: GENERAL INTRODUCTION 3

Schizophrenia 4

Historical perspective 4

Economic and psychosocial burden of schizophrenia 5

Symptom categorization 5

Importance of the prefrontal cortex in cognitive control 8

NMDARs in cognition, development, and schizophrenia 12

Subunit composition bestows unique functional properties upon NMDARs 12

Persistent expression of NR2B is distinct to PFC development 15

The NR2B-to-NR2A subunit switch does not dictate functional maturation of the PFC 18

Dopaminergic signaling modulates working memory and functional maturation of the PFC 20

The first hypothesis of SCZ: cortical hyperdopaminergia 21

The glutamate hypothesis of SCZ: NMDAR hypofunction in the PFC 22

Pharmacologically-induced NMDAR hypofunction mimics cognitive deficits of SCZ 23

Genetic mutations in SCZ patients 24

Transgenic animal models for SCZ 25

Neurodevelopmental models for SCZ 27

Epigenetics: an important level of regulation beyond genetics **29**

Basic tenets of epigenetic regulation 29

Epigenetics in SCZ 30

CHAPTER 2: SPECIFIC AIMS **33**

CHAPTER 3: EPIGENETIC MECHANISMS UNDERLYING NMDAR HYPOFUNCTION IN THE PREFRONTAL CORTEX OF JUVENILE ANIMALS IN THE MAM MODEL FOR SCHIZOPHRENIA **37**

Abstract **38**

Introduction **39**

Materials and Methods **42**

Animals 42

Tissue collection and western blot for synaptosomal proteins 42

Whole-cell patch-clamp electrophysiology 44

Spontaneous and miniature excitatory post-synaptic currents 44

Evoked excitatory post-synaptic currents 45

Western blot for histone proteins 46

Chromatin immunoprecipitation and quantitative PCR assay 47

Data analysis 48

Results **50**

NMDAR protein levels are reduced in the PFC of juvenile MAM rats 50

AMPA-s/mEPSCs are comparable between juvenile saline and MAM layer V pyramidal neurons in mPFC ... 52

Amplitude of NMDA-mEPSCs is diminished in juvenile MAM mPFC ... 52

Juvenile MAM animals have a significant reduction in NMDARs in mPFC layer V pyramidal neurons ... 53

Juvenile MAM layer V pyramidal cells exhibit a loss of NR2B-NMDARs ... 54

Synaptic AMPARs remain functionally intact in juvenile MAM mPFC ... 55

H3K27me3 is selectively increased in the PFC of juvenile MAM rats ... 62

The proximal promoter region of Grin2b is highly enriched in the transcriptional repressor REST and the histone marker H3K27me3 in juvenile MAM PFC ... 62

Discussion ... **68**

CHAPTER 4: ALTERED NMDAR SUBUNIT COMPOSITION IN ADOLESCENT PFC IN THE MAM MODEL FOR SCHIZOPHRENIA **76**

Abstract ... **77**

Introduction ... **79**

Methods ... **83**

Animals ... 83

Tissue collection and western blot for synaptosomal proteins ... 83

Whole-cell patch-clamp electrophysiology ... 84

Spontaneous and miniature excitatory post-synaptic currents ... 85

Evoked excitatory post-synaptic currents ... 86

Data Analysis ... 87

Results ... **89**

Constitutive NMDAR subunit NR1 is significantly decreased in adolescent MAM PFC *89*

NMDA-mEPSCs, but not AMPA-s/mEPSCs, have significantly diminished frequency in adolescence following prenatal MAM exposure *91*

Synaptic NMDAR function is comparable between adolescent saline and MAM mPFC *92*

Adolescent MAM animals demonstrate diheteromeric NMDAR subunit composition compared to triheteromeric NMDARs in saline animals *93*

Post-synaptic AMPARs are unaffected in adolescent MAM mPFC, yet pre-synaptic release mechanisms may be enhanced *94*

Global levels of histone modifications are not altered in adolescent MAM PFC .. *102*

Discussion **104**

CHAPTER 5: NMDAR HYPOFUNCTION IN JUVENILE PFC IN hDISC1-EXPRESSING MICE **112**

Abstract **113**

Introduction **114**

Methods **117**

Animals *117*

Inducible human DISC1 (hDISC1) mutant mouse model *117*

Tissue collection and western blot for synaptosomal proteins *120*

Whole-cell patch-clamp electrophysiology *121*

Spontaneous and miniature excitatory post-synaptic currents *122*

Evoked excitatory post-synaptic currents *123*

Data Analysis *125*

Results **126**

hDISC1 mutants exhibit reduced synaptic NR2A protein levels in juvenile PFC .. *126*

Evoked AMPA-EPSCs in juvenile hDISC1 pyramidal cells are significantly increased compared to control cells *128*

NMDARs in juvenile hDISC1 mPFC exhibit faster decay kinetics *128*

Discussion **133**

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS 138

NMDAR hypofunction in the developing PFC **139**

Compensation for NMDAR hypofunction **144**

Epigenetic mechanisms in SCZ **146**

Relevance of SCZ models to the human condition **147**

Clinical implications **150**

REFERENCES 151

LIST OF FIGURES

Figure 1 .... **11**

Figure 2 .... **17**

Figure 3 .... **51**

Figure 4 .... **57**

Figure 5 .... **58**

Figure 6 .... **59**

Figure 7 .... **60**

Figure 8 .... **61**

Figure 9 .... **65**

Figure 10 .... **66**

Figure 11 .... **75**

Figure 12 .... **90**

Figure 13 .... **96**

Figure 14 .... **97**

Figure 15 .... **98**

Figure 16 .... **99**

Figure 17 .... **101**

Figure 18 .... **103**

Figure 19 .... **111**

Figure 20 .... **119**

Figure 21 .... **127**

Figure 22 .... **130**

Figure 23 131

Figure 24 132

LIST OF TABLES

Table 1 48

LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
AMPAR(s)	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor(s)
BCA	bicinchoninic acid
ChIP	chromatin immunoprecipitation
D1Rs	dopamine D1 receptors
D2Rs	dopamine D2 receptors
DISC1	Disrupted-in-Schizophrenia-1
dlPFC	dorsolateral prefrontal cortex
DNQX	6,7-dinitroquinoxaline-2,3-dione
E	embryonic day
eEPSC	evoked excitatory post-synaptic current
EPSC(s)	excitatory post-synaptic current(s)
GWAS(s)	genome-wide association study/studies
hDISC1	human truncated DISC1 protein
IQ	intelligence quotient
LTD	long-term depression
LTP	long-term potentiation
MAM	methylazoxymethanol
mEPSC	miniature excitatory post-synaptic current
mPFC	medial prefrontal cortex
NIMH	National Institute of Mental Health

NMDAR(s)	N-methyl-D-aspartate receptor(s)
NR2A-NMDARs	NR2A-containing
NR2B-NMDARs	NR2B-containing
P	postnatal day
PCP	phencyclidine
PFC	prefrontal cortex
PPR	paired-pulse ratio
qPCR	quantitative polymerase chain reaction
RE1	response element 1
REST	RE1-silencing transcription factor
SCZ	schizophrenia, schizophrenic
sEPSC	spontaneous excitatory post-synaptic current
SNP	single nucleotide polymorphism
TTX	tetrodotoxin

ABSTRACT

NMDA Receptor Hypofunction in the Developing Prefrontal Cortex in Two Animal Models for Schizophrenia: Expression Profile, Epigenetic Mechanisms, and Physiology

Yelena Gulchina

Advisor: Wen-Jun Gao, Ph.D.

Schizophrenia is a highly debilitating illness often diagnosed in late adolescence and early adulthood following the emergence of psychotic symptoms. In addition to psychosis, negative symptoms and cognitive deficits are characteristic of the disorder, and are often evident prior to diagnosis. While positive symptoms are often attenuated with antipsychotic treatment, cognitive dysfunctions are unresponsive to therapeutic agents and persist throughout the course of the illness. As the best determinant of functional outcome, targeting the pathophysiology of cognitive deficits has become a priority for government agencies, academic research institutions, and the pharmaceutical industry. Among the prominent hypotheses attributed to schizophrenia, cortical hypofunction of the glutamate neurotransmitter system is an appealing possibility due to the importance of N-methyl-D-aspartate receptors (NMDARs), a type of ionotropic receptor, in brain development and cognition. Interestingly, blocking NMDARs during juvenile development is sufficient to elicit schizophrenia-like phenotypes in adult animals. Further, schizophrenic subjects display significant prefrontal hypofunction during performance of cognitive tasks. Compelled by the importance of the NMDAR

system in the development and maturation of the prefrontal cortex, this work explores the expression profile and functional properties of the NMDAR system during juvenile and adolescent development in two animal models for schizophrenia: the neurodevelopmental MAM model and the inducible hDISC1 mutant mouse model. We confirm that NMDAR hypofunction is a feature of prefrontal development in these two models, albeit by changes in expression of divergent subunits. Further, we explore a mechanism by which NMDAR expression changes occur in the juvenile prefrontal cortex, implicating aberrant epigenetic regulation of the NR2B subunit. These data support the neurodevelopmental hypofunction hypothesis and highlight the importance of investigating early prefrontal dysfunction to understand the complex pathology observed in the chronic stage of schizophrenia.

CHAPTER 1: GENERAL INTRODUCTION

Schizophrenia

Historical perspective

A century ago, Emil Kraeplin introduced the term "dementia praecox" to describe a common behavioral pattern he observed in his patients in the course of an illness, resulting in cognitive and executive dysfunction. Shortly after, Eugen Bleuler coined the term "schizophrenia" and constructed the earliest definition of what are now known as positive symptoms (Jablensky, 2010). In the 1970s, "positive" and "negative" symptoms were used to sub-classify schizophrenia (SCZ) into two mutually exclusive disorders: Type I (positive) was characterized by hallucinations and delusions, while Type II (negative) was defined by social withdrawal, poverty of speech, and flattened affect (Strauss et al., 1974; Crow, 1980). To this day, these symptoms are among those used to diagnose SCZ; however, this concept of mutual exclusivity between the two symptom domains has evolved into a condition where both symptom types co-occur in patients (Crow, 1985). Cases of SCZ can be separated by their familial or sporadic etiology, where the former is due to a traceable genetic mutation, and the latter can be due to environmental insults, *in vivo* somatic mutations, or both. This dichotomous etiology cannot be distinguished by differential phenotypic characteristics (Gottesman and Bertelsen, 1989; Roy and Crowe, 1994), suggesting the downstream effects in both circumstances converge on a common neurobiological dysfunction. Further, it has been difficult to identify consistent genetic vulnerabilities in SCZ by genome-wide association studies (GWASs). In fact, structural and neurochemical abnormalities are among the

most consistent features reported from analyses of post-mortem SCZ brains, but these variables lack the sensitivity to be classified as diagnostic biomarkers of the disorder (Jablensky, 2010). Over time, we have collected many pieces of the puzzle, but continue working to identify the role of each.

Economic and psychosocial burden of schizophrenia

SCZ is a severely debilitating mental disorder that persists throughout the lifetime of a patient. The economic and psychosocial burden on patients and family caregivers is substantial, as productivity is significantly diminished and family members often experience mental health disturbances as well (Awad and Voruganti, 2008). The global prevalence of SCZ is 1.1% and estimates of the annual cost of the disorder range from $63 billion to $180 billion in the United States (Ball et al., 2005). These high costs, reflective of direct and non-direct spending, were significantly higher than those of other major mental illnesses (Greenberg et al., 2003). The development of improved antipsychotics has contributed to reductions in medical expenses (Ball et al., 2005); nonetheless, the debilitating nature of SCZ and growing economic burden drives continued efforts to identify more efficient treatment strategies. To achieve this goal, efforts to understand the functionally-debilitating, physiological underpinnings of this disorder are underway.

Symptom categorization

The positive symptom domain is the most prominent diagnostic criteria for SCZ (Jablensky, 2010; Kahn and Sommer, 2015). These symptoms, commonly referred to as psychosis, are comprised of hallucinations, delusions, and thought disorder. The first presentation of psychosis is typically during late adolescence or early adulthood and pinpoints the age of onset of SCZ (Kahn and Sommer, 2015). Yet cognitive deficits, mood abnormalities, and anxiety are common features of the prodrome stage (Cannon et al., 2000; Gajwani et al., 2013; Fusar-Poli et al., 2014); therefore, strict delineation of the age of onset by one symptom cluster is too limiting.

In addition to psychosis, another set of behavioral abnormalities reported in SCZ patients are negative symptoms (Jablensky, 2010), including social withdrawal, poverty of speech, and flattened affect (Kirkpatrick et al., 2006). Individuals at high risk of converting to SCZ exhibit attenuated negative symptoms even prior to psychosis, indicating the developmental mechanisms that contribute to these symptoms may lend insight into the etiology of SCZ (Fusar-Poli et al., 2014). To streamline the diagnostic criteria, approach to clinical trial design, and therapeutic needs of SCZ patients, Kirkpatrick et al. (2006) in conjunction with the National Institute of Mental Health (NIMH) hosted a conference among clinicians, scientists, and pharmaceutical agencies to establish an industry consensus (Kirkpatrick et al., 2006). Unfortunately, imaging and neurochemical analyses of SCZ brains has led to few consistent findings in regards to negative symptoms (Galderisi et al., 2015); thus, the neurobiological contributions to this symptom cluster are still unclear.

Indeed, cognitive and executive dysfunction are among the core features of SCZ, as Kraeplin reported a century ago (Jablensky, 2010). Cognitive deficits are the best determinant of functional outcome for patients (Bowie and Harvey, 2006) because they remain consistent during early onset and chronic stages of the disorder (Hoff et al., 2005). Importantly, impairments in cognition are evident prior to psychosis (Cannon and Jones, 1996; Cornblatt et al., 1999; Rosso et al., 2000; Cannon et al., 2002), indicating pathological mechanisms begin during development in the course of SCZ. With estimates that as many as 90% or more of patients exhibit these cognitive abnormalities (Green et al., 2004), in addition to the pervasiveness of this dysfunction (Hoff et al., 2005), and limited efficacy of FDA-approved antipsychotics (Harvey and Keefe, 2001), this final symptom cluster has evolved into the primary focus of current research efforts. This culminated in the creation of MATRICS (Measurement and Treatment Research to Improve Cognition in Schizophrenia) with a similar goal to that set forth by Kirkpatrick et al. (2006) and NIMH (Green et al., 2004). An early meta-analysis of 7420 SCZ patients and 5865 control subjects revealed several neurocognitive constructs significantly impaired in SCZ, including verbal memory, attention, cognitive flexibility, working memory, processing speed, and intelligence quotient (IQ) (Heinrichs and Zakzanis, 1998). The MATRICS committee conducted a systematic assessment of the literature with the goal to identify the most replicated cognitive domains that would yield measurable outcomes during dysfunction and recovery in clinical trials. Green et al. (2004) expanded upon Heinrichs and Zakzanis (1998) findings to include social cognition, visual learning and memory, and reasoning and problem solving (commonly referred to as executive functioning, incorporating cognitive flexibility) (Green et al.,

2004). Thus, the <u>seven domains</u> of cognitive deficits in SCZ were outlined to update diagnostic criteria, and revise the guidelines for identifying and assessing therapeutic agents in clinical trials. In order to understand the importance of these domains in disease, one must appreciate their importance in normal cognitive and cellular processes.

Importance of the prefrontal cortex in cognitive control

In order to act appropriately in response to environmental demands, humans can organize their thoughts and behaviors into functional responses by integrating information from the current context with past experiences. The prefrontal cortex (PFC) is essential to this integrative process. Of particular importance for daily functioning, working memory is the ability to receive a stimulus, hold that information temporarily in mind, and subsequently respond in a goal-directed manner (Baddeley, 2012). Organisms are exposed to chaotic surroundings, incessantly bombarded with external stimuli; a system must be adept in filtering out irrelevant details and focus on pertinent information so that an appropriate response can be provoked. Working memory acts as the hub in processes necessary for higher cognitive and executive function, including thinking, planning, and comprehension (Baddeley, 1992; Goldman-Rakic, 1996). This form of short-term memory is provisionally active and serves as a middleman between the dynamic environment and long-term consolidation. Continual presentation of a relevant stimulus can lead to more permanent retention of that memory, while previously important information can be retrieved and returned to working memory manipulations to update mental schemas (D'Esposito et al., 1995; Goldman-Rakic, 1995). Working

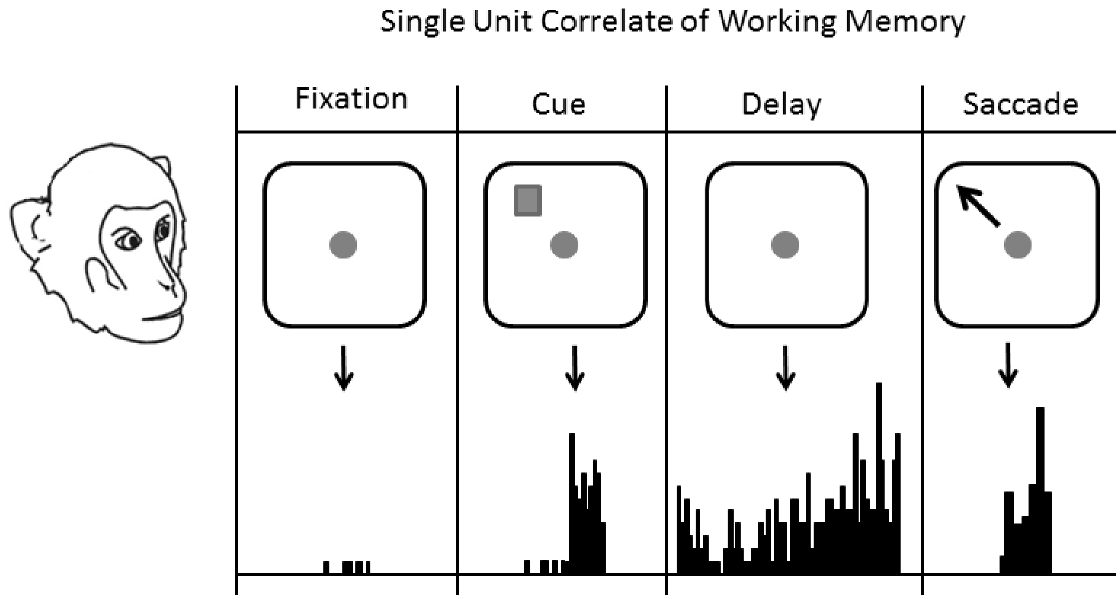
memory is a vital neurological process that allows organisms to appropriately manage the continuously changing world in a practical manner. The ability to read a story or navigate to a new destination are typical examples of working memory application. Engaging in such activities requires an individual to rapidly recall stored information from prior experiences and at the same time, focus on the influx of new information in order to understand and accomplish the current task. Overall, working memory can be considered a cerebral sieve in which the PFC first sorts incoming stimuli into essential and disposable information, recruiting and coordinating with other brain regions to manage the information. Working memory is thus a delicate process steering us through the daily chaos of modern life.

The PFC is a molecularly, neuroanatomically, and functionally distinct brain region that governs higher cognitive functions, including working memory, upon which affective recall, attention, and behavioral inhibition depend (Goldman-Rakic, 1996). As an important gatekeeper of incoming stimuli, it exerts top-down control by promoting behaviors that will benefit the individual in a context-specific manner. Increased exposure to unfamiliar environments will be stored and later retrieved when a goal-directed behavior is necessary, contributing to decision-making. Seeing that several executive processes involve PFC functioning, this area is thus crucial for guiding efficient and appropriate behavior. From this point, we will focus on describing working memory processes given their foundational role in higher-order cognition.

A growing body of literature has demonstrated the PFC's paramount role in working memory (Goldman-Rakic, 1995) and SCZ (Goldman-Rakic and Selemon, 1997). Lesion studies have continually confirmed that damage to the PFC results in working memory deficits, as assessed by performance on spatial delayed-response tasks (Goldman et al., 1971; Passingham, 1975; Mishkin and Manning, 1978; Goldman-Rakic, 1996; Buckley et al., 2009). During cognitive performance tasks, SCZ patients consistently demonstrate reduced activation of the dorsolateral PFC (dlPFC) (MacDonald et al., 2005; Lesh et al., 2011; Van Snellenberg et al., 2016). Mechanistically, electrophysiological recordings have revealed preferential sustained activity in the PFC during engagement in a delayed-response working memory task. Information perceived as important is retained transiently in mind, which requires continual robust firing in order for the PFC to properly execute top-down control (Arnsten et al., 2010). Prefrontal neuronal firing increases following presentation of a salient cue, continues throughout a delay period in which stimuli are completely absent, returning to baseline once a response has been generated (

Figure 1). This specialized activation in prefrontal cortical neurons reflects an 'on-line' sustained activity, which fosters mnemonic encoding (Fuster and Alexander, 1971; Goldman-Rakic, 1996). This unique firing property in a subpopulation of PFC neurons, referred to as Delay cells, is postulated to be the molecular underpinning of working memory. The local PFC circuitry is specialized for such sustained activity, providing a neural correlate for working memory function (Goldman-Rakic, 1995).

Figure 1



Delay cell firing supports working memory function. The schematic of the oculomotor delayed-response task illustrates the cellular mechanism of visual working memory necessary for the proper behavioral response, a saccade to a previously cued location. During the cue, Delay cell firing tuned for a specific location increases and is maintained throughout the delay period. Once the behavioral response is generated, Delay cell firing is diminished as it is no longer necessary. Figure reprinted from Monaco et al. (2015).

NMDARs in cognition, development, and schizophrenia

Subunit composition bestows unique functional properties upon NMDARs

N-methyl-D-aspartate receptors (NMDARs) are heterotetrameric complexes composed of a mandatory homodimer of NR1, and homodimers of either NR2 (NR2A-D) or NR3 (NR3A-B) subunits, or heterodimers of NR2 and NR3 subunits (Ogden and Traynelis, 2011). The NR1 subunit is vital for targeting NMDARs to discrete regions of the cell surface as well as membrane insertion; therefore, receptors lacking this subunit are not functional (Cull-Candy and Leszkiewicz, 2004). The NR2 and NR3 subtypes, however, afford functional heterogeneity to the NMDAR complex. These subunits dictate such functional characteristics as open channel time, calcium permeability, decay time, and sensitivity to Mg^{2+} block as well as pharmacological agents (Paoletti et al., 2013). Of particular interest, NR2A and NR2B are predominantly expressed in the postnatal brain with both subunits integral for synaptic plasticity and maturation mechanisms (Monyer et al., 1994; Sheng et al., 1994).

Although NMDARs are critical, not all subunits are created equal when it comes to working memory and cognitive function. NR2A- and NR2B-selective antagonists were useful in beginning to parse out the contribution of these individual subunits to cognitive processes. Long-term potentiation (LTP) was attenuated following NR2A antagonism in *ex vivo* slices, but completely blocked by an NR2B antagonist; thus, demonstrating that both NR2A and NR2B contribute to prefrontal synaptic plasticity, but NR2B plays a

more dominant role (Cui et al., 2011; Plattner et al., 2014). The importance of NR2B subunits in learning and memory processes was first brought to light by transgenic manipulations. As determined by studies utilizing NR2B overexpression methods, this subunit heavily contributes to memory function. Forebrain-restricted NR2B overexpression (Tang et al., 1999; Cui et al., 2011) results in extended NMDAR channel opening in conjunction with enhanced NMDAR activation, thereby facilitating learning and memory across an entire cognitive spectrum and enabling adult mice to exhibit "smarter" performance in cognitive tasks compared to their wild-type counterparts (Tang et al., 1999; Cui et al., 2011). Moreover, overexpression of NR2B also altered synaptic plasticity, with selective enhancement in LTP while long-term depression (LTD), basal synaptic transmission, and paired-pulse depression remained comparable between transgenic and wild-type animals (Tang et al., 1999; Cui et al., 2011). These data demonstrate that NR2B plays an important role across a gamut of memory functions and that expression of this subunit strongly impacts intellectual capabilities. Specifically, overexpression of NR2B in the forebrain not only affected long-term synaptic plasticity at a cellular level, but also translated into a behavioral change (Tang et al., 1999; Cui et al., 2011). Remarkably, altering the expression levels of this one protein can dramatically impact PFC-dependent working memory function. Thus, NMDARs are an important molecular substrate for cognitive processes, with the NR2B subunit particularly implicated in PFC-dependent cognition (Cao et al., 2007; Wang and Gao, 2009; Cui et al., 2011; Wang et al., 2013; Lett et al., 2014). Conceivably, alteration of NR2B levels in disorders presenting with cognitive deficits, such as SCZ, may contribute significantly to these functional impairments (Snyder and Gao, 2013; Plattner et al., 2014).

Computational modeling and work in the primate dlPFC has been integral in identifying the specific nature of NR2B's role in working memory. It has been proposed that NR2B grants the physiological requirements necessary for working memory function because of its slower decay time compared to NR2A. NR2B-containing receptors (NR2B-NMDARs) can conduct a large number of Ca^{2+} and Na^{2+} ions during their intrinsically longer open channel state (Wang, 1999; 2001), thereby prolonging depolarization events (Cull-Candy et al., 2001; Wang, 2001) and synaptic summation (Wang et al., 2008) essential for working memory function. More efficient temporal summation patterns help bring the neuron closer to the spike-firing threshold of the cell (Kumar and Huguenard, 2003), which in turn promotes sustained activity. The delay period between perception of a stimulus and the elicited response employs neurons to stay online, actively engaged. The NR2B subunit affects NMDAR channel kinetics so that the persistent firing pattern necessary for PFC-dependent cognitive functions, such as working memory and cognitive flexibility, can thus be maintained (Goldman-Rakic, 1995; Wang, 1999; Wang et al., 2013).

This long-held hypothesis was supported by a recent elegant study, in which Wang et al. (2013) provided the first direct evidence that NMDAR functioning underlies persistent firing specifically in primate dlPFC Delay cells (Wang et al., 2013). Generalized blockade of NMDAR activity depressed Delay cell firing and working memory function. Importantly, selectively blocking NR2B activity significantly suppressed Delay cell firing, demonstrating a direct action of NR2B in the working

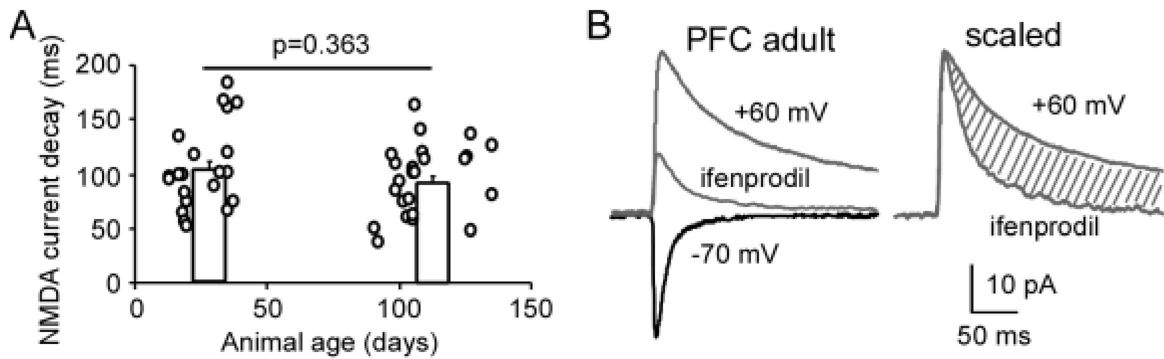
memory task. In contrast, blockade of AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors) showed a lag effect in which activity was decreased later in the Delay cell firing period, not immediately. These data suggest that AMPAR activity may serve to support depolarization events in Delay cells, whereas NMDARs, especially NR2B subunits, are required for the moment-to-moment firing necessary for working memory function (Wang et al., 2013). Therefore, we can appreciate this vital receptor is at the forefront of a highly complex network that encodes working memory and facilitates higher-order cognitive processes.

Persistent expression of NR2B is distinct to PFC development

NMDAR subunit expression is regionally- and developmentally-restricted, thereby contributing to the brain's complexity by imposing unique physiological and functional properties within individual brain regions (Cull-Candy and Leszkiewicz, 2004). A subunit switch from mostly NR2B- to NR2A-containing NMDARs occurs at a time coincident with circuit refinement necessary for maturation of synapses and learning capabilities (Dumas, 2005). Physiological changes indicative of this maturational process include reduced decay time constant, enhanced temporal summation properties, and reduced sensitivity of the NMDAR complex to NR2B-specific antagonists, such as ifenprodil and Ro25-6981. The PFC is unique relative to other brain regions because an NR2B-to-NR2A subunit switch that occurs in several other neuroanatomical regions (Dumas, 2005) appears to be absent in the PFC local circuitry (Wang et al., 2008). Over the course of neurodevelopment, NR2B levels do not decline, but remain persistently

high in the adult rat PFC (Figure 2) (Wang et al., 2008). Lack of an NR2B subunit switch serves to fit PFC function for working memory by preserving the physiological properties necessary for sustained activity. During the execution of working memory function, stimulation of glutamatergic pyramidal neurons results in persistent activity, and the temporal summation of these inputs is facilitated by the presence of NR2B subunits (Flores-Barrera et al., 2014). Seeing that the PFC retains high expression of NR2B, this brain region is ideal for supporting mnemonic encoding.

