Interactions Between Prenatal Kynurenic Acid Exposure and Adolescent Brain Development in the Emergence of Cognitive Deficits in Schizophrenia

DISSERTATION

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By
Michelle Lynn Pershing
Graduate Program in Psychology

The Ohio State University
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Dissertation Committee:
John P. Bruno - Advisor
Benedetta Leuner
Derick H. Lindquist
Abstract

The underlying mechanisms that contribute to emergence of schizophrenia (SZ) are not fully characterized and there are limited effective treatments for cognitive dysfunction in adults. Increasing evidence supports that abnormalities in the kynurenine pathway (KP) of tryptophan metabolism may be part of an early developmental mechanism of SZ as adult patients show genetic alterations in multiple KP enzymes and elevations in brain and cerebrospinal fluid levels of kynurenic acid (KYNA), an endogenous antagonist at N-Methyl-D-Aspartate (NMDA) and alpha 7 nicotinic acetylcholine (α7nACh) receptors. KYNA negatively modulates several neurotransmitters that are disrupted in SZ, including acetylcholine, glutamate, dopamine, and GABA. KYNA elevations may therefore contribute to the pathophysiology of SZ, including deficits in multiple cognitive domains such as attention, working memory, and cognitive flexibility. However, KP changes have not yet been explored prior to onset of frank illness; therefore, the role of KYNA in early development and contribution to subsequent disease is unknown. The purpose of these experiments was, therefore, to further elucidate the role of KYNA in the emergence of SZ and to utilize targeted pharmacotherapeutic intervention to improve cognition. We have developed a translationally valid rodent model to assess the effects of KP manipulations on several
relevant markers of SZ (behavior, neurotransmission, brain morphology, gene expression).

This project tested the hypothesis that elevated brain KYNA during a sensitive period of prenatal development would result in cellular/molecular changes that correspond to impairments in cognitive tasks that are dependent upon the integrity of the prefrontal cortex (PFC), a brain region critical for cognitive control and which shows distinct refinement during adolescence. The first aim determined the effect of prenatal kynurenine exposure on cortical development and trace fear conditioning (TFC; a task that is sensitive to prefrontal development) in juveniles (~PD32) and adults (~PD70). Age-dependent changes in TFC were observed in rats exposed to kynurenine in utero (EKYNs), which exhibited significantly increased cued-fear when tested as juveniles but significantly decreased cued-fear when tested as adults. The deficits noted in adult EKYNs occurred in conjunction with decreased dendritic spine density of Layer II/III mPFC neurons, which are required for TFC. The second aim of this project investigated the effects of prenatal kynurenine exposure on long-term changes in (1) peripheral and central KP metabolites (2) markers of cholinergic and glutamatergic gene expression just prior to adolescence (~PD32) and as young adults (~PD70). Remarkably, there was a post-pubertal increase in brain KYNA levels in EKYN rats, as levels were similar to controls (ECON) at PD32 but were 173% higher than controls at PD70. NMDAR subunit expression was also altered in an age-dependent manner, with significant reductions in NR2A at PD32 and PD70 and reduced NR1 expression only at PD70. Given that α7 nAChRs enhance NMDA cognitive circuits in brain regions that support
trace fear, the **third aim** determined the ability of an α7nAChR partial agonist (SSR180711) to restore cued-fear response in adult EKYNs. Administration of SSR prior to training, but not prior to testing, enhanced conditioned freezing in adult EKYNs. Taken together, these data further support the utility of KYNA elevations as a neurodevelopmental animal model of SZ, the sensitivity of the adolescent period as a window of vulnerability for the progression of neuropsychiatric disorders, and the exploration of α7nAChR/NMDAR interactions for targeted cognitive enhancement.

**KEYWORDS:** Schizophrenia, kynurenic acid, α7nAChR, NMDAR, adolescence, cognitive dysfunction, trace fear conditioning, SSR180711, prefrontal cortex
Acknowledgments

A few years ago I made the crazy decision to quit my job and return to graduate school. I’m lucky to have a wonderful network of friends and family that always provide support and encouragement, without which I would not have had the courage to make that decision or to keep moving forward when things were bleak. Many others have contributed to my personal and professional development during this time. I am appreciative to my advisor, John Bruno, for allowing me to pursue independent thought and research projects that expanded my interests and abilities. Thank you, also, to my committee members, Derick Lindquist and Benedetta Leuner, and the rest of the Behavioral Neuroscience faculty for access to lab resources and their guidance through this process. Notably, the dendritic spine analyses would not have been possible without the Leuner Lab, in particular the long hours spent at the microscope by Peter Fredericks. I also could not have completed the TFC experiments without the help of Jennifer Coppola and the willingness of the Lindquist lab to share resources with me; nor could I have run PCR without the assistance of Christinna Jørgensen and Sarah Hopp. Samantha Parsons and Damon DiSabato were instrumental in assisting with data collection; Samantha was also, without complaint, willing to come in on a weekend to feed and care for rats in order to give me a break, which was more helpful than I can likely express. Thank you, Kristin Edwards, for giving me chocolate, editorial comments, or a shoulder to cry on as
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Vita

May 2000 ...................................... B.S. Environmental Chemistry, St. Vincent College

May 2001 ...................................... B.S. Biology, St. Vincent College

December 2003 ............................ M.S., Environmental Science, The Ohio State University

December 2012 ............................ M.A., Psychology, The Ohio State University

November 2013......................... Award for Exceptional Neuroscience Research, The Ohio State University

March 2014 ............................... Psychology Department Research Excellence Fellowship, The Ohio State University
Publications


Fields of Study

Major Field: Psychology
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1.1 Schizophrenia Overview

Schizophrenia (SZ) is a neuropsychiatric disorder that emerges following adolescence with a devastating cascade of symptoms, including hallucinations and delusions; blunted emotion; and cognitive deficits. Originally considered a form of premature dementia due to the cognitive decline associated with the disorder, the term SZ was adopted beginning in the early 1900s as a reflection of the disordered thoughts that are common in these patients. Fewer than 20% of patients maintain independent living, primarily as a result of cognitive impairments (reviewed in Seeman, 1986; Green, 1996; Green et al., 2000). The economic burdens associated with this chronic disease, which typically requires life-long treatment following diagnosis, are high; costing an estimated $62.7 billion per year in the United States (Wu et al., 2005). Direct monetary costs are incurred through inpatient and outpatient therapy, assisted living facilities, case management, medications, and even criminal justice costs (McEvoy, 2007). Patients also have high unemployment and risk of early mortality, contributing to indirect costs associated with lost productivity (McEvoy, 2007).
Despite the enormous public health importance of SZ, which is among the top ten causes of disability world-wide (Auquier et al., 2007), the mechanisms underlying the dysfunctional neurocircuit trajectories that arise during development remain poorly characterized. Pharmacotherapy, as a result, has not evolved to treat underlying neuropathology, which is still not well understood. Instead, pharmaceutical intervention for years has evolved little; the efficacy (and side-effects) of antipsychotic drugs are attributed to their affinity to particular receptor subtypes. First generation side antipsychotics, noted for their efficacy in controlling positive symptoms, show particular affinity for blocking D2 receptors (Kapur and Mamo, 2003). This mechanism of action is also responsible for the adverse extrapyramidal side effects for which these drugs have generally been replaced by second generation (atypical) antipsychotics which show lower affinity for D2 receptors combined with antagonist activity at other receptors, such as 5HT2A. However, second generation antipsychotics have unique side effects as well, including weight gain and cardiovascular effects (Tschoner et al., 2007). Importantly, current pharmacotherapy is generally ineffective in addressing cognitive impairment in this disorder. Characterization of the nature and time course of cellular/molecular alterations in distributed neural systems and their relationships to cognitive performance may identify novel therapeutic targets with increased efficacy and may provide new opportunity for early intervention. Effective treatment stands to substantially reduce the impacts of this devastating disorder on the patient, their families, and on society, which is in direct alignment with NIMH goals of reducing the burden of mental illness.
1.2 Etiology and Risk Factors

SZ occurs with a prevalence of approximately 1% worldwide across geographic and economic boundaries (Lewis and Lieberman, 2000; McGrath et al., 2004; Saha et al., 2006). Meta-analyses clearly show heritability of SZ; it is estimated that genetic factors provide up to 80% of the risk for SZ (Cannon et al. 1998; Sullivan et al., 2003). The heritability of SZ suggests that the majority of variance/risk can be explained by genetic factors. For instance, third-degree relatives (cousins) of a SZ patient share approximately 12.5% of the same genes and have an approximate 2% risk of developing the disorder themselves; the proportion of shared genes and conferred risk of developing SZ increases with shared blood-line (e.g., a sibling/dizygotic twin shares approximately 50% of the same genes and has a 9% risk of developing SZ). In contrast, the risk of developing SZ in a monozygotic twin, with shared genes, is 50-80% (Farmer et al., 1987; Tsuang et al., 2001). This remarkable increase in risk with degree of biological relatedness speaks to the importance of genes; however, despite decades of research into such genetic factors, there has been no clear “smoking gun” to explain a genetic cause. Rather there is a range of susceptibility genes, including those involved in key cognitive/developmental processes (Fromer et al, 2014; Harrison and Weinberger, 2005). These susceptibility genes are proposed to interact with a multitude of environmental risk factors, including psychosocial, biological, and physical factors (Tsuang et al., 2001). Several biological and physical factors, both pre- and postnatally, are implicated in SZ. For instance, obstetric complications (hypoxia, trauma, pre-eclampsia), gestational stress, and illness are linked to increased risk of psychopathologies, including SZ (Davis et al., 2012).
Hypoxia, which may occur as a result of obstetric complications, has been shown to regulate many SZ risk genes, providing a link between enhanced SZ risk and environmental factors (Nicodemus et al., 2008; Schmidt-Kastner et al., 2006). Further, prenatal infections have been considered the primary non-genetic cause of SZ (Patterson, 2009), and epidemiological findings support a role for prenatal infections in the etiology of SZ (Brown et al., 2004; Brown and Derkits, 2010).

1.3 Hypotheses Linking Schizophrenia Etiology and Phenotype

SZ was first considered a dysfunction of dopaminergic transmission, based primarily on pharmacologic evidence from first-generation antipsychotic medications, which showed some promise in controlling positive symptoms by blocking D2-like receptors. There is much evidence to support the initial dopamine hypothesis of SZ, which in particular posited that overactive mesolimbic dopaminergic transmission results in positive symptoms and occurs in conjunction with underactive mesocortical dopaminergic transmission, resulting in negative symptoms and cognitive deficits (Abi-Dargham, 2004; Carlsson and Carlsson, 2006). These findings are consistent with single-photon emission computerized tomography (SPECT) studies and radiolabeling studies in patients, which show increased subcortical DA synthesis and D2 binding (Kugaya et al., 2000). However, the dopamine hypothesis alone is not sufficient to explain the etiology/symptomotology, nor is restoring down-stream dopaminergic transmission a viable solution for cognitive enhancement. The glutamatergic hypothesis of SZ followed the dopamine hypothesis, and has emerged from neurodevelopmental perspectives as
well as pharmacological data. Administration of N-methyl-D-Aspartate (NMDA)
receptor antagonists (PCP, ketamine, MK 801) replicate key features of SZ (Javitt and
Zukin, 1991) through multiple pathways, including increased firing of VTA
dopaminergic neurons (French, 1993; Carboni, 1989). Importantly, chronic PCP
administration results in increased mesolimbic DA in conjunction with decreased
prefrontal DA (Jentsch, 1999), which replicates the dichotomy observed in the clinical
population. NMDAR antagonists also enhance amphetamine-induced dopamine response
(Breier et al., 1998; Kegeles et al., 2000). SZ patients also exhibit reduced expression of
multiple NMDAR subunits (Weickert et al., 2013) and corresponding deficits in
cognitive tasks that are mediated by NMDAR, such as working memory (Keedy et al.,
2006).

Epidemiological data show that SZ patients smoke more (Ochoa and Lasalde-
Dominicci, 2007) and extract more nicotine per cigarette than healthy controls (Olincy et
al., 1997), which is consistent with an attempt to self-medicate to reduce negative
symptoms or to improve cognitive deficits mediated through nicotinic mechanisms
(Glassman, 1993). Nicotine has been effective in improving sensory gating, smooth eye
pursuit, and attention; phenotypic alterations in SZ that are linked to cholinergic
neurotransmission. Specifically, SZ patients show a poor capacity to filter irrelevant
stimuli and failure to habituate to repeated auditory stimuli; these deficits were transiently
normalized in a study which allowed SZ patients to smoke cigarettes prior to testing
(Adler et al., 1993). Smooth eye pursuit, saccadic eye movements that reflect failure to
coordinate eye movements during visual tracking, is also enhanced by nicotine (Olincy et
al., 1998). Diminished expression of the α7 nAChR occurs in selected brains regions of patients with SZ (Freedman et al., 2000), which may account for pathophysiological abnormalities such as deficits in attention and information processing, which are mediated by α7nAChR (for a review, see Martin et al., 2004).

Pathological alterations in PFC function in SZ have been also attributed, in part, to dysfunction in the local inhibitory circuitry mediated by GABAergic interneurons. Postmortem PFC tissue samples from patients with SZ show consistent reductions in multiple markers of GABAergic neuronal function (Lewis, 2000). GAD$_{65}$ and GAD$_{67}$, the enzymes responsible for GABA synthesis, are reliably reduced in SZ, findings which also correspond to dendritic spine abnormalities and behavioral deficits suggesting decreases in the enzyme responsible for GABA synthesis and behavioral deficits might arise from alterations in excitatory-inhibitory balance (Moyer et al., 2012; Lewis, 2012).

While there are multiple classes of GABA neurons, several lines of evidence suggest that altered GABAergic function is expressed primarily through PV-expressing interneurons. GAD$_{67}$ is not detectable in up to 50% of PV-positive GABAergic interneurons in postmortem brain tissue of SZ patients, thus providing further support for altered neuronal function of this population of GABAergic interneurons. Alterations in basket cells are suspected to affect both synaptic phasic inhibition and tonic inhibition (Beneyto and Lewis, 2011), which appear to occur primarily in Layers 3 and 4. In contrast, chandelier-cell alterations appear selective for Layers 2 and 3. Up to 30% of GABAergic neurons in Layers 2-5 of SZ patients do not express GAD mRNA (Lewis et al., 2005, 2009). Given the role of Layer 2/3 pyramidal neurons in working memory tasks,
significant dendritic spine alterations in these layers, and the sensitivity of these layers to pruning during adolescence, alterations in excitatory-inhibitory balance in Layer 2/3 of the mPFC may play key role in the emergence of this disorder during adolescence.

Adolescent emergence of GABAergic inhibition is a key process, which affects firing patterns of pyramidal neurons. LTP induction rules change during development, and the increase in GABA-ergic inhibition with maturation of the network alters the nature of the postsynaptic signal necessary to induce synaptic plasticity in (Meredith et al., 2003). These changes are reflected in age-related improvements in working memory (and other) tasks that occur in conjunction with DLPFC activation. Age-related improvements are noted in human subjects and animals assessed in attentional set-shifting (Newman & McGaughy, 2011) and working memory. PV-positive GABAergic interneurons regulate the firing of neurons, and coordinated firing of neurons is required for maintaining information during temporal delays in working memory tasks. PV-mediated inhibition of pyramidal cell activity during higher order cognitive tasks suggests that inhibition of GABAergic signaling leads to disruptions in synchronized activity. Indeed, altered GABA inhibition is correlated with reductions in gamma oscillations in animal models and SZ patients. Gamma abnormalities can be replicated by MK-801, which effectively causes disinhibition of pyramidal neurons through its affinity for interneurons.

Taken together, these data suggest that early developmental insults interact with genetic and environmental factors that disrupt brain development from a multi-systems perspective, thus dopaminergic, glutamatergic, and cholinergic systems are all critically
affected - perhaps differentially across patients. Research since approximately 2000 has focused on a possible mechanism of action which links previous disparate neurotransmitter and neurodevelopmental hypotheses, and importantly provides a naturalistic explanation for this disorder. This hypothesis is the kynurenic acid (KYNA) hypothesis of SZ, whereby alterations in the kynurenine pathway (KP) of tryptophan metabolism result in increased brain and cerebrospinal fluid levels of KYNA (see Section 1.8). KYNA, an NMDA and α7nACh receptor antagonist, has been shown to negatively modulate dopamine, glutamate, acetylcholine, and GABA (Rassoulpour et al, 2005; Konradsson-Geuken et al., 2010; Wu et al., 2010; Zmarowski et al., 2009; Beggio et al., 2014) and may thus underlie SZ symptomotology.

1.4 Symptomotology

SZ symptoms are diverse, varying in type and severity, but can be grouped into three main clusters: positive symptoms, negative symptoms, and cognitive deficits. Positive symptoms (hallucinations, delusions, and disordered thoughts) are representative of psychosis and are not present in normal patients; these symptoms tend to be episodic, with frequency and severity variable within the patient population. Conversely, negative symptoms represent a loss or lessening of normal emotions/behavior, and include affective flattening, alogia, avolition, anhedonia, and social withdrawal. Cognitive deficits are observed in multiple domains, including working memory (Goldman-Rakic, 1994, Abi-Dargham et al., 2002, Perlstein et al., 2001), sensorimotor gating (Braff and
Geyer, 1990), cognitive flexibility (Thoma et al., 2007, Everett et al., 2001), and sustained attention (Mar et al., 1996).

SZ is generally diagnosed in the late teens to early thirties, with males diagnosed earlier (21.2 ± 6.1 years) than females (24.2 ± 8.7 years) (Meltzer et al., 1997). However, despite the post-pubertal onset of psychotic symptoms, it is increasingly accepted that SZ is a neurodevelopmental disorder with both genetic and environmental risk factors. For instance, postmortem analyses show morphometric evidence of developmental abnormalities across several brain regions, including cellular disorganization/altered migration is noted in hippocampus (Kuroki and Mastushita, 1998) and cortex (Benes et al., 1991; Akbarian, 1993). Volume changes are noted in the PFC and hippocampus (Bogerts et al., 1990), and are likely reflective of decreased neuropil rather than decreased neuron number, which suggests decreased numbers of synapses and changes in dendritic/axonal organization (Harrison, 1999). Evidence of altered synaptic connectivity has been observed in the hippocampus, nucleus accumbens (NAC), and mediodorsal thalamus (MdThalm) (Weinberger, 1999). Structural magnetic resonance imaging has also demonstrated reduced amygdala volumes, which may occur premorbidly but are exacerbated in the prodrome (Lawrie et al., 2003). Further, reductions in amygdala volume occur in conjunction with abnormal amygdalar-prefrontal connectivity, which are also noted albeit to a lesser extent, in first-degree relatives (Tian et al., 2011).
1.5 Cognitive Deficits in Schizophrenia

While SZ is often associated with positive symptoms of psychosis, such as auditory hallucinations and delusions, it is underlying and persistent cognitive deficits that drive the long-term disability faced by this patient population. Cognitive impairments in SZ are pervasive, frequently precede the onset of psychosis and are now considered a core feature of the disorder. Most (90%) of SZ patients exhibit impairment in at least one cognitive domain while 75% of patients exhibit impairment in at least two domains (Palmer et al., 1997). Cognitive deficits are the most predictive of functional outcome (Green, 1996; Green et al., 2000) yet each generation of pharmaceutical intervention has remained relatively ineffective in alleviating these deficits; it is estimated that less than 20% of patients obtain full-time employment and independent living (Drake et al., 1999). Domains of impairment in SZ are considerable and depend upon cognitive control, which comprises multiple executive functions, e.g., planning, working memory, attention, inhibition, verbal reasoning, and multi-tasking (Chan et al., 2008) and affects the ability to process information and to adapt behavior.

Importantly, cognitive deficits are observed in patients prior to frank onset of illness, with impairments in a number of standard neuropsychological battery tests (Woodberry et al., 2008; Joyce et al., 2005). Thus, developmental alterations in brain circuitry that contribute to the emergence of SZ may be evidenced by cognitive dysfunction well prior to any psychotic symptoms. Unaffected first-degree relatives of SZ patients also show impairments in a variety of domains that fall under the main umbrella of cognitive control, including executive function and processing speed,
attention, and verbal memory (Egan et al., 2001; Faraone et al., 1995). Together, these findings suggest that cognitive impairment may thus be an important and consistent endophenotype (Lesh et al., 2011) and also that patients that subsequently develop SZ (versus relatives that do not) are subject to secondary factors that interact with brain maturation and which yield the SZ phenotype.

The importance of cognitive deficits as a core deficit in SZ has risen and key initiatives in the treatment of SZ now place cognition at the forefront for developing effective pharmacotherapies, with several points of emphasis including identification of cognitive domains most affected and amenable to treatment utilizing a standard set of criteria for academia, the pharmaceutical industry, and the Food and Drug Administration (Carter and Barch, 2007). The Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) and Cognitive Neuroscience Treatment to Improve Cognition in Schizophrenia (CNTRICS) initiatives identified cognitive and molecular targets, and valid clinical tests to assess cognitive impairments in a high throughput manner (Kern, 2004). The importance of cognition as key feature of SZ was highlighted and used as a platform to set forth research goals based on identifying species-appropriate tasks/paradigms to assess specific cognitive processes and their associated neural/psychological mechanisms in both animal and human models (Carter and Barch, 2007). A key premise behind cognitive-based studies in the CNTRICS initiative is translational validity. Cognitive neuroscience-based research initiatives provide a platform for preclinical translational research in disorders for which there are no clear biomarkers.
CNTRICS outlined several selection criteria, such as construct validity, linkage to known neural biology and cognitive mechanisms, availability of animal models, evidence for impairment in SZ/linkage to functional outcome, ability to use with neuroimaging, and good psychometric characteristics to identify tasks for development/use in cognitive neuroscience based assessment of 11 key cognitive domains, which can be grouped under five main constructs (see Table 1) (Barch et al, 2009).
<table>
<thead>
<tr>
<th>Cognitive Domains</th>
<th>Proposed Tasks</th>
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<tbody>
<tr>
<td>Perception</td>
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<tr>
<td>Gain Control</td>
<td>Contrast-contrast effect task</td>
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<td>Integration</td>
<td>Prepulse inhibition of startle</td>
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<td>Working Memory</td>
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<td>Goal Maintenance</td>
<td>Babble Task; Coherent Motion Detection</td>
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<td>Interference Control</td>
<td>Probabilistic Reversal Learning</td>
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<tr>
<td>Attention</td>
<td></td>
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<tr>
<td>Executive Control</td>
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<tr>
<td>Rule Generation/Selection</td>
<td>Intradimensional/Extradimensional Shift Task</td>
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<tr>
<td>Dynamic Adjustment of Control</td>
<td>Stroop Task</td>
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<tr>
<td>Long-Term Memory</td>
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<tr>
<td>Relational Encoding/Retrieval</td>
<td>Associative Inference</td>
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<tr>
<td>Item Encoding/Retrieval</td>
<td>Inhibition of Currently Irrelevant Memories</td>
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<tr>
<td>Reinforcement Learning, including Pavlovian Conditioning</td>
<td>Weather Prediction Task</td>
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<tr>
<td>Affective Recognition/Evaluation</td>
<td>Penn Emotion Recognition Task</td>
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Table 1 Summary of Cognitive Domains and Selected Proposed Tasks from CNTRICS. Selected tasks are provided; see Barch et al., 2009 for full list of proposed tasks.

1.6 Neural Substrates of Cognitive Deficits: A Role for Prefrontal Cortex Maturation

Many of the CNTRICs-recommended tasks rely upon the PFC or interactions between the PFC and hippocampus. The accumulating evidence suggests that mechanisms of cognitive dysfunction in SZ are associated with both micro-circuit dysfunction as well as macro-circuit, regional connectivity dysfunction across a larger distributed neural system that includes the hippocampus, amygdala, NAC, basal forebrain (BF), mdThalm, and PFC.

Thus, it is in context of this distributed neural system that we can explore the construct of cognitive control and integrate the numerous neurotransmitter dysfunctions
implicated in SZ on a network scale, and specifically one which arises over the course of maturation. The experiments described within this thesis emphasize interactions between the hippocampus and PFC given the importance of integration of glutamatergic inputs in PFC, which arise from ventral hippocampus and mdThlam, are modulated by acetylcholine (Hasselmo and Sarter, 2011) and are a critical component of executive functions/goal-directed behavior (Miller, 2000). There is further, particular emphasis on PFC maturation as the PFC serves as a coordination center for multiple brain regions critical to cognitive control (Asaad et al., 2000). As such, the PFC serves a primary role in top-down regulation by actively maintaining rules while incoming information is evaluated to guide responses. When there are conflicts, it is the rule maintenance of the PFC that allows successful information filtering and processing. SZ patients show deficits in information filtering and goal-oriented behaviors, which correspond to abnormal network activation including the PFC.

SZ is a disorder of cognitive neurodevelopment with characteristic abnormalities in working memory attributed, at least in part, to alterations in the circuitry of the PFC. Altered PFC circuits arise from both developmental insults in utero, and continuing in the mature brain, for example with impaired neural circuitry and synaptic connectivity in late adolescence and adulthood. Various environmental exposures from conception through adolescence increase risk for the illness, possibly by altering the developmental trajectories of prefrontal cortical circuits. Brain remodeling occurs during adolescence through many of the same early developmental mechanisms that drive the formation of functional neural circuits, including neurogenesis, apoptosis, and axonal growth and
projection; refinement of neural circuits through dendritic elaboration/elimination and synaptic pruning also defines this transitional period (Guerri and Pascual, 2010). Changes in brain volume, such as linear increases in white matter volume and inverted U-shaped changes in gray matter, result in regionally specific maturation of brain regions over time. Parietal and occipital lobes mature early followed by frontal and temporal lobes. With ongoing maturation and refinement of connectivity, the prefrontal networks start to be entrained in continuous theta–gamma rhythms (Metherate et al., 1992), whereby ACh facilitates complex interactions within a distributed neural network including the hippocampus and thalamus (Buzsaki et al., 1998). These changes correspond to the emergence of the ability to perform complex behaviors, which are guided by refined neural circuits. It is now clear that the underlying synaptic refinement process in the PFC is not completed until late adolescence and early adulthood (Andersen et al., 2003), which coincides with the period when symptoms of SZ typically begin to emerge.

1.7 Behavioral Task Selection: Trace Fear Conditioning

There is no single test of executive function. Rather, executive functions comprise the higher-level cognitive skills that allow coordinated thought, goal planning, and goal changing, inhibition and cognitive flexibility (Elliott, 2003) and draw on more fundamental cognitive skills, such as attention, language, and perception, to generate higher levels of creative and abstract thought. We have previously utilized separate hippocampus- and PFC-mediated tasks to assess cognitive dysfunction in rodents. Hippocampus-based tasks include Morris water maze (spatial navigation and reference memory) and passive avoidance (contextual memory), while PFC-based tasks include
attentional set-shifting tasks (ASST; rule learning and decision making). ASST is the rodent analog of the human Intradimensional/Extradimensional shifting task; in this task, rodents are trained to dig in terra cotta pots for a cereal reward and to subsequently discriminate between terra cotta pots based on odor, digging medium, or outside texture. Pre-/perinatal elevations of KYNA disrupt Morris water maze, passive avoidance, and rule learning and decision-making in ASST as evidenced by impaired ability to reverse rules (e.g., a previously unrewarded odor is now a rewarded odor) or to adapt when the rewarded dimension is changed (e.g., medium is rewarded instead of odor) (Alexander et al., 2013; Pershing et al., 2014). Sustained attention has also long been considered a hallmark behavioral task in assessing cholinergic/glutamatergic interactions. However, this task has a long training time making it impractical for use in juveniles. Trace Fear Conditioning (TFC), however, comprises both attention and working memory and, like attention, ACh efflux in mPFC increases during trace conditioning (Flesher, 2011). Interestingly, increased efflux of a lesser magnitude is observed in the hippocampus illustrating the distributed neural system utilized by TFC but not delay conditioning. Trace fear conditioning was selected as the cognitive measure for these experiments in order to further assess prefrontal-hippocampal interactions across development in rats prenatally exposed to KYNA. Task selection is consistent with CNTRICs-defined criteria, such as construct validity, known neurobiology, translational validity, and ability to measure impairment in several standard cognitive domains.

Trace fear conditioning is one form of classical conditioning, a well-established behavioral paradigm that is based on subjects making new associations between events in
the environment. In fear conditioning, an initially neutral conditioned stimulus (CS) such as an auditory tone is paired with an aversive unconditioned stimulus (US) such as a foot shock, which elicits a biological/reflexive response (unconditioned response; UR), such as jumping. After repeat pairings of the CS and US, the CS becomes predictive of the US and elicits conditioned fear response (CR), such as freezing. Associative learning is measured by assessing the subject’s contextual and cued-fear response. Freezing is a behavioral means to measure the animal’s ability to retain the CS-US relationship or the contextual-US relationship. Specifically, subjects are returned to the environment in which training occurred and freezing behavior in this context, in the absence of CS or US, is indicative of contextual fear memory. Conversely, when rats are placed into a novel context and exposed to the CS in the absence of shock, freezing represents CS-dependent freezing. Fear conditioning paradigms vary in terms of CS-US presentation and number of trials but can be generally grouped into delay conditioning and trace conditioning. In delay conditioning, the CS and US co-terminate, e.g., the shock is presented as the tone ends. In trace conditioning, an empty trace interval separates the CS and US. Selection of delay versus trace conditioning, and the length trace interval, has important ramifications as the underlying neurobiology and effects on learning are quite different. Delay fear conditioning is amygdala-dependent and does not require higher-order processing. Rather, the CS and US (after processing in the auditory and somatosensory cortices) converge in the basolateral amygdala (BLA). The BLA projects to the central nucleus of the amygdala; following fear conditioning, the CeA elicits fear response through hypothalamus, periaqueductal gray, and/or brain stem (Figure 1).
contrast, trace fear conditioning requires higher-order processing (attention, working memory) and depends upon amygdala, hippocampus, and prefrontal cortex (Figure 1).

Like delay conditioning, the CS and US converge in the BLA, which sends projections to the CeA, resulting in subsequent fear response via hypothalamus and brain stem. However, the addition of the trace interval recruits the hippocampus and prefrontal cortex, each of which has varying contributions to the task.
Figure 1. Distributed Neural System Underlying Fear Conditioning. Gray-shaded regions underlie delay fear conditioning, in which the CS and US are integrated from the Somatosensory and Auditory cortices to the basolateral amygdala (BLA). BLA connects to central nucleus of the amygdala (CeA), which elicits fear response through hypothalamus and brain stem regions. Addition of a trace interval recruits the hippocampus and prefrontal cortex.
Contributions of the hippocampus and PFC to the acquisition and expression of fear in trace fear paradigms are complex and under continual investigation. There are, however, some generalities: at very short trace intervals (less than 5 seconds), the task is functionally similar to delay conditioning (amygdala-dependent, not PFC- or hippocampus-dependent). Increasing the trace interval length engages mPFC and hippocampus as the subject learns both cue- and contextual-related fear; however, at long trace intervals the task is further reliant on hippocampus as contextual cues become the predominate conditioning factor. During trace fear conditioning, the CS and US are discontiguous. The hippocampus may therefore serve a bridging function. Namely, the hippocampus is critically involved in contextual conditioning (Kim and Faneslow, 1992; Phillips and LeDoux, 1992); therefore, the hippocampus may associate contextual information with the CS and US in order to overcome the discontiguity (Quinn, 2002). Indeed, the length of the trace interval has dramatic impact on recruitment of the hippocampus. The longer the trace (and the interstimulus interval) the weaker the association between the CS and US (Quinn, 2008). Indeed, the hippocampus appears to be necessary only at longer trace intervals, as inactivation of the hippocampus does not impair TFC acquisition at short trace intervals but it does impair TFC at longer trace intervals (Guimarães, et al., 2011). At very long trace intervals (>45 seconds), rats fail to associate the CS and US (Chowdhury, 2005).

The mPFC is also important for the acquisition, and possibly expression, of trace fear conditioning. This is perhaps not surprising as the PFC has been previously established to temporally integrate sensory information in behavioral paradigms (Fuster
et al., 2000) and to underlie attention and working memory (Cassaday, 2014). The specific role of the mPFC may be related to the attentional and working memory components of the task. The presence of a visual distractor during training impairs the ability of rodents to learn trace fear conditioning, but not delay or contextual fear, supporting the role of attentional processing specifically in TFC (Han et al., 2003). Lesions of the mPFC or anterior cingulate also prevent trace eye blink, but not delay, in rabbits, supporting a role for attentional processing in trace paradigms (Kronforst-Collins and Disterhoft, 1998; Weible et al., 2000). Working memory may be supported by sustained firing of Layer II/III neurons in the prelimbic region, as these neurons exhibit sustained activity in rats trained in a trace fear conditioning procedure, but not in delay conditioning or unpaired controls (Gilmartin and McEchron, 2005). It may be the sustained activity of these cells during the trace interval specifically, as optogenetic silencing of prelimbic neurons during the trace interval, but not during the CS, impairs CS-US association (Gilmartin et al., 2013).

Human studies utilizing TFC suggest that the hippocampus codes temporal information during trace conditioning, whereas brain regions supporting working memory processes maintain the CS–US representation during the trace interval (Clark and Squire, 1998; Quinn et al., 2002). SZ patients exhibit impairments in multiple classical conditioning paradigms, such as fear conditioning, measuring both autonomic and skeletomotor responses. These studies began in the 60s and 70s, expanding upon the premise that SZ is a disease of cortical/subcortical disconnectivity and/or of poor attentional processing. There are multiple paradigms in the literature used to assess
associative learning impairments in SZ, presenting a challenge for direct comparisons and for development of appropriate animal tasks. For instance, impairments in contextual processing and common hippocampus dysfunction in this population has led to contextual fear paradigms in order to establish contextual processing. Albert et al. (1970) examined fear conditioning in chronic SZ patients, and while there was no incorporation of a trace interval the methods required subjects to associate three distinct tones with foot shocks of varying intensities. While the tones and shocks and co-terminated in this experiment (delay conditioning), the inclusion of multiple tones and shocks increases task difficulty and engages PFC. The authors reported that SZ patients were unable to discriminate between non-reinforced and reinforced tones, relative to control subjects. Marenco et al. (2003) examined the effect of trace utilizing eye blink conditioning, another associative learning paradigm in which an auditory tone CS is paired with an air puff (US) to the eye, causing blinking (UR). After repeated pairings, the CS alone elicits blinking (CR). Furthermore, with repeated trials, the timing of the blink is optimized for occurrence of the US, as a blink that occurs either too early or too late would be ineffective in preventing the puff of air to the eye. Patients with SZ exhibit early, non-adaptive responses during trace eye blink conditioning, suggesting altered timing of CRs (Marenco et al. 2003).
1.8 Elevated Kynurenic Acid as a Neurodevelopmental Animal Model of Schizophrenia

While the longitudinal nature and complex interactions of risk factors underlying SZ remain unclear, changes in the kynurenine pathway (KP) of tryptophan degradation are an intriguing suspected mechanism underlying SZ pathophysiology, as multiple, previously disparate hypotheses regarding SZ etiology can be unified under KP changes. Specifically, elevations in brain levels of kynurenic acid (KYNA), an astrocyte-derived end-product of the KP, may provide a functional link between the myriad neurotransmitter hypotheses of the disorder given 1.) its ability to modulate cholinergic, glutamatergic, dopaminergic, and GABAergic neurotransmission and 2.) the ability of immune activation and stress to alter the KP, which provides links to known environmental risk factors.

KYNA is an endogenous compound, produced from dietary tryptophan, which is an essential amino acid. Dietary obtained tryptophan is utilized for subsequent neurotransmitter synthesis in the brain, including serotonin; however, the majority (99%) is utilized in the KP (Moroni, 1999). As shown in Figure 2, the initiating and rate-limiting step for either branch is the presence of kynurenine, which requires dietary tryptophan and the enzyme tryptophan 2,3 dioxygenase (TDO; located primarily in peripheral tissues such as liver and kidney) or indoleamine 2,3-dioxygenase (IDO; which is widely distributed). In microglia, kynurenine can then be further metabolized by the enzyme kynurenine 3-monooxygenase (KMO) to 3-hydroxykynurenine (3-HK), a free-radical generator and subsequently to quinolinic acid, which is excitatory and a
precursor to nicotinamide adenine dinucleotide (NAD+), a coenzyme important in redox reactions in metabolism. Kynurenine can also be catabolized in astrocytes, generally following transport from the periphery across the blood-brain barrier by the non-specific amino acid transporter, System-L (Fukui et al., 1991).

Figure 2. Kynurenine Pathway of Tryptophan Degradation. The majority (99%) of dietary obtained tryptophan is degraded in the kynurenine pathway (KP), although tryptophan also serves as a bioprecursor to serotonin. The KP is a functionally segregated two-arm pathway, in which quinolinic acid is formed in microglia and kynurenic acid is formed in astrocytes.
Within astrocytes, kynurenine is rapidly transaminated, primarily via the enzyme kynurenine aminotransferase II (KAT II) after which KYNA is released into the extracellular space (Guidetti et al., 1997). There is no known reuptake mechanism for KYNA; therefore, elimination of KYNA occurs by probenicid-sensitive excretion. There has been considerable debate regarding the pharmacologic target of KYNA at physiological concentrations. KYNA can act as a competitive antagonist at all ionotropic glutamate receptors and as a noncompetitive antagonist at α7 nAChRs, where it binds to the allosteric potentiating ligand (APL) site (Wu et al. 2010; Hilmas et al., 2001; Schwarcz and Pellicciari, 2002). The IC$_{50}$ of KYNA for the NMDA receptor is 250 µM, while in the millimolar range AMPA and kainate receptors are inhibited (Bertolino et al., 1989). Furthermore, KYNA has high affinity for the glycineB site of the NMDA receptor and would show preferential activity at this target (IC$_{50}$ of 8-16 µM) if not for the competing presence of physiologic glycine, which increases IC$_{50}$ to the high micromolar range. KYNA concentrations in rodents and cerebrospinal fluid levels in humans are in the low- to mid-nanomolar range while human postmortem brain levels are in the low micromolar range (Moroni et al., 1988; Turski et al., 1989). Therefore, under physiologically relevant conditions KYNA likely acts preferentially at the α7 nAChR, with an IC$_{50}$ of approximately 7 µM (Hilmas et al., 2001). This preferential activity at the α7nAChR is supported by experiments showing that decreased striatal dopamine release is induced by both KYNA and methyllycaconitine (MLA), an α7nAChR-specific antagonist, but not by the glycineB specific antagonist 7-chlorokynurenic acid (Rassoulpour et al., 2005). Furthermore, elevated prefrontal KYNA has also been shown
to decrease basal acetylcholine (Zmarowski et al. 2009) and glutamate (Konradsoson-Geuken et al., 2010); the KYNA-induced reductions in prefrontal glutamate were blocked by co-administration with the α7nAChR positive modulator galantamine, providing further support of KYNA’s affinity for the α7nAChR.