Figure 2



PFC pyramidal neurons do not exhibit a subunit switch from primarily NR2B- to NR2A-NMDARs as do other cognitive centers. (A) Decay of NMDAR-mediated currents is not significantly changed in pyramidal neurons throughout postnatal development of the PFC, as would be expected following the switch to predominantly NR2A-NMDARs. (B) A significant component of NMDAR-mediated current is blocked by the NR2B-selective antagonist ifenprodil, confirming a large NR2B-component in adult PFC. Figure modified and reprinted from Monaco et al. (2015).

The NR2B-to-NR2A subunit switch does not dictate functional maturation of the PFC

The NR2A/NR2B ratio is a critical regulator of synaptic plasticity function in addition to its role in cognition. Many brain regions, including the hippocampus and primary sensory cortices, undergo this subunit switch from primarily NR2B- to NR2A-NMDARs, which eventually results in a higher ratio of NR2A/NR2B (Williams et al., 1993; Sheng et al., 1994; Gonzalez-Burgos et al., 2008; Wang et al., 2008; Paoletti et al., 2013). The maturation of these regions through the increased role of NR2A allow rapid responses to incoming environmental stimuli. In mature primary visual cortex, the high NR2A/NR2B ratio endows the neural circuitry with rapid temporal summation properties and receptive field maturation (Philpot et al., 2001; Cho et al., 2009). Similarly, in the mature hippocampus where the NR2A/NR2B ratio is high, LTP is heavily mediated by NR2A-containing receptors, and can be significantly diminished in the presence of NR2A-specific, but not NR2B-specific, antagonists (Zhao et al., 2005), although this issue remains controversial (Yashiro and Philpot, 2008). Thus, in sensory cortices, hippocampus, and many other cortical regions, NR2A-NMDARs dictate cognitive functions associated with long-term synaptic plasticity in adulthood.

In the PFC, however, the NR2A/NR2B ratio is stable throughout development, which is distinctly different from these aforementioned brain areas with associative learning capabilities (Dumas, 2005; Wang et al., 2008). In the adult rat PFC, a significant NR2B-component is evident (Figure 2B, (Wang et al., 2008). Indeed, the maintenance of NR2B-NMDARs aids synaptic and functional maturation of the PFC. In late adolescence

and early adulthood, but not earlier developmental time points, LTP can be induced in the PFC upon stimulation of the ventral hippocampus. This developmental stage corresponds to a time of peak contribution from NR2B-NMDARs in the PFC, indicating this molecular component is required for circuit maturation between regions. Blockade of the NR2B subunit or NMDARs in the PFC at this later developmental stage abolishes LTP (Flores-Barrera et al., 2014). These data indicate that the NR2B subunit intimately regulates circuit refinement and functional maturation of the PFC, which is necessary for normal functioning of working memory and higher-order cognition.

Working memory is a dynamic and transient process compared to long-term memory consolidation and storage; thus, it is not surprising that the molecular mechanisms governing these processes are fundamentally different (Taylor et al., 1999; Arnsten and Jin, 2014). Differing from the PFC, other cortical areas experience a decline in NR2B protein levels during development, and at adulthood reach a level of expression that will be maintained until aging (Dumas, 2005). NR2A protein levels rise in parallel to this process such that mostly post-synaptic NR2A-NMDARs govern synaptic physiology. However, the molecular mechanisms that drive this switch are still poorly characterized. In sensory cortices and hippocampus, this switch is strongly dependent on experience and neuronal activity (Yashiro and Philpot, 2008). However, experience- and activity-dependent plasticity do not appear to guide prefrontal development in a similar fashion as the PFC lacks direct thalamocortical innervation from the sensory thalamus with the exception of the mediodorsal nucleus of the thalamus (Giguere and Goldman-Rakic, 1988; Conde et al., 1990; Ray and Price, 1992; Ferguson and Gao, 2014). Instead,

synaptic pruning and infiltration of neuromodulator systems contributes to functional maturation of the PFC. The dopaminergic system is one critical element of this process on which we will focus due to its relevance to SCZ pathology.

Dopaminergic signaling modulates working memory and functional maturation of the PFC

Thus far, we have described that neuronal activity and working memory function in the PFC are intimately linked to the NMDAR; however, this receptor cannot act in solitude. NMDARs are at the core of a dynamic network of receptor complexes and signaling cascades, which regulate neuronal activity and ultimately, working memory and higher-order cognitive capabilities (Paoletti et al., 2013). The dopamine system is one such critical regulator of NMDAR-induced activity, poised for this role by its close proximity to NMDAR complexes within dendritic spines (Arnsten and Jin, 2014). Dopamine D1 receptor (D1R) stimulation exerts modulatory effects on working memory performance in an inverted-U dose response manner (Arnsten et al., 1994; Vijayraghavan et al., 2007); moderate levels of stimulation enhance the signal-to-noise ratio by sustaining preferred direction firing while simultaneously dampening non-preferred direction firing. Thus, the dopamine system can sharpen the tuning of PFC microcircuitry to promote appropriate cognition and goal-directed behaviors. In contrast, higher levels of dopamine receptor activation induce an overall suppression of delay-related firing in both preferred and non-preferred responses (Williams and Goldman-Rakic, 1995; Vijayraghavan et al., 2007), creating a discord in communication that will impair

working memory function. Moreover, refinement of the NMDAR relies on the dopamine pathway; co-activation of NMDA and dopamine receptors facilitates the functional maturation of the PFC (Flores-Barrera et al., 2014). Thus, the development of prefrontal maturation is strongly dependent upon the dopamine system in addition to NMDARs. Dysfunction of either or both of these neurotransmitters can have devastating effects on cognition, motor function, and perception; thus, dopamine signaling is another integral player in both normal cognitive performance and in SCZ pathology (Seamans and Yang, 2004).

The first hypothesis of SCZ: cortical hyperdopaminergia

The first suggestion of dopamine contributing to SCZ was evidenced by the psychosis-like behaviors exhibited by consumers of amphetamine, a dopamine reuptake inhibitor (Matthysse, 1974). Cortical hyperdopaminergia was one of the first hypotheses regarding the neurochemical abnormalities of SCZ (Perez-Costas et al., 2010). The successful abatement of positive symptoms with neuroleptic agents, later shown to preferentially inhibit dopamine D2Rs (Ito et al., 2012), lent additional credence to this hypothesis (Konradi and Heckers, 2003; Brisch et al., 2014). Now known as typical antipsychotics, these were successful in treating positive symptoms; however, negative symptoms and cognitive deficits were often untreated or even worsened by these drugs. Their strong extrapyramidal effects on motor function (Lewis and Lieberman, 2000) left many chronically-treated patients with irreversible dyskinesia and other parkinsonian-like dysfunctions. The evolution of second-generation atypical antipsychotics provided some

relief from these harsh side effects without compromising the efficacy in treating psychosis. This new generation of treatments only partially block D2Rs and exert additional modulation on the serotonergic system (Meltzer and Massey, 2011). Unfortunately, neither typical nor atypical antipsychotics demonstrate clear improvements in cognition or daily function (Harvey and Keefe, 2001). Therefore, while the dopaminergic system clearly contributes to SCZ pathology, it alone cannot account for the pervasive nature of mood alterations and cognitive dysfunctions (de Bartolomeis et al., 2013; Laruelle, 2014), thus implicating other neurochemical systems in the etiology of the disorder.

The glutamate hypothesis of SCZ: NMDAR hypofunction in the PFC

Similar to dopamine, drug-induced exacerbation of positive and negative symptoms, and cognitive deficits implicated the glutamate system in SCZ. Phencyclidine (PCP) and ketamine, non-competitive NMDAR antagonists (Anis et al., 1983), are able to evoke psychosis in stably-treated patients (Lahti et al., 1995) and normal subjects (Krystal et al., 1994; Malhotra et al., 1996; Jentsch and Roth, 1999). Importantly, and unlike dopamine receptor antagonists, these drugs can also elicit cognitive dysfunction (Krystal et al., 1994; Newcomer et al., 1999) and negative symptoms of SCZ (Malhotra et al., 1996). A surge in development of animal models for SCZ using pharmacological blockade of NMDARs, particularly PCP, revealed a number of common behavioral phenotypes between animals and humans. Animal models are utilized not only to explore the neurobiology of SCZ-relevant behaviors, but also to identify and test the therapeutic

potential of pharmacological agents to alleviate these dysfunctions. However, this approach must be carefully considered, as many positive outcomes in preclinical models rarely demonstrate translational efficacy when tested in clinical trials.

Pharmacologically-induced NMDAR hypofunction mimics cognitive deficits of SCZ

Pharmacological blockade of NMDAR activity has been shown to diminish working memory and cognitive functions across several mammalian species, reinforcing the ubiquitous impact of these glutamate receptors. Working memory performance in conscious monkeys was impaired following chronic administration of the NMDAR antagonist MK-801 (Tsukada et al., 2005). Further, rats treated with ketamine or MK-801 show disrupted working memory performance in a delay-independent manner, (Aultman and Moghaddam, 2001), a phenotype reliably produced in human subjects (Krystal et al., 1994; Malhotra et al., 1996; Newcomer et al., 1999; Hetem et al., 2000; Parwani et al., 2005). The most common pharmacologically-induced preclinical model for SCZ uses PCP, which can more reliably reproduce deficits in the cognitive domains outlined by MATRICS than MK-801(Jentsch and Roth, 1999; Kirkpatrick et al., 2006). Adult PCP-treated animals demonstrate deficits in prefrontal-dependent cognitive domains such as working memory (Seillier and Giuffrida, 2009), reversal learning (Jentsch and Taylor, 2001), and attention (Egerton et al., 2005), among other social and sensorimotor constructs observed in SCZ patients (Jones et al., 2011). Interestingly, some of these behaviors can be attenuated by treatment with typical or atypical antipsychotics (Jentsch and Taylor, 2001). Addition of adjuvant molecules that are proposed to boost NMDAR

function have also shown positive results, although complicated by this mixed pharmacological approach. This is somewhat in conflict with evidence that neither typical nor atypical antipsychotics cause substantial amelioration of cognitive deficits (Harvey and Keefe, 2001), and have been interpreted as reinforcing the construct validity of these pharmacological models. In addition, PCP-treated animals exhibit neurobiological alterations commonly reported from post-mortem human analyses, such as reduced hippocampal volume, diminished dendritic complexity, and prefrontal hypometabolism. Therefore, these models lend valuable but limited insights into the structural and cognitive consequences of acute or chronic NMDAR blockade; however, this approach disregards the potential genetic and neurodevelopmental contributions to SCZ pathology.

Genetic mutations in SCZ patients

A unique gene encodes each subunit of the NMDAR. Polymorphisms in the genes that encode NR1, NR2A, and NR2B, in particular, are segregated with SCZ diagnosis. Chronically-ill SCZ patients who are carriers of a single nucleotide polymorphism (SNP) in *Grin2b*, the gene encoding NR2B, show a significant reduction in reasoning performance compared to controls (Weickert et al., 2013). In addition, this SNP results in a greater reduction of NR1 protein and mRNA in the dlPFC compared to carrier control subjects, which may lead to overall loss of NMDAR insertion and therefore function. Analysis of NMDAR mRNA levels in post-mortem SCZ tissue has resulted in conflicting findings, however, and studies report increases, decreases, or no change in transcript levels (Coyle, 2012). This inconsistency hinders our ability to identify whether the

pathophysiological mechanisms underlying SCZ are a direct result of diminished NMDAR subunit proteins (Weickert et al., 2013). Nevertheless, mutations in *Grin2b* are consistently associated with cognitive deficits (Endele et al., 2010). Therefore, compromised NR2B expression in SCZ can contribute significantly to cognitive aberrations characteristic of this disorder.

Transgenic animal models for SCZ

Transgenic mice manufactured to express mutations in genes implicated in the cognitive dysfunction observed in SCZ patients are another appealing tool used to develop models for SCZ. An NR1 hypomorph model in which mutant mice express a fraction (5%) of the constitutive subunit protein display cognitive deficits and motor phenotypes quite similar to pharmacological models for SCZ (Mohn et al., 1999). Further adding construct validity to this transgenic model, these behavioral phenotypes were recovered by haloperidol treatment, a typical antipsychotic (Mohn et al., 1999). However, this reinforces the lack of predictive validity given the ineptitude of these drugs to impede cognitive dysfunction in SCZ patients (Harvey and Keefe, 2001).

No single genetic aberration has yet been identified as sufficient or necessary for SCZ (Rodriguez-Murillo et al., 2012), which is surprising given the disorder's high rate of heredity. Instead, it seems likely that a complex interplay among genetic susceptibility, environment, and epigenetic mechanisms contribute to the etiology of SCZ. Nonetheless, genetic models provide useful insights into the mechanisms that may be disrupted in the

SCZ brain. Importantly, many of these animal models express mutated genes that play critical roles in synaptogenesis, dopaminergic and glutamatergic synaptic physiology, and synaptic plasticity, hallmark processes of appropriate development and maturation of the brain, particularly the PFC. Of particular interest is the Disrupted in Schizophrenia 1 (*DISC1*) gene, which has shown high co-segregation with SCZ and other major mental illnesses in a Swedish pedigree (Millar et al., 2000). This genetic abnormality was one of the first identified and implicated in SCZ (Brandon and Sawa, 2011); however, it is worth noting this model is one among many transgenic mutants that have been developed and characterized in the effort to solve the puzzle of SCZ. To date, a number of DISC1 models have been developed, including those with point mutations in the endogenous *Disc1* mouse gene, DISC1 C-terminal truncated mutants, and a model expressing the human truncated DISC1 protein isolated from the Swedish cohort (Pletnikov et al., 2008), which is used in this work. These varied genetic perturbations often yield behavioral outputs and morphological aberrations that are inconsistent across models and compared to the human condition.

Focusing on the human truncated DISC1 (hDISC1) model developed by Dr. Mikhail Pletnikov and colleagues (Hikida et al., 2007; Pletnikov et al., 2008; Abazyan et al., 2010; Ayhan et al., 2011; Holley et al., 2013), these mutant mice exhibit impairments in working memory and cognitive flexibility tasks, as well as structural and morphological alterations reminiscent of abnormalities in SCZ patients (Pletnikov et al., 2008; Jaaro-Peled et al., 2010). The specific consequences of the *DISC1* mutation are unclear, but the protein product interacts with many partners from embryonic

development, including neurogenesis and neuronal migration, until adulthood, where it is valued in synaptic formation and maintenance (Brandon and Sawa, 2011). The role of DISC1 in brain development, functional maturation, and cognition is not yet fully understood; however, the value of this transgene's models keeps them among the most commonly used tools currently available for SCZ research.

Neurodevelopmental models for SCZ

Alterations in NMDAR function during early postnatal development, such as juvenile or adolescent stages, can have profound neurodevelopmental and functional effects on cognitive capabilities in adulthood (Endele et al., 2010). Brief inhibition of NMDARs with the non-competitive antagonist ketamine in juvenile animals has long-term effects on the inhibitory microcircuitry of the medial PFC (mPFC) and on prefrontal-dependent cognition, including working memory, associative learning, and attention (Jeevakumar et al., 2015). This unique combination of NMDAR inhibition during neurodevelopment, although not a recognized model for SCZ, does support the notion that NMDAR hypofunction during a critical period of PFC development can accurately mimic SCZ pathology in adulthood.

A popular approach to model the neurodevelopmental aspect of SCZ is the use of environmental insults during gestation to evaluate the effects of this etiological factor. Among the most commonly employed neurodevelopmental models is the methylazoxymethanol (MAM) model for SCZ, which will be our focus. Mechanistically,

MAM causes transient DNA methylation selectively in actively proliferating neurons. MAM administration on progressive days of gestation (embryonic day, E), i.e. E15 and E17, results in starkly different effects on behavioral and morphological outcome measures. MAM exposure on E15 results in microcephaly and other gross brain abnormalities compared to E17-exposed offspring, which demonstrate cognitive, sensorimotor, and social perturbations specifically relevant to SCZ (Gourevitch et al., 2004; Moore et al., 2006). The latter time point restricts the effect of MAM to cortical neurons, as opposed to the effects of MAM on a greater proportion of the developing brain at E15. Focusing on the E17 treatment paradigm, enlargement of the lateral ventricles and reductions in parvalbumin immunoreactivity in the adult MAM brain are well-matched to the most commonly reported structural and biochemical abnormalities in SCZ tissue (Lodge and Grace, 2009). Moreover, MAM animals exhibit deficits in learning (Gourevitch et al., 2004; Le Pen et al., 2006; Hazane et al., 2009; Snyder et al., 2013) and rule reversal (Moore et al., 2006; Featherstone et al., 2007) in spatial working memory and attentional set-shifting tasks, respectively. Providing strong face validity for this model, MAM animals do not exhibit psychosis-like phenotypes until after puberty (Le Pen et al., 2006), yet demonstrate cognitive deficits in juvenile development (Snyder et al., 2013), mimicking the course of SCZ. However, these cognitive abnormalities are not always consistent with those reported in SCZ patients (Featherstone et al., 2007), and the MAM model has received much criticism in recent years. Nevertheless, it is another useful model for investigating the neurobiological aberrations underlying SCZ pathophysiology, providing a unique window into neurodevelopmental factors.

Epigenetics: an important level of regulation beyond genetics

DNA is the blueprint for organism development and function, encoding each critical aspect of the biological process. Another important layer contributing to this process is epigenetics, mechanisms which tightly control expression and repression of genes without any alteration to the DNA sequence. Over the past two decades, the role of epigenetic processes in chromatin stability, gene regulation, responding to environmental factors, and in disease states has grown rapidly (Allis and Jenuwein, 2016). These efforts have led to groundbreaking strides in other biomedical research fields, and provide an attractive avenue to explore the mechanisms of neuropsychiatric disorders. Given the complexity of both epigenetic processes and pathophysiology of psychiatric disorders, progress has been slow, yet promising.

Basic tenets of epigenetic regulation

Genetic material can be packaged as euchromatin, which is DNA ready to be transcribed, and heterochromatin, a state during which the accessibility to DNA by the transcriptional machinery is difficult. These chromatin states are mediated by a very complex system of epigenetic regulation, including enzymes which “write” histone and DNA modifications, “readers” to interpret these markers as permissive or repressive gene states, and “erasers” that keep the epigenetic landscape and thus gene regulation dynamic (Nestler et al., 2016).

Nucleosomes, the fundamental units of chromatin, are composed of a histone octamer core around which 147 base pairs of DNA are wrapped (Kornberg, 1974). The histone octamer consists of two copies of H2A, H2B, H3, and H4 proteins. The N-terminal tails of these histone proteins contain amino acid residues, some of which can be dynamically modified (Verdin and Ott, 2015) by enzymes adding and removing acetyl and/or methyl groups (Jenuwein and Allis, 2001). The position and valence of these histone modifications contribute to the chromatin state within a genetic region. Regions of rich cytosine-guanine dyads (CpG islands) can undergo DNA methylation, commonly a marker of repressed gene expression (Adachi and Monteggia, 2014). Further adding to the complexity of this network, seemingly antagonistic histone modifications can co-occur in promoter regions (Aranda et al., 2015), invalidating the notion that chromatin state is dictated by one class of modifications, i.e. permissive only vs repressive only. Lastly, these modifications are “erased” by enzymes or DNA excision mechanisms so that chromatin states exist only as needed, and are adapted to the changing needs of the organism. Much knowledge has been gained from ongoing research efforts, yet not all pieces of the puzzle are clear. Current efforts are focused on exploring how histone modifying enzymes are selectively recruited to target genes, how interactions among DNA methylated sites and modified histones contribute to gene regulation, how epigenetic markers at a single locus influence expression of distant and non-linear loci, among many other critical questions.

Epigenetics in SCZ

GWASs have identified numerous genetic mutations in post-mortem SCZ tissue, without a single genetic locus sufficient to induce the complex pathology seen in patients (Rodriguez-Murillo et al., 2012). The influence of epigenetic processes on gene expression patterns is transient, yet robust; thus, exploring epigenetic mis-regulation in SCZ opens provocative new possibilities for the mechanisms underlying the pathophysiology of this disorder. These efforts have led to interesting data from genes previously implicated in SCZ. One aspect of the glutamate hypothesis not explored in this work is NMDAR hypofunction on prefrontal interneurons and the associated functional implications for cognitive deficits in SCZ (Lewis et al., 2005). *GAD1*, the GABA-synthesizing enzyme glutamic acid decarboxylase, is repressed in post-mortem SCZ tissue as shown by increased DNA (Huang and Akbarian, 2007) and histone methylation (Akbarian and Huang, 2006), as well as reduced acetylation markers (Huang et al., 2007). Further, long-range gene regulation of *GAD1* is weakened in SCZ PFC, contributing to this repressed state of gene expression (Bharadwaj et al., 2013). Of note, similar long-range epigenetic processes regulate the expression of *Grin2b*, the NR2B-encoding gene, implicating these mechanisms further in the NMDAR hypofunction hypothesis of SCZ (Bharadwaj et al., 2014). An important protein for neurodevelopment and neural migration, as well as synaptic NR2B function, is encoded by *RELN,* another gene found to be hyper-methylated in SCZ tissue (Abdolmaleky et al., 2005). Finally, *SOX10* is also hyper-methylated in SCZ PFC (Yuan et al., 2013). This protein plays an important role in oligodendrocyte function, and its disruption may contribute to white matter abnormalities seen in SCZ post-mortem (Iwamoto et al., 2005).

Together, these findings support a role for epigenetic mis-regulation in the PFC in SCZ, a region highly implicated in the cognitive deficits of the disorder. Surprisingly, this is a relative wealth of data compared to the absence of information from animal models. As this research progresses, we must take a careful and critical approach to the relevance of data from rodent models to that from humans. The unique complexity of epigenetic processes differs among neuronal sub-types and may be species-dependent as well. However, this shortcoming is already being addressed by work utilizing transgenic mouse models and human SCZ tissue concomitantly (Kurita et al., 2012).

CHAPTER 2: SPECIFIC AIMS

Schizophrenia (SCZ) is a severe mental disorder characterized by cognitive disruption, even prior to the initial psychotic episode. Cognitive dysfunction is the best indicator of functional outcome; however, current pharmacological therapies only alleviate positive symptoms of SCZ. In order to identify viable treatments for the cognitive symptoms, we must understand the underlying mechanisms of these deficits. The NMDAR has been widely implicated in SCZ pathology; pharmacological and transgenic models of NMDAR hypofunction recapitulate cognitive impairments seen in SCZ, such as working memory, attention, and cognitive flexibility. Importantly, these cognitive deficiencies manifest during early postnatal development, particularly in juvenile and adolescent stages. The NMDAR subunit NR2B is particularly important for working memory function. The NR2B-to-NR2A subunit switch is an epigenetically regulated process, and is critical in functional maturation of many brain regions. However, the NR2B subunit remains high in the PFC throughout development and into adulthood. In order to investigate how NMDARs are affected during development in SCZ, we employ two animal models: the neurodevelopmental MAM rat model and the transgenic hDISC1 mouse model. Our findings show aberrant NMDAR- and AMPAR-neurotransmission in the PFC of developing rats and mice. This proposal aims to further characterize the consequences of aberrant glutamatergic neurotransmission on synaptic physiology and to explore the mechanisms by which NMDAR hypofunction occurs in developing MAM animals and hDISC1 mice. We elucidated a mechanism of epigenetic mis-regulation that is aberrantly co-opted in the MAM PFC during development and contributes to juvenile NMDAR hypofunction.

Specific Aim 1: Determine the functional consequences of reduced synaptic NR2B protein levels in juvenile and adolescent MAM mPFC.

Part 1: Evaluate the functionality of glutamatergic neurotransmission in the juvenile and adolescent mPFC following prenatal MAM exposure. Hypothesis: significant reductions in synaptic NMDAR protein will impair NMDAR-mediated currents but not AMPAR-mediated currents.

Part 2: Characterize post-synaptic NMDAR and AMPAR function in juvenile and adolescent MAM animals and determine the contribution of NR2B subunits to NMDAR-mediated currents using a selective NR2B antagonist, Ro25-6981. Hypothesis: post-synaptic NMDARs will be functionally impaired due to loss of the persistently expressed NR2B subunit in juveniles, as well as decreased levels of the constitutive NR1 subunit in adolescent MAM PFC. MAM animals will display diminished NMDAR activation, which will occlude the effect of Ro25 blockade due to substantial loss of NR2B-containing NMDARs. AMPAR function will remain functionally intact during these developmental time points.

Specific Aim 2: Elucidate the mechanism of epigenetic mis-regulation underlying NMDAR hypofunction during early postnatal development in the MAM model for schizophrenia.

Explore the epigenetic mechanisms by which NR2B protein is downregulated in juvenile MAM animals using chromatin immunoprecipitation (ChIP). Hypothesis: enrichment of repressor proteins and histone modifications at the *Grin2b* promoter will be aberrantly increased in juvenile MAM PFC resulting in hyper-repression of the gene. Other NMDAR subunit genes, *Grin1* and *Grin2a*, will not show altered regulation by these epigenetic mechanisms.

Specific Aim 3: Characterize NMDAR expression and assess synaptic physiology in the prefrontal cortex of juvenile hDISC1 mutant mice

Part 1: Characterize synaptic NMDAR expression in juvenile PFC of hDISC1 mice constitutively expressing the truncated human DISC1 protein. Hypothesis: subunit expression patterns will indicate NMDAR hypofunction occurs during juvenile development of the mPFC in hDISC1 mice.

Part 2: Assess the consequences of altered NMDAR subunit expression on synaptic glutamatergic neurotransmission in juvenile hDISC1 mPFC. Hypothesis: NMDAR-mediated currents will be reduced in hDISC1 animals as a result of diminished synaptic NR2A protein. AMPAR-mediated currents will also be compromised in the context of hDISC1 expression and NMDAR hypofunction.

CHAPTER 3: EPIGENETIC MECHANISMS UNDERLYING NMDAR HYPOFUNCTION IN THE PREFRONTAL CORTEX OF JUVENILE ANIMALS IN THE MAM MODEL FOR SCHIZOPHRENIA

Abstract

Schizophrenia (SCZ) is characterized not only by psychosis, but also by working memory and executive functioning deficiencies, processes that rely on the prefrontal cortex (PFC). Because these cognitive impairments emerge prior to psychosis onset, we investigated synaptic function during development in the neurodevelopmental methylazoxymethanol (MAM) model for SCZ. In particular, we focused on NMDA receptors (NMDARs), which are an important substrate for learning and memory throughout the brain and are extensively implicated in PFC-dependent cognition as well as SCZ pathogenesis. Using Western blot and whole-cell patch-clamp electrophysiology, we found that the levels of synaptic NR2B protein are significantly decreased in juvenile MAM animals, and the function of NMDARs is substantially compromised. Both NMDA-mEPSCs and synaptic NMDA-eEPSCs are significantly reduced in layer V pyramidal neurons. This protein loss during the juvenile period is correlated with an aberrant increase in enrichment of the epigenetic transcriptional repressor REST and the repressive histone marker H3K27me3 at the *Grin2b* promoter, as assayed by ChIP-qPCR. Glutamate hypofunction has been a prominent hypothesis in the understanding of SCZ pathology; however, little attention has been given to the NMDAR system in the developing PFC in models for SCZ. Our work is the first to confirm that NMDAR hypofunction is a feature of early postnatal development, with epigenetic hyper-repression of the *Grin2b* promoter being a contributing factor. The selective loss of NR2B protein and subsequent synaptic dysfunction weakens PFC function during development and may underlie early cognitive impairments in SCZ models and patients.

Introduction

Glutamatergic hypofunction in the prefrontal cortex (PFC) is speculated to underlie cognitive symptoms (Lipska and Weinberger, 2000; Gainetdinov et al., 2001; Kantrowitz and Javitt, 2012) and contribute to the pathophysiology of schizophrenia (SCZ) (Coyle et al., 2002; Lau and Zukin, 2007; Snyder and Gao, 2013). Particularly, the N-methyl-D-aspartate receptor (NMDAR), a major glutamate receptor subtype, has received great attention (Martucci et al., 2006; Kristiansen et al., 2010; Weickert et al., 2013). NMDARs are heterotetrameric complexes composed of the requisite NR1 subunit, as well as two subunits of either NR2 (A-D) or NR3 (A-B) (Paoletti et al., 2013). The subunit composition of NMDARs dictates their physiological properties, pharmacological sensitivity, and functional complexity (Paoletti et al., 2013). NR2A and NR2B subunits are predominantly expressed in the adult brain and are essential for synaptic plasticity (Monyer et al., 1994). In the PFC, NR2B-containing NMDARs (NR2B-NMDARs) on pyramidal neurons are necessary for the sustained firing activity required to maintain working memory function in response to incoming stimuli (Goldman-Rakic, 1995; Wang et al., 2008). Moreover, NR2B subunits are persistently expressed throughout PFC maturation (Wang et al., 2008), whereas other brain regions undergo an NR2B-to-NR2A subunit switch. This switch is an essential element of experience-dependent maturation of many brain areas (Dumas, 2005); however, PFC maturation is characterized by different developmental milestones (Monaco et al., 2015).

Deficits in NMDAR function, particularly NR2B-NMDARs, impair working memory (Wang et al., 2013), which is a constituent process of many higher-order cognitive capacities including cognitive flexibility and executive functioning (Goldman-Rakic, 1995). These cognitive deficits, among others, are commonly reported in the SCZ population (Reichenberg, 2010) and are the best predictor of functional outcome (Bowie and Harvey, 2006). Notably, behavioral manifestations of impaired cognition are evident prior to psychosis in patients (Jones et al., 1994; Cannon et al., 2000; Rosso et al., 2000). Thus, a greater understanding of the developmental vulnerabilities that produce this early cognitive dysfunction is critical.

We use the methylazoxymethanol acetate (MAM) neurodevelopmental model for SCZ to investigate PFC postnatal development, with a focus on NMDAR mis-expression (Gilmour et al., 2012). Lending both face and predictive validities to this model for SCZ, MAM animals demonstrate a greater degree of MK801-induced hyper-locomotion that is sensitive to treatment with antipsychotics (Moore et al., 2006; Lodge and Grace, 2009). Additionally, deficiencies in PFC-dependent cognition, including working memory and cognitive flexibility, are also evident in this model (Gourevitch et al., 2004; Flagstad et al., 2005; Lavin et al., 2005; Moore et al., 2006). Furthermore, MAM animals have altered hippocampal NMDAR expression, and impaired learning and memory function in juvenile and adolescent stages of development (Hradetzky et al., 2012; Snyder and Gao, 2013), demonstrating the relevance of this model in assessing NMDAR hypofunction in early postnatal development.

Epigenetic mechanisms regulate glutamatergic gene expression in normal brain development (Stadler et al., 2005), with alterations in histone and DNA modifications implicated in neuropsychiatric disorders, including SCZ (Akbarian, 2010a; b; 2014; Nestler et al., 2016). A possible mechanism underlying NMDAR hypofunction is repression of glutamatergic genes within the PFC, as reported in post-mortem SCZ brain tissue (Numata et al., 2014). In fact, in the MAM model, aberrant histone modification patterns are evident in the developing medial PFC (mPFC) (Mackowiak et al., 2014). However, two major questions remain: (1) is NMDAR hypofunction evident in early postnatal development of the PFC in this SCZ model, and (2) is there an epigenetic mechanism that may contribute to this endophenotype? In the following experiments, we seek to address these questions by characterizing the expression profile, synaptic physiology, and regulation by epigenetic mechanisms of NMDARs in the PFC of juvenile MAM and saline animals.

Materials and Methods

Animals

All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Drexel University College of Medicine Institutional Animal Care and Use Committee. Adult pregnant Sprague-Dawley rats were purchased from Charles River Laboratories International, Inc. (Wilmington, MA) and were intraperitoneally (I.P.) injected with 25 mg/kg methylazoxymethanol acetate (MAM) or vehicle (saline) on embryonic day 17 (E17), as previously reported (Hradetzky et al., 2012; Snyder et al., 2013; Xing et al., 2016). Pups were usually born on E21/P0, and were weaned and rehoused on postnatal day 21 (P21). Only male animals aged P17-23 (classified as juveniles (Spear, 2000; Wang and Gao, 2009; 2010)) were used in all experiments.

Tissue collection and western blot for synaptosomal proteins

Animals were anesthetized with Euthasol (0.2 ml kg^{-1}, I.P.) until unresponsive to toe pinch, and then the brain was extracted. The brain region containing the PFC was dissected, homogenized in sucrose buffer (in mM: 320 sucrose, 4 HEPES-NaOH buffer, pH 7.4, 2 EGTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 50 sodium fluoride, 10 sodium pyrophosphate, 20 glycerophosphate, with 1 μg/ml leupeptin and 1 μg/ml aprotinin), and then centrifuged at 1000 g for 10 minutes to remove large cell

fragments and nuclear materials. The resulting supernatant was centrifuged at 17,000 g for 15 minutes to yield cytoplasmic proteins in the supernatant. The pellet from this spin was re-suspended in homogenization buffer and centrifuged at 17,000 g for an additional 15 minutes to yield washed synaptosomes. The synaptosomal fraction then was hypo-osmotically lysed and centrifuged at 25,000 g for 20 minutes to yield synaptosomal plasma membranes in the pellet.

A bicinchoninic acid (BCA) protein assay was performed to determine protein concentration. The protein sample was mixed with Laemmli sample buffer, boiled for 5 minutes, and separated on a 7.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (EMD-Millipore, Billerica, MA). The membrane was blocked in 5% nonfat milk and probed with primary anti-serum. Each blot was probed for anti-mouse NR1 (Invitrogen, 32-5000, 1:5000, Carlsbad, CA), anti-rabbit NR2A (EMD-Millipore, 04-901, 1:4000), anti-mouse NR2B (EMD-Millipore, 05-920, 1:2000), anti-rabbit NR3A (EMD-Millipore, 07-356, 1:4000), anti-rabbit NR3B (Tocris, 2060, 1:4000), and anti-mouse actin (Sigma, A5316, 1:100,000, St Louis, MO), which was used as a loading control. The blots were incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG secondary antibody (Vector Laboratories), and proteins were visualized using enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Piscataway, NJ). Protein expression of each subunit was evaluated by densitometry using Image-J software. Additionally, samples from each animal were run at least 4 times to minimize interblot variance. Raw values for NMDAR subunit proteins were normalized to actin and this ratio was further normalized to the first

usable band on each membrane. The normalized values for each protein were averaged per animal to yield the mean and standard error per group.

Whole-cell patch-clamp electrophysiology

Male juvenile (aged P17-23) saline and MAM animals were anesthetized with Euthasol (0.2 ml kg−1, I.P.), the brains were removed, and coronal slices through the prelimbic PFC were cut (300 μm) into an ice-cold bath of oxygenated artificial cerebrospinal fluid (aCSF, in mM: 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, 26 $NaHCO_3$, and 10 dextrose, pH 7.4) using a VT-1200S vibratome tissue slicer (Leica Microsystems, Wetzlar, Germany). Slices were transferred to a holding chamber, submerged in oxygenated aCSF at 35°C for one hour and then remained at room temperature until used for recording. Slices were placed into a recording chamber mounted on an Olympus upright microscope (BX51, Olympus America, Center Valley, PA), where they were continuously bathed in oxygenated aCSF. Neurons were visualized with infrared differential interference video microscopy. All experiments were conducted with an Axon MultiClamp 700B amplifier (Molecular Devices), and data were acquired using pCLAMP 9.2 software and analyzed using Clampfit 10.2 software (Molecular Devices, Novato, CA).

Spontaneous and miniature excitatory post-synaptic currents

To record AMPAR- and NMDAR-mediated spontaneous or miniature excitatory post-synaptic currents (EPSCs), somatic whole-cell voltage-clamp recordings were obtained from layer V pyramidal cells using patch electrodes with an open tip resistance of 5–7 MΩ (internal solution, in mM: 110 D-gluconic acid, 110 CsOH, 10 $CsCl_2$, 1 EGTA, 1 $CaCl_2$, 5 QX-314, 1 ATP-Mg, 10 HEPES, at pH 7.3, adjusted with CsOH). In voltage-clamp mode, the membrane potentials were held at -70 mV in the presence of picrotoxin (50 μM) to record AMPAR-mediated spontaneous EPSCs (AMPA-sEPSCs), or with both picrotoxin and tetrodotoxin (TTX, 0.5 μM) for miniature EPSCs (AMPA-mEPSCs). mEPSCs were recorded for at least 5 minutes and then DNQX (20 μM) was washed on to block AMPARs. When all currents ceased, the membrane potential was slowly ramped up to 60 mV. NMDA-mEPSCs were then recorded for at least 5 minutes. A typical s/mEPSC event was selected to create a sample template for event detections within a 5-min period for each data file. The frequency (number of events) and amplitude of the individual events were examined with the threshold set at the medium level (i.e., 5 within a range of 1 to 9) in Clampfit. The detected events were then visually inspected to ensure specificity.

Evoked excitatory post-synaptic currents

To record evoked NMDAR-mediated EPSCs (NMDA-eEPSCs), a bipolar stimulating electrode was placed in layer II/III of the mPFC approximately 200-300 μm from visually-identified layer V pyramidal cells. Picrotoxin (50 μM) was used to block $GABA_A$R-mediated neurotransmission. Cells were held at 60 mV to record NMDA-

eEPSCs for 5 min before and 5 min after bath application of a selective NR2B antagonist Ro25-6981 (Ro25, 1 μM). EPSCs were evoked through the stimulating electrode using a physiologically relevant 10-pulse, 20 Hz train with a 0.1 Hz stimulating interval. Stimulus intensity was adjusted until 50-100 pA NMDA-eEPSC amplitude responses were achieved. Cells with an unstable baseline or unusual response patterns throughout recording were removed from the analysis. The EPSC recordings were monitored throughout each experiment by implementing a -100 pA current pulse with a 200 ms duration, and all cells with >20% change in series resistance during recording were discarded. Traces were analyzed for amplitude relative to the first post-stimulus baseline and cumulative charge (charge transfer, nA*ms), as well as paired-pulse ratio (PPR).

Western blot for histone proteins

PFC tissue was collected as described above. The Epiquik Total Histone Extraction Kit (OP-0006, Epigentek) was used to isolate the histone fraction. A BCA assay was used to determine protein concentration. After boiling in Laemmli buffer for 5 minutes, 15 μg of protein were loaded on a 15% SDS-PAGE gel. After electrophoresis, blots were transferred to pore size 0.20 μm polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk and separately probed with the following primary antibodies: H3K9ac (Abcam, ab10812, 1:5000, Cambridge, UK), H3K18ac (Abcam, ab1191, 1:5000), H3K27ac (Abcam, ab4729, 1:5000), H3K27me3 (Abcam, ab6002, 1:2000), and total histone H3 (Novus, 1:100,000) was used as a loading control. Blots were incubated with horseradish peroxidase-coupled

anti-rabbit or anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA), and proteins were visualized using enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Piscataway, NJ). Protein expression for each histone modification was evaluated by densitometry using Image-J software. Samples from each animal were run at least 4 times to minimize interblot variance.

Chromatin immunoprecipitation and quantitative PCR assay

PFC tissue was dissected from saline and MAM animals at P21 and pooled for the chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR) assays. Pooled samples from 4 saline and 4 MAM animals yielded the total tissue quantity (120 mg/group) necessary for shearing using the truChIP™ Chromatin Shearing Kit (Covaris, PN520083, Woburn, MA) and E220 evolution Focused-ultrasonicator (Covaris, 500239UPE). The chromatin was immunoprecipitated using the EZ-Magna ChIP™ A-Chromatin Kit (EMD-Millipore; 17-408) and three separate reactions with 50 mg of sheared chromatin were carried out using: (i) 2 μg of REST antibody (EMD-Millipore; 07-579), (ii) 1 μg H3K27me3 antibody (Abcam; ab6002), and (iii) a mock reaction containing all reagents with IgG as a control. To isolate the chromatin from these experiments, the antibodies and cross-linked histone modifications and modifying proteins were removed with repetitive washes and Proteinase K-mediated digestion. The eluted material was purified using a QIAquick PCR kit (Qiagen; 28104, Manchester, UK) and was directly used for qPCR. Positive and negative control primer sets were purchased from Active Motif (71027, 71024, Carlsbad, CA). PCRs were performed in a 20-μL

reaction containing purified chromatin DNA, 1 mM Power SYBR Green PCR Master Mix (Applied Biosystems; 4367659), and 10 mM of both forward and reverse primers (Table 1). PCR was performed in triplicate using an ABI 7500 system (Applied Biosystems, 4406985), yielding 6 data points per group. Fold change in DNA was determined by the ΔΔCt method.

Table 1

	Forward primers	Reverse primers
Grin2b (PR1)	GGTCAAGCTGCCTCTCCAT	GCAGAGCAGAAGGAAATGTATTCG
Grin2a	TCCGGAGTGGAACAGAAAGC	CTCATCCAGCCCCATGCT
Grin1	TCCCTGCTTCCTCTCTTGGA	AATGACTGCTGGGAGCAAGAC

Forward and reverse primer sequences for *Grin2b* (proximal promoter region), *Grin2a*, and *Grin1* used in ChIP-qPCR experiments.

Data analysis

Statistical analyses were carried out using SPSS Statistics version 24.0 (IBM, Armonk, NY). Normality and homogeneity of variance testing determined the use of parametric or non-parametric tests for each dataset. Protein expression analyses, AMPA- and NMDA-s/mEPSC amplitude and frequency, and ChIP-qPCR enrichment levels were compared using unpaired Student's t-test or Mann-Whitney U tests. Enrichment of REST and H3K27me3 at *Grin1*, *Grin2a*, and *Grin2b* between groups and across promoter regions were analyzed using a One-Way ANOVA with a Tukey's post-hoc. Evoked data were analyzed using repeated measures ANOVA to compare amplitude or charge transfer

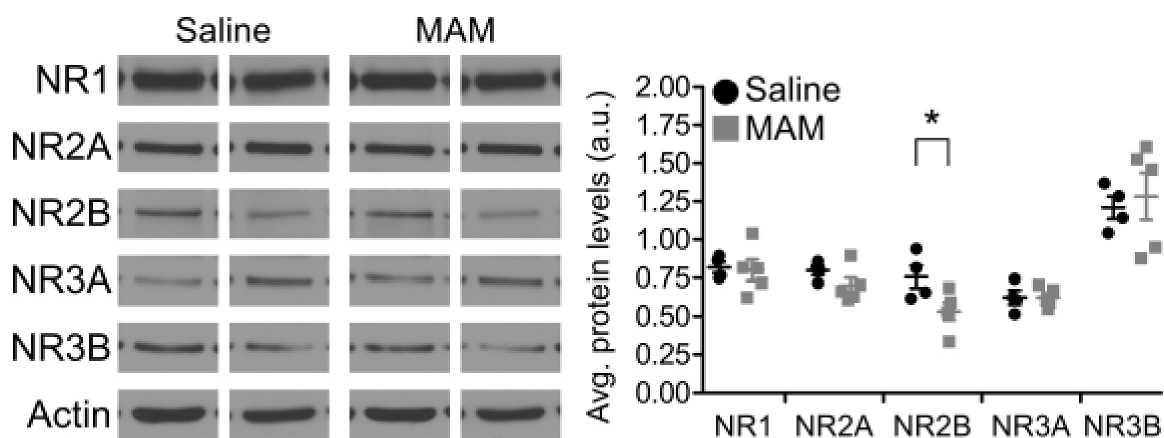
between groups and across pulses. Student's t-tests or Mann-Whitney U tests were used for analysis of amplitude or charge transfer at each pulse between groups. All data are presented as mean ± standard error (S.E.). Single, double, and triple asterisks represent $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Results

NMDAR protein levels are reduced in the PFC of juvenile MAM rats

To determine if NMDARs are altered during prefrontal development in this SCZ model, we first examined how prenatal MAM exposure affects NMDAR protein levels in juvenile (P21) animals. We collected synaptic plasma membrane fractions from saline and MAM animals and probed for NMDAR subunit levels via Western blot. Actin was used as a loading control. We found no significant differences between saline and MAM animals in NR1, NR2A, NR2B, NR3A, or NR3B subunit levels at P14 (data not shown; Saline n=3-5 rats, MAM n=3-5 rats; p=0.651, p=0.360, p=0.892, p=0.172, and p=0.230, respectively). However, in the PFC of late juvenile (P21) MAM animals, there was a significant decrease in NR2B protein compared to saline animals (Figure 3; saline 0.76±0.07, n=4; MAM 0.53±0.06, n=5; p=0.045). No difference was found for NR1, NR2A, NR3A, or NR3B levels at P21 (Saline n=4, MAM n=5; p=0.842, 0.170, 0.994, and 0.681, respectively). Together, these data indicate that prenatal MAM exposure alters NMDAR protein levels during the critical juvenile period of the developing PFC. Moving forward, experiments in juvenile animals encompassed the late juvenile (P21) time point.

Figure 3



NR2B protein is reduced in synaptosomes from PFC of juvenile MAM animals. Left, representative Western blot bands, and right, summary graph, show NR2B protein is significantly reduced in synaptosomes from juvenile MAM PFC (NR2B: saline 0.76±0.07, n=4; MAM 0.53±0.06, n=5; p=0.045). No difference at P21 was found for the other NMDAR subunits (saline n=4, MAM n=5; NR1 p=0.842, NR2A p=0.170, NR3A p=0.994, and NR3B p=0.681).

AMPA-s/mEPSCs are comparable between juvenile saline and MAM layer V pyramidal neurons in mPFC

We next sought to determine if the reduced NMDAR subunit protein levels in juvenile MAM animals resulted in altered glutamatergic neurotransmission. We utilized whole-cell patch-clamp to record from layer V pyramidal neurons in the mPFC and examined AMPA-s/mEPSCs, allowing us to understand the functional state of AMPARs in response to inputs from multiple brain regions. We first recorded sEPSCs that presumably resulted from action potentials of pre-synaptic neurons, as well as the spontaneous release of neurotransmitter at synapses, and then washed on tetrodotoxin to block action potentials in the slice and recorded mEPSCs. We held the cell at -70 mV with $GABA_AR$ function blocked to assess if MAM exposure had an effect on functional AMPARs and if there were changes in pre-synaptic function. We found no differences in either the frequency or amplitude of the AMPA-sEPSCs or -mEPSCs in juvenile (P17-23) MAM and saline rats (Figure 4A; sEPSCs: frequency, Hz, saline 2.12±0.42, n=14 cells; MAM 1.99±0.31, n=23 cells; p=0.731. Amplitude, pA, saline 17.80±1.29; MAM 16.26±0.54, p=0.506. Figure 4B; mEPSCS: frequency, Hz, saline 1.34±0.32, n=13; MAM 2.28±0.66, n=15; p=0.387. Amplitude, pA, saline 14.27±0.59; MAM 14.33±1.02, p=0.961).

Amplitude of NMDA-mEPSCs is diminished in juvenile MAM mPFC

We next held the cell at 60 mV while blocking $GABA_A$Rs and AMPARs to examine changes in NMDAR function. In juveniles, we saw a significant decrease of NMDA-mEPSC amplitude, but not frequency, in MAM layer V mPFC neurons compared with those in saline animals (Figure 5; mEPSCs: frequency, Hz, saline 1.16±0.18, n=14; MAM 1.51±0.38, n=11; p=0.424. Amplitude, pA, saline 32.06±2.98, MAM 20.73±2.12; p=0.007). Thus, prenatal MAM exposure impairs functional NMDARs, but not AMPARs, in the developing mPFC; however, it is unclear whether pre- or post-synaptic dysfunction contributes to deficient NMDAR neurotransmission.

Juvenile MAM animals have a significant reduction in NMDARs in mPFC layer V pyramidal neurons

To determine whether synaptic function of layer V pyramidal cells is affected by prenatal MAM exposure, we recorded NMDAR-mediated EPSCs evoked by stimulating layer II/III afferents with a 10-pulse, 20 Hz train. To evaluate the functional state of NMDARs during development, we compared the amplitude and charge transfer of the evoked EPSCs between saline and MAM animals. $GABA_A$R-mediated neurotransmission was blocked with picrotoxin, and stimulation was adjusted until EPSC amplitudes of 50-100 pA were achieved. We found that amplitudes of NMDA-eEPSCs are reduced in juvenile MAM animals (Figure 6; amplitude, pA, Saline n=10, MAM n=10, Repeated Measures ANOVA: group: $F(1,18)=9.64$, $p=0.006$, pulse: $F(1.69, 30.43)=5.39$, $p=0.013$; interaction: $F(1.69, 30.43)=1.17$, $p=0.316$). Post-hoc analysis reveals amplitude is significantly diminished in MAM animals at all 10 pulses ($p<0.05$). Similarly, charge

transfer of NMDA-eEPSCs is significantly reduced in MAM animals (Figure 6; charge transfer, nA*ms, Repeated Measures ANOVA: group: $F(1,18)=7.36$, $p=0.014$, pulse: $F(1.06, 19.08)=55.51$, $p<0.001$; interaction: $F(1.06, 19.08)=6.25$, $p=0.020$). Simple main effects analysis shows charge transfer is significantly decreased in juvenile MAM mPFC at all pulses ($p<0.05$). We did not find any changes in paired-pulse ratio (PPR), a measure of pre-synaptic release probability, which indicates these reductions in synaptic transmission are restricted to alterations in post-synaptic receptor function (Figure 6; $t(18)=1.08$, $p>0.05$). We also did not find any significant differences in stimulation intensity between the saline and MAM groups (Figure 6; $U=42$; $p>0.05$). Together, these data demonstrate a loss of post-synaptic NMDARs in layer V pyramidal neurons in juvenile MAM mPFC.

Juvenile MAM layer V pyramidal cells exhibit a loss of NR2B-NMDARs

To evaluate whether the NR2B protein loss seen in Western blot experiments and the reduction of synaptic NMDA-eEPSC responses reflects a loss of post-synaptic NR2B-NMDARs specifically, we bath-applied Ro25-6981 (Ro25, 1 μM), a selective NR2B antagonist, while holding the cell at 60 mV. Reductions in evoked NMDA amplitude and charge transfer following Ro25 treatment indicate a blockade of solely NR2B-NMDARs. Following bath application of Ro25 in saline cells, we found a significant reduction of NMDA-eEPSC amplitude (Figure 7A; amplitude, pA; Saline before Ro $n=10$, Saline after Ro $n=8$, group: $F(1, 16)=5.04$; $p=0.039$; pulse: $F(1.36, 21.69)=5.32$, $p=0.022$; interaction: $F(1.36, 21.69)=0.38$, $p=0.608$; post-hoc: pulses 3-5,

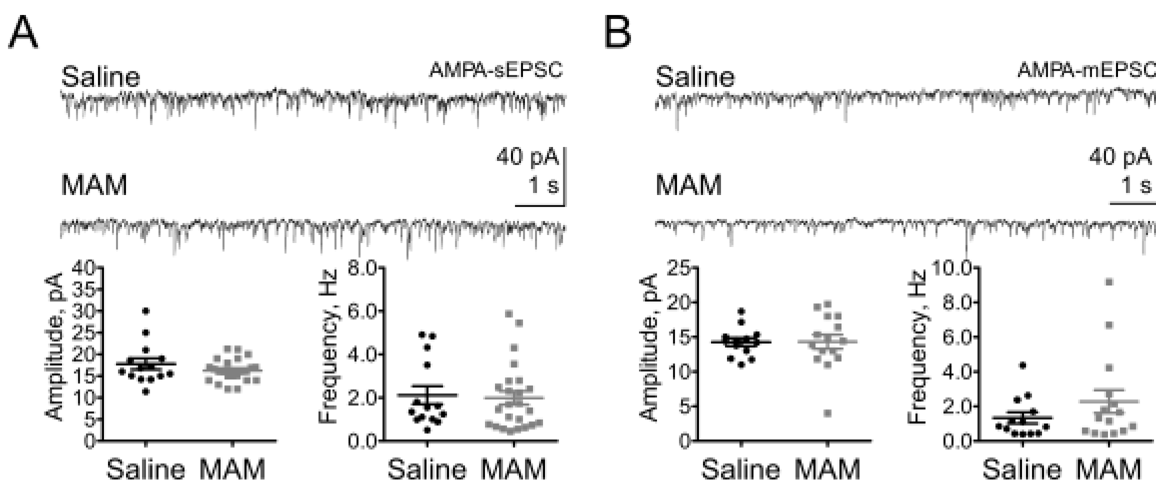
$p<0.05$, pulses 1-2, 6-10, $p>0.05$). Surprisingly, charge transfer is only marginally diminished following Ro25 wash-on in saline cells (Figure 7A; charge transfer, nA*ms; group: $F(1, 16)=3.06$, $p=0.10$; pulse: $F(1.05, 16.73)=38.02$, $p<0.001$; interaction: $F(1.05, 16.73)=2.88$, $p=0.107$; post-hoc: pulses 1&4, $p<0.05$, pulses 2-3, 5-10, $p>0.05$). However, bath application of Ro25 does not reduce amplitude or charge transfer in juvenile MAM animals, likely due to the significant loss of NMDARs (Figure 7B; amplitude, pA; MAM before Ro $n=10$, MAM after Ro $n=9$, group: $F(1, 17)=0.43$; $p=0.519$; pulse: $F(1.83, 31.18)=3.42$, $p=0.049$, interaction: $F(1.83, 31.18)=0.25$, $p=0.763$; charge transfer, nA*ms; group: $F(1, 17)=0.19$, $p=0.671$; pulse: $F(1.05, 17.79)=38.85$, $p<0.001$; interaction: $F(1.05, 17.79)=13.90$, $p=0.691$). Post-hoc analyses reveal no significant differences in amplitude or charge transfer between saline or MAM animals after Ro25 treatment. Thus, we can conclude synaptic NR2B-NMDARs are reduced in juvenile MAM animals as confirmed by the occlusion of Ro25's effect on layer V pyramidal cells in juvenile MAM mPFC.

Synaptic AMPARs remain functionally intact in juvenile MAM mPFC

To further confirm AMPAR function is unchanged following prenatal MAM exposure, we evaluated the effect of layer II/III stimulation on AMPA-eEPSCs in saline and MAM layer V pyramidal neurons. To record AMPA-eEPSCs, the cells were held at -70 mV in the presence of picrotoxin (50 μM) to block $GABA_A$R-mediated neurotransmission. EPSCs were evoked as described above; stimulation intensity was increased until 50-100 pA responses were elicited. We found no significant differences in

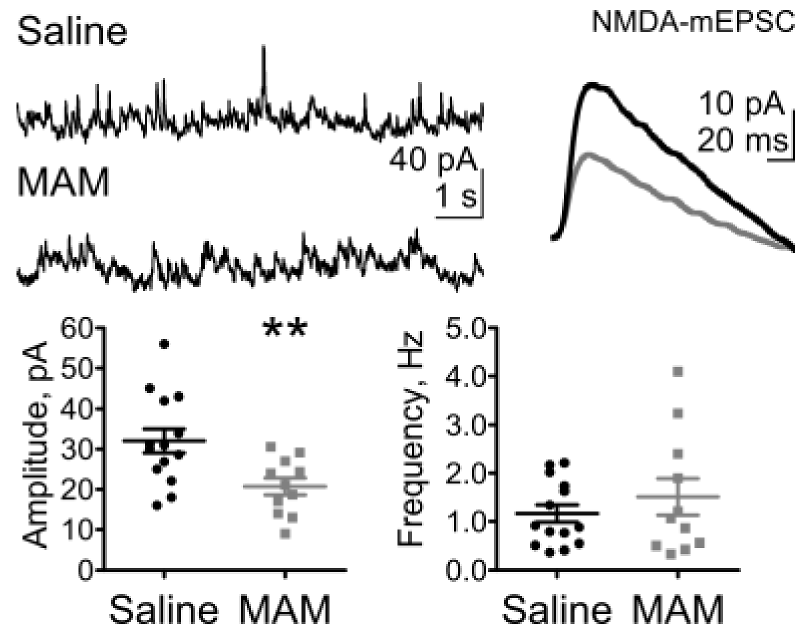
amplitude or charge between juvenile saline and MAM animals (Figure 8; amplitude, pA, Saline n=11, MAM n=9, Repeated Measures ANOVA: group: $F(1,18)=3.09$, $p=0.096$, pulse: $F(2.49, 44.84)=17.16$, $p<0.001$; interaction: $F(2.49, 44.84)=0.88$, $p=0.441$. Charge transfer, nA*ms, Repeated Measures ANOVA: group: $F(1,18)=0.77$, $p=0.391$, pulse: $F(1.03, 18.57)=64.09$, $p<0.001$; interaction: $F(1.03, 18.57)=0.77$, $p=0.395$). Post-hoc analysis further confirmed no significant differences in amplitude or charge transfer between saline and MAM animals. In addition, there were no significant differences in PPR or stimulation intensity between the two groups (Figure 8; PPR: saline 1.03 ± 0.05, MAM 0.89 ± 0.11, $t(11.66)=1.25$, $p=0.236$. Stimulation intensity: saline 247.36 ± 45.40, MAM 256.44 ± 79.65, $U=37.5$, $p=0.370$). Together, these data confirm that hypofunction of NR2B-NMDARs is unique to juvenile MAM mPFC.

Figure 4



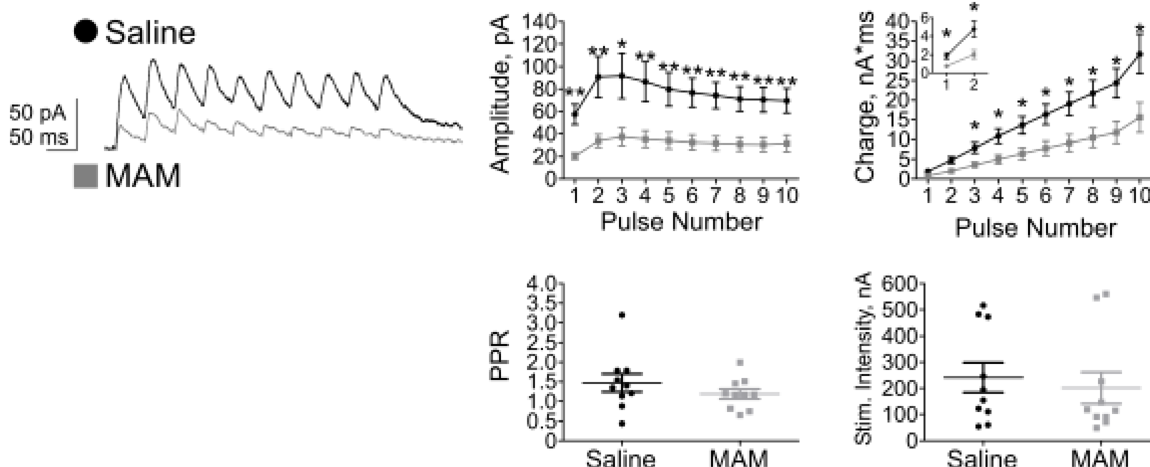
AMPAR-mediated neurotransmission is unaffected in juvenile mPFC by prenatal MAM exposure. (A) AMPA-sEPSCs are not significantly affected by prenatal MAM exposure. sEPSCs: frequency, Hz, saline 2.12±0.42, n=14 cells; MAM 1.99±0.31, n=23 cells; p=0.731; amplitude, pA, saline 17.80±1.29; MAM 16.26±0.54, p=0.506. (B) AMPA-mEPSCs are also not significantly different between saline and MAM mPFC. mEPSCS: frequency, Hz, saline 1.34±0.32, n=13; MAM 2.28±0.66, n=15; p=0.387; amplitude, pA, saline 14.27±0.59; MAM 14.33±1.02, p=0.961.