Cerebrospinal fluid and postmortem brain levels of KYNA are approximately two-fold higher in SZ patients than in control subjects (Erhardt et al., 2001, 2007; Schwarcz et al., 2001). CSF kynurenine is similarly increased; however, tryptophan is unchanged (Erhardt et al., 2007). These changes are suggestive of enhanced conversion of bioprecursor along the KP to yield KYNA. Such enhanced metabolism is supported by changes in multiple KP enzymes, particularly TDO and KMO. TDO, one of the enzymes responsible for the rate-limiting conversion of tryptophan to kynurenine, is up-regulated, corresponding to genetic changes (Miller et al., 2004). Wonodi et al. (2011) found reduced KMO gene expression and enzyme activity postmortem brain tissue of patients; polymorphisms in the gene encoding KMO also correspond to behavioral deficits in predictive pursuit and visuospatial working memory.

KYNA increases as observed in the clinical population can be replicated in a variety of pharmacologic and genetic animal models. Mice with genetic alterations in kynurenine pathway, such as KMO knockouts (KO) show significantly increased brain KYNA while KATII deficient mice have significantly reduced brain KYNA (Alkondon et al., 2004; Amori et al., 2009a,b; Giorgini et al., 2013; Yu et al., 2004). Pharmacologic manipulation of these enzymes is also available and KMO inhibition with Ro61-8048 results in increased brain KYNA levels (Forrest et al., 2013a, 2013b; Röver et al., 1997)
and administration of newly available systemic KATII inhibitors decreases KYNA levels, enhances glutamatergic transmission in the mPFC, and improves cognitive performance (Cherian et al., 2014; Wu et al., 2014). Further, intraperitoneal (i.p.) injection of the immediate bioprecursor, L-kynurenine at 25-, 50-, and 100-mg/kg results in 4-, 10-, and 12-fold increases in brain KYNA, respectively (Konradsson-Geuken et al., 2010; Alexander et al., 2012). However, metabolic adaptations may occur following repeated injections that limit KYNA production following daily i.p. injections (Vecsei et al., 1992). In contrast, subchronic dietary administration of L-kynurenine results in robust increases in brain KYNA (Alexander et al., 2013; Pocivavsek et al., 2012, 2014; Pershing et al., 2014). This method, relying upon each subject’s own enzymatic and astrocytic capacity, is thus a naturalistic and translationally valid experimental tool to assess the effects of developmental KP changes.

The experiments in this thesis were conducted to further elucidate mechanisms of cognitive control deficits in SZ and validate the KYNA animal model of the disorder. The three related and overlapping specific aims were directed at understanding: (1) the interaction of KP changes and adolescent brain maturation and resulting emergence of cognitive dysfunction; (2) how prenatal KYNA exposure results in long-term changes in gene expression; and (3) the potential of α7nAChR modulation to improve cognition.
Chapter 2: General Methods

There are three experimental aims outlined in this dissertation. The first aim was designed to determine the effect of prenatal kynurenine exposure on trace fear conditioning (TFC) prior to and following adolescence. Because this task is particularly sensitive to prefrontal development, dendritic spines were also examined. The second aim of this project determined the effects of prenatal kynurenine exposure on long-term changes in selected peripheral and central kynurenine pathway metabolites and markers of cholinergic and glutamatergic gene expression. The third aim investigated the effects of acute administration of a partial α7 nAChR agonist on TFC.

2.1 Animals and Husbandry

Animals for all experiments were bred in The Ohio State University vivarium. Adult Wistar rats were purchased from Charles River Laboratories, Inc. (Kingston, NY) from separate barrier rooms to avoid sibling mating. Rats were 225-250 g at the time of receipt and were housed for a minimum of 7 days for acclimation purposes. Animals were housed in plastic cages with ground corncob bedding (Bed-O’Cobs, The Andersons,
Maumee, OH), which was changed at least once per week. Rats were maintained on a 12:12h light/dark cycle (lights on at 0600 h) in temperature- and humidity-controlled, AAALAC-approved animal facilities with *ad libitum* access to food and water. All procedures were approved by the Institutional Animal Care and Use Committees of The Ohio State University in accordance with the NIH Guide for Care and Use of Laboratory Animals.

2.2 Breeding Procedures

Rats were cohabited on a 1:1 basis, in the home cage of the male. Females were checked daily and positive evidence of mating was confirmed by evidence of a copulatory plug or the presence of sperm in a vaginal lavage. The day on which evidence of mating was observed was termed embryonic day (ED) 0 and the animals were separated and housed individually in plastic cages with ground corncob bedding (Bed-O’Cobs, The Andersons, Maumee, OH) and Nestlets™ (Ancare, Bellmore, NY). Females were a minimum of 90 days old at the start of breeding.

2.3 Kynurenine Supplementation and Treatment Groups

Rats were fed a mash diet spiked with L-kynurenine or an unadulterated mash. Beginning on embryonic day (ED) 0, rats were habituated to rodent mash (Teklad Diets, Madison, WI) by providing 30 g mash mixed with water (1:1 w/v). Food intake was observed daily and the amount of food provided was adjusted (± 5 g) based on amount
eaten. Beginning on ED15 rats continued on unadulterated mash and were designated as controls (ECON) or were provided with diet that contained 100 mg L-kynurenine sulfate (99.4% purity; Sai Advantium, Hyderabad India) each day from ED15-22 (EKYN). After ED22, all rats were given standard rodent pellets (Teklad Diets, Madison, WI). The day on which dams gave birth was denoted postnatal day (PD) 0. On PD 2, litters were culled to 9 to 11 pups to standardize growth rates across all litters and to maximize the number of males per litter.

2.4 Body Weights

Females were weighed during gestation (ED6, 9, 12, 15, 18, and 20). Offspring were weighed weekly from postnatal day (PD7) through PD56 and on PD70. Litter means were calculated separately by sex for ECON (n=16) and EKYN (n=17) litters that subsequently contributed to biochemistry/behavior endpoints.

2.5 Weaning And Selection

Each dam and litter remained together until weaning on PD21. Females were discarded at weaning or used for experiments that are beyond the scope of this dissertation. Males were pair-housed by litter and assigned to three experimental aims as follows (see Figure 3 and Table 2 below): Two males/litter (n = 10-12/group) were randomly assigned to neurobehavioral testing PD32 or PD70 (Aim 1) and 1-2 males/litter (n = 9-10/group) were assigned to neurobehavioral testing (Aim 3) at PD70. The remaining males from each litter were assigned to biochemical assessment at PD32 or
PD70 (Aim 2). Because the number of males per litter was not always sufficient to allocate males to all endpoints (behavior and biochemistry at two ages), additional litters were generated and offspring (1-3 pups/litter) were assigned for biochemical assessment at PD32 or PD70 (n=9-13 litters per group). At the start of testing, preadolescent rats ranged in age from PD31-34 (designated PD32 hereafter) while adult rats ranged in age from PD65-75 (designated PD70 hereafter). Rats selected for dendritic spine analyses represent an additional 6-7 rats per group (2-3 rats/litter) analyzed at PD56-80 (designated PD70) that were previously presented in Pershing et al., 2014, but which represent litters generated under the same conditions as the remaining litters and for which biochemistry data were also previously reported. Table 2 below summarizes the allocation of offspring for behavioral tests and breeding.
Table 2. Summary of Offspring Allocation

<table>
<thead>
<tr>
<th>No. Selected</th>
<th>Age</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3 pups/litter (A, C)</td>
<td>PND32 and 70</td>
<td>Biochemistry</td>
</tr>
<tr>
<td>2 pups/litter (A)</td>
<td>PD32 and 70</td>
<td>Trace Fear Conditioning</td>
</tr>
<tr>
<td>1-3 pups/litter (B)</td>
<td>PD 70</td>
<td>Dendritic Spines</td>
</tr>
<tr>
<td>1-2 pups/litter (C)</td>
<td>PD 70</td>
<td>Trace Fear Conditioning/Rescue with SSR</td>
</tr>
</tbody>
</table>

A, B, C Represents litter cohorts from which animals were selected. Different pups were evaluated at each age.
Chapter 3: Effects of Prenatal Kynurenic Acid Augmentation on PFC Development and Trace Fear Conditioning

3.1 Brief Rationale

The central hypothesis guiding the experiments outlined in this document is that early developmental alterations in the kynurenine pathway (KP) interact with brain maturation processes to yield altered cholinergic/glutamatergic neurotransmission and corresponding behavioral deficits following adolescence. This hypothesis is examined in the current Aim utilizing trace fear conditioning (TFC), a Pavlovian conditioning procedure that is dependent upon intact cholinergic/glutamatergic signaling in the forebrain. The ability to assess TFC prior to and following adolescence in conjunction with associated neurochemical/cellular substrates provided an opportunity to determine sensitive windows of development and possible windows for therapeutic intervention. Specifically, TFC is dependent upon sustained firing of Layer II/III pyramidal neurons in mPFC. It is this layer which shows 1.) Remarkable remodeling during adolescence and 2.) Consistent changes in dendritic spine density and morphology in the clinical population. It was therefore hypothesized that KYNA-induced impairments would not be functionally expressed until adolescent maturation of neural systems that underlie cognitive flexibility.
**Hypothesis:** If KYNA-induced behavioral deficits emerge as a function of prefrontal maturation, then conditioned fear responding in a trace fear conditioning task will be similar to controls prior to adolescence (~PD32) and reduced following adolescence (~PD70). Furthermore, given that TFC depends upon sustained firing of Layer II/III pyramidal neurons, it is expected that PD70 TFC deficits will correspond to pyramidal cell dysfunction (changes in spine density).
3.2 Introduction

3.2.1 Functional Consequences of KYNA Elevation during Development

Alterations of the kynurenine pathway of tryptophan degradation have received increasing interest in schizophrenia (SZ) and myriad other disorders, including, bipolar disorder, depression and Alzheimer’s disease among others (Schwarcz et al., 2012). This endogenous pathway of tryptophan metabolism yields end-products that help to maintain excitatory-inhibitory balance through modulation of multiple neurotransmitters, including dopamine, acetylcholine, glutamate, and gamma-aminobutyric acid (GABA). For instance, the kynurenine pathway (KP) produces quinolinic acid, an N-Methyl-D-Aspartate (NMDA) receptor agonist in microglia and kynurenic acid (KYNA), an NMDAR and α7nAChR antagonist in astrocytes (see Section 1.8). KYNA is elevated in the CSF and postmortem brains of SZ patients, but it is unknown when in the course of disease progression these increases occur. While little is known regarding the nature and time course of KYNA elevations prior to disease onset, the functional consequences of such elevations can be explored in the context of neurodevelopmental animal models, with particular emphasis on the roles of α7nAChR and NMDAR receptors during brain development. In particular, the question arises whether a developing fetus, exposed to elevated levels of KYNA due to maternal stress, inflammation, or infection, will exhibit resulting long-term functional changes.

The sensitivity of fetal tissue to elevated levels of KYNA has not been empirically determined; therefore, functional implications can be explored on the basis of direct nicotinic and NMDAR antagonists. Nicotinic AChRs have several roles during
development and neuronal plasticity (Broide and Leslie, 1999; Role and Berg, 1996). The density of nAChRs varies during the course of development; binding sites and mRNA for α7 increase in rodents from ED13 through birth, then subsequently decrease following birth to reach adult levels (Gotti, 2004). The increase in α7 nAChR expression during synapse formation indicated to Berg and colleagues that α7nAChRs play a fundamental role in the development of neuronal architecture and synapse formation/stabilization (Pugh and Berg, 1994; Role and Berg, 1996). In relation to the current experiments, this means that α7 receptor expression is increasing at a time when KYNA is elevated. Several key papers have recently illustrated the importance of the α7nAChR in nervous system development: A key characteristic of developmental α7 dysfunction is synaptic dysfunction, evidenced by structural deficits (dendritic spine abnormalities), decreased functional glutamatergic synapses, and subsequently altered excitatory-inhibitory balance in the hippocampus. Specifically, Lozada et al. (2012) utilized α7 knock out (KO) mice and determined, through electron microscopy analysis of synapse abundance, that α7 KOs have fewer synapses than wild types; furthermore, there were no additional decreases in double knockouts (e.g. α7/β2) suggesting the effect was driven by α7. While Lozada et al. examined the effect of early α7 dysfunction and long-term disruptions in excitatory-inhibitory balance in juveniles (through PD25), Campbell and Berg (2010) examined the role of α7 KO in adulthood and further examined the excitatory-inhibitory balance following α7 deletion. Importantly, α7 KO mice have significant dendritic abnormalities (truncated arbors), likely due to receiving less synaptic input. Liu et al. (2006) also showed that even a transient disruption in
cholinergic signaling has long-term impact on excitatory-inhibitory balance, as a single prenatal exposure to α7-specific antagonists (α bungarotoxin or Methyllycaconitine) are sufficient to delay GABAergic maturation in conjunction with alterations in synaptic contacts and neurite outgrowth.

NMDA receptors are also critically involved in CNS development; thus, KYNA-mediated NMDAR antagonism is expected to have long-lasting consequences. Namely, NMDA-mediated antagonism may result in fewer or non-functional connections (Haberny et al., 2005). NMDARs are composed of two obligatory NR1 subunits and two NR2 subunits, which in forebrain are most commonly of the NR2A or NR2B type. The relative proportions of these subunits change with brain development: NR1 expression begins as early as ED14, peaks around the third postnatal week, and then declines slightly to adult levels. NR2B is the only NR2 subunit expressed prenatally; minimal expression is noted in mid-gestation (ED14) and levels peak in cortex and hippocampus during the third postnatal week. In contrast, NR2A is observed only following birth, with low levels at PD0 followed by an upregulation to maximal levels in the third postnatal week. The developmental profile observed with NMDAR complexes suggests active roles brain development. NR1/NR2B complexes, for instance, are highly permeable to calcium and also have slow deactivation kinetics (seconds). These characteristics make NR1/NR2B subunits ideal during brain growth and development, and indeed this subunit predominates early in life. In contrast, the NR1/NR2A complex has a lower calcium permeability and rapid decay kinetics, and is the predominate adult phenotype (Cull-Candy et al., 2001). The distinct roles of NMDAR subtypes could have several
ramifications in the current context. First, dendritic arbor growth is dependent upon synaptic activity (mediated by NMDARs) in addition to extracellular cues and intracellular signaling (Ewald and Cline, 2009). NMDAR antagonism early in development has been previously shown to cause both increases and decreases to dendritic growth, depending upon brain region examined, developmental time period, and experimental study design (McKinney, 2010). Apoptosis has also been observed following brief periods of fetal NMDAR antagonism (Ikonomidou et al., 1999). NMDAR-mediated synaptic activity is also required for the integration of neurons into an existing circuit (Tashiro et al., 2006); thus NDMAR-mediated activity is critical throughout the life span, and alterations in either the function of NMDARs or subunit composition could have long-lasting consequences.

3.2.2 Utility of TFC in Establishing Pre- and Post-Adolescent Cognitive Performance

The experimental design of cognitive studies has evolved, from initial studies of chronically ill patients housed in long-term facilities, to cross-sectional and longitudinal studies that recruited first-episode patients in order to address cognitive decline over disease progression. From these studies, there is general consensus that cognitive deficits are pervasive and predictive of functional outcome (see General Introduction). However, the nature and time course of cognitive deficits relative to disease emergence is less understood. Emerging evidence suggests that cognitive deficits are not part of the post-adolescent disease sequelae but are present well before psychosis (Woodberry et al., 2008) and may worsen in the prodrome (Bilder et al., 2006; Jahshan et al., 2010). Indeed
cognitive deficits may be the first predictor of frank illness, representing a reliable endophenotype and early predictor (Keefe et al., 2006). Recent neurocognitive studies in high risk or ultra high risk (UHR) individuals have established that cognitive deficits observed during first-episode or chronic schizophrenia are present in the prodrome (Bora and Murray, 2014). HR subjects, which include individuals with first-degree relatives that have SZ, are more likely to be diagnosed with a DSM schizophrenia spectrum disorder or exhibit psychotic symptoms (Keefe et al., 2006; Fusar-Poli et al., 2013). These studies indicate that vulnerability to psychosis is associated with cognitive impairments in executive function, verbal fluency, attention, and verbal/working memory; cognitive constructs that are affected in both first-episode and chronically ill patients. Fusar-Poli et al. (2013) further established that transition to psychosis in HR subjects is most associated with deficits in particular domains (verbal/working memory, verbal fluency, and general intelligence). These data provide evidence that cognitive assessments, particularly in key cognitive domains, may provide key early intervention and therapeutic strategies. Indeed in a large, prospective, longitudinal study evaluating children from 3 to 32 years of age, Reichenberg et al. (2010) determined that children who later developed schizophrenia had early and persistent attentional processing, and working memory, deficits.

Based upon CNTRICs guidelines (see General Introduction), which recognize the utility of Pavlovian conditioning, TFC was considered an ideal task to explore the emergence of deficits following neurodevelopmental manipulations. TFC comprises attention, working memory, and context, all of which are impaired in the clinical
population (Carter and Barch, 2009) and have characterized neurobiology. TFC can be assessed in adolescent and adult animals (Barnet and Hunt, 2005; Hunt et al., 2007), an important selection criteria that limits several other tasks that may take weeks or months to train (such as operant-based working memory tasks) or tasks in which juveniles and adults show distinct ontogenetic differences. For instance, attentional set-shifting (ASST) is an ideal task for assessing prefrontal-dependent executive function. We have previously shown that rats exposed to elevated KYNA during prenatal development are impaired in the ASST (Alexander et al., 2013; Pershing et al., 2014). However, executive function required for this task emerges in adulthood, with juvenile rats exhibiting inflexibility through the 7th postnatal week (Newman and McGaughy, 2011). Given that rats with elevated brain levels of kynurenic acid exhibit inflexibility in the ASST, we were unable to utilize this task to explore the emergence of cognitive deficits. In contrast, TFC emerges following weaning and can be reliably measured by PD32 (Barnet and Hunt, 2005; Moye and Rudy, 1987). Furthermore, the association between the conditioned stimulus (CS) and unconditioned stimulus (US) across a temporal delay requires cholinergic/glutamatergic signaling across a distributed neural network including the hippocampus and PFC (Barnet and Hunt, 2005; Raybuck and Gould, 2010).

With the exception of a TCF4 mouse knock-out model of SZ (Brzózka and Rossner, 2013), TFC has rarely been examined in animal models of SZ. TCF4 is a transcription factor expressed in the developing brain and which is associated with several sensory/cognitive functions such as verbal learning/memory, sensory gating, and pre-pulse inhibition. TCF4 abnormalities and associated gating deficits in SZ suggested
that mice lacking TCF4 would also show deficits in trace fear conditioning. To that end, TCF4 KOs exhibited reduced contextual and cued-fear following conditioning with a 30-second trace fear protocol. There are well-established reports of contextual and delay fear conditioning in several animal models with particular relevance here, namely prenatal stress (Wilson et al., 2010), post-weaning isolation (Gresack et al., 2010), and acute KYNA manipulations (Chess, Landers and Bucci, 2009; Iaccarino et al, 2013). Specifically, Bucci and colleagues have established that acute KYNA elevations during PD7-10 or 27-53 alter contextual fear conditioning. Contextual fear conditioning is hippocampus-dependent and models contextual impairments noted in the clinical population (Hemsley, 2005). The major purpose of this experiment, then, is to expand the currently available research in three major directions by: 1. Focusing on prenatal elevation of KYNA, 2. Assessing the emergence of cognitive deficits utilizing a cognitively demanding task that is both hippocampus- and PFC-dependent, and 3. Exploring underlying cellular/mechanisms related to cognition.

3.2.3. NMDA and α7nAChRs in Trace Fear Conditioning

Early developmental alterations of the cholinergic/glutamatergic system could clearly lead to long-term changes in circuit excitability, neurotransmitter release, and learning and memory. Pharmacologic studies have illustrated the functional significance of nicotinic and NMDARs in TFC. Importantly, Barnet and Hunt (2005) showed that maturation of TFC in juvenile rats is mediated by the cholinergic system and the cued-fear response can be enhanced by administration of the cholinesterase inhibitor
physostigmine. Raybuck and Gould (2010) further explored the role of cholinergic signaling in the PFC and hippocampus in supporting trace fear conditioning, and found that cued-fear is indeed modulated by cholinergic signaling but in a complex manner. That is, nicotine, depending upon dose and infusion location enhanced or impaired cued-fear without affecting contextual fear following TFC. Interestingly, similar effects were observed with local infusion of α7 specific antagonist methyllycaconitine (MLA), suggesting that either positive or negative manipulations of cholinergic signaling can critically affect TFC. This may be due to the influence of α7 receptors on glutamatergic synapses (Yang et al, 2013). Specifically, NMDAR activation in both the hippocampus and PFC is necessary for TFC. Infusion of NMDA antagonists have been shown to disrupt TFC (Gilmartin and Helmstetter, 2010; Quinn et al., 2005; Czerniawski et al., 2012).

3.2.4 Role of Dendritic Spines

Given the role of Layer II/III in the acquisition of TFC, this layer was selected for examination of dendritic spine density. Dendritic spines contain the majority of glutamatergic synapses and are the primary sites of excitatory neurotransmission (McKinney 2010). Therefore, alterations in dendritic spine density and/or morphology are suggestive of multiple functional disturbances. A primary and consistent finding in SZ is decreased dendritic spine density, localized to layer 3 of the prefrontal cortex (Glantz and Lewis, 2001; Glausier et al., 2013). The lamina-specific spine abnormalities in SZ may be particularly important from a neurodevelopmental perspective.
Specifically, there are particularly strong refinements of dendritic spines during adolescence, with Layer II/III showing increased synaptic pruning during adolescence relative to other cortical layers (Bourgeois et al., 2005).

The functional importance of Layer II/III neurons was initially elucidated from the seminal work of Goldman-Rakic and colleagues, who showed that these neurons generate persistent firing in the absence of sensory stimulation, allowing representation of the stimulus over a delay. Baeg et al (2001) further illustrated the importance of these neurons in relation to task performance relevant to fear conditioning. Specifically, single unit recordings in the prelimbic and infralimbic cortex during the auditory CS show sustained increases in firing, supporting a role for the mPFC in processing tone and contextual information. Gilmartin and McEchron (2005) further clarified the role of the mPFC by specifically examining mPFC neuron firing in response to trace and delay condition, in which the CS and US co-terminate. Sustained firing was observed in the mPFC neurons across the CS and trace intervals in trace-fear conditioned animals, but not in delay conditioned or unpaired controls. These data support the role of the PFC in working memory or bridging the CS-US gap. In a follow-up study, Gilmartin and colleagues further clarified that this sustained activity was required for learning by inactivating the prelimbic cortex prior to training. Indeed, inactivation of the prelimbic cortex impairs the acquisition of trace fear conditioning, but spares delay fear conditioning (Gilmartin and Helmstetter, 2010).
3.3 Methods

3.3.1 Shock Sensitivity/Reactivity

To ensure that TFC data were not due to differences in sensory perception, shock reactivity/sensitivity was assessed for separate juvenile and adult ECON and EKYN rats (n= 3 per condition per age). Each rat was placed into a conditioning chamber (as described above) and allowed to habituate for 120 seconds. An ascending series of ten foot shocks (0.1 to 1 mA) was administered in 0.1-mA increments with a $30 \pm 5$ second ITI. Rats were recorded using a digital camera and responses were scored off-line by observers that were blind to treatment condition. The following criteria, adapted from Nielsen and Crnic (2002), were used: 0 = No Response (no reaction to shock); 1 = Flinch/Rear (jerky movement; shift in body posture; attentional orienting; but with two paws remaining on floor); 2 = Hop/Run (forward or backward movement, less than half the length of the testing chamber or small vertical movement less than body height); 3 = Jump (horizontal motion greater than half of the chamber or vertical motion greater than body height with all four paws leaving the floor in a springing motion). It was also noted whether animals vocalized. The shock reactivity/sensitivity for each group was determined by calculating the range and mean shock intensity at which each response occurred for each group. That is, the mean intensity was determined at which flinching, hopping/running, jumping, and vocalizing occurred in ECON and EKYN juveniles and adults.
3.3.2 Trace Fear Conditioning

Trace fear conditioning (TFC) and testing were conducted for ECON and EKYN offspring over 3 days. Ten ECON and 12 EKYN were tested as juveniles (PD32) or as adults (PD70). Day 1 training was conducted between 0900 and 1400 hours; testing was initiated within ± 2 hours of training on the subsequent 2 days. **Day 1:** Rats were transported to the testing suite in opaque transport boxes. Training was conducted in two operant boxes (Coulbourn Instruments, Allentown, PA), enclosed in sound-attenuating chambers (Figure 4).

![Figure 4](image-url)  
**Figure 4.** Trace Fear Conditioning Chambers. Training and Context Testing were conducted in Chamber A (left) while Cue-Dependent freezing was tested in Chamber B (right).
Each operant box was composed of stainless steel side walls, clear Plexiglass front and back walls, and a grid floor composed of 0.5-cm stainless steel bars placed approximately 1.5 cm apart. A small animal shock generator (model H13–15; Coulbourn Instruments) and grid scrambler connected to the floor provided the foot shock unconditioned stimulus (US). A speaker located at the top of each chamber provided the conditioned stimulus (CS), a 15-sec, 2.8-kHz, 85-dB tone. Each chamber was wiped with a 20% vinegar solution at the start of testing and between animals in order to provide distinct olfactory cues specific to the training chamber and to reduce odor-cues between animals. The TFC training procedure is illustrated in Figure 5. Training consisted of a 120-second habituation period followed by 10 paired CS-US presentations. The US was a 1.0-sec, 1.0-mA scrambled foot shock. This US intensity was selected based off of pilot data in intact juvenile and adult Wistar rats showing that conditioned freezing response varies with US stimulus intensity but that juveniles and adults show similar cue-dependent freezing following training with a 1-mA, 1-sec shock (Appendix A). The US was delivered 10 sec after the CS offset, resulting in a 25-sec interstimulus interval (ISI). This trace interval (10 seconds) was selected in order to maximize the utility of this task as one that is both hippocampus- and PFC-dependent and which was similarly difficult for both juveniles and adults. Shorter trace intervals were disregarded as short trace intervals are not considered to be hippocampus-dependent (Chowdhury, Quinn, and Faneslow, 2005; Misane et al., 2005) and CS-US association varies with temporal contiguity (Rescorla, 1988). A variable intertrial interval (ITI) of 240 ± 30 sec was selected so that amount of time between shocks could not be used as a cue for foot-shock
onset and in order to maximize the CS-US association, as longer ITIs have been found to maximize the CS-US association in TFC (Detert et al, 2008). Following the last CS-US pairing, rats were removed from the chambers and transported back to their home cages. 

**Day 2:** Twenty-four hours following training (± 2 hours), rats were returned to the training chamber for a context test, which measured fear in the absence of the CS and US. Rats were given 120 seconds to habituate to the chambers and then fear to the original context was measured by recording freezing behavior, defined as the cessation of movement except that required for breathing (Blanchard & Blanchard, 1969). Freezing was recorded and scored automatically (see below for details) for 14 minutes and then returned to their home cages. **Day 3:** Twenty-four hours later (± 2 hours) rats were assessed for conditioned fear response to the CS independent of contextual cues. To that end, rats were placed in a novel context, which varied in transport, odor, visual, and tactile cues (Figure 5). The testing suite was illuminated using red light and the operant boxes were illuminated by 12-W white lights. Operant boxes were further modified from Day 1 with Windex® (S. C. Johnson & Son, Inc., Racine, WI), Plexiglass inserts, which changed the interior wall angle and covered the grid bars, Paper Shapes, and fans, which provided airflow and background noise. After a 120-second baseline period, rats were presented with ten CS-alone presentations (180 ± 30 sec ITI).
Freezing was defined as cessation of all movement except that required for respiration (Blanchard & Blanchard, 1969). Freezing behavior was recorded with a black and-white video camera (Model WDSR-2005SC; Circuit Specialists, Inc., Mesa, AZ) mounted at the top of the conditioning chamber. The interior was illuminated by an infrared light source to observe freezing behavior. The video signal was input to FreezeScan (CleverSys, Inc., Reston, VA), a video-based tool that can detect and quantify when subjects are motionless. Freezing is presented as % time freezing during the CS and trace intervals (for Day 1 and Day 3). Day 2 data are presented in 2-minute bins, following the 120-second habituation period. In addition, freezing during the Day 3 baseline period was examined to verify no differences in generalized freezing to the novel context between treatment groups. Therefore, the amount of freezing during the 1 minute prior to CS onset is also presented for Day 3. Due to procedural error, Day 3
data were collected for two EKYN rats using the incorrect program. These data were excluded; therefore, Day 3 data represent n=10 per group.

3.3.3 Dendritic Spine Density

A separate group of rats (ECONs, n = 7; EKYNs, n = 6) was used to quantify spine density at PD70. The staining procedures described below have previously been established as successfully staining pyramidal cells (Kleen et al., 2006). Rats were deeply anesthetized with Euthasol (Virbac Animal Health, Ft. Worth, TX) and transcardially perfused with 120 mL of 4.0% paraformaldehyde in 0.1 M phosphate buffer and 1.5% picric acid (v/v). Blocks containing the PFC were incubated in rapid golgi solutions (GolgiStain Kit™, FD Neurotechnologies, Columbia, MD), based on previously described methods (Leuner and Gould, 2010). Serial coronal sections (150 µm) were cut on a vibratome (Leica Instruments) and mounted on gelatin-coated slides with Permount mounting medium (Fisher Scientific) and allowed to dry before quantitative analysis.

Golgi-stained tissue was analyzed with a Nikon 90i microscope (Nikon, Tokyo, Japan). Four to five Golgi-impregnated pyramidal neurons from the prelimbic/infralimbic cortex were selected from each animal and analyzed blind to experimental condition. To be selected for analysis, Golgi-impregnated pyramidal neurons had to possess the following characteristics: (1) The neuron had to be within layer 2/3 and within the boundaries of the prelimbic/infralimbic areas. (2) Cell bodies had to be located in the middle third of the tissue section to avoid analysis of impregnated neurons that extended largely into other sections. (3) Golgi-impregnation had to be dark
and consistent throughout the extent of all dendrites. (4) Cells had to be relatively isolated from neighboring impregnated cells.

Counting required focusing in and out with the fine adjustment of the microscope using 1000x magnification and oil immersion. Measurement of apical and basal dendrites was conducted on secondary and tertiary branches; apical dendrites were measured at three distances from the cell body: 25-75 µm, 75-150 µm, and 150-225 µm. Basal dendrites were measured at least 50 µm from the cell body. Spine density was calculated by dividing the number of spines on a segment by the length of the segment and was expressed as the number of spines per 10 µm of dendrite. Densities of spines on three to five segments of a cell were averaged for a cell mean, and the cells from each animal were averaged for an animal mean. Spine density values using this method are underestimates, because spines protruding either above or beneath the dendritic shaft are not accounted for (Leuner and Gould, 2010).

A separate, comparator, region of interest was also selected and analyzed from the same rats. The dentate gyrus was selected as this brain region has been studied extensively by the investigators in this laboratory and thus serves as a methodological control and also serves to identify changes in this model on a global scale. Granule cell dendrites from the middle third of the molecular layer of the dorsal blade of the dentate gyrus were examined from three to five granule cells per animal. Densities of spines on three to five segments were averaged for a cell mean, and the cells from each animal were averaged as described above for pyramidal neurons.
3.3.4 Statistical Analyses

Maternal and offspring body weights are presented with the litter as the experimental unit. All other endpoints utilized the rat as the experimental unit. **Body Weights** (maternal and offspring): mean litter weights were analyzed by two-way repeated measures ANOVA, with prenatal CONDITION as between subjects factor and body weight as a within-subjects factor. **Trace Fear Conditioning**: percentage time freezing during the CS and trace intervals were analyzed on Day 1 by Three-Way Repeated Measures ANOVA, with prenatal CONDITION and AGE as between subjects factors and TRIAL as within-subjects factors. The source of a significant TRIAL x AGE interaction in the absence of main effects of AGE or CONDITION was elucidated by an ANOVA comparing TRIAL between juveniles and adults (collapsed across condition) by AGE (e.g., the percentage of time freezing was compared for juveniles and adults, regardless of prenatal condition). Percentage time freezing for the entire session (Days 1, 2, and 3) and baseline freezing (Day 3) were analyzed using a Two-Way (prenatal condition x age) ANOVA. Following significant interactions, t-tests were conducted comparing ECON and EKYN within each AGE. Shock Reactivity/Sensitivity data are presented as descriptive statistics (Means, Standard Errors, Ranges) but were not otherwise analyzed. **Dendritic Spine Density**: mean spine density was analyzed by t-test. Significance was defined as P<0.05, and the Huynh-Feldt correction was utilized to reduce Type 1 errors associated with repeated measures ANOVAs (Vasey and Thayer, 1987). All statistical tests were performed using SPSS for windows (versions 19-22; Chicago, IL).
3.4 Results

3.4.1 Maternal Food Consumption and Body Weights

The route of kynurenine administration in this study was oral (dietary) exposure from ED15-22. To ensure both maximal and consistent exposure, dams were habituated to wet mash beginning on ED0 then each day thereafter the amount of food was adjusted ± 5 g. Given the nature of the diet (wet mash), a true quantitative assessment of food intake was not possible. However, the amount of food given each day and all instances of food remaining, which would result in presumably lessened KYN exposure, were recorded (Appendix B). Because the normal gestation length of rats is 21-23 days (Peters, 1986), food consumption was calculated for ED15-20. ECON and EKYN females ate similar amounts of food during ED15-20 during the KYN treatment period (36.4 ± 0.79 g/dam/day vs. 36.0 ± 0.91 g/dam/day, respectively; t27 = 0.361, P = 0.359). Instances of food left were generally limited to one or two non-consecutive days for six EKYN females. We have previously established that prenatal treatment with kynurenine results in robust (6-fold) increases in fetal brain KYNA (published in Pershing et al, 2014); thus, rare instances of females not consuming all food is not considered to detrimentally impact the goals of this research.

There were also no effects of kynurenine administration on maternal body weights during gestation as determined by repeated measures ANOVA comparing prenatal condition and body weights over time (F3,60 = 0.695, P = 540). As shown in Figure 6, ECON and EKYN females gained weight similarly during gestation (129 ± 8.4 g vs. 126 ± 6.5 g, respectively, from ED6-20; t26=0.357, P=0.724). No obvious
differences in maternal care (i.e., nest-building, nursing posture, pup retrieval) were observed between the EKYN and ECON groups.

Figure 6 Summary of Maternal Body Weights during Gestation. Data represent litter means ($G \pm SE$).
3.4.2 Offspring Body Weights

Offspring body weights were recorded weekly from PD7 through PD56 and on PD65 for all litters, regardless of which postnatal endpoint to which they were ultimately assigned. Thus, litter mean body weights for all cohorts were analyzed together for preweaning (PD7-21) and postweaning (PD28-65) periods. Prenatal treatment with kynurenine had no effect on offspring growth (Figure 7). There were no significant differences between ECON and EKYN rats during the preweaning (PD7-21) and postweaning (PD28-65) periods when assessed via repeated measures analysis (P ≥ 0.242).

![Figure 7. Summary of Offspring Body Weights. Data represent litter means (G ± SE)](image-url)
3.4.3 Trace Fear Conditioning

Shock Reactivity/Sensitivity

To ensure that TFC data were not due to differences in sensory perception, shock reactivity/sensitivity was assessed for separate juvenile and adult ECON and EKYN rats. There was no apparent effect of prenatal condition or age of testing on typical shock-induced behaviors (flinch, hop/run, jump, vocalize). As shown in Table 3, the mean shock intensity at which typical shock-induced behaviors (flinch, hop/run, jump, and/or vocalizing) occurred was similar between ECON and EKYN rats across ages. With the exception of one ECON and EKYN rat at PD70, rats were insensitive to the lowest shock intensity. Shock scores increased similarly across groups with increasing shock intensity, and co-occurring vocalization was noted similarly at shock intensities of 0.4 mA and above.

<table>
<thead>
<tr>
<th>Shock Score</th>
<th>PD32</th>
<th>PD70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECON</td>
<td>EKYN</td>
</tr>
<tr>
<td>1</td>
<td>0.3 (0.2-0.5)</td>
<td>0.3 (0.2-0.5)</td>
</tr>
<tr>
<td>2</td>
<td>0.7 (0.4-1.0)</td>
<td>0.7 (0.5-0.9)</td>
</tr>
<tr>
<td>3</td>
<td>0.9 (0.5-1.0)</td>
<td>0.9 (0.7-1.0)</td>
</tr>
<tr>
<td>Vocalize</td>
<td>0.7 (0.4-1.0)</td>
<td>0.8 (0.4-1.0)</td>
</tr>
</tbody>
</table>

Table 3. Summary of Shock Sensitivity Scores at PD32 and PD70
1 = Flinch/Rear; 2 = Hop/Run; 3 = Jump
Day 1 Conditioning

Trace fear conditioning was conducted at PD32 or 70 in order to assess the effect of prenatal kynurenine exposure on the developmental profile of cued-fear responding. As shown in Figure 8, juveniles and adults of both conditions showed similar, low levels of freezing (approximately 20%) during Trial 1, which occurred prior to the first US and is considered a measure of baseline freezing. The amount of time animals spent freezing subsequently increased as the animals learned the CS-US association, as evidenced by a significant effect of trial following repeated measures analysis of freezing during the CS ($F_{9,360} = 14.506, P = 0.000$) and trace intervals ($F_{9,360} = 17.743, P = 0.000$). This effect occurred regardless of prenatal condition ($P \geq 0.074$). There was, however, an effect of rat age on freezing over time to the CS ($F_{9,360} = 1.992, P = 0.039$) and trace interval ($F_{9,360} = 2.017, P = 0.037$). This effect was transient, as juveniles regardless of prenatal condition, exhibited less freezing than adults during the Trial 2 CS ($F_{1,42} = 11.341, P = 0.002$) and trace intervals ($F_{1,42} = 14.274, P = 0.000$), but similar freezing to adults by Trial 3 ($P \geq 0.521$). These data suggest slower rate of CS-US association in juveniles compared to adults. The slightly slower rate of CS-US association had no effect on overall freezing levels for the entire session, which were comparable between ECON juveniles and adults (51 ± 5.5% and 54 ± 3.9%, respectively) and EKYN juveniles and adults (45 ± 4.1% and 44 ± 4.2%, respectively); there was no effect of age ($F_{1,40} = 1.579, P = 0.216$, condition ($F_{1,40} = 1.399, P = 0.244$, nor an interaction ($F_{1,40} = 2.112, P = 0.154$; Figure 9).
Figure 8. Summary of Trace Fear Conditioning Data by Trial (Day 1). Percentage time freezing during the 15-second CS (A) and the 10-second trace (B) for PD32 juveniles and PD70. Juveniles take slightly longer than adults to learn the CS-US association as evidenced by significantly reduced freezing at Trial 2 ($a = P<0.05$).
Figure 9. Summary of Percent Time Freezing for the Entire Training Session (Mean ± SE). Juveniles and adults froze similarly following ten CS-US pairings, regardless of prenatal condition (all P’s > 0.05).