Figure 5



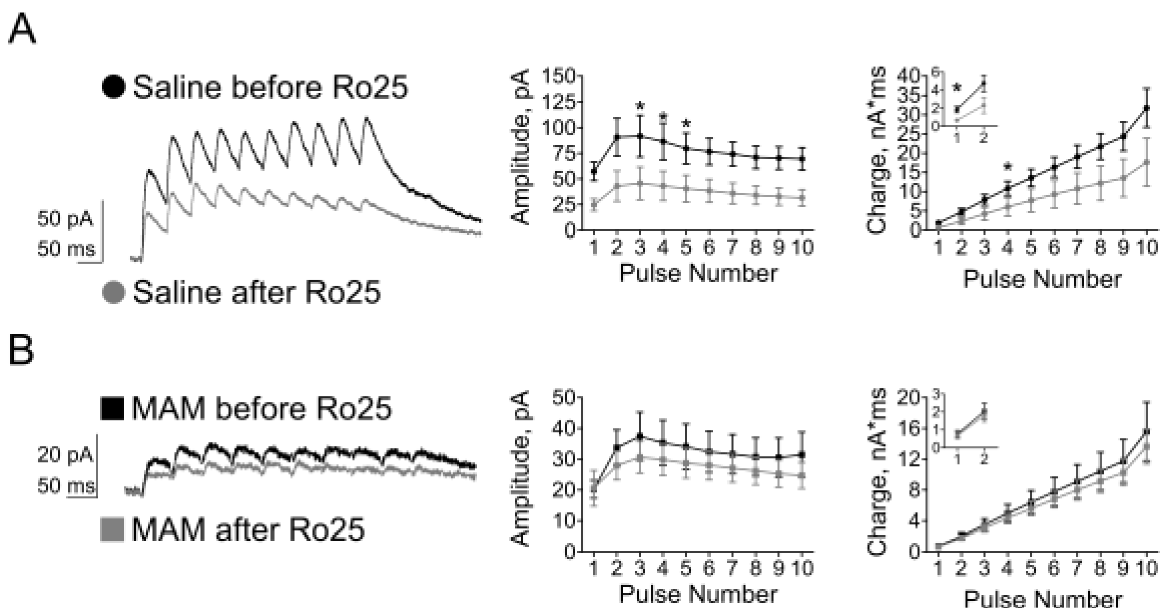
NMDAR neurotransmission is selectively impaired in juveniles following prenatal MAM exposure. Juvenile NMDA-mEPSCs have significantly reduced amplitude (right traces), but no change in frequency of currents. mEPSCs: frequency, Hz, saline 1.16±0.18, n=14; MAM 1.51±0.38, n=11; p=0.387; amplitude, pA, saline 32.06±2.98, MAM 20.73±2.12; p=0.007.

Figure 6



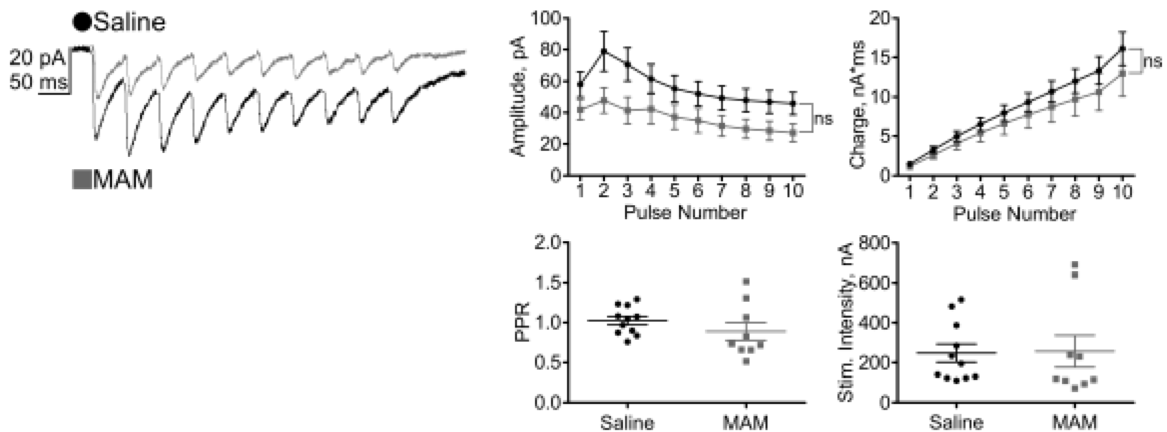
The number of NMDARs is diminished in layer V pyramidal neurons of juvenile MAM mPFC. Using repeated measures ANOVA with pulse as a within-subject factor and group as a between-subjects factor, there is a significant group effect on both amplitude and charge transfer of NMDA-eEPSCs. At each pulse, there is a significant decrease in both amplitude and charge transfer in juvenile MAM animals. Amplitude, pA, Saline n=10, MAM n=10, Repeated Measures ANOVA: group: F(1,18)=9.64, p=0.006, pulse: F(1.69, 30.43)=5.39, p=0.013; interaction: F(1.69, 30.43)=1.17, p=0.316; post-hoc: pulses 1-10, p<0.05. Charge transfer, nA*ms, Repeated Measures ANOVA: group: F(1,18)=7.36, p=0.014, pulse: F(1.06, 19.08)=55.51, p<0.001; interaction: F(1.06, 19.08)=6.25, p=0.020; post-hoc: pulses 1-10, p<0.05. There were no significant differences in PPR (t(18)=1.08, p>0.05) or stimulation intensity between the saline and MAM groups (U=42; p>0.05).

Figure 7



Synaptic NR2B-NMDARs are selectively diminished in juvenile MAM animals. (A) Ro25, an NR2B antagonist, significantly decreases the amplitude of saline NMDA-eEPSCs, with strongly trending effects on charge transfer. Amplitude, pA; Saline before Ro n=10, Saline after Ro n=8, group: F(1, 16)=5.04; p=0.039; pulse: F(1.36, 21.69)=5.32, p=0.022; interaction: F(1.36, 21.69)=0.38, p=0.608; post-hoc: pulses 3-5, p<0.05, pulses 1-2, 6-10, p>0.05. Charge transfer, nA*ms; group: F(1, 16)=3.06, p=0.10; pulse: F(1.05, 16.73)=38.02, p<0.001; interaction: F(1.05, 16.73)=2.88, p=0.107; post-hoc: pulses 1&4, p<0.05, pulses 2-3, 5-10, p<0.10. (B) Ro25 does not significantly decrease amplitude or charge transfer of NMDA-eEPSCs in MAM animals. Amplitude, pA; MAM before Ro n=10, MAM after Ro n=9, group: F(1, 17)=0.433; p=0.519; pulse: F(1.83, 31.18)=3.42, p=0.049; interaction: F(1.83, 31.18)=0.25, p=0.763. Charge transfer, nA*ms; group: F(1, 17)=0.187, p=0.671; pulse: F(1.05, 17.79)=38.85, p<0.001; interaction: F(1.05, 17.79)=13.90, p=0.691. Post-hoc analyses reveal no significant differences in amplitude or charge transfer in MAM mPFC after Ro25 treatment.

Figure 8



AMPA-eEPSCs are functionally intact in MAM layer V pyramidal neurons compared to saline mPFC. Using repeated measures ANOVA, we found no significant differences in amplitude or charge across pulses between juvenile saline and MAM animals. Amplitude, pA, Saline n=11, MAM n=9, group: $F(1,18)=3.09$, $p=0.096$, pulse: $F(2.49, 44.84)=17.16$, $p<0.001$; interaction: $F(2.49, 44.84)=0.88$, $p=0.441$. Charge transfer, nA*ms, group: $F(1,18)=0.77$, $p=0.391$, pulse: $F(1.03, 18.57)=64.09$, $p<0.001$; interaction: $F(1.03, 18.57)=0.77$, $p=0.395$). Post-hoc analysis further confirmed no significant differences at each pulse in amplitude or charge transfer between saline and MAM animals. In addition, there were no significant differences in PPR (saline 1.03±0.05, MAM 0.89±0.11, $t(11.66)=1.25$, $p=0.236$) or stimulation intensity (saline 247.36±45.40, MAM 256.44±79.65, $U=37.5$, $p=0.370$) between the two groups.

H3K27me3 is selectively increased in the PFC of juvenile MAM rats

To evaluate whether epigenetic mechanisms may be implicated in regulating NMDAR expression during postnatal development, we assayed a number of histone modifications relevant to learning and memory processes. This exploratory approach would lend insight into whether a mis-regulation of the epigenome underlies juvenile NMDAR hypofunction in the MAM model for SCZ. We collected PFC tissue from saline and MAM animals at P21 and processed the tissue to isolate the histone fraction. Using Western blotting to determine levels of acetylation and methylation on histone H3, we found juvenile MAM animals had a significant increase in H3K27me3 (Figure 9; Saline 0.93±0.04, n=5; MAM 1.05±0.01, n=6; p=0.045), but no change in the acetylation markers assayed (H3K9ac, p=0.263; H3K18ac, p=0.524; H3K27ac, p=0.294). These data suggest epigenetic modifications are facilitating the NR2B protein loss observed in the juvenile MAM PFC. We further tested this possibility using ChIP-qPCR as described below.

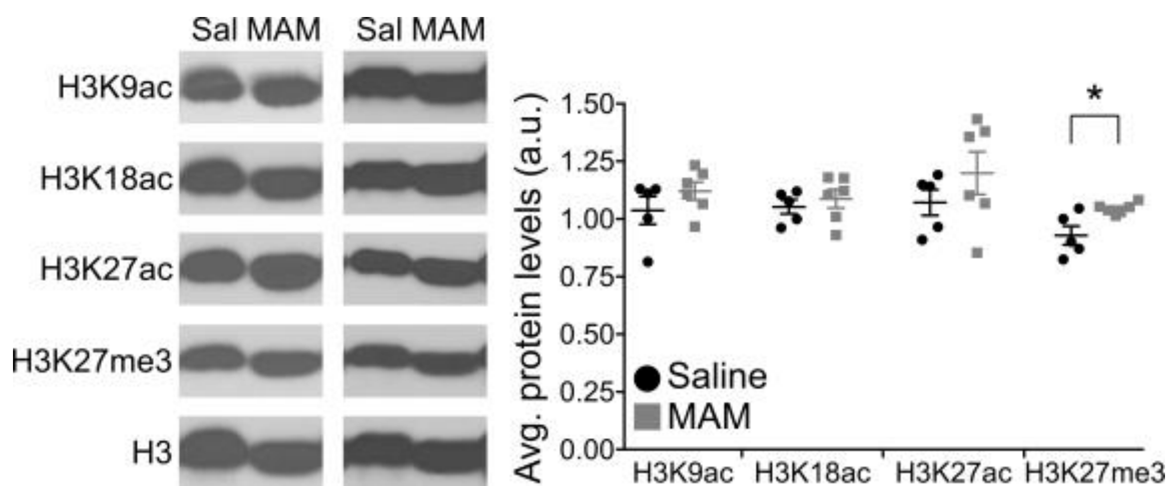
The proximal promoter region of Grin2b is highly enriched in the transcriptional repressor REST and the histone marker H3K27me3 in juvenile MAM PFC

Our findings thus far reveal a marked increase of the repressive epigenetic marker H3K27me3 with concomitant reduction of NR2B protein levels and functional NR2B-NMDARs in juvenile MAM PFC. Using the evidence of increased H3K27me3 in juvenile MAM PFC, we carried out ChIP-qPCR assays to measure the enrichment levels

of the repressor protein REST and repressive histone marker H3K27me3 at the proximal promoter region of the *Grin2b* gene (NR2B-encoding). *Grin2b* has response element (RE1) motifs within its promoter region to which REST can be recruited (Rodenas-Ruano et al., 2012). To test the specificity of REST-mediated repression, we also assessed the enrichment levels of REST at the RE1 sites of *Grin1* (NR1-encoding) and *Grin2a* (NR2A-encoding). For saline controls, we found REST was significantly enriched at *Grin2b*, but not at *Grin1* or *Grin2a* RE1 sites (Figure 10A; saline REST, One-Way ANOVA, group: *Grin2b* 20.86±5.23, n=4 rats yielding 6 data points from qPCR; *Grin2a* 3.85±1.04; *Grin1* 3.05±1.13; $F(2, 17)=9.17$, $p=0.003$; Tukey's post-hoc: saline *Grin2b* vs *Grin2a*, $p=0.005$; saline *Grin2b* vs *Grin1*, $p=0.007$; saline *Grin2a* vs *Grin1*, $p=0.985$). Similarly, repressive marker H3K27me3 was significantly enriched at the *Grin2b* site, but not at the *Grin1* or *Grin2a* RE1 sites in saline PFC (Figure 10B; saline H3K27me3, One-Way ANOVA, group: *Grin2b* 230.03±19.24, n=4; *Grin2a* 102.82±28.49; *Grin1* 57.88±12.41; $F(2, 17)=17.91$, $p<0.001$; Tukey's post-hoc: saline *Grin2b* vs *Grin2a*, $p=0.002$; saline *Grin2b* vs *Grin1*, $p<0.001$; saline *Grin2a* vs *Grin1*, $p=0.316$). Together, these data demonstrate that REST is normally more highly enriched within the proximal promoter region of *Grin2b* than at either *Grin1* or *Grin2a* RE1 sites. This confirms the selective role of REST in regulating *Grin2b* expression, as has been shown in the hippocampus (Rodenas-Ruano et al., 2012). Further, repressive marker H3K27me3 enrichment at the *Grin2b* promoter region, but not at *Grin1* or *Grin2a*, lends evidence to the notion that *Grin2b* expression is tightly regulated by these mechanisms compared to these other major NMDAR subunit proteins.

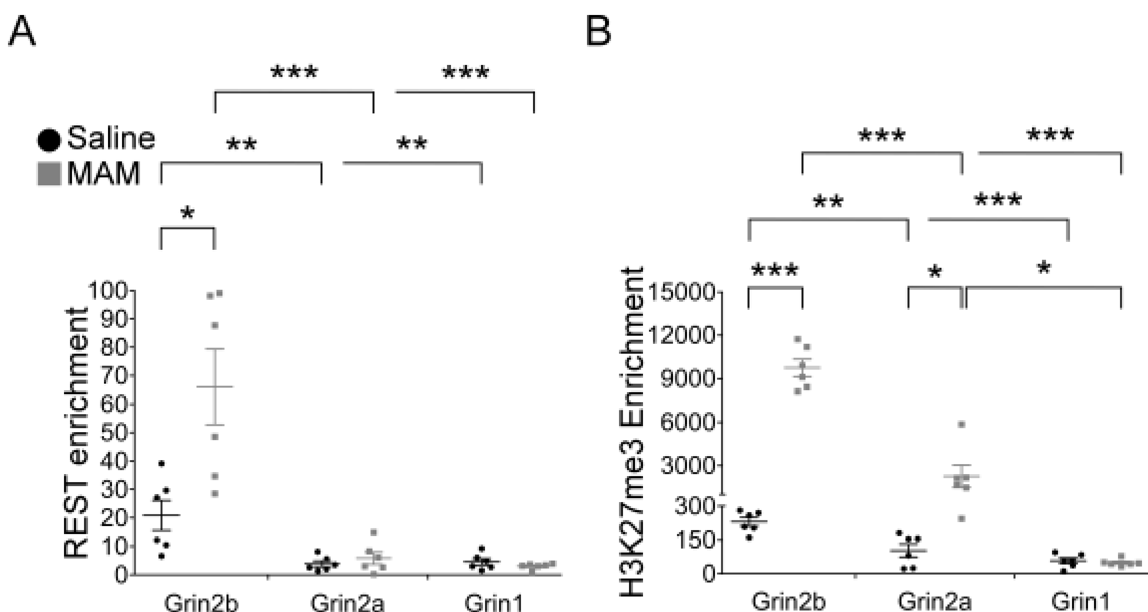
In contrast, the proximal promoter of *Grin2b* in MAM animals has a significant increase of REST enrichment in the PFC compared to saline controls (Figure 10A; REST *Grin2b*: saline 20.86 ±5.23, n=4; MAM 66.10 ± 13.28, n=4; p=0.017). We found that enhanced enrichment of REST binding at *Grin2b* is accompanied by a significant increase in H3K27me3 levels (Figure 10B; H3K27me3 *Grin2b*: saline 230.03 ± 19.24; MAM 9752.23 ± 598.3; p<0.001). Of note, we did observe an increased level of H3K27me3 in the MAM PFC at the *Grin2a* RE1 site (Figure 10B; H3K27me3 *Grin2a*: saline 102.82 ± 28.49, MAM 2258.43 ± 774.18; p=0.019). Such enrichment is not associated with increased REST enrichment at this *Grin2a* RE1 site (Figure 10A; REST *Grin2a*: saline 3.85±1.04, MAM 5.85±2.14; p=0.420). Moreover, we did not observe a significant difference in REST or H3K27me3 enrichment at the *Grin1* promoter region in saline versus MAM animals, suggesting MAM exposure does not induce transcriptional regulation at this *Grin1* RE1 site (Figure 10; REST *Grin1*: saline 4.59±1.13, MAM 3.05±0.40; p=0.230; H3K27me3 *Grin1*: saline 57.88±12.41, MAM 49.18±6.27; p=0.546). Thus, in comparing levels of REST and H3K27me3 at RE1 sites of NMDAR-expressing genes *Grin1*, *Grin2a*, and *Grin2b* between saline and MAM animals, it is evident that the extent of REST and H3K27me3 enrichment is far greater than that observed in control PFC, indicating that the *Grin2b* promoter region is in a hyper-repressed state in juvenile MAM PFC. We conclude that this hyper-repression in MAM PFC in part contributes to the decrease in synaptic NR2B protein and NR2B-NMDAR hypofunction evident in prior experiments.

Figure 9



Epigenetic histone marker H3K27me3 is selectively increased in developing MAM animals. Left, representative Western blot bands and, right, scatterplot demonstrates that histone marker H3K27me3 is selectively upregulated in juvenile MAM PFC. Saline 0.93±0.04, n=5; MAM 1.05±0.01, n=6; p=0.045, but no change in the acetylation markers assayed (H3K9ac p=0.263, H3K18ac p=0.524, and H3K27ac p=0.294).

Figure 10



REST-mediated repression of *Grin2b* at the proximal promoter region correlates with reduced synaptic NR2B protein in juvenile MAM animals. (A) Representative scatterplots depicting elevated REST repression of *Grin2b* in saline and MAM animals compared to other promoter regions, *Grin2a* and *Grin1*. REST *Grin2b*: saline 20.86±5.23, MAM 66.10±13.28, p=0.017. REST *Grin2a*: saline 3.85±1.04, MAM 5.85±2.14; p=0.420. REST *Grin1*: saline 4.59±1.13, MAM 3.05±0.40; p=0.230. In addition, REST enrichment is specific to *Grin2b* in the juvenile PFC. Saline REST, One-Way ANOVA, group: F(2, 17)=9.17, p=0.003; Tukey's post-hoc: saline *Grin2b vs Grin2a,* p=0.005; saline *Grin2b vs Grin1*, p=0.007; saline *Grin2a vs Grin1,* p=0.985. MAM REST, One-Way ANOVA, group: F(2, 17)=21.01, p<0.001; Tukey's post-hoc: MAM *Grin2b vs Grin2a,* p<0.001; MAM *Grin2b vs Grin1*, p<0.001; MAM *Grin2a vs Grin1,* p=0.965. (B) H3K27me3 levels are significantly elevated at *Grin2b* and *Grin2a* in MAM animals, but not at *Grin1*, compared to saline animals. H3K27me3 *Grin2b*: saline 230.03±19.24; MAM 9752.23±598.3; p<0.001. H3K27me3 *Grin2a*: saline 102.82±28.49,

MAM 2258.43±774.18; $p=0.019$. H3K27me3 *Grin1*: saline 57.88±12.41, MAM 49.18±6.27; $p=0.546$. H3K27me3 enrichment is significantly increased in saline animals at *Grin2b*, compared to *Grin2a* and *Grin1*. Saline H3K27me3, One-Way ANOVA, group: $F(2, 17)=17.91$, $p<0.001$; Tukey's post-hoc: saline *Grin2b vs Grin2a,* $p=0.002$; saline *Grin2b vs Grin1*, $p<0.001$; saline *Grin2a vs Grin1,* $p=0.316$. MAM animals show a similar trend in H3K27me3 enrichment at the subunit RE1 sites, as well as a significant enrichment at the *Grin2a* promoter. MAM H3K27me3, One-Way ANOVA, group: $F(2, 17)=81.05$, $p<0.001$; Tukey's post-hoc: MAM *Grin2b vs Grin2a,* $p<0.001$; MAM *Grin2b vs Grin1*, $p<0.001$; MAM *Grin2a vs Grin1,* $p=0.036$.

Discussion

In these experiments, we explored the glutamate hypofunction hypothesis by measuring NMDAR subunit expression and function in the developing PFC using the neurodevelopmental MAM rat model for SCZ. Our findings provide the first direct evidence that NMDAR hypofunction is occurring at an early (juvenile) stage of development in the MAM model for SCZ. Specifically, synaptic NR2B protein expression is significantly decreased in the mPFC. Concomitantly, NMDAR-mediated neurotransmission is deficient as evidenced by reduced amplitude of NMDA-mEPSCs and diminished synaptic NMDAR-mediated current in juvenile MAM PFC layer V pyramidal neurons. Mechanistically, epigenetic mechanisms demonstrate a key role in this NR2B-related dysfunction. REST repressor protein is preferentially localized to the RE1 site in the *Grin2b* proximal promoter, but not to RE1 sites in the promoters of other major NMDAR subunit genes, *Grin1* or *Grin2a*. Additionally, a functional marker of transcription, H3K27me3, is significantly increased at the *Grin2b* promoter in juvenile MAM PFC. Together, these data suggest that epigenetic mis-regulation of NR2B expression underlies NMDAR hypofunction in the PFC of juvenile animals in the MAM model for SCZ (Figure 11).

Pharmacological, neurodevelopmental, and transgenic mouse models are used extensively to understand the role of NMDARs in SCZ (Kantrowitz and Javitt, 2010; Gilmour et al., 2012; Kantrowitz and Javitt, 2012; Snyder and Gao, 2013). However, conclusions drawn from previous work remain incomplete given that this research often

focuses on investigation of the adult brain, albeit cognitive dysfunction emerges early in development and precedes psychosis (Jones et al., 1994; Cannon et al., 2000; Rosso et al., 2000). Indeed, transient antagonism of NMDARs during the juvenile period can elicit commonly reported SCZ-like endophenotypes in adulthood (Jeevakumar et al., 2015). These results corroborate our findings in the MAM model; NMDAR hypofunction in juveniles, in the form of reduced NR2B-NMDARs, precedes dopaminergic hypersensitivity, diminished social interaction (Flagstad et al., 2004), sensorimotor gating deficits, and perseverative behavior in cognitive flexibility (Moore et al., 2006), akin to positive, negative, and cognitive symptoms reported in SCZ patients (Elliott et al., 1995; Laruelle, 2000; Geyer et al., 2001). This is an important indicator that NMDAR hypofunction may emerge in early postnatal development, such as the juvenile stage of PFC development in SCZ.

NMDAR hypofunction in juvenile MAM PFC is specifically due to reduced NR2B protein expression and function at the post-synaptic site, as evidenced by the smaller amplitude of NMDA-mEPSCs and evoked NMDAR-mediated currents. The NR2B subunit is integral for promoting persistent activity, which is the proposed molecular correlate of working memory function (Goldman-Rakic, 1995; Wang et al., 2013). Because NR2B levels remain persistently high in the PFC throughout development and adulthood (Wang et al., 2008), the selective loss of NR2B-NMDARs in MAM PFC results in a vulnerability to working memory impairments (Snyder et al., 2013; Xing et al., 2016). NR2B protein levels are substantially reduced, as shown by Western blot analysis, but this approach cannot distinguish the laminar specificity of this

deficiency. To this end, we used a selective blocker of NR2B, Ro25, to confirm the loss of synaptic NR2B-NMDARs in layer V neurons. In the saline group, Ro25 treatment reduces the amplitude of NMDAR-mediated currents by 57% at the first pulse, and approximately 50% at each proceeding pulse (range 49-55%). However, in the MAM group, NMDA amplitude is reduced by only 22% (range 14-26%). The significant loss of post-synaptic NMDARs in MAM cells occludes the predicted effect of Ro25 compared to saline cells, confirming a significant reduction in specifically NR2B-NMDARs in MAM PFC (Figure 7B).

Since glutamate hypofunction emerged as a potential neurobiological underpinning of SCZ, efforts have focused on identifying the mechanism underlying this molecular disruption. Single-nucleotide polymorphisms (SNPs) and other genetic disturbances implicate *Grin2b* in psychiatric disorders, including SCZ (Qin et al., 2005; Endele et al., 2010; Jiang et al., 2010; Jia et al., 2012; Weickert et al., 2013; Akbarian, 2014; Fromer et al., 2014). Indeed, complex higher-order chromatin conformations are involved in regulating gene expression and eventually, cognitive function. For example, rare SNPs in distal gene locations can, in fact, disrupt *Grin2b* expression and affect working memory function in both humans and mice (Bharadwaj et al., 2014). Therefore, a highly complex network of genetic and epigenetic processes is implicated in how glutamatergic genes may become dysregulated in SCZ.

We sought to identify how the NR2B subunit was diminished in MAM PFC without substantial alterations of other NMDAR subunit protein levels. To address this

question, we looked to a well-characterized molecular process, the NR2B-to-NR2A subunit switch, in which NR2B protein levels are significantly decreased while NR2A protein expression is augmented to promote functional maturation of critical brain regions (Dumas, 2005; Rodenas-Ruano et al., 2012; Monaco et al., 2015). This process is tightly controlled by an epigenetic mechanism that is dependent upon the repressor REST and its corepressor protein partners (Tamminga and Zukin, 2015). Though it is unclear what dictates the specificity of REST for *Grin2b* compared to other NMDAR promoters, this pattern has been shown in these experiments and others (Rodenas-Ruano et al., 2012). Thus, we hypothesized that this endogenous repressive mechanism of *Grin2b* expression is responsible, at least in part, for the selective reduction in NR2B protein observed in juvenile MAM animals via aberrant hyper-repression of the *Grin2b* promoter. Our work is the first to reveal REST is a selective regulator of *Grin2b* expression in the juvenile PFC in the MAM model for SCZ, with no significant enrichment at the *Grin1* or *Grin2a* promoters (Figure 10A).

Histone modifications can serve as functional readouts of chromatin state (Akbarian, 2010b). Altered histone modification patterns have been reported in the developing PFC of MAM animals (Mackowiak et al., 2014), indicating aberrant epigenetic processes contribute to abnormal PFC development in this model. Specifically, the repressive histone modification H3K27me3 is implicated in cellular vulnerability in SCZ tissue (Kano et al., 2013), reinforcing the contribution of this modification to neural dysfunction in the human condition. However, information regarding the specific location of these histone modification changes in the genetic landscape is lacking. Here, we

confirm that the increase in H3K27me3 in juvenile MAM PFC, as shown in Western blots, is also evident at the *Grin2b* site. This is likely due to the REST-mediated recruitment of Polycomb complex PRC2, which contains a catalytic enzyme domain, EZH2, responsible for the H3K27me3 modification (Kim and Kim, 2012). Importantly, these processes are transient regulators of gene expression. Thus, our focus was on the intermediate stage (P21) of juvenile development in order to measure H3K27me3 enrichment levels concomitant with NR2B protein loss in the PFC.