Contextual Fear (Day 2 Testing)

Twenty four hours following conditioning, rats were returned to the training chamber and assessed for contextual fear (fear response to the context in absence of CS and US). Contextual fear was unaffected by prenatal kynurenine treatment at PD32 and PD70. Juveniles and adults of both conditions showed similar levels of freezing throughout the 14-minute context test (34 ± 2.6% to 41 ± 3.2% freezing across ages and
conditions; Figure 10). There were no significant effects of prenatal condition ($F_{1,40} = 0.742, P = 0.394$), Age ($F_{1,40} = 0.009, P = 0.926$), nor interactions of Age and Prenatal Condition ($F_{1,40} = 0.941, P = 0.338$).

Figure 10. Summary of Contextual Fear Response. Percent time freezing to the training chamber in the absence of CS or US at PD32 and PD70. Contextual fear was similar across all ages and conditions (all $P$’s $> 0.05$).
Cued-Fear Response (Day 3)

Rats were then assessed (24 hours later; Day 3) for percentage freezing during the CS and trace intervals. As shown in Figure 11, a 2 x 2 ANOVA supports that prenatal kynurenine treatment resulted age-dependent alterations freezing to the tone, an assessment of cue-dependent freezing ($F_{1,38} = 13.664, P = 0.001$) and the equivalent 10-second trace interval ($F_{1,38} = 9.908, P = 0.003$). ECONs and EKYNs were subsequently compared within each age, revealing that PD32 EKYNs exhibited significantly higher freezing than ECONs during the tone ($66 \pm 3.5\%$ vs $54 \pm 2.7\%; t_{18} = -2.872, P = 0.010$) and trace intervals ($69 \pm 4.1\%$ vs $56\% \pm 3.7\%; t_{18} = -2.349, P = 0.030$). In contrast, PD70 EKYN rats exhibited significantly less freezing than ECON rats during the tone ($58 \pm 3.4\%$ vs $72 \pm 4.9\%; t_{20} = 6.259, P = 0.021$) and trace intervals ($57 \pm 3.8\%$ vs $69 \pm 4.3\%; t_{20} = 4.414, P = 0.049$). This effect was not due to baseline freezing, which was similar between ECON and EKYN rats at each age ($P \geq 0.228$).
Figure 11. Summary of Cued-Fear Response (Day 3). Percent time freezing to the CS in a novel context at PD32 and PD70. EKYNs froze significantly more than controls as juveniles, but significantly less than controls as adults. * = P < 0.05; ** = P < 0.01
3.4.4 Dendritic Spine Density

Dendritic spine density was assessed in a separate cohort of ECON (n=7) and EKYN (n=6) rats at PD70. Prenatal kynurenine exposure was associated with decreased dendritic spine density on Golgi-impregnated pyramidal neurons located in layer 2/3 of the mPFC in adults, as illustrated in the photomicrographs of Golgi-stained dendritic segments shown in Figure 12. Compared to ECONs, dendritic spine density in EKYN rats was decreased 11% on apical dendrites ($t_{11} = 5.731, P < 0.001$) and 14% on basal dendrites ($t_{11} = 2.713, P = 0.020$). Figure 13 shows total dendritic spine density on apical and basal dendrites in Layer 2/3 of the mPFC. The spine density reduction noted on apical dendrites occurred at a similar magnitude at proximal, intermediate, and distal segments evaluated (e.g., 25-75, 75-150, and 150-225 µm from the soma; Figure 13; all differences were significant ($P \leq 0.002$). Notably, all but one EKYN rat had apical dendritic spine densities that were lower than the minimum observed ECON density (11.9 spines/10 µm). Basal dendritic spine densities were distinct for two reasons: basal spine density was more variable than apical spine density in both groups and there was resulting more overlap across ECON and EKYN. That is, the mean basal spine density was decreased (as discussed above); however, the range of values overlapped those observed in the ECON group to a greater degree than observed for apical dendrites. The potential importance of differences in apical versus basal spines will be addressed in the Discussion Section.
The dentate gyrus was also examined in the same rats. There was no effect of prenatal kynurenine treatment on spine density in the dentate gyrus when rats were examined as adults (Figure 13). Mean spine density was similar in the ECON group (12.5 ± 0.43 spines/10 µm) and the EKYN group (11.9 ± 0.44 spines/10 µm); \( t_{11} = 0.885, P = 0.395 \).
Figure 13. Summary of Mean Dendritic Spine Density in mPFC and Dentate Gyrus. Top panel represents apical spine density as a function of distance from the soma (50-µm increments proximal to distal). Bottom panel represents mean spine density for apical dendrites (all distances from soma), basal dendrites, and the dentate gyrus (DG).
3.5. Discussion

The present experiment examined the effects of prenatal KYNA elevation on the emergence of cognitive dysfunction utilizing trace fear conditioning (TFC), a task which depends upon cholinergic/glutamatergic functioning in the mPFC. To this end, following in utero KYNA elevation, offspring were assessed in a TFC task as juveniles (PD32) or as adults (PD70). Several findings can be reported. Notably, there were distinct age-dependent differences in fear conditioning, with juvenile EKYNs exhibiting increased cued-fear response and adult EKYNs exhibiting decreased cued-fear response. These effects occurred in the absence of contextual fear impairments, which was an unexpected finding. Taken together with observed age-dependent changes in NMDAR subunit expression (Section 4.4.2) and the finding that adult rats that were exposed to KYNA in utero also had significant reductions in dendritic spine density in Layer II/III pyramidal cells, these data suggest that KP manipulations could affect TFC in myriad ways, but all of which may be considered to converge upon changes in excitatory-inhibitory balance by 1.) Modulating presynaptic glutamate release, 2.) Altering postsynaptic plasticity, 3.) Altering α7nAChR/NMDAR receptor expression. Given the increased vulnerability of adolescents to a variety of a variety of neuropsychiatric disorders, the emergence of distinct age-dependent differences in fear conditioning following prenatal kynurenine exposure is particularly interesting and provides further support for examination of juvenile/adolescent brain development in the emergence of cognitive dysfunction in SZ.

Assessing the emergence of cognitive deficits in humans or animal models is challenged by the innate developmental profile exhibited as subjects age. That is,
cognitive performance is different between juveniles and adults, thus “normalizing” results to interpret developmental differences can be challenging. For instance, like humans, juvenile rats and mice show higher levels than adults in measures of anxiety-like behavior, risk-seeking, and stress responsivity (Andersen et al., 2003; Hefner and Holmes, 2007). In contrast, these behaviors in adults are associated with pathologies (Swann, 2001). Animal models are confounded by a second important limitation, which is shorter development. In humans, the juvenile and adolescent periods extends into the second decade of life. In rodents, this window is limited to a number of days. Thus, trace fear conditioning (TFC) was selected in order to assess juveniles versus adults, as TFC takes only 3 days and can be rapidly acquired in both juveniles and adults. We further selected task parameters for which intact juveniles and adults show similar conditioned fear response in order to assess the emergence of conditioned fear response following prenatal kynurenine exposure. Given this normalized set of task parameters, we hypothesized that prenatal KYNA elevation would interact with other known plasticities in hippocampus and PFC at adolescence to yield the adult phenotype. In contrast, distinct changes were observed in juvenile EKYNs, which exhibited significantly increased cued-fear response whereas adult EKYNs showed significantly reduced cued-fear response. Mechanisms that may underlie the age-dependent differences in cued-fear response in relation to changes in dendritic spines are discussed below, as are relative lack of contextual fear deficits.
Sensory Perception

The issue of sensory perception is inherently important in an age-dependent assessment. Shock reactivity/sensitivity was determined by exposing juvenile and adult ECONs and EKYNs to shocks of increasing intensity and recording the response (flinching/rearing, hopping/running, jumping, and/or vocalizing). ECON and EKYN rats showed similar shock reactivity/sensitivity at both ages, suggesting that there were no differences in sensory perception that contributed to the differential TFC results. However, in pilot experiments conducted in intact juvenile and adult Wistar rats, juveniles and adults showed distinct differences in TFC performance as a function of shock intensity. That is, juveniles showed low cued-fear response following training at 0.6 mA in comparison to adults. In contrast, juveniles and adults show similar, high levels of freezing in response to a high-intensity US (1 mA, 1 s). These results suggest learning differences in the absence of functional sensory differences between juveniles and adults. This interpretation is further supported by the slower rate of CS-US acquisition in juveniles, which was observed in the current experiments regardless of prenatal condition. That is, adult rats show high levels of freezing to the CS following the second trial; however, juveniles do not exhibit high levels of freezing until the third trial. Thereafter, freezing levels during training were comparable between juveniles and adults. This difference in CS-US association in the absence of changes of sensory perception may be explained by slower maturation of executive function and sensory association relative to primary sensory function (Sisk and Zehr, 2005).
Mechanisms That Support Increased Cue-Dependent Freezing

While differing maturational profiles in brain regions that underlie primary sensory and association sensory/executive function may explain differences in the rate of CS-US association between juveniles and adults they do not explain why juvenile EKYNs exhibit increased cue-dependent freezing relative to their age-matched controls. Fear conditioning deficits generally manifest as decreased freezing, as this represents a failure to learn or recall biologically salient information. However, despite similar overall freezing during training, juvenile EKYNs show increased freezing relative to ECONs whereas adult EKYNs showed reduced cue-dependent freezing. The emergence of SZ-like deficits is a key characteristic of multiple neurodevelopmental animal models, including neonatal ventral hippocampus inactivation (Lipska and Weinberger, 2002) gestational stress (Markham et al., 2010, 2013) and maternal immune activation (Harvey and Boksa, 2012). The juvenile and adolescent characterization of these models is often limited in regard to cognitive assessments; however, pre- and post-adolescent differences in freezing responses have been reported by Zimmerman and Grace (2013). Utilizing a rodent model of SZ in which the anti-mitotic agent MAM was administered to gestating rodents to disrupt fetal brain development resulted in increased freezing and ultrasonic vocalizations following repeated shocks in juvenile offspring (PD22), but not young adult offspring (PD54). Abnormal corticosterone response relative to sham controls indicated that rodents with disrupted brain development differentially responded to stressful experiences as juveniles and adults. Developmental changes in stress response are not uncommon. Hefner and Holmes (2007) had a particularly telling experiment, in which
juvenile mice were subject to variable restraint stress, resulting in enhanced fear conditioning as adolescents but not as adults. There are fewer studies which examine 1.) The effects of prenatal manipulations and 2.) Distinct ontogenetic changes that follow in adolescence and adults. These experiments may be particularly important considering evidence that SZ patients exhibit abnormal reactivity to stress such that stressful situations during childhood and adolescence may be triggering environmental factors in disease onset.

Given the initial hypothesis that cholinergic/glutamatergic signaling would be impacted by KYNA elevation during development, it is possible that developmental alterations in glutamatergic/cholinergic transmission underlie increased freezing in juveniles and decreased freezing and adults. Indeed, as discussed in Chapter 4, prenatal KYNA also resulted in age-dependent changes NMDAR expression. EKYN rats had significantly lower mRNA levels of NR2A at both PD32 and PD70; at PD70, these reductions occurred in conjunction with decreased NR1. Postsynaptic induction and expression of several forms of synaptic plasticity require calcium influx through NMDARs and the initiation of calcium-dependent signaling in the dendritic spine. In TFC, NR2A-containing subunits support both CS-US association and generalized learning and NR2-B containing subunits support maintenance of the trace interval. One possibility for the current experiment is that decreases in NR2A-containing subunits in the absence of changes in NR2B-containing subunits allowed for an overall change in excitability due to the characteristics of NR2B-containing subunits. Such a change has been previously noted to result in a prolonged decay time of decay activation (Vicini et
al., 1998), thus indicating greater excitability. Gilmartin and Helmstetter (2012) explored the relative contributions of NR2A and NR2B to acquisition of trace, delay, and contextual fear conditioning. Infusion of NR2A- and NR2B-specific antagonists NVP and Ro25, respectively, showed that NR2A subunits affect both cued and contextual fear conditioning while NR2B-containing NMDARs were specific to cued fear and may thus be particularly important for associating the CS and US across the trace interval. While protein expression was not assessed in the current studies (future experiments are planned), these findings are consistent with the ratio hypothesis of long-term potentiation (LTP) induction, whereby changes in the relative levels of NR2A and NR2B subunits affect LTP induction threshold. Indeed, the increased LTP has been observed in juvenile rats to elevated KYNA *in utero* (Forrest et al., 2013), a phenomenon which reverses in adulthood in conjunction with alterations in NMDAR expression and dendritic spines (Forrest et al., 2013b).

**Dendritic Spines**

The working hypothesis for this aim was that behavioral abnormalities in EKYNs would emerge following abnormal PFC maturation. A primary goal of this aim was to evaluate dendritic spines in Layer 2/3, given the clinical relevance to SZ and the dependence of TFC on this population of neurons. Dendritic spine density was significantly decreased on both apical and basal dendrites from Layer II/III of the medial prefrontal cortex of adult EKYN rats. Given that dendritic spines are necessary for the acquisition and consolidation of information acquired during trace fear conditioning,
these decreases may underlie the reduced cue-dependent freezing. Of particular interest, both apical and basal dendritic spine density was decreased. Spine abnormalities are replicated in multiple animal models, including post-weaning social isolation and repeated stress (Silva-Gómez et al., 2003; Radley et al., 2006; Leuner et al., 2014). However, in contrast to chronic stress, for example, which predominately appears to affect apical dendrites with maximal impact on distal spines, prenatal kynurenine exposure resulted in significant reductions in apical dendrites at all distances examined proximal to distal, in addition to reductions on basal dendrites.

The functional significance of dendritic spine anomalies throughout the dendritic tree are multi-fold, perhaps providing evidence to the nature of the insult and functional significance to which spine abnormalities may underlie cognitive impairments. That is, the rather extensive and global decreases in spine density suggest macro-circuit dysfunction, considering that basal and proximal apical dendrites receive local excitatory input while distal dendrites receive long-range cortical and subcortical input (Shepherd and Svoboda, 2005; Spruston, 2008). For instance, Amygdala input to mPFC undergoes re-organization during adolescence, such that afferents to all cortical layers are eliminated/refined leaving amygdala input to Layers II and V in adults. GABA-ergic interneurons also target pyramidal cells in a regionally specific manner (Kubota, 2014). The effects of KYNA could presumably alter GABAergic networks and subsequent synchronization of pyramidal neuronal activity (Banerjee et al., 2012).

The dentate gyrus was selected as a separate, comparison region as 1.) A methodological control and 2.) To evaluate global changes in dendritic spine density.
Importantly, there were no changes in dendritic spine density in the dentate gyrus. The effect of KYNA elevation on hippocampus development has been extensively explored by Stone and colleagues, who utilize the KMO inhibitor Ro61-8048 to elevate KYNA levels prenatally. Prenatal KMO inhibition has been shown to result in unique dendritic spine changes: in CA1, basal spine density was decreased, whereas there was no effect on apical spine density, but there was a change in the proportion of mushroom:thin spines. In the dentate gyrus, there were decreases in the total numbers of dendrites and in dendrite length/complexity. However, there were no actual measures of spines themselves. Future experiments are planned utilizing ballistic labeling to examine spine morphology in greater detail. These experiments could be expanded to examine dendritic length/complexity as well to resolve the current discrepancy. However, the current discrepancy does not impact the utility of prenatal kynurenic acid elevation in modeling the cognitive deficits associated with SZ.

Given that dendritic spines are critical for biochemical compartmentalization and plasticity, it is not surprising that decreases in spine density correspond to cognitive impairments in this animal model. There are three critical issues that need to be addressed in subsequent experiments. Specifically, the main hypothesis of this experiment was that cognitive deficits would emerge as a function of PFC maturation. Spine density was measured in the current experiments in a separate cohort of rats (e.g., non-behaving rats) and only at PD70. A separate cohort of rats was selected because the behavioral paradigm selected, TFC, in and of itself changes spine density/morphology. These changes do not occur in paradigms in which the CS and US are explicitly unpaired or in the presence of
CS or US alone (Leuner and Shors, 2004) suggesting that spine growth is specifically due to learning the CS-US association. Intrinsic neuronal modifications occur as a result of learning, including those that prevent excessive activity (Hasselmo et al., 1995). Thus, animals that are exposed to kynurenic acid \textit{in utero} have significantly reduced spine density \textit{as adults}, which may reflect decreased numbers of glutamatergic synapses and altered excitatory-inhibitory balance. Because juvenile rats were not examined it cannot be elucidated whether these deficits emerge from or are exacerbated by maturational processes. Future experiments are planned to examine dendritic spine density and morphology both prior to and following adolescence in order to better elucidate maturational changes and impact on learning and behavior.

\textit{Context-Dependent Freezing}

An unexpected finding in the current experiments was that contextual fear conditioning was unaltered in rats exposed to kynurenine \textit{in utero}. Indeed, contextual freezing was similar across all ages and conditions. Contextual impairments, including contextual fear, are a key feature in humans (Hemsley, 2005) and in animal models of SZ. Specifically, Bucci and colleagues demonstrated that early elevation in kynurenic acid (PD7-10) resulted in impaired contextual fear in adult offspring. There are several possible reasons to explain the apparent discrepancies. We consider the differences to be most likely related to task design. For instance, Bucci and colleagues have established contextual impairments using rodent models of kynurenic acid elevation (Chess, Landers and Bucci, 2009; Iaccarino et al, 2013). However, the procedures therein differ
substantially, with two or three tone-shock (0.75 mA) pairings in which the tone and shock co-terminate (delay conditioning); trials were separated by a 64-second ITI. In contrast, we utilized trace fear conditioning following ten tone-shock pairings with a stronger US (1 mA/1 sec) and a significantly longer ITI (240 ± 30 s). Preliminary data in intact rats (Appendix A) showed that cue and context-dependent freezing are highly dependent upon the shock intensity, with context-dependent freezing enhanced at lower shock intensity and cue-dependent freezing enhanced at higher shock intensity. Further, ITI has a significant impact on outcome; a longer ITI during training increases cue-dependent freezing at the expense of context-dependent freezing (Detert et al., 2008). Thus, training parameters change the extent to which animals associate the US with either the context or the CS and the training paradigm utilized in these experiments likely examines primarily, if not exclusively, cue-dependent freezing.
Chapter 4: Age-Dependent Changes in the Kynurenine Pathway and 
α7nAChR/NMDAR Gene Expression

4.1 Brief Rationale

Kynurenic acid (KYNA) levels are elevated in the brains and cerebrospinal fluid of patients with schizophrenia (SZ; Erhardt et al., 2001; Schwarcz et al., 2001); however, the nature and time course of these changes are relatively unknown. Given the post-pubertal emergence of SZ, questions remain regarding whether developmental disruptions to the kynurenine pathway (KP) underlie cognitive deficits observed in adults. The previous chapter explored the emergence of such deficits using a trace fear conditioning task that depends upon cholinergic and glutamatergic signaling in the medial prefrontal cortex. The purpose of this aim was to characterize KP metabolites and markers of cholinergic/glutamatergic gene expression during a protracted period of development in order to elucidate the suspected link between KP disruptions and the emergence of cognitive deficits, as well as to provide a novel basis for targeted pharmaceutical intervention. This is important, as there are currently no reliable cognitive enhancing adjunctive pharmaceuticals and no early interventions available for this disorder. Kynurenic acid (KYNA) has the ability to antagonize NMDA and α7nACH receptors (Hilmas et al., 2001; Stone, 1993), which are not only critical for brain
development and executive function, but are down-regulated in SZ (Freedman et al., 2001; Schwartz et al., 2012).

**Hypothesis:** Prenatal kynurenine exposure results in changes to the kynurenine pathway and corresponding alterations in cholinergic/glutamatergic neurotransmission via its ability to antagonize NMDA and/or α7nACh receptors.
4.2 Introduction

Prenatal exposure to L-kynurenic acid, via administration in the maternal diet, was previously shown to raise fetal brain KYNA levels by 6000% (Pershing et al., 2014). Shortly following birth, offspring exposed to kynurenic acid in utero had brain KYNA levels similar to control offspring, an effect attributed to lack of continued precursor loading. Curiously, however, offspring exhibited subsequent 75% elevations in brain KYNA levels in adulthood. This adult-onset increase, in the absence of continued precursor loading, raises several important questions. Most relevant to this Aim, transient KYNA elevation could induce a cascade of effects that manifest in adulthood, through KYNA’s ability to act as an α7nACh and NMDAR antagonist. Specifically, NMDARs affect neuronal migration, synapse formation, neurite growth, and dendritic spine formation (Behar et al., 1999; Rajan and Cline, 1998; Ultanir et al. 2007), while α7nAChRs modulate NMDAR expression and postsynaptic maturation of glutamatergic synapses (Lin et al., 2012; Lozada et al., 2012). Short-term antagonism has been shown to have long-lasting effects on brain growth and development, as evidenced by prenatal exposure to NMDA antagonists or α7nAChR-specific antagonists (Section 3.2). However, the adult emergence of elevated KYNA suggests an even more complex interaction. How does prenatal KYNA lead to elevated KYNA in adulthood? At what postnatal age do KYNA levels increase? The cascade of events initiated by prenatal KYNA elevations could interact with elevated KYNA during the course of development, which would serve to decrease acetylcholine, glutamate, dopamine, and GABA (Zmarowski et al., 2009; Konradsson-Geuken et al., 2009; Rassoulpour et al., 2005; Beggiato et al., 2014). This
Aim therefore explores changes in two KP metabolites, kynurenine and KYNA, and corresponding changes in the expression of mRNA levels of α7nAChR and NMDAR subunits in juveniles and adults.

### 4.2.1 Kynurenine Pathway during Development

Brain KYNA levels are developmentally regulated in mammals, and studies in rodents and sheep have shown that brain KYNA levels show a U-shaped curve, with highest levels observed immediately prior to birth (Cannazza et al, 2001; Walker et al, 1999). Following birth, KYNA levels fall dramatically, then rise slowly to reach adult levels (mid-nanomolar rodents; low micromolar humans) during the course of development. The increased KYNA activity prior to birth may be a protective mechanism against brain injury that would otherwise occur from oxygen deprivation during the birthing process. Indeed, KYNA has been shown to be neuroprotective in neonatal animal models of ischemia and hypoxia via its ability to reduce glutamate release (Schwarcz et al., 1999; Ceresoli-Borroni et al., 2001). While brain KYNA levels in neonatal humans are unknown, Kazda et al. (1998) measured the levels of KYNA’s bioprecursor (kynurenine) in maternal venous and umbilical cord blood for females that delivered vaginally versus those that underwent cesarean-section and found that labor results in increased placental transfer of tryptophan and fetal synthesis of kynurenine. The increase in the bioprecursor in venous/umbilical tissue for females allowed to labor suggests a strong role of kynurenines for neuroprotection in the neonate. Little is known regarding subsequent changes in kynurenine pathway during normal human development. Adult
brain levels (determined from postmortem tissue) are in the low micromolar range, which appear to remain relatively stable under homeostatic conditions, with the exception of aging-related increases (Kepplinger et al., 2005). In contrast, KYNA levels change considerably under pathological conditions, including SZ in which levels increase with disease duration (Carlborg et al., 2013). KP changes occur in other pathological conditions, including Huntington’s Disease, Alzheimer’s Disease, depression, and bipolar disorder (Schwarcz et al., 2012; Müller, 2014). Notably, it has become increasingly evident that the KP is directly activated by both stress and inflammation, providing a potential causal link to environmental factors and neuropsychiatric disorders.

4.2.2 The Kynurenine Pathway and Inflammation

The KP is a multi-enzymatic pathway that results in formation of quinolinic acid (excitatory) in microglia or KYNA (inhibitory) in astrocytes. There are several key enzymes in this pathway that are 1.) Activated by stress and inflammation and 2.) Genetically altered in SZ patients. Prenatal infections have been considered the primary non-genetic cause of SZ (Patterson, 2009). Infection during pregnancy results in a 3- to 7-fold increased risk of SZ in the offspring (Brown et al., 2004). Interestingly, the type of infection appears to be rather unimportant, with association to multiple viruses, in particular influenza herpes simplex and influenza most implicated (Brown and Derkits, 2010) and bacteria such as Toxoplasmosis gondii (Schwarcz and Hunter, 2007; Torrey and Yolken, 2003). A common factor, then, is immune system activation, either for the mother during the second trimester or for the child during later stages of CNS
development. Markers of immune activation (pro-inflammatory cytokines) are elevated in both mothers (Buka et al., 2001; Brown et al., 2004; Fineberg, 2013; Howard, 2013) and offspring who subsequently develop SZ (Potvin et al., 2008; Soderlund et al. 2009). Immune system activation may result in life-long immune reactivity (Müller, 2014) and is associated with numerous neuropsychiatric disorders, likely through the mediation of pro- and anti-inflammatory cytokines. A link to immune dysfunction and KYNA production is evidenced by several clinical reports, including the finding that IFNα, a proinflammatory cytokine associated with immune activation, results in robust increases in peripheral kynurenine and CSF KYNA (Raison et al., 2010). The KP is directly modulated by both immune system activation and stress; specifically, enzymes responsible for the rate-limiting conversion of dietary tryptophan to L-kynurenine (the bioprecursor of KYNA) are up-regulated during immune activation or stress (Schwarcz et al., 2012; Müller, 2014). Up-regulation in these enzymes increase the availability of kynurenine and occur in conjunction with down-regulated kynurenine monooxygenase (KMO), which limits microglial catabolism. The net result is increased kynurenine shunted to astrocytic KYNA production. As such, providing L-kynurenine to rodents mimics the natural increases in substrate observed in patients and allows assessment of critical periods of vulnerability.
4.2.3 KYNA-Induced Disruptions in Cholinergic and Glutamatergic Neurotransmission

Cholinergic/glutamatergic dysfunction is suspected to underlie the devastating cognitive impairments in SZ. KYNA has the ability to antagonize NMDA and α7nACh receptors, which are not only critical for brain development and executive function, but are down-regulated in SZ. Manipulation of the KP has been shown in other animal models to alter offspring KYNA levels, with resulting changes in brain development that are of particular relevance in exploring SZ. For instance, Stone and colleagues utilize in utero inhibition of kynurenine monoxygenase (KMO) to raise KYNA in fetuses. Following this manipulation, offspring subsequently show age-dependent alterations in NMDA receptor subunit expression, with corresponding changes in synaptic plasticity including increased long-term potentiation (LTP) in weanlings and decreased LTP in adults (Forrest et al. 2013a; Forrest et al., 2013b). Developmental data suggest that timing and subsequent changes in NMDAR subunits may underlie cognitive deficits in SZ. For instance, transient NMDAR antagonism during development results in adult-emergence of a SZ-like phenotype (Stefani and Moghaddam, 2005; Baier et al., 2009), which may be due to changes in NR2A and NR2B subunits during the course of development (Wang et al., 2011).

KYNA, once thought to be an exclusive NMDAR inhibitor, also acts as a negative allosteric modulator of α7nACh receptors. Hilmas and colleagues (2001) utilized electrophysiological studies (patch-clamp) to determine that physiologically relevant
concentrations of KYNA (0.1-1 µM) reduced somatodendritic and presynaptic α7nAChR activity by 20-40%. Stone (2001) further elucidated KYNA’s role in cholinergic transmission in juvenile rats. Utilizing hippocampal slices from juvenile rats, KYNA reduced EPSP amplitude in a dose-responsive manner, an effect that was mimicked by the α7nAChR-specific antagonist methyllycaconitine (MLA) but not by the α4β2-specific antagonist dihydro-β-erythroidine (DHBE). Elucidating relationships between KYNA concentrations and nAChR blockade have been of continuing interest, as genetic deletion of the enzyme that converts kynurenine to KYNA results in significantly enhanced α7nAChR activity (Alkondon et al., 2004). This suggested functional interaction between KYNA and α7nAChRs is supported by several in vivo pharmacologic studies, including the reversal of KYNA-induced decreases in prefrontal glutamate by the α7nAChR positive modulator galantamine (Konradsson-Geuken et al., 2009).
4.3 Methods

Rats for Aim 2 were juvenile and adult ECON and EKYN offspring; 1-3 rats/litter were selected for analysis at PD32 (n = 8 ECON and 6 EKYN) and PD70 (n = 11 ECON and n = 13 EKYN). Ideally, rats would have been selected in all cases from litters that also contributed to behavioral endpoints in order to compare KYNA levels from a given litter to the behavior of that litter. However, due to the number of endpoints in this study (i.e., behavior and biochemistries at two ages), the number of males per litter was not always sufficient to fully balance littermates across all endpoints. Thus, biochemistries were conducted on litters that contributed to behavioral endpoints (84% of samples) and those that did not (16% of samples), but which were treated in all other regards the same (prenatal treatment, culling, housing conditions, etc.).

4.3.1 Biochemical Analyses

Plasma Kynurenine

Blood was collected into tubes containing 0.5 M disodium EDTA (Promega, Madison, WI) by cardiac puncture from anesthetized (CO₂) rats at PD32 and PD70. The supernatant plasma was isolated by centrifugation (12 000 x g, 10 min) and transferred to new Eppendorf tubes, frozen on dry ice and stored at -80°C until shipped on dry ice to our collaborator at the University of Maryland Psychiatric Research Center, Baltimore, MD, for analysis as follows. On the day of the assays, the samples were thawed, and 100 μL of plasma was acidified with 25 μL of 6% perchloric acid. After centrifugation (12 000 g, 10 min), 20 μL of the supernatant was subjected to high-performance liquid
Kynurenine was isocratically eluted from a 3-µm C18 reverse-phase column (80 mm · 4.6; ESA, Chelmsford, MA, USA), using a mobile phase containing 250 mm zinc acetate, 50 mm sodium acetate and 3% acetonitrile (pH adjusted to 6.2 with glacial acetic acid), using a flow rate of 1.0 mL/min. In the eluate, kynurenine was detected fluorimetrically (excitation: 365 nm; emission: 480 nm; Perkin Elmer Series 200 fluorescence detector, Waltham MA, USA). The retention time of kynurenine was approximately 6 min. Plasma levels for 1-3 offspring per litter were averaged to generate a litter mean, and the litter means were averaged to generate group means.

Kynurenic Acid (KYNA) In Tissue Homogenates

At the time of blood collection for each age, brains were rapidly removed and sectioned through the sagittal midline. Hemisections from offspring were dedicated (sides counterbalanced) for determination of either KYNA levels or qPCR analysis. Counterbalanced hemi-sections were collected and stored at -80°C until analysis. Hemisections for KYNA analysis were shipped on dry ice to the University of Maryland Psychiatric Research Center, Baltimore, MD, for analysis as follows. Tissues were thawed and sonicated in ultrapure water (1:10 w/v). One hundred µL of the homogenate were acidified with 25 µL of 6% perchloric acid. After centrifugation (10 min; 12,000 x g), 20 µL of the supernatant were applied to a 3-µm C18 reverse-phase column (80 mm x 4.6; ESA, Chelmsford, MA), and KYNA was isocratically eluted using a mobile phase containing 250 mM zinc acetate, 50 mM sodium acetate, and 3% acetonitrile (pH adjusted to 6.2 with glacial acetic acid), using a flow rate of 1.0 mL/min. KYNA was
then detected fluorimetrically (excitation: 344 nm, emission: 398 nm). The retention time of KYNA was approximately 7 min. Tissue KYNA levels for 1-3 offspring per litter were averaged to generate a litter mean, and the litter means were averaged to generate group means.

4.3.2 Quantitative Real Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from forebrain/frontal cortices of juvenile and adult ECON and EKYN using PureZol reagent (Bio-Rad, Hercules, CA, USA) followed by NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturers’ instructions. Genomic DNA contamination was eliminated by performing DNase digestion using RNase-free DNase (Macherey-Nagel, Düren, Germany). cDNA was obtained from the extracted RNA (up to 1 μg/reaction) using iScript reverse transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA) under the following conditions: priming at 25°C for 5 min, reverse transcription for 30 min at 42°C, and inactivation of transcriptase by heating at 85°C for 5 min. The first-strand cDNA solution was diluted 5-fold with 0.5X Tris-EDTA buffer (Sigma-Aldrich, MO, USA) prior to qPCR.

qPCR was performed using the CFX96, C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a reaction mixture containing 10 μL Sso Advanced SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 5 μL of each cDNA sample solution, 15 pmol of forward and reverse gene-specific primer-pairs (Integrated DNA Technologies, Coralville, IA, USA), and DNase-free water to a total volume of 20 μL. The thermal
profile for PCR was as follows: initial denaturation at 95°C for 30 sec followed by 39 temperature cycles of denaturation at 95°C for 5 s, annealing/extension (55°C for 30 s for mGluR2, GAPDH, and NMDAR subunits; 62°C for 30 s for α7nAChR), and melting curves. Fluorescence was acquired at the end of each extension phase. Fold changes in mRNA expression levels were calculated according to the comparative cycle threshold (CT) method and normalized using the level of GAPDH mRNA (Schmittgen and Livak, 2008). The primers used for each of the three genes are located in Table 4: Primer quality was evaluated by standard curve, melting curve and the presence of a single PCR product after gel electrophoresis.

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligo Primers</th>
<th>Expected Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>FW: 5’-CAT CAA GAA GGT GGT GAA GCA-3’</td>
<td>93</td>
<td>Rao et al., 2006</td>
</tr>
<tr>
<td></td>
<td>RV: 5’-CTG TTG AAG TCA CAG GAG GAC ACA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha7</td>
<td>FW: 5’-TGC ACG TGT CCC TGC AAG GC-3</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RV: 5’-GTA CAC GGT GAG CGG CTG CG-3</td>
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</tr>
<tr>
<td>NR1</td>
<td>FW: 5’-GTT CTT CCG CTC AGG CTT TG-3</td>
<td>225</td>
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<tr>
<td></td>
<td>RV: 5’-AGG GAA ACG TCC TGC TAC CA-3</td>
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<tr>
<td>NR2A</td>
<td>FW: 5’-AGC CCC CTT CGT CAT CGT-3</td>
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<tr>
<td>NR2B</td>
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<td>77</td>
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<td></td>
<td>RV: 5’-GAT CTT CCG GTC AGA CAT-3</td>
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</tr>
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</table>

Table 4. PCR Primers
4.3.3 Statistics

The litter was used as the experimental unit for all analyses of plasma kynurenine and brain kynurenic acid. Because single rats per litter were selected for qPCR, the rat was the experimental unit. Group comparisons (ECON vs EKYN at each age) were made using a minimum number of unpaired $t$-tests.
4.4 Results

4.4.1 Plasma Kynurenine and Brain Kynurenic Acid Analyses

The long-term effects of prenatal exposure to kynurenine on subsequent KP metabolism were assessed by measuring plasma kynurenine and brain KYNA in juveniles (PD32) and adults (PD70) (see Figure 14). Plasma kynurenine was similar between ECON and EKYN juveniles (2.5 ± 0.22 µM and 2.5 ± 0.17 µM, respectively; t_{12} = -0.126, P = 0.902) and adults (1.84 ± 0.19 µM and 1.2 ± 0.13 µM, respectively; t_{21} = -0.774, P = 0.447). Brain KYNA levels were also similar in juvenile ECON and EKYN rats (160.5 ± 43.7 and 159.8 ± 32.2 fmol/mg protein, respectively; t_{12} = -0.557, P = 0.588). However, brain KYNA levels in adult EKYNs (412 ± 96.2 fmol/mg protein) were 173% higher than ECON (151 ± 31.3 fmol/mg protein) levels at PD70, approximately 10 weeks following the end of precursor loading. The difference from the control group was significant (t_{22} = -2.403, P = 0.025).
Figure 14. Summary of Plasma Kynurenine and Brain Kynurenic Acid at PD32 and PD70. Plasma kynurenine was similar between ECON and EKYN juveniles and adults. Brain levels of KYNA were also similar between control and treated animals at PD32. However, KYNA levels in PD70 EKYN rats were 173% higher than ECON rats. *P<0.05.
4.4.2 Gene Expression (qPCR)

mRNA expression of several genes implicated in the etiology of SZ, that are antagonized by KYNA, and which underlie TFC (i.e., α7nAChR, and NMDAR subunits NR1, NR2A, and NR2B) were analyzed by qPCR at PD32 and PD70 (Figure 15). The predominate finding was a 30% reduction ($t_{17} = 2.216, P = 0.041$) in NR2A at PD32, relative to age-matched controls. This decrease persisted in adult EKYNs ($t_{18} = 2.314, P = 0.033$), but occurred in conjunction with a 50% reduction in NR1 ($t_{18} = 2.550, P = 0.020$). In order to further elucidate the interactions of NMDAR subunits over time, the ratios of NR2A:NR1, NR2B:NR1, and NR2A:NR2B were calculated for ECON and EKYN at each age (Table 5). The developmental changes in NR2A and NR2B for ECON rats, in this case as evidenced by changes in relative proportions of NR2A:NR1 and NR2B:NR1 suggested that NR2A levels increased slightly from PD32 to PD70, while NR2B levels remained constant. In contrast, the marked adult-onset reduction in NR1 observed for EKYN rats resulted in a dramatically different ontogenetic profile of NMDAR subunit expression, with approximately 1.5- to 2.4-fold increases in NR2B and NR2A. The ratio of NR2A:NR2B decreased slightly in EKYNs from PD32 to PD70, and this measure was lower than that observed in ECONs across ages. The potential implications of developmental alterations in NMDAR subunits are addressed in the discussion section.

Alpha 7 nAChR mRNA was unaffected at PD32. A 16% reduction in $\alpha7$ mRNA was observed at PD70; this difference was not statistically significant ($t_{18} = 1.153, P =$
0.264). However, similar slight reductions have been previously noted in rats exposed to KYNA in utero (Pershing et al., 2014).