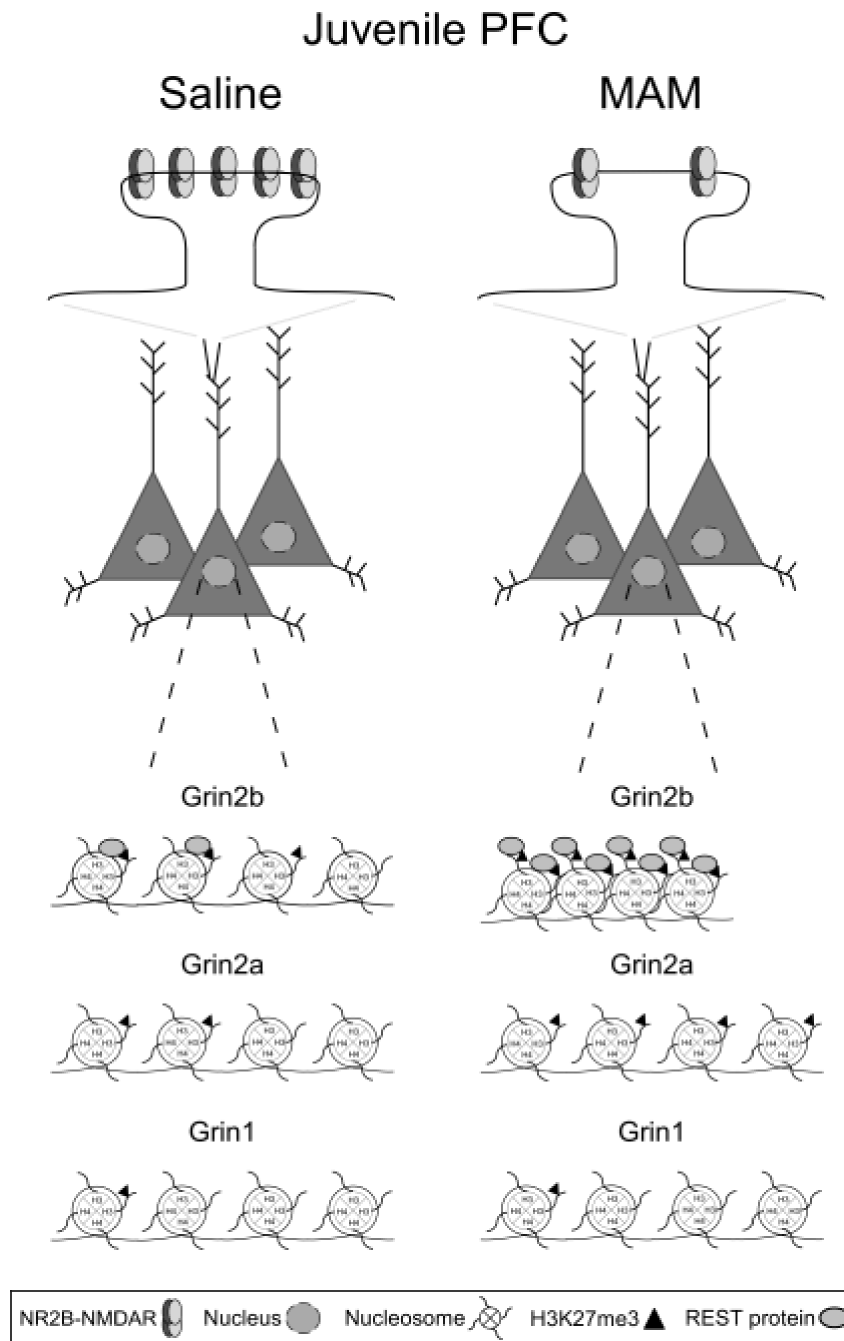
Surprisingly, there is also a significant increase of H3K27me3 enrichment at *Grin2a* in MAM PFC, although the levels of REST are not significantly enriched at this promoter and protein levels of NR2A are not altered by this enrichment. As shown by our ChIP experiments, a pattern of H3K27me3 enrichment exists in saline animals across promoters; that is, levels of this histone modification are highest at *Grin2b*, lower at *Grin2a*, and lowest at *Grin1* (*Grin2b* > *Grin2a* > *Grin1*). Interestingly, MAM animals show a similar pattern of H3K27me3 enrichment in the PFC, although to a much higher degree. Compared to saline PFC, the level of H3K27me3 enrichment is 41-fold higher at *Grin2b*, while the enrichment at *Grin2a* is only 21-fold higher in MAM PFC. We hypothesize levels of H3K27me3 are elevated by a REST-independent mechanism at the *Grin2a* promoter. In fact, shRNA-mediated knockdown of REST does not significantly affect NR2A mRNA levels (Rodenas-Ruano et al., 2012), supporting the notion that significant enrichment of H3K27me3 levels at *Grin2a* in MAM PFC are likely independent of REST. Alternatively, modifications such as H3K4me3 and H3K36me3 balance the chromatin state by mitigating the effects of repressive histone modifications,

prohibiting alterations to gene expression (Aranda et al., 2015). We propose an initial increase of H3K27me3 levels at the *Grin2b* promoter disrupts the balance of histone modifications, eventually recruiting REST for further EZH2-mediated enrichment of H3K27me3, resulting in reduced NR2B protein levels. However, at the *Grin2a* promoter, this threshold has likely not been surpassed by the 21-fold increase in H3K27me3. Further, the levels of other histone modifications, including the mutually exclusive marker H3K27ac, are unknown at these promoter regions. Therefore, more complex mechanisms are likely regulating expression of *Grin2a*, cumulatively resulting in no change to NR2A protein levels.

We have previously demonstrated the persistent expression of NR2B in PFC compared to other cortical regions (Wang et al., 2008). Therefore, alterations in NR2B protein in the PFC throughout development and adulthood can have a great impact on circuitry and behavior (Tang et al., 1999; Cui et al., 2011; Monaco et al., 2015). The aberrant decrease in synaptic NR2B-NMDARs during juvenile development of MAM animals can affect signaling cascade activation, facilitation in response to incoming stimuli, and working memory and higher-order cognitive abilities (Monaco et al., 2015). These disruptions to the NMDAR system in the PFC and hippocampus (Snyder et al., 2013), particularly during brain development, impose a vulnerability upon the critical fine-tuning of the network necessary for normal cognition in adulthood. Given the importance of the hippocampus-mPFC pathway in cognitive functioning (Marquis et al., 2006) (Marquis et al., 2008), future experiments will explore the cognitive profile of juvenile MAM rats with a focus on PFC-dependent processes, such as working memory

and cognitive flexibility. Due to the continued lack of successful treatments for cognitive impairments in SCZ (Lett et al., 2014), even in the face of improved positive and negative symptoms, these deficits will persist as will the psychological and monetary burden of this disorder. These data are a contribution to the ever-growing force to understand the neurobiological underpinnings of the highly complex and devastating etiology of SCZ.

Figure 11



Schematic depiction of synaptic dysfunction in juvenile MAM layer V pyramidal neurons (top) and epigenetic hyper-repression of the *Grin2b* promoter by REST and H3K27me3 (bottom).

CHAPTER 4: ALTERED NMDAR SUBUNIT COMPOSITION IN ADOLESCENT PFC IN THE MAM MODEL FOR SCHIZOPHRENIA

Abstract

Development of the prefrontal cortex (PFC) is characterized by refinement of the local microcircuitry, pruning of excitatory synapses, and the final stages of myelination. Adolescence is a critical period during which these processes are ongoing, and are particularly vulnerable to genetic or environmental perturbations. The prolonged time course of PFC maturation during late adolescence and early adulthood further increases its susceptibility to molecular aberrations. In addition to pruning and cellular maturation, the neurobiological mechanisms of adolescent brain development include alterations in N-methyl-D-aspartate receptors (NMDARs). In particular, NMDARs exhibit increased incorporation of the NR2B subunit, a proposed molecular correlate of working memory function. Thus, interrupting these developmental processes can have devastating effects on cognition and executive functioning. Schizophrenia (SCZ) is a debilitating mental illness often diagnosed during late adolescence and early adulthood, coinciding with these critical maturational processes. Considered a neurodevelopmental disorder, at-risk individuals exhibit cognitive and mood abnormalities during these critical developmental periods. However, it is yet unknown the molecular processes which may be affected during development. Thus, we employed the neurodevelopmental methylazoxymethanol (MAM) model to investigate synaptic NMDAR physiology during adolescent development. We found glutamatergic neurotransmission is significant affected by gestational MAM exposure, specifically due to downregulation of synaptic NMDAR subunits NR1 and NR2B. Further, NMDAR-mediated currents are significantly diminished in adolescent MAM animals. Interestingly, we revealed the subunit

composition of synaptic NMDARs in adolescent MAM animals are predominantly diheteromeric, while NMDARs in saline animals exhibit triheteromeric subunit arrangement. This functional alteration implicates NMDAR dysfunction during adolescent development in the neurodevelopmental MAM model for SCZ, and indicate complex mechanisms of glutamatergic dysfunction may be evident in the human illness.

Introduction

Proper brain development at critical prenatal and postnatal stages is critical for appropriate function in adulthood (Cannon et al., 2002). During early development, synapses are strengthened at variable rates depending on their frequency of activation by external stimuli. This period of high neuroplasticity results in an overabundance of synapses that can be highly functional or less-often utilized, resulting in an energy-inefficient network (MacAskill et al., 2010). Thus, synaptic pruning is a mechanism by which relatively weak synaptic contacts are removed so the brain is able to communicate in an energy-efficient manner once fully mature. Synaptic pruning is evident throughout the brain during each developmental stage; however, the prefrontal cortex (PFC) is unique in that it undergoes the latest and most protracted functional maturation (Kolb et al., 2012). Pruning is influenced by environmental inputs and is tightly regulated by changes in gene expression. PFC development during adolescence is a landmark stage of functional maturation characterized by refinement of the cortical circuitry; these processes include the final stages of myelination, maturation of interneurons, innervation of dopaminergic afferents, and pruning of excitatory synapses (Jaaro-Peled et al., 2009; Kolb et al., 2012). Thus, adolescence represents a highly vulnerable period of PFC development due to these critical changes in synaptic connectivity among excitatory neurons, interneurons, and neuromodulator systems (Kolb et al., 2012; Arnsten and Jin, 2014). Although this stage is transient, environmental inputs or genetic aberrations can strongly affect these processes such that abnormalities in decision-making, behavioral

flexibility, and working memory, among many other PFC-dependent cognitive functions, emerge in adulthood (Gilmour et al., 2012).

In addition to circuit-level changes in the PFC during adolescence, molecular changes are a critical component of functional maturation. The glutamatergic system is an integral mediator of neuroplasticity during development, as well as appropriate behavioral responses to stimuli in adulthood. Pre-synaptic release of glutamate coincident with AMPAR- and NMDAR-mediated depolarization of the post-synaptic membrane serves to maintain and strengthen synaptic connections. Importantly, the composition of these receptors dictates the physiological properties of neurons across development (Paoletti et al., 2013). In the adult PFC, NR2B-containing NMDARs (NR2B-NMDARs) maintain sustained firing of Delay cells, the proposed molecular mechanism of normal working memory function (Goldman-Rakic, 1995; Wang et al., 2008; Wang et al., 2013; Flores-Barrera et al., 2014). A delicate balance of NR2B-NMDAR activation promotes Delay cell firing in the preferred direction without overstimulating activity of non-preferred direction firing. Pharmacological inhibition of NR2B-NMDARs reduces the signal-to-noise ratio of persistent activity during the delay phase of working memory tasks (Wang et al., 2013), disrupting the fine-tuned network of the PFC (Goldman-Rakic, 1996). Alternatively, aberrant increases in NR2B protein can increase NMDAR activity, enhance long-term potentiation (LTP), and augment working memory and other cognitive functions (Tang et al., 1999; Cui et al., 2011). Thus, changes in NMDAR subunit protein expression and composition has a dynamic role in cognition; increased NR2B levels in adulthood can strengthen pre-existing cortical connections and promote cognition.

However, such alterations during juvenile and adolescent development can sensitize the brain to cognitive dysfunction later in life (Jeevakumar et al., 2015; Monaco et al., 2015; Xing et al., 2016; Li et al., 2017).

Schizophrenia (SCZ) is a devastating, chronic mental illness that clinically manifests during late adolescence or early adulthood, during and immediately after these critical stages of NMDAR and PFC development (Konradi and Heckers, 2003). This neurodevelopmental disorder is characterized by psychosis, deficits in social cognition, and cognitive impairments. Although imaging studies demonstrate brain abnormalities during onset of SCZ (Cannon et al., 2002), behavioral and neurological abnormalities are evident even prior to diagnosis (Cannon and Jones, 1996; Marenco and Weinberger, 2000; Chung et al., 2017). PFC tissue from post-mortem adult SCZ patients reveal reductions in spine density (Glantz and Lewis, 2000), neuropil volume (Goldman-Rakic and Selemon, 1997), and defective connectivity (Lewis and Lieberman, 2000), which are thought to result from aberrant brain development, manifesting as functional changes during adolescence and adulthood (Konradi and Heckers, 2003). Post-mortem analysis further reveals synaptic abnormalities in adolescent cortex and hippocampus, supporting the hypothesis that crucial neural circuits are altered in SCZ prior to adulthood (Goldman-Rakic and Selemon, 1997; Lewis et al., 2005; Tamminga et al., 2010). Neurobiological evidence of normal adolescent PFC development and how this differs from neurodevelopmental disorders is lacking (Lee et al., 2014). Particularly, it is yet unclear whether changes in NR2B and NMDAR expression and function occur in SCZ during adolescent PFC development. To address this question, we used the

neurodevelopmental methylazoxymethanol (MAM) model, which recapitulates structural and behavioral endophenotypes seen in adult SCZ patients (Moore et al., 2006; Lodge and Grace, 2009), to evaluate the physiological consequences of alterations in NMDAR subunit expression on the adolescent PFC.

Methods

Animals

All animal procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Drexel University College of Medicine Animal Care and Use Committee. Food and water were provided *ad libitum* throughout the experiments. Pairs of adult pregnant Sprague-Dawley rats were purchased from Charles River Laboratories International, Inc. (Wilmington, MA) to arrive on embyronic day 15 (E15). Dams were injected IP with either 25 mg/kg MAM or saline on E17. Pups were usually born on E21 or postnatal day 0 (P0) and were weaned on P21. Male and female rats were group-housed by gender after weaning. Both female and male tissue was collected; however, only male tissue was used in the experiments outlined below. For all experiments, offspring aged P42-49 were characterized as adolescents (Kolb et al., 2012).

Tissue collection and western blot for synaptosomal proteins

PFC tissue was collected and processed as described in Chapter 3. Briefly, adolescent animals were perfused with 60 mL HEPES-buffered sucrose (in mM: 320 sucrose, 4 HEPES-NaOH buffer, pH 7.4, 2 EGTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 10 sodium fluoride, and 10 sodium pyrophosphate). The

brain region containing the PFC was dissected and homogenized in sucrose buffer. The homogenate was process to yield a synaptosomal fraction.

A BCA protein assay was performed to determine protein concentration. The protein sample was mixed with Laemmli sample buffer, boiled for 5 minutes, and separated on a 7.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to pore size 0.45 μm polyvinylidene difluoride membranes (PVDF, EMD-Millipore, Billerica, MA). The membrane was blocked in 5% nonfat milk and probed with primary anti-serum. Each blot was probed for anti-mouse NR1 (1:5000), anti-rabbit NR2A (1:4000), anti-mouse NR2B (1:2000), anti-rabbit NR3A (1:4000), anti-rabbit NR3B (1:4000), and anti-mouse actin (1:100,000), which was used as a loading control. The blots were incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG secondary antibody (1:5000), and proteins were visualized using ECL Plus. Protein expression of each subunit was evaluated by densitometry using ImageJ software. Samples from each animal were run at least 4 times to minimize interblot variance. Raw values for NMDAR subunit proteins were normalized to actin and this ratio was further normalized to the first usable band on each membrane. These normalized values were averaged per animal to yield the final values which comprised the mean and standard error per group.

Whole-cell patch-clamp electrophysiology

Animals were anesthetized with Euthasol (0.2 ml kg^{-1}, IP) until unresponsive to toe pinch and perfused with ice-cold sucrose solution (in mM: 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 75 sucrose, 25 glucose, 0.5 $CaCl_2$, 7.0 $MgSO_4$, pH 7.4) to preserve tissue quality. The brains were dissected, and 300 µm coronal slices containing the prelimbic PFC were cut into an ice-cold bath of oxygenated ice-cold sucrose solution using a VT-1200S vibratome tissue slicer (Leica Microsystems, Wetzlar, Germany). Slices were transferred to a holding chamber, submerged in oxygenated (95% O_2, 5% CO_2) aCSF (in mM: 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$, and 10 dextrose, pH 7.4) at 35^oC, for one hour and then remained at room temperature until used for recording. Slices were placed into a recording chamber mounted on an Olympus upright microscope (BX51, Olympus America, Center Valley, PA), where they were continuously bathed in oxygenated aCSF and maintained at 36^oC. Neurons were visualized with infrared differential interference video microscopy. All experiments were conducted with an Axon MultiClamp 700B amplifier (Molecular Devices), and data were acquired using pCLAMP 9.2 software and analyzed using Clampfit 10.2 software (Molecular Devices).

Spontaneous and miniature excitatory post-synaptic currents

To record spontaneous or miniature EPSCs, somatic whole-cell voltage-clamp recordings were obtained from layer V pyramidal cells using patch electrodes with an open tip resistance of 8-11 MΩ ($CsCl_2$ intracellular solution, in mM: 110 D-gluconic acid, 110 CsOH, 10 $CsCl_2$, 1 EGTA, 1 $CaCl_2$, 5 QX-314, 1 ATP-Mg, 10 HEPES, at pH

7.3, adjusted with CsOH). Resting membrane potential was observed in current clamp mode for 1 to 2 minutes to confirm cell health. In voltage clamp mode, the membrane potentials were first held at -70 mV in the presence of picrotoxin (50 μM) to record AMPAR-mediated spontaneous EPSCs (AMPA-sEPSCs), which are a result of both action potential-mediated and stochastic release of glutamate, in the absence of $GABA_A$R-mediated neurotransmission. After 5 minutes of recording, tetrodotoxin (TTX, 0.5 μM) was added to the wash-on buffer to isolate miniature EPSCs (AMPA-mEPSCs). TTX blocks Na^{2+} channels, thus prohibiting action potential-stimulated release of neurotransmitter. mEPSCs were recorded for at least 5 minutes and then DNQX (20 μM) was washed on to block AMPARs. When all currents ceased, the membrane potential was slowly ramped up to 60 mV. NMDAR-mediated mEPSCs (NMDA-mEPSCs) were then recorded for at least 5 minutes.

A typical s/mEPSC event was selected to create a sample template for event detections within a 5-minute period for each data file. The frequency (number of events) and amplitude of the individual events were examined with the threshold set at the medium level (i.e., 5 within a range of 1 to 9) in Clampfit. The detected events were then visually inspected to ensure specificity.

Evoked excitatory post-synaptic currents

To record evoked EPSCs (eEPSCs), a bipolar stimulating electrode was placed in layer II/III of the mPFC approximately 200-300 μm from visually-identified layer V

pyramidal cells. Picrotoxin (50 μM) was used to block $GABA_AR$-mediated neurotransmission. Cells were held at -70 mV to record AMPA-eEPSCs for 5 mins in the presence of picrotoxin (50 μM). Then the membrane potential was held at 60 mV to record NMDA-eEPSCs for 5 minutes before and 5 minutes after bath application of a selective NR2B antagonist Ro25-6981 (Ro25, 1 μM). EPSCs were evoked through the stimulating electrode using a physiologically relevant 10-pulse, 20 Hz train with a 0.1 Hz stimulating interval. Stimulus intensity was adjusted until 50-100 pA amplitude responses were achieved. Cells with an unstable baseline or unusual response patterns throughout recording were removed from the analysis. The EPSC recordings were monitored throughout each experiment by implementing a -100 pA current pulse with a 200 ms duration, and all cells with >20% change in series resistance during recording were discarded. Traces were analyzed for amplitude (pA) relative to the first post-stimulus baseline and cumulative charge (charge transfer, nA*ms), as well as paired-pulse ratio (PPR).

Data Analysis

Statistical analyses were carried out using SPSS Statistics version 24.0 (IBM). Normality and homogeneity of variance testing determined the use of parametric or non-parametric tests for each dataset. Protein expression analyses, and AMPA- and NMDA-s/mEPSC amplitude and frequency were compared using unpaired Student's t-test or Mann-Whitney U test. Evoked data were analyzed using repeated measures ANOVA to compare amplitude or charge transfer between groups and across pulses. In the event of a

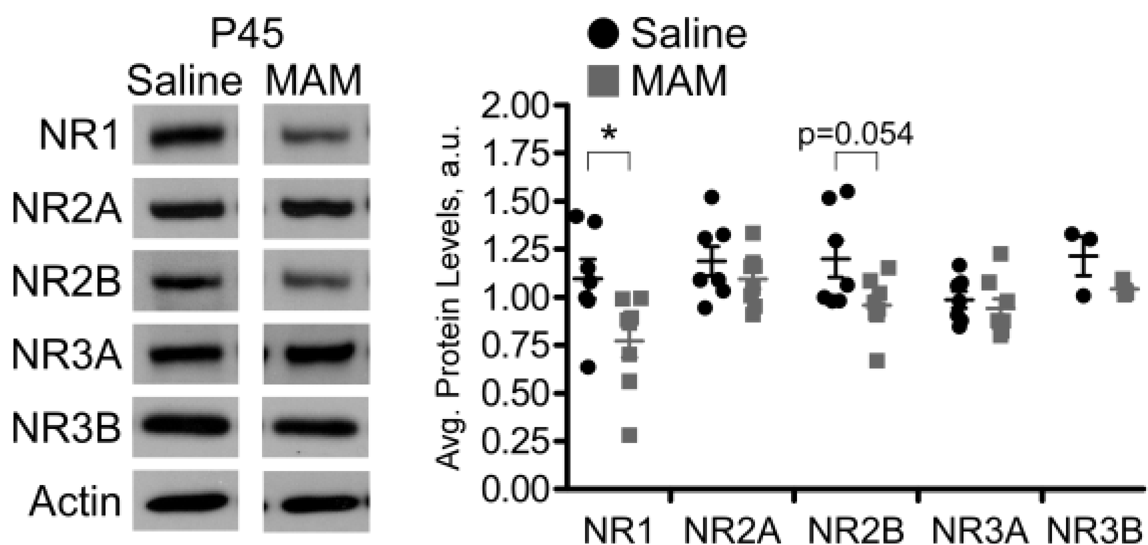
significant interaction effect, the results of a simple main effects analysis are reported. All data are presented as mean ± standard error (S.E.). Single, double, and triple asterisks represent $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Results

Constitutive NMDAR subunit NR1 is significantly decreased in adolescent MAM PFC

To determine if NMDARs are altered during prefrontal cortical development in this neurodevelopmental SCZ model, we first examined how prenatal MAM exposure affects NMDAR protein levels in adolescent (P42-P49) saline and MAM PFC. We collected synaptic plasma membrane fractions from saline and MAM animals, and probed for NMDAR subunits, including NR1, NR2A, NR2B, NR3A, and NR3B. Actin was used as a loading control. At this developmental time point, MAM animals had significantly lower synaptic NR1 protein levels (Figure 12; NR1: saline 1.10±0.10, n=7; MAM 0.77±0.09, n=8; $t(13)=2.43$, $p=0.030$). Additionally, synaptic NR2B levels are decreased, although not significantly (NR2B: saline 1.20±0.10, n=7; MAM 0.96±0.05, n=8; $t(9.23)=2.29$, $p=0.054$). We found no changes in synaptic NR2A, NR3A, or NR3B proteins between groups ($p>0.05$).

Figure 12



Synaptic NR1 protein is significantly reduced in adolescent MAM PFC, with a non-significant decrease in NR2B protein. Left, representative Western blot bands, and right, summary scatterplots illustrating a significant reduction in synaptic NR1 levels in adolescent MAM PFC (NR1: saline 1.10±0.10, n=7; MAM 0.77±0.09, n=8; p=0.030). We observed a strongly trending reduction in NR2B levels that did not reach significance, and no significant differences in other synaptic NMDAR subunits between adolescent saline and MAM animals (NR2B: saline 1.20±0.10; MAM 0.96±0.05; p=0.054. NR2A: saline 1.19±0.08; MAM 1.09±0.05; p=0.311. NR3A: saline 0.99±0.04; MAM 0.94±0.05; p=0.515. NR3B: saline 1.21±0.10; MAM 1.04±0.02; p=0.237).

NMDA-mEPSCs, but not AMPA-s/mEPSCs, have significantly diminished frequency in adolescence following prenatal MAM exposure

We next sought to determine if the reduced NMDAR subunit protein levels in adolescent MAM animals altered glutamatergic neurotransmission. We utilized whole-cell patch clamp electrophysiology to record from layer V pyramidal neurons in the mPFC and examined both NMDA- and AMPA-EPSCs. We first recorded sEPSCs that presumably resulted from action potentials of pre-synaptic neurons as well as the spontaneous release of neurotransmitter at synapses, and then washed on tetrodotoxin (TTX, 0.5 μM) to block action potentials in the slice and recorded mEPSCs. We held the cell at -70mV while blocking $GABA_AR$ function, to assess if MAM exposure had an effect on functional AMPARs and pre-synaptic function. We found no significant changes in AMPA-sEPSCs or -mEPSCs in adolescent MAM compared to saline animals (Figure 13A; AMPA-sEPSCs: frequency, Hz, saline 2.72±1.05, n=10; MAM 2.17±0.58, n=14; p=0.285; amplitude, pA, saline 21.8±3.19, MAM 19.12±1.37, p=0.285. Figure 13B; mEPSCs, frequency, Hz, saline 1.49±0.26, n=11; MAM 2.06±0.69, n=12; p=0.566; amplitude, pA, saline 17.80±0.86, MAM 17.63±0.70, p=0.878).

We next held the cell at +60mV while blocking $GABA_ARs$ and AMPARs to examine changes in NMDAR function. Adolescent MAM animals showed significantly reduced NMDA-mEPSC frequency, and a trend toward increased amplitude (Figure 14; NMDA-mEPSCs: frequency, Hz, saline 1.47±0.45, n=10; MAM 0.627±0.13, n=10; p=0.005; amplitude, pA, saline 24.71±2.82; MAM 31.4±2.0; p=0.069).

Synaptic NMDAR function is comparable between adolescent saline and MAM mPFC

To further evaluate the functional consequences of prenatal MAM exposure on NMDARs, we recorded NMDA current in layer V pyramidal neurons evoked by stimulation of layer II/III cells with a 10-pulse, 20 Hz train. After 50-100 pA responses were stable for 5 mins, we recorded NMDA-eEPSCs in the presence of picrotoxin (50 μM). Surprisingly, we found no significant differences in amplitude and charge transfer between adolescent saline and MAM animals (Figure 15; amplitude, pA, Saline n=9, MAM n=11, Repeated Measures ANOVA: group: $F(1,18)=0.18$, $p=0.678$, pulse: $F(2.39, 43.02)=40.21$, $p<0.001$; interaction: $F(2.39, 43.02)=2.26$, $p=0.107$. Charge transfer, nA*ms, Repeated Measures ANOVA: group: $F(1,18)=0.80$, $p=0.383$, pulse: $F(1.04, 18.66)=59.20$, $p<0.001$; interaction: $F(1.04, 18.66)=0.41$, $p=0.538$). Further, the stimulation intensity required to elicit this magnitude of responses was not significantly different between groups (saline 82.77±13.71, MAM 101.46±15.37, $t(18)=-0.89$, $p=0.386$). In addition, pre-synaptic release probability is not affected by MAM exposure as we found no difference in PPR compared to saline cells (saline 0.65±0.06, MAM 0.66±0.09, $t(18)=-0.12$, $p=0.904$). Given the loss of synaptic NR1, substantial reduction of NR2B protein, and hypofunction of NMDA-mEPSCs, these findings are unexpected. However, we continued to probe the function of NR2B-NMDARs in the adolescent MAM mPFC with the NR2B-selective antagonist, Ro25.

Adolescent MAM animals demonstrate diheteromeric NMDAR subunit composition compared to triheteromeric NMDARs in saline animals

To compare the levels of NR2B-NMDARs between adolescent saline and MAM layer V pyramidal neurons, we bath-applied Ro25 (1 μM), a selective NR2B antagonist. Knowing NR2B levels are persistent in PFC through development, we found Ro25 blocked a significant portion (45-60%) of NR2B-mediated NMDA current in the control condition (Figure 16A; amplitude, pA, Saline before Ro25 n=9, saline after Ro25 n=7, Repeated Measures ANOVA: group: $F(1,14)=5.16$, $p=0.039$, pulse: $F(2.54, 35.50)=19.61$, $p<0.001$; interaction: $F(2.54, 35.50)=5.40$, $p=0.005$). Simple main effects analysis showed saline layer V pyramidal cells treated with Ro25 had a significant reduction in amplitude at pulses 1 to 4 ($p<0.05$), but not at pulses 5 to 10. Similarly, the charge conducted through NR2B-NMDARs was significant diminished following Ro25 wash-on (Figure 16A; charge transfer, nA*ms, Repeated Measures ANOVA: group: $F(1,14)=4.33$, $p=0.056$, pulse: $F(1.02, 14.24)=28.14$, $p<0.001$; interaction: $F(1.02, 14.24)=2.42$, $p=0.141$). Saline cells treated with Ro25 demonstrated a significant loss of current during pulses 1 to 6 ($p<0.05$), but not at pulses 7 to 10.