Figure 15. Summary of PCR Data at PD32 and PD70. Prenatal kynurenine exposure resulted in significant reductions in mRNA levels of NR2A at PD32 and PD70, while NR1 was decreased at PD70. *P<0.01.
<table>
<thead>
<tr>
<th></th>
<th>NR2A:NR1</th>
<th></th>
<th>NR2B:NR1</th>
<th></th>
<th>NR2A:NR2B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PD32</td>
<td>PD70</td>
<td>PD32</td>
<td>PD70</td>
<td>PD32</td>
</tr>
<tr>
<td>ECON</td>
<td>0.92 ± 0.11</td>
<td>1.06 ± 0.07</td>
<td>1.22 ± 0.19</td>
<td>1.22 ± 0.17</td>
<td>1.02 ± 0.14</td>
</tr>
<tr>
<td>EKYN</td>
<td>0.71 ± 0.08</td>
<td>1.69 ± 0.57</td>
<td>0.96 ± 0.15</td>
<td>2.51 ± 0.82</td>
<td>1.44 ± 0.72</td>
</tr>
</tbody>
</table>

Table 5. NMDAR Subunit Ratios at PD32 and PD70.
4.5 Discussion

The purpose of this experiment was to determine whether prenatal kynurenine exposure resulted in age-related changes in the kynurenine pathway (KP) and cholinergic/glutamatergic gene expression. Data presented herein support a post-pubertal increase in brain KYNA, as juvenile KYNA levels were comparable between ECON and EKYN while adults exhibited a 173% increase in brain KYNA, relative to ECON. Persistent decreases (30-40%) in NR2A occurred in EKYNs relative to age-matched controls at both PD32 and PD70. The decrease in adults occurred in conjunction with a 50% reduction in NR1. A slight (16%) reduction in \( \alpha_7 \) nAChR mRNA was also observed in adults. This reduction was not statistically significant, but is consistent with prior observations noting that EKYN rats have reduced \( \alpha_7 \)nAChR expression at PD2 and PD56-80 (Pershing et al., 2014).

Post-Pubertal Emergence of Elevated KYNA

One particularly striking finding that has emerged from these sets of experiments is the post-pubertal increase in brain KYNA. Offspring in these studies were not exposed to kynurenine-loading subsequent to the end of gestation; therefore, this increase appears to reflect changes in endogenous KP that manifest following puberty. There were no differences between ECON and EKYN plasma kynurenine levels, which may indicate a change in the rats’ ability to convert kynurenine to KYNA. This finding is consistent with recent clinical studies in both SZ and depression in which plasma KYNA levels are
similar to control subjects, despite consistent findings of increased brain and CSF KYNA in patients. However, these studies have shown a significant increase in the tryptophan breakdown index, a measure of plasma kynurenine to tryptophan, which suggests that patients exhibiting SZ (Barry et al, 2009) and depression (Myint et al., 2012) more efficiently produce kynurenine (the bioprecursor of KYNA) from dietary tryptophan. Such a situation could arise from changes in underlying KP enzymes, namely decreased kynurenine 3-monoxygenase (KMO) activity, which would shunt kynurenine to the KYNA pathway or increased KATII activity, which would increase KYNA production from available kynurenine. Initial investigations support the former premise, that increased KYNA production may be related to down-regulation in KMO, as mRNA levels and KMO activity are decreased in adult EKYN rats whereas KATII activity is similar between ECON and EKYN (Pocivavsek et al., 2013). The correlation of decreased KMO activity provides one functional link to support the emergence of post-pubertal increases in brain KYNA. During puberty it possible that changes in gonadotrophins serve to activate the KP. For instance, KP activity has been shown to be sensitive to hormonal changes, with gonadotropins/glucocorticoids altering tryptophan metabolism by inducing IDO/TDO activity (Abdel-Tawab et al., 1975; Danesch et al., 1983, 1987; Gibney et al., 2014).

Elevated KYNA levels could also be observed as a result of altered efflux/elimination. Elimination of KYNA occurs via a probenicid-sensitive mechanism, utilizing organic anion transporters. These transporters can be saturated, although likely at higher than physiological concentrations (Uwai, Hara, and Iwamoto, 2013). There is
relatively little information on the role of efflux mechanisms to elevated brain KYNA levels. Several lines of research could provide unique insight to the differences between peripheral and central KP metabolites, particularly the finding that plasma KYNA is not elevated while brain levels are.

**Changes in NMDARs During Development**

Developmental changes in NMDAR expression were also observed following prenatal kynurenine exposure, with significant reductions (30-40%) in NR2A at both PD32 and PD70 and concurrent 50% reduction in NR1 at PD70. NMDAR hypofunction corresponding to reduced gene expression is noted in SZ (Akbarian et al., 1996; Beneyto and Meador-Woodruff, 2008; Coyle et al., 2003). These findings have been replicated in animal models, including NR1- and NR2A-deficient mice, which show multiple cognitive impairments (pre-pulse inhibition, novel object recognition) in conjunction with changes in neuronal excitability (Cui et al., 2004; Nabekura et al., 2002). How does prenatal KYNA exposure result in long-term functional changes in NMDAR gene expression? NMDAR gene expression is regulated by physiological, pharmacological, and pathological factors in a cell- and tissue-specific manner (Bai, 2009). There are six steps at which gene expression can be controlled: 1. Transcription, 2. RNA processing, 3. RNA transport, 4. Translation, 5. mRNA degradation, and 6. Protein activity. Transcription is sensitive to cellular needs and environmental cues and as the initiating step is a primary mechanism in controlling gene expression (Wray et al., 2003).
Indeed, NMDAR hypofunction in the clinical population is linked to lowered NR2A promoter activity. This NR2A promoter abnormality correlated with reduced mRNA levels or NR2A and severity of deficits (Itokawa et al., 2003; Iwayama-Shigeno et al., 2005). A sequence in the promoter region of NR2A is necessary for the developmental expression of NR2A (Bai and Hoffman, 2009). The activity of these elements, in general, is not well understood. Alterations in the NR1 gene are also correlated to SZ (Weikert et al., 2012). The neuronal specificity of NR1 expression has been shown to reside in the proximal promoter. If the activity of these promoter sequences are impacted by prenatal KYNA elevation then expression would be reduced. The functional consequences of such reductions are diverse, affecting NMDAR kinetics, cellular mechanisms of learning and memory, etc. (discussed below).

Other possibilities which remain to be explored in both the current animal model and in the clinical population are whether polymorphisms of regulatory regions play a role in altered NMDAR expression. Pathological NMDAR effects may also occur as a result of abnormal receptor activity stemming from altered agonist availability or alterations in membrane-associated receptors. KYNA can act as an NMDAR and α7nAChR antagonist, which may have functionally significant impacts on long-term glutamate release. NMDAR antagonism results in an initial increase in glutamate; the shift to a greater proportion of NR2B-containing subunits than NR2A-containing subunits may also result in increased excitability in juveniles. NMDAR-mediated excitotoxicity by high levels of glutamate has been hypothesized in several pathological disorders, including SZ (Moghaddam, 2013). However, NMDAR hypofunction/reduced gene
expression has been replicated and correlates with symptom severity. Gascón et al. found that treatment of cultured cortical neurons with NMDA or glutamate resulted in a reduction of the NR1 protein and mRNA (Gascón et al., 2005). Perhaps changes in cortical excitability in the pubertal period lead to enhanced susceptibility to excitotoxicity and subsequent down-regulation of NR1 as a compensatory mechanism in adults.

**Functional Consequences of Changes in NMDAR Subunit Composition**

Postsynaptic induction and expression of several forms of synaptic plasticity require calcium influx through NMDARs and the initiation of calcium-dependent signaling in the dendritic spine. The precise NMDAR subunit composition has a significant impact on pharmacological and signaling properties (Paoletti et al., 2013); therefore, the developmental changes in NR2A and NR1 may have several consequences. Indeed, it is suggested that NR2B with slow deactivation kinetics provides for dynamic synaptic changes whereas NR2A, with fast deactivation kinetics provides temporal resolution, allowing the adult-onset CNS refinement (reviewed in Sepulveda et al., 2010). For instance, NR2A-deficient neurons have increased EPSC decay times, supporting the idea that developmental increases in NR2A are responsible for developmental decreases in NMDAR EPSC duration (Flint et al., 1997). In the visual cortex, appropriate synapses are strengthened and maintained while inappropriate connections are eliminated. This bidirectional synaptic plasticity is mediated by the ratio of NR2A/NR2B receptors, as genetic knockout or knockdown of NR2A impairs bidirectional synaptic plasticity (Cho et al., 2008). Such a change has been previously noted to result in a prolonged decay time
of decay activation (Vicini et al., 1998), thus indicating greater excitability. In contrast, adult EKYNs had significantly reduced NR1 expression in conjunction with NR2A. This post-pubertal decrease in NR1 expression may be particularly important in elucidating mechanisms of action. Maternal administration of Poly I:C to mice results in decreased NR1 expression in adult offspring, which corresponds to working memory impairments - a core deficits observed in SZ (Meyer and Feldon, 2008).

Developmental changes in NMDAR subunits have also been observed by Stone and colleagues, who utilize the KMO inhibitor Ro61-8048 to elevate KYNA levels prenatally. Within several hours following KMO inhibition, fetuses exhibited 43% reductions in NR2A and 395% increases in NR2B, relative to controls. At weaning, both NR2A and NR2B were increased relative to controls, corresponding to increased LTP observed in weanlings (Forrest et al., 2013a). As adults, however, Ro-treated exhibited decreased NR2A levels in the absence of changes in NR2B, with accompanying decreased LTP and delayed recovery from LTD (Forrest et al., 2013b). Ro-treated rats also exhibit decreased dendritic spine density (Khalil et al., 2014), which may be explained by altered NR2A:NR2B, as knockdown of endogenous NR2A reduces dendritic branching (Ewald et al., 2008). However, myriad developmental proteins were also altered as function of KMO inhibition, including RhoB, a RhoGTPase that mediates the effects of NMDAR on dendritic spines.
Chapter 5: The α7 nAChR as a Therapeutic Target

5.1 Brief Rationale

Aims 1 and 2 explored the functional consequences of prenatal kynurenine exposure on the emergence of cognitive deficits, and results suggest that age-dependent alterations in trace fear conditioning (TFC) may be mediated by changes in NMDAR subunits. Specifically, decreased expression of NR1/NR2A subunits emerged following adolescence and corresponded to decreased dendritic spine density and decreased cue-dependent freezing in adult EKYNs. Aim 3 was therefore designed as an initial investigation to explore a pharmacotherapeutic intervention to improve cued-fear response in EKYNs. The α7 partial agonist (SSR180711) was selected because nicotinic receptors are consistently associated with glutamatergic synapses, and there has long been a suggested mutual interaction that serves to fine-tune cellular activity through nAChR’s ability to regulate the functional properties of glutamatergic and GABAergic neurons by modulating neurotransmitter release and activity dependent plasticity (Mansvelder et al., 2006; Yang et al., 2013; Griguoli et al., 2012). Previous data indicate enhancement of TFC or trace eye blink with nicotine, physostigmine (an acetylcholinesterase inhibitor) or galantamine (an α7nAChR positive modulator) (Hunt & Richardson, 2007; Raybuck & Gould, 2010; Weible et al., 2004; Simon et al., 2004). This
aim will therefore further elucidate the role of α7nAChR in mediating TFC by administering SSR180711 prior to TFC training (an assessment of acquisition/consolidation) or prior to testing (an assessment of recall).

**Hypothesis:** If reduced cue-dependent freezing in EKYN adults is mediated by α7nAChR, then administration of the partial agonist SSR180711 will increase cue-dependent freezing.
5.2 Introduction

The majority of FDA-approved medications for schizophrenia (SZ) have primary efficacy through dopamine (D2-like) receptor antagonism (Correll and Kane, 2014). These drugs may be effective for positive symptoms such as hallucinations or delusions, but have adverse effects on the cardiovascular, digestive, and nervous systems (Üçok and Gaebel, 2008) and subsequently result in compliance issues for many patients (Freedman, 2005; Lieberman et al., 2005). These drugs are also generally poor at addressing cognitive dysfunction, which is a critical unmet need in pharmacotherapy considering that cognitive deficits are the primary predictor of functional outcome (Green et al., 1996; Green et al., 2001). Due to the side effects and poor efficacy of drugs that target D2-like receptors, several new classes of drugs have been explored based upon recommendations by the National Institute of Mental Health-funded program Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS). These drugs are mechanistically diverse and include dopaminergic (D1) agonists, cholinergic (muscarinic and nicotinic) agonists, GABA modulators, NMDAR modulators (glycineB site agonists/antagonists), and KATII inhibitors. None of these medications have been approved by the FDA for cognitive enhancement in SZ; however, postmortem/neuropathological findings, including altered NMDAR and nAChR expression (Freedman et al., 1995; Crook et al., 2000; Dean et al., 2002; Weikert et al., 2012) in multiple brain regions, and altered dendritic spine density (Glantz and Lewis, 2000), findings replicated in the current experiments by prenatal KYNA exposure, suggests a continued focus on glutamatergic/cholinergic interactions in development of
adjunctive therapeutics. Indeed, in recent clinical trials the most promising data reported were for galantamine, an α7nAChR positive modulator/cholinesterase inhibitor that is currently approved for use in Alzheimer’s Disease; EVP-6124 an α7nAChR partial agonist, and D-cycloserine, a partial agonist of the glycineB site of the NMDAR (Keefe et al, 2013; Forum Pharmaceuticals Clinical Trial, 2012).

The α7nAChR is of specific interest as a pharmacologic target because it is widely distributed in the CNS but not in the periphery, which indicates that drug development for this target is ideal for exploration in enhancing cognition without side effects mediated by other cholinergic receptors, such as addiction, convulsions, gastrointestinal upset, and adverse cardiovascular events (Martin, Kem, and Freedman, 2004). The utility of the α7nAChR as a therapeutic target for cognition is further supported from multiple converging lines of evidence including receptor dysfunction in the clinical population and pharmacologic data from animal models suggesting a unique ability of α7nAChRs to modulate other neurotransmitter systems. The diverse expression of α7nAChRs, which are located presynaptically on nerve terminals and postsynaptically on dendritic spines and soma, suggests strong roles in neurotransmission including gene expression, modulation of neurotransmitter release, and fast excitatory transmission (Fabian-Fine et al, 2001; Marchi et al, 2002; Alkondon et al, 2000; Frazier et al, 1998). Activation of α7nAChRs can result in immediate, short-term, and long-term effects (Shen and Yakel, 2009). Immediate release of neurotransmitter occurs via the nAChR’s high calcium permeability and the direct influx of calcium into the channel pore. Down-stream events and regulatory feedback mechanisms, which depend upon cellular signaling without gene
expression, result in short-term effects (seconds to minutes) following α7nAChR activation. These include regulation of neurotransmitter release and receptor desensitization, which are controlled through the activation of second messenger systems (primarily protein kinases) and intracellular calcium signaling (calcium release, calmodulin, and CaMKII activity. The nAChR-mediated intracellular calcium subsequently activates down-stream pathways that alter gene expression/protein synthesis (ERK/MAPK/CREB). All mechanisms (immediate, short, and long-term) have profound influence on the ability of α7nAChRs to modulate excitatory/inhibitory balance. Indeed, presynaptic α7nAChR stimulation can increase glutamate release in the prefrontal cortex (Aramakis and Metherate, 1998) and the hippocampus (McKay and Dani, 2007; Gotti et al., 2009). The activation of α7nAChRs by nicotine or by endogenously released acetylcholine has been shown to convert silent synapses into functional ones (Maggi et al., 2003) and desensitization of α7 nAChRs on inhibitory GABAergic interneurons can cause disinhibition of glutamatergic neurons (Alkondon et al., 2000). The activation of pre-synaptic α7 nAChRs has also been shown to enhance long-term potentiation (LTP), an electrophysiological model of learning and memory formation (Sawada et al, 1994; Hunter et al, 1994).

Activation of LTP by α7nAChR induces long-lasting increases in intracellular calcium and activation of second-messenger systems such as cAMP, elevated levels of gene products, and enhanced neurotransmitter release (Bucafasco et al, 2005), thus providing a possible neurochemical mechanism through which α7 modulation could enhance cognition. As such, learning-induced changes in excitatory/inhibitory synaptic
strength may be modulated by α7nAChR. Given the importance of this receptor in cognitive domains that are particularly impaired in SZ, including attention (Freedman et al., 2003; Young et al., 2007) and working memory (Jacobsen et al., 2004; Timofeeva and Levin, 2011), understanding the cholinergic modulation of learning-induced synaptic plasticity may provide a targeted platform for therapeutic intervention.

Numerous α7 modulators are in development and grouped according to their affinity/mode of action and are categorized as agonists, partial agonists, or allosteric modulators. Development of differential mode-of-action pharmacotherapeutics is based on the unique kinetic properties of nAChRs in conjunction with dysfunction noted in the clinical population. Alpha 7 nicotinic receptors are ligand-gated ion channels composed of five polypeptide subunits (α7) around a central, water-filled pore (Dani, 2001). Receptor activation occurs when the endogenous ligand ACh (at least two molecules) or nicotinic agonists bind to the orthosteric site. When activated, receptors are stabilized in an open conformation and cations (calcium and sodium) rapidly diffuse into the open channel causing a depolarization of the postsynaptic membrane. Following ligand binding, α7nAChRs remain open for seconds to minutes, allowing cations to pass through the ion channel (Dani, 2001). Following this period of cation exchange, the α7nAChR channel closes and the receptor enters a desensitized state, whereby there is a loss of functional response upon repeated or continuous exposure to agonist (Quick and Lester, 2002). Complex receptor kinetics, including propensity for receptor desensitization, has led to focused research. For instance, positive allosteric modulators (PAMs) lack agonist activity but potentiate response of endogenous ligands acetylcholine and choline. There
are several PAMs in development, based on their ability to increase agonist-evoked response in the absence (Type I) or presence (Type II) of affecting desensitization (Thomsen, El-Sayed, and Mikkelsen, 2011).

Impairments observed in animal models of SZ in multiple tasks (novel object recognition, social recognition, and latent inhibition) can be improved with α7 nAChR agonists (Pichat, 2007; Barak, 2009; Thomsen et al. 2010) and positive modulators (Timmerman et al., 2007; Thomsen, El-Sayed, and Mikkelsen, 2011). Galantamine, the α7 nAChR positive modulator that has shown clinical promise for cognitive deficits in Alzheimer’s disease, has shown cognitive enhancing properties in two animal models of SZ in our laboratory. Namely, galantamine (3 mg/kg) restores attentional set-shifting performance and working memory in rats exposed to elevated KYNA chronically during development or acutely as adults (Alexander et al., 2013; Vunck et al., 2013). However, galantamine has a narrow effective dose range in trace fear and trace eye blink (Weible et al. 2004; Simon et al. 2004) and may act as acetylcholinesterase inhibitor at higher doses (Samochocki, 2003). Given the narrow effective dose range for galantamine and evidence that α7nAChR partial agonists, but not positive modulators, can upregulate receptor expression (Thomsen, El-Sayed, and Mikkelsen, 2011), the utility of SSR180711 as a cognitive enhancer was explored in the current experiments.
5.2.1 SSR180711 as a Pharmacotherapeutic

SSR180711 (1,4-Diazabicyclo[3.2.2]nonane-4-carboxylic acid, 4-bromophenyl ester) is a partial agonist, possessing 39% - 51% of the intrinsic activity of the endogenous ligand acetylcholine (Biton et al., 2007). SSR has high affinity for α7 nAChR in the absence of functional activity at other nicotinic subtypes in either the CNS or in the periphery, which indicates that this drug is ideal for exploration in mediating cognitive deficits without side effects. SSR has been thoroughly characterized in vivo at dose levels of 0.1-10 mg/kg (Barak et al., 2009; Pichat et al., 2007; Kristensen et al. 2007). Repeated dose administration showed no sign of tachyphylaxis, minimizing concerns over effects on receptor desensitization (Pichat et al., 2007). Effective dose levels vary according to task, dosing regimen (acute vs. chronic) and outcome measure, including measures of binding or measures of behavior, and for specific behavioral assays.