In adolescent MAM mPFC, Ro25 wash-on resulted in drastic effects to NR2B-mediated NMDA current. The amplitude and current of NMDA-eEPSCs was significantly diminished in the presence of Ro25 (Figure 16B; amplitude, pA, MAM before Ro25 n=11, MAM after Ro25 n=8, Repeated Measures ANOVA: group:

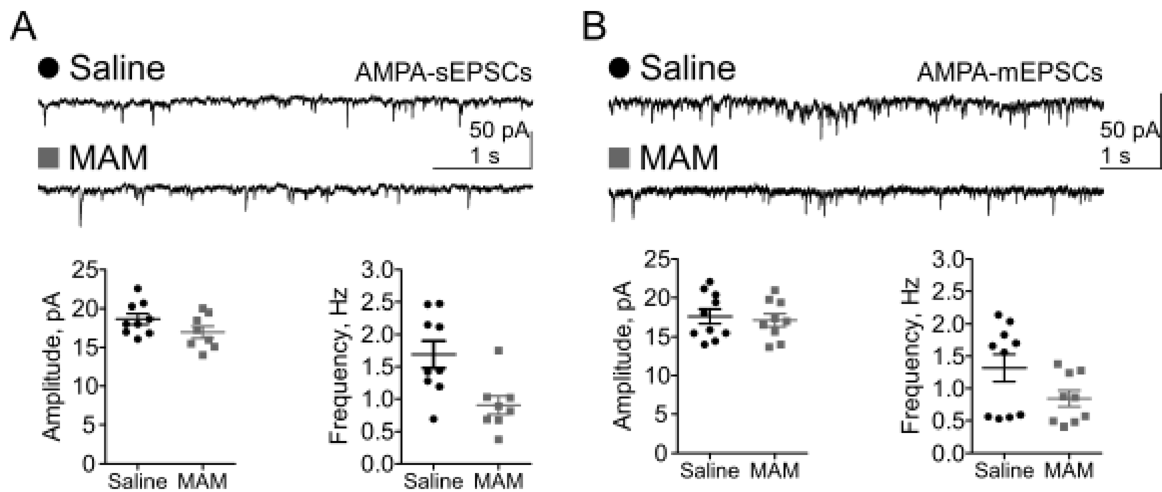
F(1,17)=19.76, p<0.001, pulse: F(2.18, 37.07)=18.26, p<0.001; interaction: F(2.18, 37.07)=16.70, p<0.001. Charge transfer, nA*ms, Repeated Measures ANOVA: group: F(1,17)=21.79, p<0.001, pulse: F(1.04, 17.67)=24.82, p<0.001; interaction: F(1.04, 17.67)=14.84, p=0.001). Further, simple main effects analysis revealed substantial loss of NMDA-eEPSC amplitude (p<0.01) and current (p<0.001) following Ro25 treatment of MAM cells. That Ro25 exerts a much greater effect on NMDA current in MAM animals compared to saline animals strongly suggests the subunit composition of NMDARs is altered in MAM mPFC. In fact, it indicates that NMDARs in MAM mPFC are homodimers of NR1 and NR2B subunits, while NMDARs in saline animals are heterotetramers of NR1/NR2B/NRX subunits.

Post-synaptic AMPARs are unaffected in adolescent MAM mPFC, yet pre-synaptic release mechanisms may be enhanced

To evaluate whether MAM exposure has detrimental effects on AMPAR-mediated neurotransmission in the adolescent mPFC, we compared EPSCs in layer V pyramidal neurons evoked by layer II/III stimulation between saline and MAM animals. We found AMPA-eEPSCs remain uncompromised during the adolescent period in MAM mPFC. There were no significant differences in amplitude or charge of AMPA-eEPSCs between the two groups (Figure 17; amplitude, pA, Saline n=11, MAM n=10, Repeated Measures ANOVA: group: F(1,19)=0.07, p=0.798, pulse: F(2.07, 39.28)=32.70, p<0.001; interaction: F(2.07, 39.28)=0.17, p=0.853. Charge transfer, nA*ms, Repeated Measures ANOVA: group: F(1,19)=0.18, p=0.674, pulse: F(1.06, 20.18)=64.09, p<0.001;

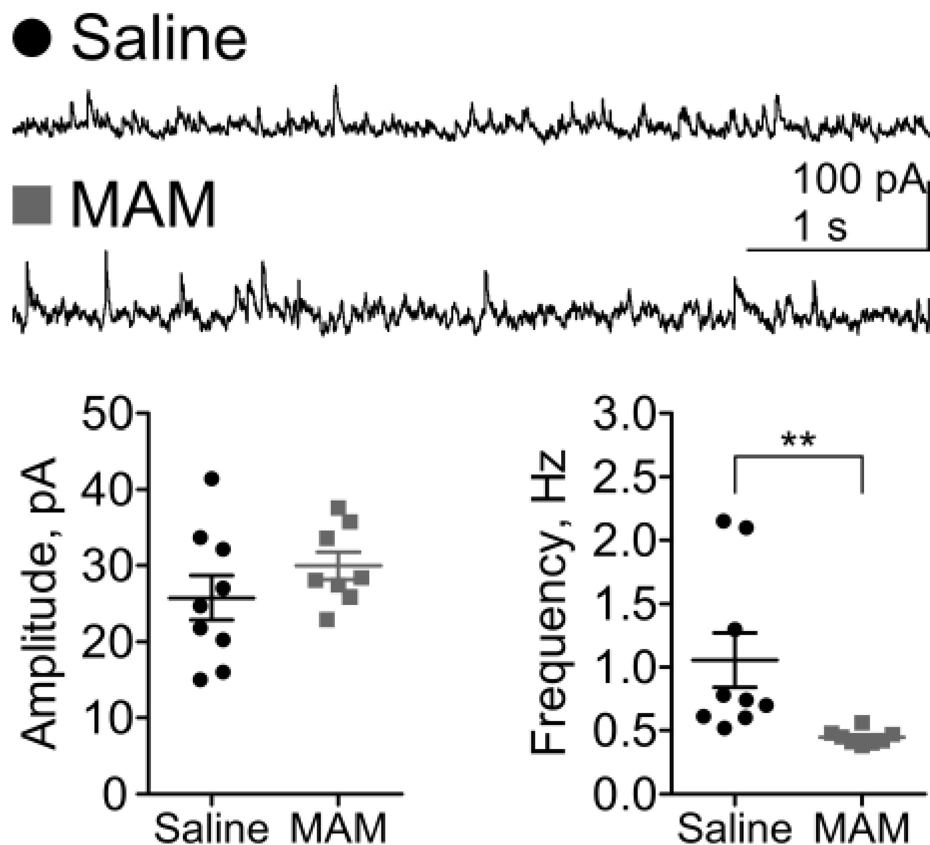
interaction: $F(1.06, 20.18)=0.32$, $p=0.592$). In addition, there was no significant difference in stimulation intensity required to elicit AMPA-eEPSCs in MAM cells compared to saline cells (saline 264.76±65.90, MAM 224.80±74.83, $t(19)=0.90$, $p=0.359$). Interestingly, we did observe a significant increase in PPR in adolescent MAM mPFC compared to saline cells (saline 0.88±0.05, MAM 1.09±0.05, $t(18)=-2.80$, $p=0.012$).

Figure 13



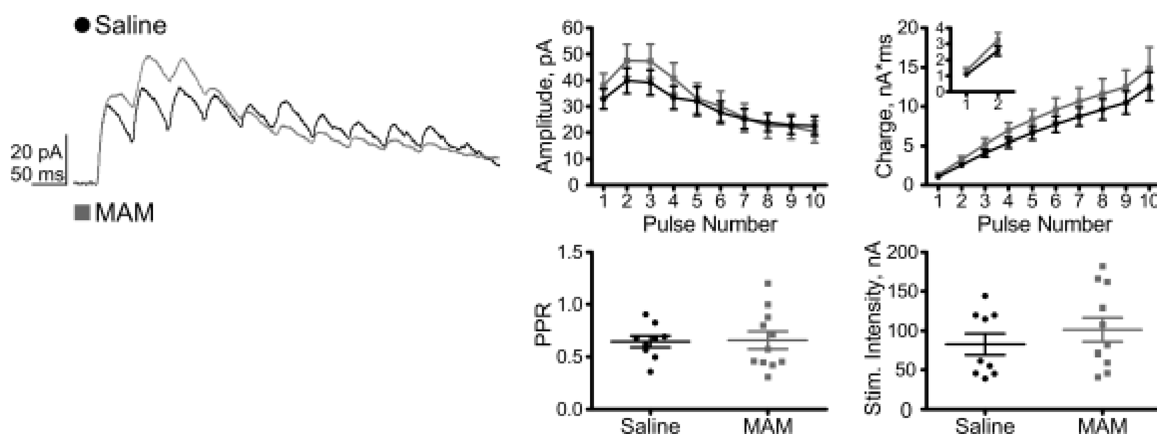
AMPA-s/mEPSCs are functionally intact in adolescent MAM mPFC. We found no significant changes in (A) AMPA-sEPSCs or (B) -mEPSCs in adolescent MAM compared to saline animals (A; AMPA-sEPSCs: frequency, Hz, saline 2.72±1.05, n=10; MAM 2.17±0.58, n=14; p=0.285; amplitude, pA, saline 21.8±3.19, MAM 19.12±1.37, p=0.285. B; mEPSCs, frequency, Hz, saline 1.49±0.26, n=11; MAM 2.06±0.69, n=12; p=0.566; amplitude, pA, saline 17.80±0.86, MAM 17.63±0.70, p=0.878).

Figure 14



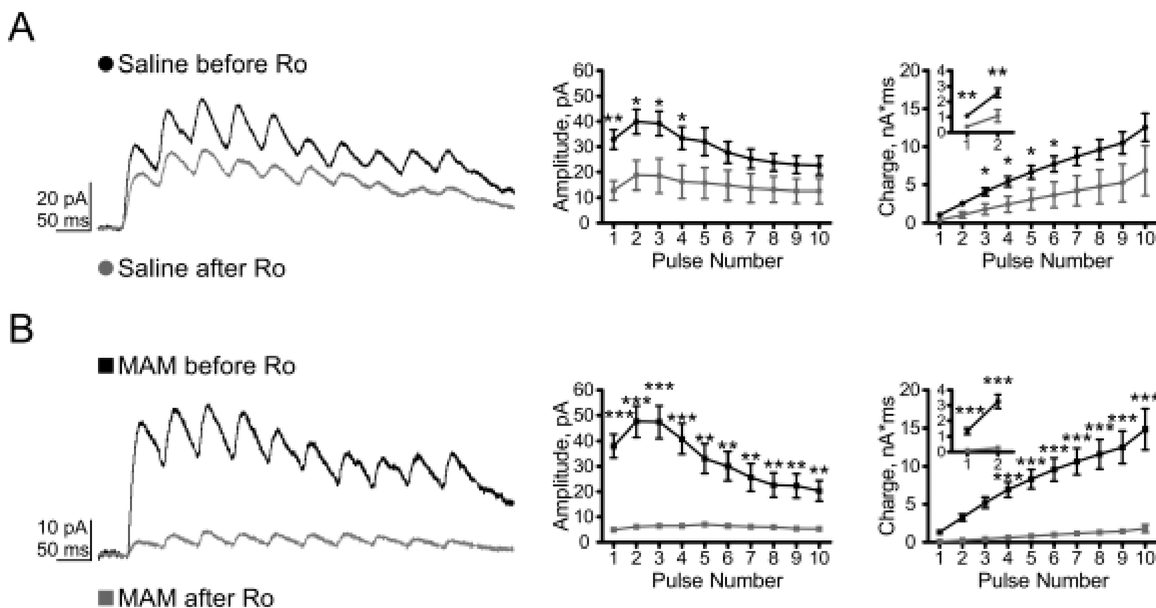
Adolescent MAM animals showed significantly reduced NMDA-mEPSC frequency, and a trend toward increased amplitude. We found a significant increase in NMDA-mEPSC frequency, and a trend of increased amplitude in NMDA currents from layer V pyramidal cells in MAM mPFC compared to saline cells (NMDA-mEPSCs: frequency, Hz, saline 1.47±0.45, n=10; MAM 0.627±0.13, n=10; p=0.005; amplitude, pA, saline 24.71±2.82; MAM 31.4±2.0; p=0.069).

Figure 15



Synaptic NMDARs in saline and MAM adolescent mPFC are functionally comparable. We found no significant differences in amplitude and charge transfer between adolescent saline and MAM animals (Amplitude, pA, Saline n=9, MAM n=11, Repeated Measures ANOVA: group: F(1,18)=0.18, p=0.678, pulse: F(2.39, 43.02)=40.21, p<0.001; interaction: F(2.39, 43.02)=2.26, p=0.107. Charge transfer, nA*ms, Repeated Measures ANOVA: group: F(1,18)=0.80, p=0.383, pulse: F(1.04, 18.66)=59.20, p<0.001; interaction: F(1.04, 18.66)=0.41, p=0.538). The stimulation intensity required to elicit this magnitude of responses was not significantly different between groups (saline 82.77±13.71, MAM 101.46±15.37, t(18)=-0.89, p=0.386). In addition, pre-synaptic release probability is not affected by MAM exposure as we found no difference in PPR (saline 0.65±0.06, MAM 0.66±0.09, t(18)=-0.12, p=0.904).

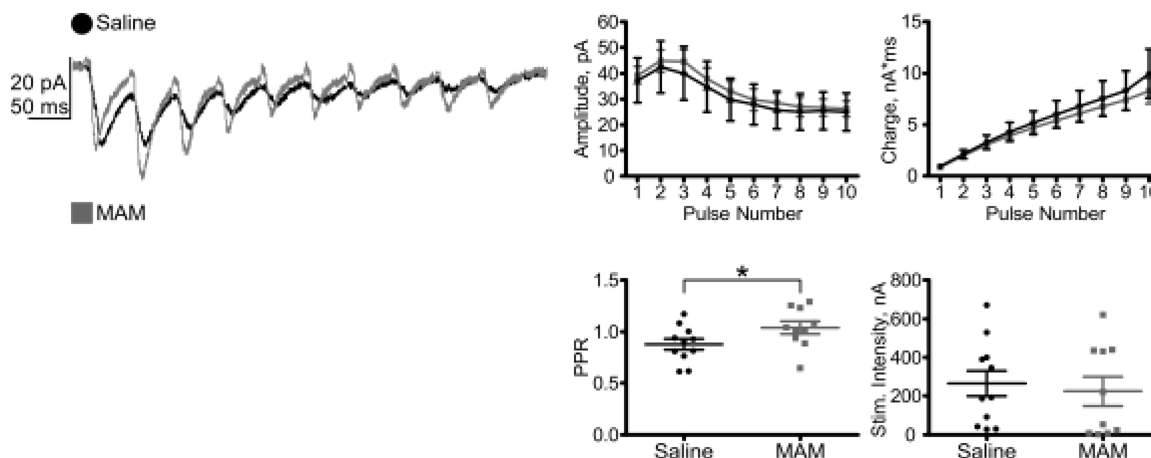
Figure 16



Adolescent MAM animals display properties of diheteromeric NR2B-NMDARs, while saline animals exhibit functional properties of triheteromeric NR1/NRX/NR2B-NMDARs. (A) We found Ro25 blocked a significant portion (45-60%) of NR2B-mediated NMDA current in the control condition (amplitude, pA, Saline before Ro25 n=9, saline after Ro25 n=7, Repeated Measures ANOVA: group: F(1,14)=5.16, p=0.039, pulse: F(2.54, 35.50)=19.61, p<0.001; interaction: F(2.54, 35.50)=5.40, p=0.005; simple main effects: pulses 1-4 p<0.05). Similarly, the current conducted through NMDARs was significant diminished following Ro25 wash-on of saline cells (charge transfer, nA*ms, Repeated Measures ANOVA: group: F(1,14)=4.33, p=0.056, pulse: F(1.02, 14.24)=28.14, p<0.001; interaction: F(1.02, 14.24)=2.42, p=0.141; Student's t-test post-hoc: pulses 1-6, p<0.05). (B) In adolescent MAM mPFC, the amplitude and current of NMDA-eEPSCs was significantly diminished in the presence of Ro25 (amplitude, pA, MAM before Ro25 n=11, MAM after Ro25 n=8, Repeated Measures ANOVA: group: F(1,17)=19.76, p<0.001, pulse: F(2.18,

37.07)=18.26, $p<0.001$; interaction: $F(2.18, 37.07)=16.70$, $p<0.001$; simple main effects: all pulses, $p<0.01$. Charge transfer, nA*ms, Repeated Measures ANOVA: group: $F(1,17)=21.79$, $p<0.001$, pulse: $F(1.04, 17.67)=24.82$, $p<0.001$; interaction: $F(1.04, 17.67)=14.84$, $p=0.001$; simple main effects: all pulses: $p<0.001$).

Figure 17

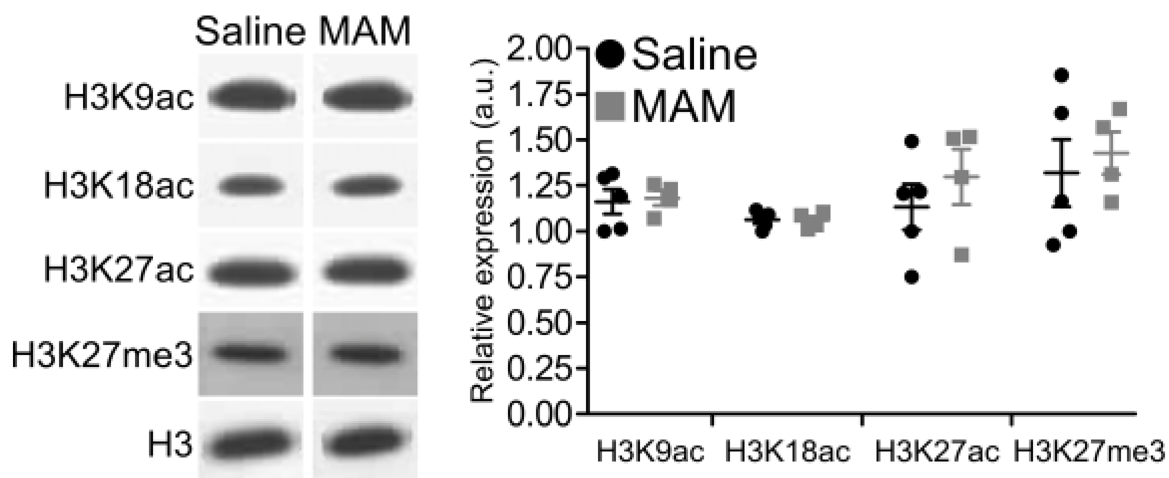


Synaptic AMPAR physiology is not affected by MAM exposure, yet pre-synaptic glutamate release is enhanced. We found no differences in amplitude or change of AMPA-eEPSCs between adolescent saline and MAM animals (amplitude, pA, Saline n=11, MAM n=10, Repeated Measures ANOVA: group: $F(1,19)=0.07$, $p=0.798$, pulse: $F(2.07, 39.28)=32.70$, $p<0.001$; interaction: $F(2.07, 39.28)=0.17$, $p=0.853$. Charge transfer, nA*ms, Repeated Measures ANOVA: group: $F(1,19)=0.18$, $p=0.674$, pulse: $F(1.06, 20.18)=64.09$, $p<0.001$; interaction: $F(1.06, 20.18)=0.32$, $p=0.592$). In addition, there was no significant difference in stimulation intensity between saline and MAM cells (saline 264.76±65.90, MAM 224.80±74.83, $t(19)=0.90$, $p=0.359$). However, we observed a significant increase in PPR in adolescent MAM mPFC (saline 0.88±0.05, MAM 1.09±0.05, $t(18)=-2.80$, $p=0.012$).

Global levels of histone modifications are not altered in adolescent MAM PFC

In order to explore one possible mechanism underlying the reduced expression of NR1 and NR2B, we used Western blot to probe levels of histone modifications that have been implicated in cognitive processes as well as psychiatric disorders. Adolescent saline and MAM PFC was used to probe for H3K9ac, H3K18ac, H3K27ac, H3K27me3, with total H3 protein serving as the normalizing protein. We found no significant differences in any of these modifications during adolescent development between saline and MAM PFC (Figure 18: H3K9ac: saline 1.16±0.07, n=5; MAM 1.18±0.04, n=4; $t(7)=-0.25$, $p=0.810$; H3K18ac: saline 1.06±0.02, MAM 1.06±0.02; $t(7)=0.10$, $p=0.924$; H3K27ac: saline 1.13±0.12, MAM 1.30±0.15; $t(7)=-0.853$, $p=0.422$; H3K27me3: saline 1.32±0.18, MAM 1.43±0.12; $t(7)=-0.472$, $p=0.651$).

Figure 18



No significant differences in histone modifications between adolescent saline and MAM PFC. Permissive acetylation markers and a repressive methylation marker was assayed using Western blot to compare histone modification patterns between saline and MAM animals during adolescent PFC development (H3K9ac: saline 1.16±0.07, n=5; MAM 1.18±0.04, n=4; p=0.810. H3K18ac: saline 1.06±0.02, MAM 1.06±0.02; p=0.924. H3K27ac: saline 1.13±0.12, MAM 1.30±0.15; p=0.422. H3K27me3: saline 1.32±0.18, MAM 1.43±0.12; p=0.651).

Discussion

Mental health care costs present the largest economic burden worldwide, and diseases that emerge before adulthood cost approximately 10% more than those diagnosed in later life (Lee et al., 2014). However, it is estimated the National Institutes of Health allotted less than 1% of its FY2014 budget to adolescent brain research (Lee et al., 2014). This disparity between the economic and psychosocial burden of an early-life diagnosis and limited research funds restrains our ability to develop advanced tools for diagnosis and treatment of mental health disorders, such as SCZ. A greater appreciation of the adolescent critical period can expand our understanding of molecular dysfunctions and physiological aberrations underlying abnormal cognition and behavior in adulthood. NMDARs are of particular interest due to their central role in developmental neuroplasticity and adult cognitive functioning. Thus, we investigated the expression and function of NMDARs in adolescent saline and MAM animals to determine how the PFC may become vulnerable during postnatal development.

During adolescence, MAM animals demonstrate complex changes in NMDAR subunit expression and synaptic physiology. As shown by Western blotting, synaptic NR1 protein is significantly reduced in adolescent MAM PFC, and NR2B protein levels are greatly decreased, although not statistically significant ($p=0.056$). As the constitutive subunit of the NMDAR complex (Paoletti et al., 2013), reduced NR1 protein levels indicate a decrease in post-synaptic NMDARs. Thus, we expected further investigation of synaptic NMDAR function on layer V pyramidal cells to be compromised following a

physiologically-relevant 10-pulse, 20 Hz train in layer II/III. However, we were surprised to find no significant differences in amplitude or charge of NMDA-eEPSCs between adolescent saline and MAM animals. In fact, Figure 15 shows a slight increase in summation in response to the initial pulses of the stimulation train in adolescent MAM mPFC. Additionally, PPR and stimulation intensity were not different between the two groups. However, our findings indicate subunit composition of NMDARs of layer V pyramidal cells in the adolescent MAM PFC differ from those in saline PFC. Given the importance of the NR2B subunit in synaptic plasticity and working memory (Wang et al., 2013), we evaluated the contribution of NR2B subunits to NMDA-eEPSCs with a selective NR2B inhibitor, Ro25 (1 μM). Pyramidal cells from saline mPFC exhibited a 45-60% reduction in NMDA-eEPSC amplitude and charge following Ro25 wash-on. However, Ro25 had a much stronger effect on adolescent MAM layer V pyramidal cells, dramatically reducing amplitude and charge of NMDA-eEPSCs by 73-87% at all pulses. Therefore, we postulated that adolescent MAM mPFC has a larger proportion of NR2B-NMDARs at the post-synaptic site of layer V pyramidal neurons while saline mPFC is characterized by triheteromeric NR1/NR2B/NRX receptors.

The extent of pharmacological inhibition by Ro25 depends on (1) the number of NR2B subunits contained within the NMDAR complex, as well as (2) the activity level of NMDARs. First, diheteromeric receptors containing the constitutive NR1 subunit as well as one other subunit (i.e. NR2B) will exhibit far greater reductions in NMDAR current than triheteromeric receptor complexes (NR1/NR2A/NR2B-containing) due to the increased number of Ro25 binding sites on the N-terminal domain (Hatton and

Paoletti, 2005). Therefore, the larger inhibitory effect of Ro25 on layer V pyramidal cells in adolescent MAM mPFC indicates predominantly NR2B-containing diheteromeric complexes are present at the post-synaptic membrane. Conversely, the moderate blockade of NMDA-eEPSCs by Ro25 in saline cells suggests mixed NR2B-containing triheteromeric complexes are more abundant during normal adolescent development. Alternatively, the magnitude of NMDAR activation positively correlates with the degree of pharmacological inhibition by Ro25 (Kew et al., 1996; Zhang et al., 2000). Due to the diheteromeric composition of NR2B-NMDARs in MAM mPFC, these receptors exhibit longer decay kinetics (Paoletti et al., 2013; Flores-Barrera et al., 2014). Therefore, the greater degree of receptor activation due to prolonged open channel time can further explain the much stronger inhibitory effect of Ro25 on NMDARs in MAM compared to saline animals.

Our findings indicate diheteromeric NR2B-NMDARs predominate in the MAM mPFC whereas triheteromeric NR1/NR2A/NR2B-NMDARs are prevalent in the mPFC of adolescent saline animals. Although this seems antithetical to previous reports linking persistent NR2B expression to working memory function (Wang et al., 2008; Wang et al., 2013), we hypothesize the incorporation of NR2A subunits serves to modulate Ca^{2+} influx to prevent excitotoxicity in the adult brain (Monaco et al., 2015), while the retention of NR2B subunits in the MAM PFC is a compensatory mechanism to prolong neuroplasticity. Our previous work in juvenile saline and MAM PFC (Chapter 3) shows NMDARs are significantly decreased in MAM layer V pyramidal cells. The substantial loss of NMDA-eEPSC amplitude and charge in layer V pyramidal cells from MAM

mPFC occluded the inhibition by Ro25 indicating NR2B-NMDARs are specifically downregulated during juvenile development. Thus, we hypothesize the retention of diheteromeric NR2B-NMDARs in adolescent MAM mPFC serves to extend the window of neuroplasticity in an attempt to compensate for the earlier loss of NR2B-NMDARs in juveniles. Visual processing, somatosensation, and motor functions rely on experience-dependent synaptic plasticity as a platform for maturation (Dumas, 2005; Monaco et al., 2015). In the corresponding cortices, NR2B-NMDARs are highly expressed during this critical learning phase until the NR2B-to-NR2A subunit switch occurs, which closes the critical period and limits further neuroplasticity. In the PFC, this subunit switch is absent leading to persistent expression of NR2B (Wang et al., 2008; Flores-Barrera et al., 2014), promoting the functional maturation of layer V pyramidal neurons and PFC-dependent cognitive functions. Therefore, the reduction in synaptic NR2B-NMDARs in juvenile MAM mPFC dampens plasticity, limiting synaptogenesis and synaptic strengthening (Davies et al., 1998; Konradi and Heckers, 2003). We propose the retention of NR2B-NMDARs in adolescent MAM mPFC serves to extend the juvenile critical window in an attempt to lengthen the period of functional plasticity. However, compared to layer V pyramidal neurons in saline mPFC, the high number of NR2B-NMDARs exposes the adolescent brain to excitotoxicity due to excessive Ca^{2+} influx and may lead to pruning of damaged or weakened synapses (Iafrati et al., 2014). Globally, an elevation of NR2B subunit levels can sensitize the brain to hyperexcitability (Jantzie et al., 2015). During early postnatal development, stimulation of glutamate receptors with seizure-inducing doses of NMDA, a time during which NR2B is highly expressed throughout the forebrain, results in cognitive impairment in adulthood (Stafstrom and Sasaki-Adams,

2003). This evidence further confirmed that over-stimulation of NR2B-NMDARs in early postnatal development can result in cognitive impairments, likely due to an overabundance of intracellular calcium signaling. In this way, the NR2B subunit exposes the brain's vulnerability and sensitivity to environmental perturbations during early postnatal development that can have direct effects on cognition in adulthood. Indeed, adult MAM animals demonstrate significant reductions in spine density in the mPFC compared to saline animals (Xing et al., 2016).

Thus, we propose that a threshold of NR2B expression exists (Figure 19). In this case, age seems to set this threshold. In early development, bidirectional changes in NR2B levels can result in lasting impairment of cognitive performance and synaptic plasticity. When this same aberrant increase in NR2B is placed in the context of the adult brain, we see many pro-cognitive effects of its overexpression (Tang et al., 1999; Cui et al., 2011). This is intimately tied to the conductance of calcium ions through NMDARs. High calcium in early development results in excitotoxic events, whereas in adulthood, high calcium seems to restore the plasticity of the brain. How can this be? NR2B levels are relatively high in the PFC throughout development, suggesting a greater calcium influx is an integral part of PFC maturation and cognitive functioning. Thus, other critical developmental milestones, such as synaptic pruning and maturation of neuromodulator systems, contribute to prefrontal development and eventually working memory and cognitive functioning.

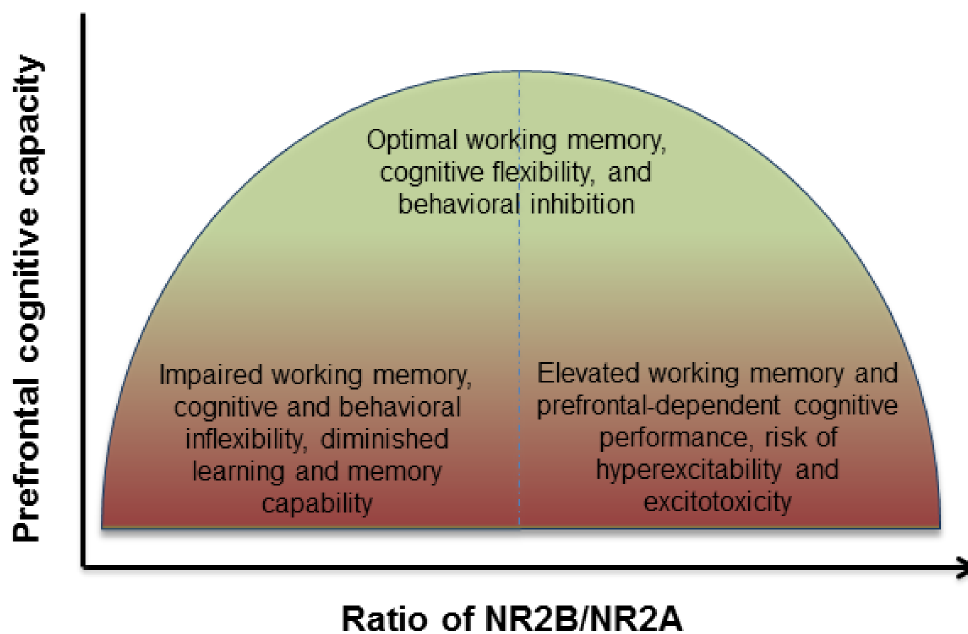
Our findings that post-synaptic AMPAR function is not affected in juvenile (Chapter 3) or adolescent MAM mPFC suggests this ionotropic glutamate receptor subtype is functionally intact. However, we did find a significant increase in the PPR of AMPA-eEPSCs, suggesting a facilitation of pre-synaptic release probability. This may be further evidence of compensation by the pre-synaptic machinery to facilitate depolarization of the post-synaptic membrane. To confirm this possibility, we will characterize AMPAR subunit expression, as well as levels of pre-synaptic vesicle packaging and release proteins to evaluate whether pre-synaptic mechanisms are also dysregulated in adolescent MAM PFC.

Lastly, we evaluated the global levels of histone modifications, which are involved in learning and memory processes, in the adolescent PFC of saline and MAM animals. We found no significant differences in any of the modifications assayed. However, these Western blot findings do not preclude a role for epigenetic mechanisms in regulating NMDAR subunit expression and composition. Chromatin immunoprecipitation is a very sensitive technique for evaluating the enrichment of proteins at the promoter region of interest, and would be better suited for evaluating the levels of epigenetic repressor and facilitator proteins at NMDAR subunit genes. Further experiments will allow us to explore this possibility.

Adolescence represents a critical developmental window during which the PFC undergoes the final stages of maturation, including excitatory and inhibitory circuitry strengthening, synaptic pruning, and dopaminergic innervation (Arnsten and Jin, 2014).

Insults during earlier periods of postnatal development increase the vulnerability of the PFC to dysfunction as it continues to mature, eventually resulting in cognitive impairments. In SCZ, these deficiencies in cognition are evident during the juvenile and adolescent periods, leading to the hypothesis that SCZ is a neurodevelopmental disorder. The MAM model is a reliable resource for evaluating these developmental alterations as it recapitulates many endophenotypes relevant to SCZ in adulthood. Thus, the findings herein lend insight into the neurobiology underlying cognitive delays exhibited by individuals prior to a diagnosis of SCZ, and contribute to the investigation of potential therapeutic targets during neurodevelopmental vulnerability to this debilitating disorder.

Figure 19



Schematic representation of the effects of optimal and imbalanced NR2B/NR2A ratio on cell health and cognitive functions.

CHAPTER 5: NMDAR HYPOFUNCTION IN JUVENILE PFC IN HDISC1-EXPRESSING MICE

Abstract

The Disrupted in Schizophrenia 1 (*DISC1*) gene was originally discovered in a Scottish pedigree in which family members with this genetic perturbation demonstrate high co-segregation with mental illnesses, primarily schizophrenia (SCZ). The DISC1 interactome is vast, and many protein partners are vital for neural development and adult synaptic physiology. Though the function of DISC1 is still under investigation, its implications in the earliest stages of brain development and adult-stage cognitive functions is clear. Perturbations of this protein in either developmental stage results in brain morphology abnormalities and cognitive impairments, respectively. However, the effect of dysfunctional DISC1 on early postnatal development and synaptic function has not yet been elucidated. This time point is of particular interest in elucidating the mechanisms at play in SCZ due to evidence of cognitive impairments in early development, prior to the typical age of onset in late adolescence or early adulthood. Previously, we have described impairment of N-methyl-D-aspartate receptor (NMDAR) synaptic physiology in the prefrontal cortex (PFC) in a neurodevelopmental model for SCZ. Thus, in addition to NMDAR hypofunction in the chronic stage of SCZ, dysfunction of this critical synaptic player begins as early as juvenile development of the PFC. Therefore, we investigated the consequences of the truncated human DISC1 protein on synaptic NMDAR expression and function in the juvenile PFC. Our results confirm NMDAR hypofunction is a feature of juvenile development in the PFC in this model for SCZ as well.

Introduction

Originally discovered and studied in a Scottish pedigree in the 1960s and 1970s (Blackwood et al., 2001), the Disrupted in Schizophrenia 1 (*DISC1*) gene is the result of a balanced chromosomal translocation between non-homologous chromosomes 1 and 11 (Millar et al., 2000). Importantly, family members with this genetic perturbation demonstrate high co-segregation of mental illnesses, primarily schizophrenia (SCZ), bipolar disorder, and depression (Millar et al., 2000; Brandon and Sawa, 2011). However, in genome-wide association studies (GWASs), *DISC1* failed to produce any significant associations with SCZ (Mathieson et al., 2012). Nevertheless, the DISC1 interactome includes proteins that are critical mediators of neural development, migration, synaptic formation and maintenance (Brandon and Sawa, 2011). Therefore, genetic perturbation of *DISC1* at these critical junctions sensitizes the brain to genetic or environmental insults, which contribute to the etiology of neurodevelopmental disorders (Brandon and Sawa, 2011). SCZ is characterized as a neurodevelopmental disorder due to early neuroanatomical and behavioral abnormalities evident in patients (Jones et al., 1994; Cannon et al., 2000; Rosso et al., 2000; Chung et al., 2017). Thus, DISC1 is well-positioned to play a role in early postnatal development, contributing to cognitive and behavioral deficiencies that persist into adulthood.

Although the function of DISC1 is unclear, various *Disc1* perturbations in animal models consistently recapitulate endophenotypes of SCZ, including dysfunction of PFC-dependent cognitive functions (Clapcote et al., 2007; Hikida et al., 2007; Li et al., 2007;

Pletnikov et al., 2008), enlarged lateral ventricles (Pletnikov et al., 2008), reduced parvalbumin immunoreactivity (Abazyan et al., 2010), and dopaminergic dysfunction (Niwa et al., 2010). Of particular interest, N-methyl-D-aspartate receptors (NMDARs) are implicated in the cognitive impairments characteristic of SCZ pathology (Gilmour et al., 2012; Snyder and Gao, 2013), and play a critical role in neuroplasticity and functional development (Dumas, 2005; Yashiro and Philpot, 2008). Interestingly, DISC1 and NMDARs reciprocally regulate each other and synaptic physiology (Ayhan et al., 2011; Namba et al., 2011; Ramsey et al., 2011; Wei et al., 2014). RNAi-mediated knockdown of DISC1 results in enhanced NMDA currents in cortical cultures and prefrontal pyramidal neurons (Wei et al., 2014) as well as in hippocampus (Wang and Zhu, 2014), and a dominant negative construct of DISC1 leads to working memory impairments (Li et al., 2007). Conversely, chronic NMDAR antagonism during embryonic development of the dentate gyrus (Namba et al., 2011) and in the adult striatum (Ramsey et al., 2011) results in diminished DISC1 function. These findings implicate DISC1 in the earliest stages of neuronal development and in the mature brain. However, it is yet unknown the effects of mutant DISC1 on early postnatal development.

Insufficient attention is paid to the developing brain and factors which may lead to dysfunction in later life (Lee et al., 2014); therefore, our goal in this study is to reveal the effect of truncated mutant DISC1 on NMDAR expression and synaptic physiology in juvenile prefrontal development. Specifically, the transgenic mouse line used herein results in constitutive expression of the same truncated mutant *DISC1* reported in the Swedish cohort (Millar et al., 2000) restricted to glutamatergic neurons of the forebrain

(Pletnikov et al., 2008). This particular model is proposed to result in a dominant negative effect; endogenous DISC1 protein is significantly diminished in perinatal development (Ayhan et al., 2011), but not in juvenile or adult stages (Pletnikov et al., 2008). Our findings indicate pre-synaptic release mechanisms, NMDAR and AMPAR function are compromised in juvenile hDISC1 PFC.

Methods

Animals

All animal procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Drexel University College of Medicine Animal Care and Use Committee. Food and water were provided *ad libitum* throughout the experiments.

Inducible human DISC1 (hDISC1) mutant mouse model

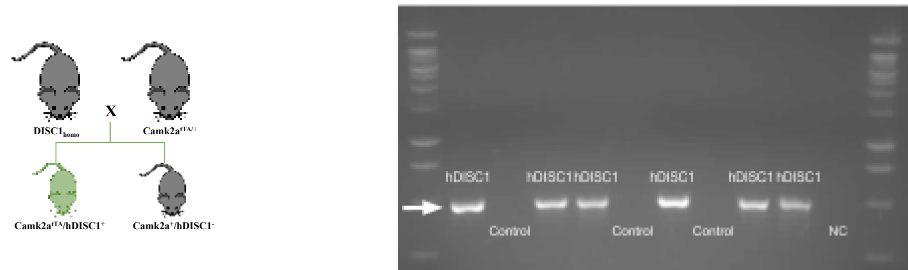
Homozygous mice expressing the truncated human Disrupted-in-Schizophrenia-1 gene (hDISC1, line 1001, C57BL6/J strain) were generously donated by Dr. Mikhail Pletnikov (Johns Hopkins University, Baltimore, MD) (Pletnikov et al., 2008; Abazyan et al., 2010). B6;CBA-Tg(Camk2a-tTA)1Mmay/J (Camk2a-tTA) hemizygous mice were purchased from The Jackson Laboratory (stock no. 003010, Bar Harbor, ME). Expression of Camk2a results in expression of the tetracycline-transactivor, tTA. In the hDISC1 line, a tetracycline-response element (TRE) site to which tTA will bind is positioned upstream of the transgene. Therefore, successful breeding of Camk2a-tTA hemizygotes and hDISC1 homozygotes yields two types of transgenic mice: 1) experimental mice with both Camk2a-tTA and hDISC1 genes will yield expression of the hDISC1 transgene, and 2) control animals wherein the tTA element is not present prohibiting the expression of the hDISC1 transgene. This is a TET-off system, where presentation of doxycycline

binds tTA and prohibits expression of hDISC1. In our experiments, hDISC1 gene expression is uninterrupted as our animals are never exposed to doxycycline. Further, because hDISC1 gene expression relies on the expression of tTA driven by Camk2a, forebrain- and neuron-specific expression of the mutant protein is possible.

To obtain control and experimental littermates, homozygous DISC1 mice were paired with hemizygous Camk2a-tTA mice (Figure 20). Breeding strategies included pairs and trios to maximize breeding efficiency. In trio scenarios, pair-housed females ensured successful care for all pups. Pups were usually born at E21/P0 and weaned between P21 to P28, at which time offspring were group-housed according to gender and not genotype. Tissue from both male and female mice was collected, but only male tissue was used for the experiments described below.

At P14, tail tip collection for genotyping and ear punch identification was carried out. Pups were briefly anaesthetized with isoflurane. While anesthetized, a small section of tail tip was cut using a clean razor blade and a small hole was punched in the ear based on a predetermined identification scheme. Pups were placed back with the dam and no further procedures were carried out until weaning or use for experiments. Pups aged P17-P23 were classified as juveniles, P32-P39 were classified as adolescents, and P60 or older were classified as adults.

Figure 20



Schematic representation of the hDISC1 breeding strategy. Left, homozygous DISC1 mice were paired or trio-housed with Camk2a-tTA hemizygotes. Offspring were typically born at E23/P0. At P14, tail tips were collected and mice received ear-punch identifications. Right, genotyping was performed using primers for the tTA transgene sequence (Jackson Laboratory). Animals were classified as mutants (green mouse) if a band appeared at 500 base pairs whereas control littermates (grey mouse) were identified by the absence of this band. NC, negative control

Tissue collection and western blot for synaptosomal proteins

PFC tissue was collected and processed as described in Chapter 3. Briefly, animals were perfused with 60 mL HEPES-buffered sucrose (in mM: 320 sucrose, 4 HEPES-NaOH buffer, pH 7.4, 2 EGTA, 1 sodium orthovanadate, 0.1 phenylmethylsulfonyl fluoride, 10 sodium fluoride, and 10 sodium pyrophosphate). The brain region containing the PFC was dissected and homogenized in sucrose buffer. The homogenate was centrifuged at 1000 g for 10 minutes at 4°C to remove large cell fragments and nuclear materials. The resulting supernatant was centrifuged at 15,000 g for 15 minutes at 4°C to yield cytoplasmic proteins in the supernatant. The pellet from this spin was re-suspended in homogenization buffer and centrifuged at 15,000 g for an additional 15 minutes to yield washed synaptosomes. The synaptosomal fraction then was hypo-osmotically lysed in 9 volumes of ddH_2O containing protease and phosphatase inhibitors and 4 mM HEPES-NaOH buffer, pH 7.4 and centrifuged at 25,000 g for 30 minutes to yield synaptosomal plasma membranes in the pellet.

A bicinchoninic acid (BCA) protein assay was performed to determine protein concentration. The protein sample was mixed with Laemmli sample buffer, boiled for 5 minutes, and separated on a 7.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to pore size 0.45 μm polyvinylidene difluoride membranes (PVDF, EMD-Millipore, Billerica, MA). The membrane was blocked in 5% nonfat milk and probed with primary anti-serum. Each blot was probed for anti-mouse NR1 (Invitrogen, 32-5000, 1:5000, Carlsbad, CA), anti-rabbit NR2A (EMD-Millipore, 04-901, 1:4000), anti-mouse

NR2B (EMD-Millipore, 05-920, 1:2000), anti-rabbit NR3A (EMD-Millipore, 07-356, 1:4000), anti-rabbit NR3B (Tocris, 2060, 1:4000, Bristol, UK), and anti-mouse actin (Sigma, A5316, 1:100,000, St Louis, MO), which was used as a loading control. The blots were incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG secondary antibody (1:5000, Vector Laboratories, Burlingame, CA), and proteins were visualized using enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Little Chalfont, UK). Protein expression of each subunit was evaluated by densitometry using ImageJ software (NIH, Bethesda, MD). Samples from each animal were run at least 4 times to minimize interblot variance. Raw values for NMDAR subunit proteins were normalized to actin and this ratio was further normalized to the first usable band on each membrane. These normalized values were averaged per animal to yield the final values which comprised the mean and standard error per group.

Whole-cell patch-clamp electrophysiology

Animals were anesthetized with Euthasol (0.2 ml kg^{-1}, IP) until unresponsive to toe pinch, the brains were dissected, and 300 μm coronal slices containing the prelimbic PFC were cut into an ice-cold bath of oxygenated artificial cerebrospinal fluid (aCSF, in mM: 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$, and 10 dextrose, pH 7.4) using a VT-1200S vibratome tissue slicer (Leica Microsystems, Wetzlar, Germany). To preserve tissue quality in adolescent animals, rats were perfused with ice-cold sucrose solution (in mM: 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 75 sucrose, 25 glucose, 0.5 $CaCl_2$, 7.0 $MgSO_4$, pH 7.4). Slices were transferred to a holding

chamber, submerged in oxygenated (95% O_2, 5% CO_2) aCSF at 35°C, for one hour and then remained at room temperature until used for recording. Slices were placed into a recording chamber mounted on an Olympus upright microscope (BX51, Olympus America, Center Valley, PA), where they were continuously bathed in oxygenated aCSF and maintained at 36 °C. Neurons were visualized with infrared differential interference video microscopy. All experiments were conducted with an Axon MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), and data were acquired using pCLAMP 9.2 software and analyzed using Clampfit 10.2 software (Molecular Devices).

Spontaneous and miniature excitatory post-synaptic currents

To record spontaneous or miniature excitatory post-synaptic currents (EPSCs), somatic whole-cell voltage-clamp recordings were obtained from layer V pyramidal cells using patch electrodes with an open tip resistance of 8-11 MΩ ($CsCl_2$ intracellular solution, in mM: 110 D-gluconic acid, 110 CsOH, 10 $CsCl_2$, 1 EGTA, 1 CaCl2, 5 QX-314, 1 ATP-Mg, 10 HEPES, at pH 7.3, adjusted with CsOH). Resting membrane potential was observed in current clamp mode for 1 to 2 minutes to confirm cell health. In voltage clamp mode, the membrane potentials were first held at -70 mV in the presence of picrotoxin (50 µM) to record AMPAR-mediated spontaneous EPSCs (AMPA-sEPSCs), which are a result of both action potential-mediated and stochastic release of glutamate, in the absence of $GABA_A$R-mediated neurotransmission. After 5 minutes of recording, tetrodotoxin (TTX, 0.5 µM) was added to the wash-on buffer to isolate miniature EPSCs (AMPA-mEPSCs). TTX blocks Na2+ channels, thus prohibiting action

potential-stimulated release of neurotransmitter. mEPSCs were recorded for at least 5 minutes and then DNQX (20 μM) was washed on to block AMPARs. When all currents ceased, the membrane potential was slowly ramped up to 60 mV. NMDAR-mediated mEPSCs (NMDA-mEPSCs) were then recorded for at least 5 minutes.

A typical s/mEPSC event was selected to create a sample template for event detections within a 5 minute period for each data file. The frequency (number of events) and amplitude of the individual events were examined with the threshold set at the medium level (i.e., 5 within a range of 1 to 9) in Clampfit. The detected events were then visually inspected to ensure specificity.

Evoked excitatory post-synaptic currents

To record evoked EPSCs (eEPSCs), a bipolar stimulating electrode was placed in layer II/III of the mPFC approximately 200-300 μm from visually-identified layer V pyramidal cells. Picrotoxin (50 μM) was used to block $GABA_A$R-mediated neurotransmission, and DNQX (20 μM) to block AMPAR-mediated currents when recording NMDA-eEPSCs. Cells were held at -70 mV to record AMPA-eEPSCs, and 60 mV to record NMDA-eEPSCs, which were evoked through the stimulating electrode using a paired pulse protocol at 50 Hz, and a single pulse, each with a 0.1 Hz stimulating interval. Stimulus intensity was adjusted until 50-100 pA eEPSC amplitude responses were achieved. Cells with an unstable baseline or unusual response patterns throughout recording were removed from the analysis. The EPSC recordings were monitored

throughout each experiment by implementing a -100 pA current pulse with a 200 ms duration, and all cells with >20% change in series resistance during recording were discarded. Traces were analyzed for amplitude (pA) relative to the first post-stimulus baseline and cumulative charge (charge transfer, nA*ms), as well as paired-pulse ratio (PPR). Single-pulse recordings were additionally analyzed for decay tau (ms) to compare channel kinetics between groups.

Tissue collection and western blot for histone proteins

PFC tissue was dissected as described above. The Epiquik Total Histone Extraction Kit (OP-0006, Epigentek, Farmingdale, NY) was used to isolate the histone fraction. Tissue was cut using a fresh razor blade and homogenized in Pre-Lysis Buffer. Homogenate was centrifuged at 10,000 rpm for 1 minute at 4°C. The pellet was resuspended in 3 volumes of Lysis Buffer and incubated for 30 minutes before another centrifugation at 12,000 rpm for 5 minutes at 4°C. The supernatant was mixed with Balance-DTT Buffer to yield the final histone fraction. A BCA assay was used to determine protein concentration. After boiling in Laemmli buffer for 5 minutes, 15 μg of protein were loaded on a 15% SDS-PAGE gel. After electrophoresis, blots were transferred to pore size 0.20 μm PVDF membrane (EMD-Millipore). Membranes were blocked with 5% nonfat milk and separately probed with the following primary antibodies: H3K9ac (Abcam, ab10812, 1:1000, Cambridge, UK), H3K18ac (Abcam, ab1191, 1:2000), H3K27ac (Abcam, ab4729, 1:2000), H3K27me3 (Abcam, ab6002, 1:1000), and total histone H3 (Novus, 1:100,000, St. Louis, MO) was used as a loading

control. Blots were incubated with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG secondary antibody (1:5000, Vector Laboratories), and proteins were visualized using enhanced chemiluminescence (ECL Plus, Amersham Biosciences). Protein expression for each histone modification was evaluated by densitometry using ImageJ software (NIH, Bethesda, MD). Samples from each animal were run at least 4 times to minimize interblot variance. Raw values for each histone modification were normalized to H3 and this ratio was further normalized to the first usable band on each membrane. These normalized values were averaged per animal to yield the final values which comprised the mean and standard error per group.

Data Analysis

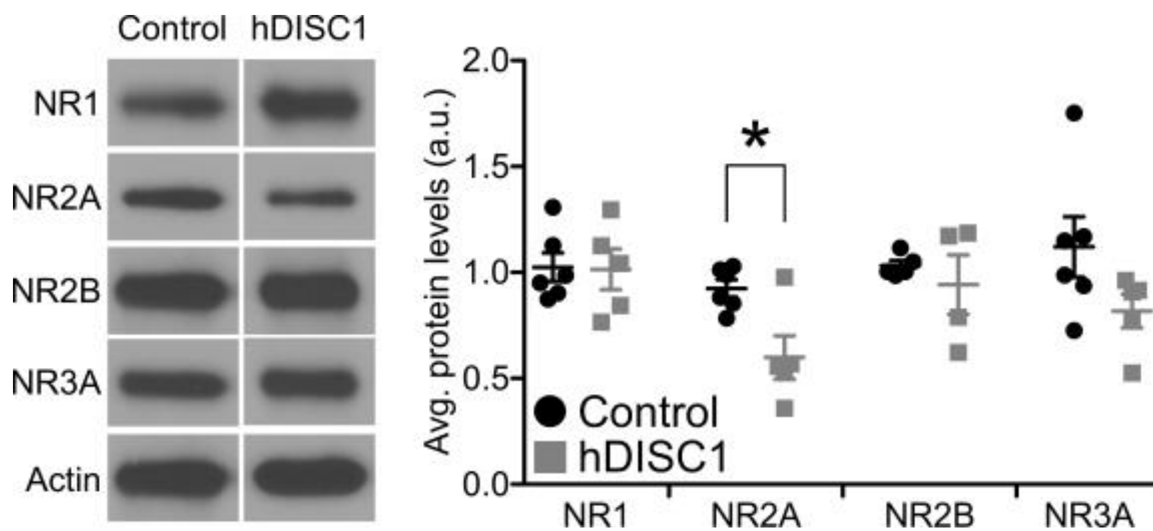
Statistical analyses were carried out using SPSS Statistics version 24.0 (IBM, Armonk, NY). Normality and homogeneity of variance testing determined the use of parametric or non-parametric tests for each dataset. Protein expression analyses, AMPA- and NMDA-s/mEPSC amplitude and frequency, and outcome measures from evoked recordings were compared using unpaired Student's t-test or Mann-Whitney U tests. All data are presented as mean ± standard error (S.E.). Single, double, and triple asterisks represent $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Results

hDISC1 mutants exhibit reduced synaptic NR2A protein levels in juvenile PFC

To characterize synaptic NMDAR subunit expression, we isolated synaptosomes from PFC of juvenile control and hDISC1 littermates to probe protein expression using antibodies for NR1, NR2A, NR2B, and NR3A. Actin served as the loading control. We found a significant decrease in synaptic NR2A protein in hDISC1 mutants compared to controls (Figure 21; NR2A: control 0.92±0.04, n=6 mice, hDISC1 0.60±0.10, n=5 mice; $t(9)=3.18$, $p=0.011$). We found no significant differences in other synaptic NMDAR proteins NR1, NR2B, or NR3A (NR1: control 1.02±0.07, hDISC1 1.01±0.10; $t(9)=0.09$, $p=0.932$; NR2B: control 1.03±0.02, hDISC1 0.94±0.14; $t(3.17)=0.71$, $p=0.571$; NR3A: control 1.12±0.14, hDISC1 0.82±0.80; $t(9)=1.75$, $p=0.115$).

Figure 21



Synaptic NR2A protein is significantly diminished in synaptosomes of juvenile hDISC1 PFC compared to control PFC. Synaptosomes isolated from juvenile control and hDISC1 PFC demonstrated reduced synaptic NR2A protein (NR2A: control 0.92±0.04, n=6 mice, hDISC1 0.60±0.10, n=5 mice; p=0.011). We found no significant differences in other synaptic NMDAR proteins (NR1 p=0.932; NR2B p=0.571; NR3A p=0.115).

Evoked AMPA-EPSCs in juvenile hDISC1 pyramidal cells are significantly increased compared to control cells

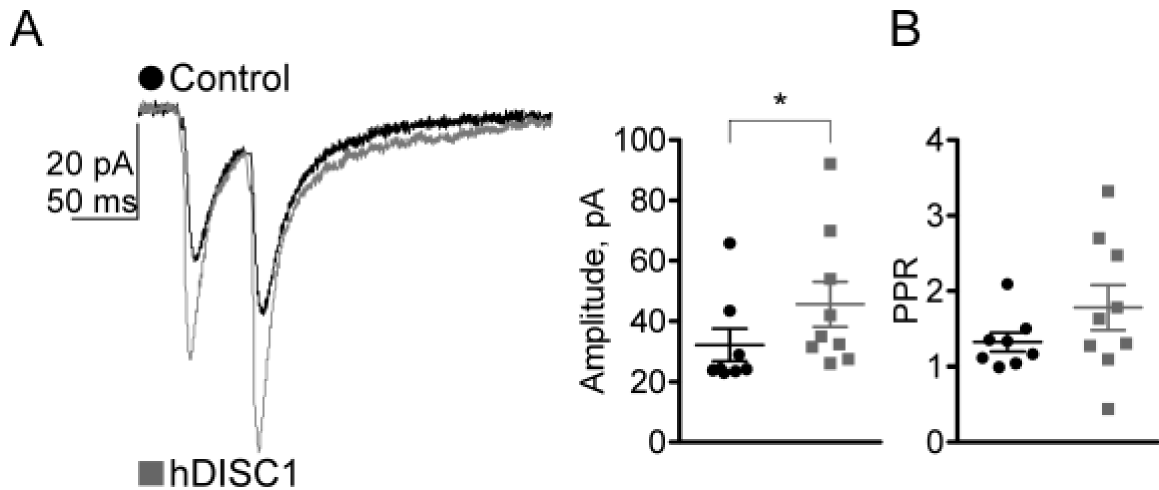
To directly investigate pre-synaptic release probability as well as post-synaptic AMPAR function, we recorded layer V pyramidal neurons while activating layer II/III afferents in the PFC using a paired-pulse 20 Hz stimulation paradigm. Picrotoxin (50 μM) was bath-applied to block $GABA_A$R-mediated neurotransmission as the cell was held at -70 mV. Amplitude, paired-pulse ratio (PPR), and stimulation intensity were compared between juvenile control and hDISC1 cells. With no significant difference in stimulation intensity (control 88.68±15.36, n=8 cells, hDISC1 93.87±16.07, n=9 cells; $t(15)=-0.23$, $p=0.820$), the amplitude of AMPA-eEPSCs was significantly increased in hDISC1 layer V pyramidal cells compared to controls (Figure 22; control 32.06±5.39, hDISC1 45.53±7.49; $U=15$, $p=0.046$). Pre-synaptic release probability was increased, although not significantly, in the mutants as well (PPR: control 1.32±0.13, hDISC1 1.78±0.30; $t(10.68)=-1.40$, $p=0.189$).

NMDARs in juvenile hDISC1 mPFC exhibit faster decay kinetics

To further explore the function of post-synaptic NMDARs, we recorded evoked EPSCs as describe for AMPA-eEPSCs with two notable exceptions. Membrane potential was held at 60 mV to account for spatial clamp issues, and DNQX (20 μM) was used in addition to picrotoxin to inhibit both $GABA_A$R- and AMPAR neurotransmission. We first recorded responses elicited by the paired-pulse stimulation paradigm, and then switched

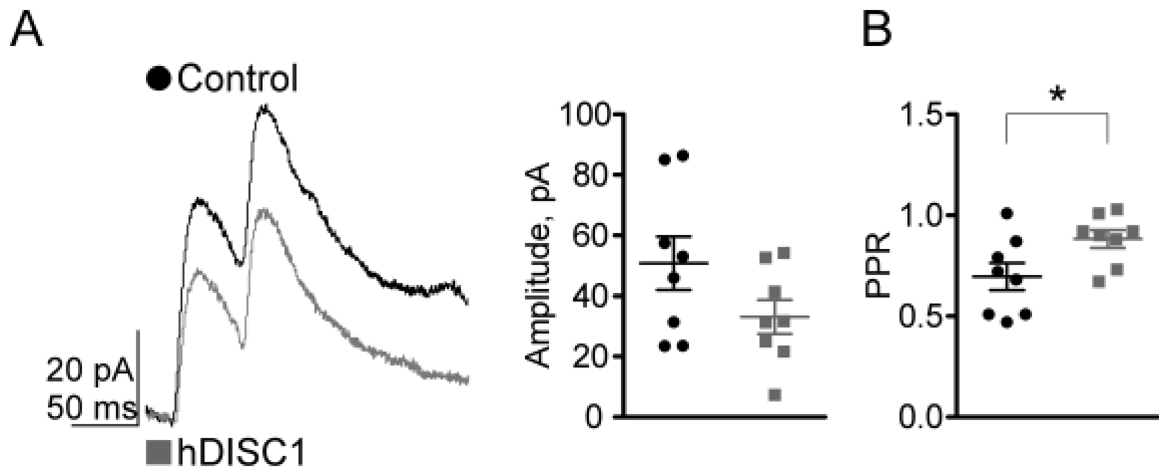
to a single-pulse paradigm to evaluate decay properties of post-synaptic NMDARs. Again, we found no differences in the stimulation intensity required to elicit NMDA-eEPSCs (control 78.56±14.59, n=8 cells, hDISC1 90.65±19.29, n=8 cells; $t(14)=-0.50$, $p=0.625$). In addition, an observed reduction in amplitude of NMDA-eEPSCs from hDISC1 cells did not reach significance (Figure 23; control 50.74±8.84, hDISC1 33.10±5.62; $t(14)=1.68$, $p=0.114$). Surprisingly, we revealed a significant increase in PPR in mutant animals compared to controls (Figure 23; control 0.69±0.07, hDISC1 0.88±0.05; $t(14)=-2.31$, $p=0.037$). Further, single-pulse stimulation elicited NMDA-eEPSCs with faster decay kinetics in juvenile hDISC1 mutant mice compared to control littermates (Figure 24; control 134.21±13.99, hDISC1 94.76±9.73; $t(14)=2.32$, $p=0.036$).

Figure 22



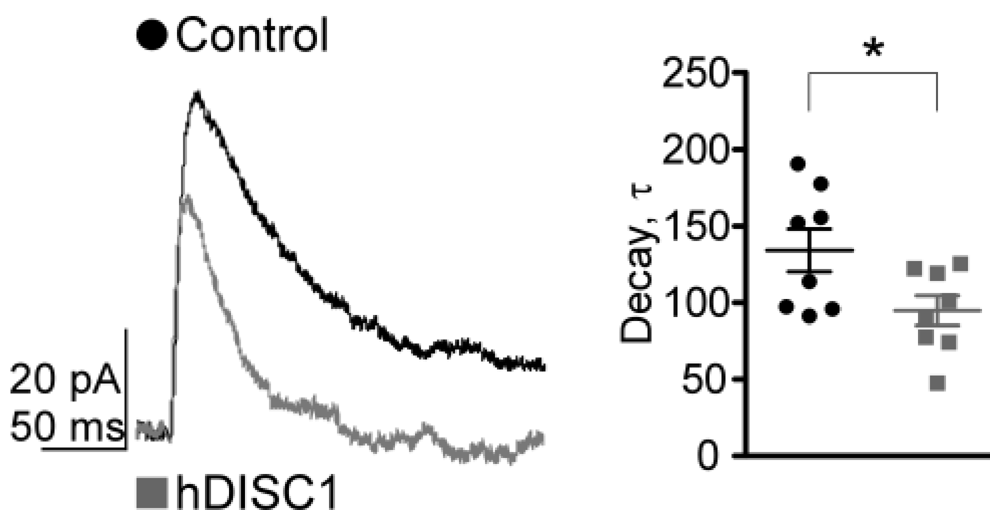
Synaptic AMPAR function is increased in hDISC1 mutant animals compared to control littermates. (A) Representative traces and (B) summary scatterplots from control littermates and hDISC1 mutants demonstrate a significant increase in amplitude of AMPA-eEPSCs without a significant difference in stimulation intensity (control 32.06±5.39, hDISC1 45.53±7.49; p=0.046). Pre-synaptic release probability was increased, although not significantly, in the hDISC1 mutants (control 1.32±0.13, hDISC1 1.78±0.30; p=0.189).

Figure 23



Enhanced pre-synaptic release in juvenile mPFC of hDISC1 mutants. (A) Representative traces, we observed a reduction in amplitude of NMDA-eEPSCs from hDISC1 cells, which did not reach significance (control 50.74±8.84, hDISC1 33.10±5.62; p=0.114). (B) We also revealed a significant increase in PPR in mutant animals compared to controls (control 0.69±0.07, hDISC1 0.88±0.05; p=0.037).

Figure 24



Synaptic NMDA currents display faster decay kinetics in juvenile mPFC compared to control littermates. Left, representative traces and right, summary scatterplot demonstrating a single-pulse stimulation paradigm elicited NMDA-eEPSCs with faster decay kinetics in juvenile hDISC1 mutant mice compared to control littermates (control 134.21±13.99, hDISC1 94.76±9.73; $p=0.036$).

Discussion

Numerous constructs of mutated *DISC1* exhibit behavioral and structural features characteristic of SCZ (Cash-Padgett and Jaaro-Peled, 2013). However, many of these silencing tools have been used *in vitro* or in the adult brain. The neurodevelopmental nature of SCZ begs the question, what are the effects of the truncated hDISC1 construct on early postnatal brain development? The cognitive impairments observed in individuals who are later diagnosed with SCZ indicate postnatal development is of particular relevance to the neurobiological underpinnings of early dysfunctions (Cannon et al., 2000; Rosso et al., 2000; Chung et al., 2017). Our work is the first to demonstrate the relevance of the NMDAR system in early postnatal development of the PFC in this hDISC1 model. We found significant effects of hDISC1 on levels of the synaptic NR2A subunit, and synaptic physiology of both NMDARs and AMPARs. Our findings suggest alterations to glutamatergic neurotransmission during juvenile development may sensitize the PFC to cognitive deficits later in life (Hikida et al., 2007; Li et al., 2007; Pletnikov et al., 2008; Abazyan et al., 2010).

In hDISC1 mutant mice, we found synaptic NR2A subunit protein is significantly reduced in the juvenile PFC compared to control animals. Somatic recordings of NMDA-eEPSCs from layer V pyramidal neurons during stimulation of layer II/III afferents revealed a significant increase in PPR, a measure of pre-synaptic release probability, as well as a non-significant reduction in amplitude. Reductions in NR2A protein and NMDA-eEPSC amplitude support our hypothesis that NMDAR hypofunction is evident

in the developing PFC of this model for SCZ. The NR2A subunit plays an important role in synaptic plasticity during development; typically NR2B-NMDARs are highly expressed during early stages of development, followed by a switch from primarily NR2B- to NR2A-containing receptors, signifying functional maturation of those regions (Sheng et al., 1994; Dumas, 2005; Zhao et al., 2005; Yashiro and Philpot, 2008; Monaco et al., 2015). In contrast, the PFC persistently expresses NR2B throughout development and adulthood (Wang et al., 2008; Monaco et al., 2015); thus, a shift in NR2B/NR2A ratio during the juvenile critical developmental period can have significant effects on cognitive capacities (Snyder et al., 2013; Xing et al., 2016; Li et al., 2017). Indeed, deletion of the NR2A subunit during development reduces the threshold for long-term potentiation (LTP) and depression (LTD), compromising metaplasticity in the visual cortex (Philpot et al., 2007). Conversely, dysregulated elevation of NR2B levels during juvenile development impairs LTP induction and maintenance (Iafrati et al., 2014) and maturation of GABAergic interneurons in the PFC (Bouamrane et al., 2016). In addition, a reduction, although non-significant, in NMDA-eEPSC amplitude in these mutant mice carries biological implications for the improper development of the PFC. Indeed, these findings may underlie the cognitive and synaptic dysfunction reported in adult hDISC1 mice (Li et al., 2007; Pletnikov et al., 2008; Abazyan et al., 2010; Ayhan et al., 2011; Holley et al., 2013).

Surprisingly, we also observed a significant speeding of decay kinetics of NMDA-EPSCs. Given the decrease in NR2A and purported increase in NR2B subunits, we expect decay to be significant slowed in these receptor complexes (Fu et al., 2005;

Gray et al., 2011). Indeed, Wei et al. (2014) demonstrated increased NMDA-eEPSC decay in addition to increased NR2A protein levels and enhanced NMDA-EPSC amplitude in cortical cultures treated with DISC1 shRNA (Wei et al., 2014). However, comparing these data directly to our own is difficulty given the disparities in experimental design between these works: our transgenic model constitutively expresses hDISC1 protein beginning from embryonic development, whereas Wei et al. (2014) targeted endogenous mouse DISC1 protein using short-term shRNA knockdown. Conceptually, both approaches should yield the same result: hDISC1 is proposed to exert a dominant negative effect on endogenous DISC1 protein, resulting in diminished DISC1 function (Li et al., 2007; Ayhan et al., 2011); however, the chronic expression of hDISC1 allows for compensatory mechanisms to occur in the intact biological system compared to the rapid affects afforded by RNAi-mediated methods. Therefore, our findings in juvenile hDISC1 mice reflect the effect of developmental hDISC1 expression, which is more relevant to the human population (Millar et al., 2000; Brandon and Sawa, 2011). Alternatively, these changes in evoked NMDAR-mediated responses may be localized to different dendritic compartments of the pyramidal cell, which would not be distinguishable by our global assessment of NR2A protein. Kumar and Huguenard (2003) elegantly demonstrated that NMDARs of differential composition are distributed according to afferent inputs; local cortical connections are mediated by diheteromeric NR1/NR2B-NMDARs whereas calloso-cortical synapses contain triheteromeric NR1/2A/2B or NR3 subunits (Kumar and Huguenard, 2003). Thus, layer-specific differences in synaptic NMDAR composition may occur in developing hDISC1 PFC. Here, we recorded layer II/III synapses onto layer V pyramidal cells; however, layer V to

layer V synapses are worth exploring to compare afferent-specific effects of hDISC1 in juvenile PFC circuitry.

In addition to NMDAR hypofunction, juvenile hDISC1 mPFC demonstrates a significant increase in PPR, a measure of pre-synaptic release probability. Facilitation of currents in response to a train of stimuli can occur by multiple pre-synaptic mechanisms such as residual calcium accumulation in the pre-synaptic bouton after the first stimulus, saturation of calcium-binding sites on calcium-buffering proteins, and calcium-dependent facilitation in a use-dependent manner (Fioravante and Regehr, 2011). Indeed, facilitation of NMDA currents is mediated by pre-synaptic effects of hDISC1 expression on the probability of vesicular release (Maher and LoTurco, 2012). RNAi-mediated DISC1 knockdown results in facilitation of post-synaptic NMDA currents whereas overexpression of wild-type DISC1 protein reliably results in paired pulse depression; thus, the role of pre-synaptic DISC1 may be to control the reliability of glutamate release (Maher and LoTurco, 2012). Compromising this system alters the synaptic plasticity dynamics in the developing PFC, upon which the proper development of cognitive faculties relies. However, these data are in contrast to others' findings demonstrating weakened pre-synaptic architecture and impaired vesicular transport by motor proteins in cultured neurons (Flores et al., 2011) as well as patient-derived induced pluripotent stem cells (Wen et al., 2014). Further elucidation of pre-synaptic function in the context of developmental hDISC1 expression is warranted.

PPF in layer V pyramidal neurons in hDISC1 mPFC may account for the augmented amplitude of AMPA-eEPSCs. NMDAR hypofunction can result in higher levels of synaptic AMPARs as a result of homeostatic plasticity changes (Hall et al., 2007; Hall and Ghosh, 2008; Gray et al., 2011). Holley et al. (2013) observed significant increases in excitatory spontaneous AMPA-EPSCs without significant alteration of spontaneous inhibitory events on layer II/III pyramidal cells in PFC, suggesting hyperexcitability of the PFC circuitry in adult hDISC1 mice (Holley et al., 2013). Thus, pre-synaptic alterations in release may be augmented persistently through development and into adulthood, contributing to dysregulation of PFC microcircuitry, which is critical for PFC-dependent cognition (Goldman-Rakic, 1995; Monaco et al., 2015). Evaluation of synaptic AMPA-EPSCs in adolescent hDISC1 mice will yield further insights into this hypothesis.

The complex etiology of SCZ is a result of both genetic and environmental perturbations. Here, we explore the effects of the hDISC1 genetic aberration found to highly co-segregate with neuropsychiatric disorders, SCZ among them (Millar et al., 2000; Blackwood et al., 2001; Brandon and Sawa, 2011). Much attention has been dedicated to the effects of mutated DISC1 protein on cellular dysfunction in early neuronal development, or synaptic physiology and cognitive dysfunction in the adult brain; thus, we aim to fill a gap in understanding the effects of hDISC1 on PFC development in juvenile animals and how this may contribute to cognitive deficits and SCZ phenotypes in adulthood.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The overarching hypothesis of this thesis is NMDAR hypofunction in the PFC, a postulated mechanism of cognitive dysfunction in the adult SCZ brain, extends to early stages of postnatal development, specifically evident in juveniles and adolescents in animal models for SCZ. In particular, our focus was on the cellular physiology and mechanisms underlying this early postnatal dysfunction of the PFC, a region vital for cognitive faculties. Because working memory is a constituent process of higher-order cognition (Goldman-Rakic, 1995) and is intimately dependent on NR2B-NMDARs (Wang et al., 2013; Monaco et al., 2015), this work evaluates the functional consequences of NR2B mis-expression on the developing PFC. Supporting this hypothesis, we observed significant alterations in synaptic NR2B/NR2A ratios as well as deficient physiology of NMDA currents in layer V pyramidal neurons, the critical cell layer exerting top-down control by the PFC, in both the MAM and hDISC1 models for SCZ. Further, we explored mechanisms that may underlie this dysfunction and determined epigenetic hyper-repression of *Grin2b* contributes to reduced synaptic NR2B protein in juvenile MAM animals. Finally, we have identified, for the first time, a potential compensatory mechanism of juvenile NMDAR hypofunction in adolescent MAM PFC. The implications of this finding remain to be fully appreciated until further experimentation can provide clarification.

NMDAR hypofunction in the developing PFC

NMDAR hypofunction is a proposed mechanism of SCZ, due to the emergence of cognitive dysfunction and other SCZ-relevant endophenotypes following

pharmacological NMDAR antagonism. However, less information can be gathered from the developing brain because SCZ is only diagnosed in late adolescence and early adulthood, and probing the function of the NMDAR system in humans is limited to imaging technology (Lee et al., 2014). Gross lesions of the developing PFC result in executive dysfunction and disinhibited behavioral outbursts that persist from early development to adulthood (Eslinger et al., 2004). These cognitive-behavioral abnormalities during school-age years undoubtedly impair an individual's ability to meet developmental milestones. In addition to these delays, cognitive impairment reaches a plateau at the age of onset of SCZ (Hoff et al., 2005). Thus, before a formal diagnosis and treatment plan can be employed, patients are already severely affected by the neurophysiological deficits underlying cognitive impairments in SCZ. Again, it is difficult with current technology to evaluate the physiological underpinnings of these dysfunctions in the human brain. Therefore, we must cautiously use animal models for SCZ to guide our understanding of this pathology.

The NMDAR system plays a critical role in synaptic plasticity processes during juvenile and adolescent development that are necessary to establish proper cognition in adulthood. Because PFC functional maturation occurs later and longer than other brain regions, this region is exposed to genetic vulnerabilities and/or environmental insults well into adolescence (Kolb et al., 2012; Monaco et al., 2015). Furthermore, the innervation of the dopaminergic system occurs during this critical developmental period (Arnsten and Jin, 2014), and impairment of receptor signaling complexes compromise the

neuromodulator systems that are another critical factor of cognitive regulation in the adult PFC (Monaco et al., 2015).

Intervention during PFC development in patients at-risk for SCZ presents a fundamental process that is currently untapped. As shown here, NMDAR hypofunction occurs during the early stages of postnatal development, and is proceeded by disruption of the dopaminergic system. The current FDA-approved therapeutic approach is to target this later dopaminergic dysfunction in an effort to minimize the symptoms and functional impairments characteristic of SCZ. However, therapeutic agents that have previously failed to demonstrate efficacy in clinical trials may be more effective when employed during the earlier stages of development, specifically during the window of NMDAR hypofunction. Indeed, treatment during juvenile development with an mGluR2 agonist/mGluR3 antagonist has demonstrated ameliorative effects on working memory function and NMDAR-mediated neurotransmission (Li et al., 2017). This finding highlights the importance of developmental NMDAR function in the maturation of cognitive functions, a process which is disrupted and can be subsequently recovered in the MAM model for SCZ by a brief pharmacological intervention during juvenile development. Further, current antipsychotic medications demonstrate minimal therapeutic efficacy in treating cognitive deficits in SCZ, suggesting the dopaminergic system is not capable of repaired the pathological processes that have left lasting damage to the PFC.

To model the neurodevelopmental aspect of NMDAR hypofunction, acute pharmacological blockade of NMDARs with PCP during gestation was found to elicit SCZ-like phenotypes in adulthood (Mouri et al., 2007; Jones et al., 2011; Coyle, 2012). More specifically, transient NMDAR antagonism during early juvenile development in mice also results in SCZ-relevant phenotypes, including diminished parvalbumin immunoreactivity, impaired latent inhibition, and poor behavioral flexibility (Jeevakumar et al., 2015). Interestingly, several studies have shown treatment during the juvenile period for various cellular aberrations is sufficient to restore cognitive and structural abnormalities in adult animals (Iafrati et al., 2014; Jeevakumar et al., 2015; Xing et al., 2016; Li et al., 2017). However, in models for SCZ where these receptors are not directly targeted, less evidence has been put forth regarding the state of NMDAR function during postnatal development. This work fills that gap; we show imbalance of the NR2B/NR2A ratio, via alterations in either subunit protein, is evident in juvenile MAM and hDISC1 animals, models in which psychosis-like phenotypes and cognitive impairments are reported in the adult stage (Flagstad et al., 2004; Flagstad et al., 2005; Moore et al., 2006; Li et al., 2007; Pletnikov et al., 2008; Abazyan et al., 2010). We have also found that NMDAR levels are not affected in early juvenile (P14) animals, suggesting the critical window of juvenile development begins at the P21 time point (Xing et al., 2016).

In juvenile MAM and hDISC1 animals, NMDAR hypofunction is due to an imbalance of NR2B/NR2A ratio, the two prominent NMDAR subtypes implicated in developmental processes (Paoletti et al., 2013). In MAM animals, synaptic NR2B levels are significantly reduced, and in hDISC1 animals, synaptic NR2A protein is significantly

reduced. These differential synaptic protein reductions result in diminished synaptic NMDA current, leading to dysfunctional glutamatergic neurotransmission at one of the critical periods of PFC development.

Future experiments exploring the subunit composition of juvenile NMDARs in hDISC1 animals are necessary to confirm the reduction in synaptic NR2A. Inhibition with TCN-201, a selective NR2A subunit antagonist, should significantly reduce NMDA-eEPSCs in control animals, but a substantial loss of synaptic NR2A protein in hDISC1 PFC will occlude the pharmacological effect. Further, the increase in pre-synaptic release probability evident in a paired-pulse stimulation paradigm muddies the potential post-synaptic effect on NMDARs. Bath application of NMDA would circumvent the possible effects of hDISC1 on the pre-synaptic vesicle machinery and allow us to explore NMDAR function when glutamate binding sites are saturating and presumably all NMDARs are activated. To explore pre-synaptic changes in hDISC1 PFC, immunohistochemistry using mPFC-containing slices to assess levels of vesicular packaging proteins and pre-synaptic markers, such as synaptophysin, will lend insight into the functional condition of these synaptic connections. A full functional profile of both pre- and post-synaptic sites is still unlikely to give us a complete picture of the pathophysiology underlying SCZ. Instead, it can lead us to explore mechanisms by which these synapses become hypofunctional and further our understanding of the highly complex development of a brain region as vital as the PFC.

Compensation for NMDAR hypofunction

By investigating NMDAR function during juvenile and then adolescent development, we are able to demonstrate the progressive effects of NMDAR hypofunction on this receptor system in the PFC. In saline-exposed PFC, we observe similar changes to NMDAR function when probing the subunit composition with a selective NR2B antagonist, Ro25. That is, Ro25 is able to inhibit NMDA-eEPSC amplitude and charge moderately. Further, we observed that an initial reduction in synaptic NR2B-NMDARs during juvenile development in the MAM PFC is followed by a rise in synaptic NR2B-mediated current in adolescence, as shown by the substantial effect of Ro25 on NMDA-eEPSC amplitude and charge. Thus, we concluded that synaptic NMDARs in adolescent MAM PFC are primarily diheteromeric NR2B-NMDARs. We propose that this is an over-compensation due to the low NR2B-NMDAR levels in juvenile MAM PFC. In fact, increased NR2B-NMDARs in the apical dendrites of layer V pyramidal cells during adolescent development promotes functional maturation of the PFC, specifically due to increased dopamine D1 receptor tone (Li et al., 2010) and is mediated by protein kinase A signaling (Flores-Barrera et al., 2014) and/or protein kinase C signaling (Li et al., 2010). Interestingly, post-mortem evaluation of PFC tissue from SCZ patients as well as chronic ketamine users reveals an increase in D1R levels (Narendran et al., 2005), supporting the possibility that NR2B-NMDARs are increased in the adult SCZ brain. However, the maintenance of high NR2B-NMDAR levels compared to normal development exposes the PFC to excitotoxicity (Monaco et al., 2015). NR2B subunits confer a longer open channel time than NR2A subunits,

resulting in higher Ca^{2+} influx (Paoletti et al., 2013). Thus, aberrant NR2B-NMDAR elevation in adolescent MAM PFC may serve a compensatory role, but also sensitize the still-developing PFC to excitotoxic damage (Monaco et al., 2015). Indeed, oxidative stress is proposed as another major hypothesis of neurodevelopmental dysfunction in SCZ (Do et al., 2009).

Another important question to be addressed is whether these receptors are primarily synaptic or extrasynaptic. To test insertion dynamics of NMDARs between saline and MAM animals, complete blockade of all activated synaptic NMDARs by the activity-dependent and irreversible antagonist MK-801 (Li et al., 2010) followed by stimulation of D1R activity (Li et al., 2010; Flores-Barrera et al., 2014), any restored NMDAR-mediated activity would be due to lateral diffusion of extra-synaptic NMDARs. Using this approach, we can evaluate the degree of NMDAR insertion in saline compared to MAM animals. Further, using Ro25 to selectively block NR2B-NMDARs from this newly inserted pool, we can evaluate the proportion of extrasynaptic NMDARs that exhibit this subunit composition. Insights from this experiment will reveal the preferential composition of NMDARs in adolescent PFC of MAM compared to saline animals, as well as the readily available pool of extrasynaptic receptors in MAM animals. The significant reduction in synaptic NR1 protein suggests total NMDAR levels may indeed be diminished in adolescent MAM PFC.

Furthermore, to reveal the identity of the third subunit in triheteromeric NMDARs in adolescent saline PFC, we will use a pharmacological approach to inhibit NR2A and

NR2B subunits. If NR2A subunits are indeed present in these receptors, no NMDAR-mediated current should remain in the presence of both TCN-201 and Ro25, NR2A and NR2B selective blockers, respectively. Future experiments may employ electron microscopy, which is the best technique to confirm the composition of these receptors definitively.

Epigenetic mechanisms in SCZ

In addition to elucidating the synaptic physiology of juvenile and adolescent PFC in MAM and hDISC1 animals, we explored a potential mechanism for dysregulated expression of NMDAR subunit protein. We were able to identify an epigenetic mechanism of *Grin2b* repression in the juvenile MAM PFC that contributes to NMDAR hypofunction. Epigenetic regulation of gene expression as a mechanism of dysfunction in neuropsychiatric disorders is receiving increasing attention (Nestler et al., 2016). These complex mechanisms of gene regulation provide an interesting new approach to understanding these illnesses because they indicate environmental insults can be translated into altered gene expression patterns without affecting the genetic code. We are far from employing therapeutic agents to successfully treat mental health disorders; however, they provide a relatively novel series of mechanistic insights that continues to be explored.

We have revealed an epigenetic mechanism that regulates *Grin2b* expression in the endogenous NR2B-to-NR2A subunit switch (Rodenas-Ruano et al., 2012; Tamminga

and Zukin, 2015) is aberrantly employed in the developing PFC to repress *Grin2b* expression in juvenile MAM PFC. REST (RE1-silencing transcription factor) is an endogenous repressor protein of *Grin2b* expression, with selectivity for this gene among the NMDAR subunits. REST enrichment at the *Grin2b* promoter, along with H3K27me3 enrichment, represses *Grin2b* in juvenile MAM animals. This finding is of particular interest because others have reported altered histone modification patterns in developing MAM PFC (Mackowiak et al., 2014), but have not demonstrated the specificity of an aberrant epigenetic program for any genes, as we have here.

Nonetheless, a more thorough investigation of histone markers and repressor enzymes at the promoter regions of *Grin1*, *Grin2a*, and *Grin2b* in juvenile animals will allow for a better understanding of epigenetic regulation in the developing MAM PFC throughout development. Thus, although Western blot analysis revealed no significant differences between histone modifications in adolescent saline and MAM PFC, chromatin immunoprecipitation may reveal protein-chromatin interactions that can be masked by global protein assessments.

Relevance of SCZ models to the human condition

There is a long-standing debate about whether rats have what could be a structural homolog to the primate PFC (Preuss, 1995; Seamans et al., 2008). The questions still remain: do rats have Delay cells as have been observed and studied in the primate dlPFC, and how can findings in rodents be extrapolated to primates? In order to evaluate whether

rats have a region comparable to the primate dlPFC, a set of criteria are commonly used to study the homology of these species' cortices. Focusing on (1) the pattern and relative density of specific connections among the PFC and other brain regions, as well as (2) the electrophysiological and behavioral properties that make up the functional PFC (Uylings et al., 2003). Firstly, based on anatomical definitions, it has been shown that both nonhuman primate and rat PFC receive their most dense projections from the mediodorsal nucleus of the thalamus (Ferguson and Gao, 2014), a major PFC identifier. Furthermore, both rat and primate PFC receive vast afferent projections from other cortical areas, primarily those of sensory and limbic origins (Van Eden et al., 1992). Anatomical evidence supports the notion that rat mPFC is related to primate anterior cingulate cortex as well as dlPFC (Seamans et al., 2008). Thus, previous literature suggests that rats do have an anatomical region relevant to the study of PFC-dependent cognitive functioning. However, it is still unclear whether rat PFC contains a discrete region comparable to primate dlPFC, and therefore, whether rats can provide a useful tool to model dorsolateral function and dysfunction, specifically (Preuss, 1995). Comparing the neuroanatomical correlates of behavior among species is more difficult given the unique survival mechanisms of each species (Uylings et al., 2003). Behavior is essentially the expression of an animal's capability to respond to environmental demands (Uylings et al., 2003). Through this simplified definition, it becomes easier to identify the brain regions specifically responsible for weeding out distracting stimuli, placing incoming information in a previously developed context, and allowing for an appropriate behavioral response, be it new or old. We cannot deny that this function serves a critical role in the survival of both rats and nonhuman primates.

In primates, lesions to dlPFC result in deficits to working memory function. In rats, lesions to the mPFC, also result in severe working memory deficits, specifically in acquisition and retention of a task (Uylings et al., 2003). We can therefore expect that neurons with properties similar to Delay cells as described in the dlPFC may also exist in the rat mPFC. In fact, such cells have recently been identified in the rat mPFC via electrophysiological recordings made during a modified delayed alternation Y-maze task (Yang et al., 2014). Through this technique, Yang et al. (2014) identified a subset of pyramidal neurons within the mPFC that respond in a transient, but not persistent, manner during the delay period of this paradigm of Y-maze. Interestingly, they identified three types of delay-like cells: those that fire during the early, middle, or late stages of the delay period (Yang et al., 2014). Therefore, from an electrophysiological perspective, we can conclude that rats do indeed possess a cellular correlate for encoding working memory information. It should be noted that in the eight-arm radial maze, a more cognitively complex task, such delay-like cells were not identified in the rat mPFC (Jung et al., 1998) (Jung et al., 1998). Thus, these characteristic firing patterns in the rat mPFC may be dependent on the cognitive load imposed by each behavioral task. Interestingly, the activity of discrete cells throughout the delay stage of a working memory task has also been identified in primate dlPFC (Rainer et al., 1999; Funahashi and Inoue, 2000). Thus, there appears to be a network of cells in the rat mPFC capable of encoding working memory-relevant information (Yang et al., 2014), as in the primate dlPFC. However, it remains to be determined whether these cells express NR2B-NMDARs, and whether they

are similarly sensitive to NR2B antagonism during a working memory task, as those described in primate dlPFC.

Clinical implications

Operating under the hypothesis that SCZ is a disorder of synaptic dysfunction, one may believe a single treatment, or combination of therapeutic agents, will be able to completely recovery cognitive deficits in this disorder. However, we must not forget the overt structural damage to the SCZ brain. Decreases in grey and white matter, as observed by fractional anisotropy, demonstrate reductions in myelination (Green et al., 2004). Therefore, we must consider cognitive impairments, and even positive and negative symptoms, can likely only recover to a certain degree, given our current inability to treat these structural alterations (Green et al., 2004). Nonetheless, functional improvement for patients will lessen the economic burden of this debilitating illness as well as the psychosocial burden on family and caregivers. These steps to independence for SCZ patients are the ultimate goal of our research endeavors.

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