Barak et al. (2009) utilized SSR to normalize abnormal latent inhibition (LI) in two animal models of SZ. LI is the delay in associative learning that occurs when a previously neutral stimulus is subsequently reinforced with a salient stimulus. LI requires a subject to allocate attentional resources and the ability to discriminate between relevant and irrelevant stimuli, and is thus a useful measure of cognition. In rodents, administration of MK-801 to adults or N-nitro-L-arginine (NOS inhibitor, which affects neuronal migration and brain development) to neonates results in low LI, as evidenced by reduced association of tone with a shock following pre-exposure to that tone. Administration of SSR at doses of 1 and 3 mg/kg (but not 0.3 mg/kg) restored LI in both
of these models. The authors attribute this effect, a restoration of flexible responding, on SSR’s ability to increase prefrontal and limbic glutamate. Pichat et al. (2007) further characterized the dose-response profile of SSR in a variety of behavioral tasks, including novel object recognition, Morris water maze, and linear maze sequential memory. Novel object recognition in intact adult rats was enhanced to a similar magnitude by doses of SSR at 0.3-10 mg/kg, suggesting a ceiling effect, perhaps imposed by the use of non-impaired rats. Pichat et al. subsequently explored the ability of SSR to improve novel object recognition in rats exposed to phencyclidine (PCP) as neonates, and rats administered MK-801 as adults. Each of these compounds disrupts glutamatergic signaling and serves to model NMDAR dysfunction in SZ (Geyer and Moghaddam, 2002). Rats exposed to PCP as neonates or MK-801 as adults exhibited reduced novel object recognition, evidence of impaired short-term episodic memory; an effect which was reversed by SSR at 0.3-1 mg/kg. An easy explanation for SSR’s apparent pro-cognitive abilities would be alpha7-mediated increases in behaviorally relevant neurotransmitters such as dopamine, glutamate, and acetylcholine. SSR has, indeed, been shown to dose-dependently increase prefrontal acetylcholine, dopamine (Biton et al., 2007) and glutamate (Bortz et al., 2013). However, it is hard to imagine that cognitive impairment arising from neurodevelopmental disruptions are easily remedied by simple elevations in extracellular concentrations of neurotransmitters. Biton et al. (2007) also observed SSR-mediated increases in hippocampal LTP in conjunction with acetylcholine release, providing a cellular mechanism by which SSR may enhance cognition. Furthermore, Mikkelsen and colleagues have performed an extensive series of
experiments to establish the underlying cellular mechanisms relating to SSR efficacy and established that systemic injection of SSR (3-20 mg/kg) activates c-fos and Arc in a regionally specific manner (Hansen et al., 2007; Kristensen et al., 2007; Thomsen et al., 2007; Thomsen et al., 2010). Immediate early gene activation is critical for learning and memory, which may explain why some α7nAChRs have reported efficacy that cannot be explained by plasma pharmacokinetics alone (Werkheiser et al., 2009).

A dose level of 1 mg/kg was selected for use in this study. A higher dose (3 mg/kg) was effective in restoring attentional set-shifting ability in a rodent neurodevelopmental model of TTX hippocampus inactivation (Brooks et al., 2012). However, initial investigations in rats exposed to kynurenine from GD15-PD21 indicated that this dosage level was ineffective. When a subsequent higher dosage (10 mg/kg) resulted in possible anxiolytic signs in these rats, it was hypothesized that lower dosages should be explored. Complicated dose-response profiles have been observed for SSR and other α7nAChR agonists. Barak et al. (2009) observed that spatial water maze impairments caused by neonatal exposure to PCP were dose-dependently improved by SSR in early trials as evidenced by a decreased in the time required to find a hidden platform. However, the highest SSR dose evaluated (10 mg/kg) increased the time to complete the maze in later trials. In addition, cognitive enhancing effects in the Barak study did not correspond to consistent neurophysiological changes. For instance, while SSR at dose levels of 1 and 3 mg/kg resulted in robust behavioral improvements, across tasks, SSR at 1 mg/kg decreased spontaneous firing rate in the retrosplenial cortex while 3 mg/kg increased spontaneous firing rate.
The experiment in this aim therefore utilized offspring exposed to prenatal elevations of KYNA, which has been previously shown to modulate acetylcholine, dopamine, and glutamate, as an animal model of the cognitive deficits in SZ, with TFC as a measure of cognitive function that incorporates attention, working memory, and long term memory. This Pavlovian task is recognized by CNTRICs and examines cognitive domains for which patients show deficits and which predict functional outcome. Based on the observed adult-onset decreases in cued-fear response in EKYN rats, this experiment was designed to explore the ability of SSR to restore cued-fear response in adults. In order to assess possible mechanisms of action SSR was administered either prior to TFC (an assessment of acquisition/consolidation), prior to testing (an assessment of recall) or on all 3 days in order to account for state-dependent effects.
5.3 Methods

5.3.1 Animals

Animals for this experiment were ECON and EKYN offspring obtained as described in the General Methods (Chapter 2). Rats were reared to adulthood (PD70) then assessed in trace fear conditioning (TFC). Animals in this experiment were dosed, as adults, using one of four dosing paradigms. Thus, a within-litter design was utilized and 1-2 rats/litter (N = 9-10 per group) were assigned to one of four treatment groups, described below. Remaining rats per litter were assigned to biochemistry (plasma kynurenine and brain KYNA) assessments (See Section 4.3).

5.3.2 Drug and Group Assignment

SSR180711 (hereafter designated SSR) was obtained from NeuroSearch A/S, Denmark, Copenhagen and stored at -20°C. Solutions of the test article (1 mg/mL) were prepared in physiological saline, and adjusted to approximately pH 7 using 0.1 N NaOH. Sterile-filtered aliquots of SSR and vehicle (pH-matched 0.9% saline for injection, USP) were stored at -20°C. On each day of TFC, aliquots of SSR or vehicle were removed from the freezer, and thawed to room temperature. Dose administration was intraperitoneal (i.p.) injection based on body weight at a dosage level of 1 mg/kg/day for a total of three doses, one each day of TFC. Groups of adult (PD70) ECON and EKYN rats were dosed 50 minutes prior to the start of each day of TFC with either vehicle or SSR in the following combinations: Group 1: SAL/SAL/SAL (Saline); Group 2: SSR/SAL/SAL (SSR - Pre-Training); Group 3: SAL/SAL/SSR (SSR - Pre-Testing),
Group 4: SSR/SSR/SSR (SSR - All Days). Following injection of saline or SSR, TFC was conducted as described in Aim 1. Following completion of behavioral testing, rats were euthanized by carbon dioxide inhalation and discarded.

### 5.3.3 Statistical Analyses

Given that the purpose of this Aim was to assess the ability of SSR to restore cue-dependent freezing, results are framed in response to freezing during the auditory tone conditioned stimulus (CS) without focus on freezing during the trace interval. Therefore, percentage time freezing during the CS was analyzed as a function of VEHICLE or SSR administration on Day 1 by a Two-Way Repeated Measures ANOVA, with prenatal CONDITION as a between-subjects factor and TRIAL as within-subjects factors. Percentage time freezing for the entire session on Days 1, 2 and 3; and baseline freezing during the cued-fear test (Day 3) were analyzed using a 2 (prenatal condition) x 4 (drug condition) Way ANOVA, where prenatal condition is ECON and EKYN and drug condition is vehicle, SSR - Pre-Training, SSR - Pre-Test, or SSR - All Days. Following a significant interaction of prenatal condition and drug condition, a minimum number of t-tests were conducted based on *a priori* hypotheses. Significance was defined as P<0.05, and the Huynh-Feldt correction was utilized to reduce Type 1 errors associated with repeated measures ANOVAs (Vasey and Thayer, 1987). All statistical tests were performed using SPSS for windows (versions 19-22; Chicago, IL).
5.4 Results

5.4.1 Trace Fear Conditioning

Day 1 Conditioning

As shown in Figure 16, cue-dependent freezing increased as animals learned the CS-US association, as evidenced by significant effect of Trial following repeated measures ANOVA for rats receiving vehicle (F_{9,35} = 6.545, P = 0.000) or SSR (F_{9,36} = 14.509, P = 0.000) prior to training. This effect was not influenced by prenatal exposure to kynurenine, as there were no interactions with prenatal condition in rats receiving vehicle (F_{1,35} = 2.876, P = 0.104) or SSR (F_{1,36} = 2.117, P = 0.154). Similarly, as shown in Figure 17 overall freezing levels for the entire session were not significantly different, regardless of prenatal condition and drug (50 ± 4.3% to 63 ± 4.6%; F_{3,70} = 2.655, P = 0.055). Freezing levels in the saline control groups (ECON - SAL: 57 ± 3.1%; EKYN - SAL: 48.02 ± 4.22%) were also comparable to non-injected rats from Aim 2 (ECON: 54.1 ± 3.9%; EKYN: 44.4 ± 4.2%), which serves measure of behavioral control for the saline injection.
Figure 16. Summary of Trace Fear Conditioning Data (Day 1). Rats received either vehicle or SSR prior to training (Pre-Train), prior to testing (Pre-Test), or both (All Days). Percentage time freezing (mean ± SE) during the CS is presented for rats that received vehicle (A) or SSR (B) prior to training. There was no effect of prenatal condition or SSR administration on cue-dependent freezing on Day 1.
Figure 17. Summary of Total Percentage Time Freezing (mean ± SE). The percentage of time freezing for the entire session on Day 1 is presented for separate cohorts of rats, e.g., those that received vehicle or SSR prior to training on Day 1, prior to testing on Day 3, or prior to training and testing. There was no effect of prenatal condition or SSR administration on cue-dependent freezing on Day 1.
Contextual Fear Responding (Day 2)

Twenty four hours (±2 hours) following conditioning, rats were returned to the training chamber and assessed for contextual fear (fear response to the context in absence of CS and US). Similar levels of freezing were noted throughout the 14-minute context test across groups (38 ± 4.7% to 51 ± 3.6% freezing across groups; Figure 17). There were no significant effects of prenatal condition (F\textsubscript{1,70} = 0.078, P = 0.781), Drug Treatment (F\textsubscript{3,70} = 0.1465, P = 0.231), nor interactions prenatal condition and drug treatment (F\textsubscript{3,70} = 0.790, P = 0.504).

![Day 2: Context Test](image)

Figure 18. Summary of Contextual Fear Response. Percent time freezing to the training chamber in the absence of CS or US on PD70, following injection of either saline or SSR. Contextual fear was unaffected by prenatal kynurenine or SSR administration (all P’s > 0.05).

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Cued-Fear Response (Day 3)

Rats were then assessed (24 hours later; Day 3) for percentage freezing during the CS in order to determine the effect of SSR administration on cued-fear response. Baseline freezing ranged from 18 ± 9.2% to 28 ± 5.4%; differences were not significantly different as a function of prenatal condition or drug treatment (F_{3,70} = 0.163, P = 0.921). As shown in Figure 19, a 2 x 2 ANOVA supports that SSR administration results in prenatal treatment-dependent alterations freezing to the tone, an assessment of cue-dependent freezing (F_{3,70} = 3.567, P = 0.018). A series of planned t-tests were conducted in order to assess whether SSR affected TFC via acquisition/consolidation or retrieval. EKYNs administered saline (EKYN-SAL) prior to training and testing exhibited significantly less cue-dependent freezing than ECON-SALs (49 ± 5.7% vs 68 ± 6.2%, t_{17} = 2.341, P = 0.032). Freezing levels were similar between saline-injected and non-injected rats in Aim 2, suggesting that there was no effect of the injection itself. Remarkably, administration of SSR to EKYN rats prior to training on Day 1 (EKYN-SSR Pre-Train) resulted in a significant increase in cued-fear relative to EKYN-SAL rats (67 ± 4.3% vs. 49 ± 5.7%, t_{17} = -2.606, P = 0.018). This increase was also sufficient to restore cued-fear response in EKYNs to ECON levels (t_{17} = 0.861, P = 0.861). In contrast, pre-testing administration of SSR to EKYNs was not effective; EKYNs in this group froze significantly less (47 ± 6.2%) than both ECON-SAL (68 ± 6.2%, t_{17} = 2.395, P = 0.028) and EKYNs given SSR prior to training (67 ± 4.3%; t_{18} = 2.647, P = 0.016). Administration of SSR to EKYNs for 3 days, an assessment of state dependent effects, revealed similar freezing levels as rats administered SSR prior to training only (t_{18} =
0.094, P = 0.926). There were, similarly, no effects of SSR administration on control
group performance, as ECONs froze similarly regardless of drug administration paradigm
(all P’s > 0.05).
Figure 19. Summary of Cued-Fear Response following SSR Administration at PD70. Mean percentage time freezing (± SE) to the CS at PD70. EKYN rats receiving vehicle (EKYN-SAL) froze significantly less than ECON-SAL, replicating the findings of non-injected rats in Aim 1. EKYN-SAL rats also froze significantly less than rats that received SSR prior to training (SSR Day 1) or prior to training and testing (SSR All Days), but not prior to testing alone (SSR Day 3). Thus, administration of SSR on Day 1 prior to training restored cued-fear response in EKYNs to that of ECONs. a = Significantly different from ECON - Saline Group; b = Significantly different from EKYN - SSR Prior to Training and Testing; c = Significantly different from EKYN - Pre-Training, P<0.05
5.5 Discussion

The experiment in this aim utilized offspring exposed to prenatal elevations of KYNA, which has been previously shown to modulate acetylcholine, dopamine, and glutamate, as an animal model of the cognitive deficits in SZ, with trace fear conditioning (TFC) as a measure of cognitive function that incorporates attention, working memory, and long-term memory. This Pavlovian task is recognized by CNTRICs and examines cognitive domains for which patients show deficits, and which predict functional outcome. Based on the observed adult-onset decreases in cued-fear response in EKYN rats (Chapter 3), this experiment was designed to explore the ability of an α7nAChR partial agonist (SSR) to restore cued-fear response in EKYN adults. In order to assess possible mechanisms of action, SSR was administered either prior to TFC training (an assessment of acquisition/consolidation), prior to testing (an assessment of recall) or prior to training and testing in order to account for state-dependent effects. The most relevant finding observed in this Aim was that SSR administered prior to training, but not prior to testing, restored cue-dependent freezing in EKYNs to control levels. The finding that SSR administered prior to testing alone does not enhance cue-dependent freezing suggests that the deficits observed in EKYNs occur as a result of, and are rescued through, mechanisms relating to acquisition/consolidation rather than recall. Because dose administration in this study is systemic, specific mechanisms cannot be elucidated but may relate to broad cognitive domains (attention, working memory) or behavioral processes (encoding/consolidation) and their underlying cellular mechanisms (immediate early gene activation; protein synthesis).
**Possible Mechanisms**

Attention

Unlike delay conditioning, in which the CS and US co-terminate, trace conditioning requires attentional mechanisms in order to maintain the CS-US representation over the empty trace interval. The addition of this trace interval specifically engages signaling through nicotinic receptors in the mPFC, which are not engaged in delay fear conditioning (Raybuck and Gould, 2010). Much like the sustained attention task in rodents, Han et al. (2003) showed that a distractor during conditioning significantly impairs cued-fear response. The underlying cellular mechanisms in this study were elucidated and indicated a 50% increase in c-fos-positive cells in rats that acquire TFC, but not in rats that were distracted. This increase in c-fos-positive cells is particularly intriguing given the collaborative findings of Pichat et al. (2007) and Mikkelsen and colleagues, who found that SSR restored selective attention deficits in a juvenile PCP model of SZ in conjunction with region-specific increases in early activation genes, including c-fos and Arc, following SSR administration (Hansen et al., 2007; Kristensen et al., 2007). However, Mikkelsen and colleagues found immediate early gene (IEG) activation in the prelimbic region of the mPFC at doses only 3 mg/kg or higher. Further, distinct subregions of the distributed neural system that underlie TFC required even higher doses. Namely, the cingulate cortex, which underlies attentional components of TFC and the infralimbic cortex, which underlies extinction, exhibited c-fos activation at 10–20 mg/kg SSR. The observation in the current experiment, namely, that of behavioral improvement at doses lower than that required for early gene induction is consistent with
previously reported findings. This may arise from functional differences/interactions of endogenous cholinergic tone in performing animals versus non-performing animals (Parikh et al., 2007; 2008; Howe et al., 2010).

Phasic increases in acetylcholine or glutamate may be particularly important in a task that requires the association of cues. Acetylcholine increases as a result of attentional demands, as shown by microdialysis conducted in rats performing sustained attention tasks (Arnold et al., 2002). In operant sustained attention tasks, rodents are required to respond to the presence of visual signals. The visual signals vary in duration and may be accompanied by distractors, factors that affect that rats’ ability to discriminate between signal and non-signal events (McGaughy and Sarter, 1995). The nature of the operant task allowed Sarter and colleagues to further elucidate the role of cholinergic and glutamatergic transmission in cue detection with microelectrode-based detection, which has much higher temporal and spatial resolution than microdialysis procedures. Parikh et al. (2007) utilized a cue-detection task in rodents that were implanted with choline-sensitive microelectrode arrays (MEA) in the mPFC. MEAs, through a series of enzymatic reactions, detect choline generated from the hydrolysis of endogenously generated acetylcholine. In that experiment, there were two notable findings with particular relevance to the current Aim. First, transient (seconds) increases in cholinergic activity were observed when rats detected cues but not when they missed cues. Secondly, these cholinergic transients occurred on top of slower (minutes) performance-related increases in cholinergic signals, with higher tonic levels resulting in greater phasic response and enhanced cue-detection. Importantly, the generation of cue-evoked
cholinergic transients also depends upon prefrontal glutamate release and stimulation of ionotropic glutamate receptors (Parikh et al., 2008; Howe et al., 2010). Cue detection, a top-down process, requires representation of the presence of the cue and information about the associative significance of the cue (Sarter et al., 2005, 2006). It is possible that in rats exposed to KYNA in utero there is low cholinergic/glutamatergic tone and that administration of SSR180711 enhances the ability of rats to associate the CS to the US. Previous data from this laboratory have shown that prefrontal glutamate release in EKYN rats is significantly attenuated (Pershing et al., 2014). Not only is this attenuated glutamate release mediated by α7nAChRs, it is dependent on the salience of stimulation. That is, with sufficient stimulus, glutamate release in these rats is comparable to control rats (Alexander et al., 2011).

Working Memory

Working memory, the ability to maintain/manipulate information over short periods of time, depends upon feed-forward excitation and local circuits in the mPFC. Goldman-Rakic and colleagues (1995) established that working memory, evaluated in delayed-response tasks, depends upon sustained firing of Layer II/III neurons of the mPFC. Yang et al. (2013) clarified the importance of cholinergic signaling in the sustained firing of these neurons, showing not only the necessity of α7-nAChR stimulation for an NMDAR-dependent working memory task but also that the amount of stimulation is critical for task performance. Namely, the authors assessed the effects of three different α7 agonists (PHA543613, DMAB-A, and TC1698) or antagonists
(mecamylamine or MLA) on the ability of monkeys to perform a delayed-response task in which the location of a visual cue (which moves from trial to trial) must be remembered over a delay in order to receive a reward. In accordance with the previously established role of the mPFC in working memory (Goldman-Rakic, 1995), Layer II/III neurons exhibited sustained firing over the delay in this task. Importantly, low-dose stimulation with α7 agonists results in improved performance in the manual delayed-response task via “spatial tuning”, which increases delay cell firing specific to the cell’s preferred direction (e.g., firing in response to correct location of visual target). In contrast, high-dose stimulation with an α7 agonist results in general increases in excitability and resulting decreases in performance. Similarly, application of α7nAChR-specific antagonist reduced delay-related firing for the neurons’ preferred direction while mecamylamine resulted in general reductions in firing. Sustained firing of Layer II/III pyramidal cells is also observed in trace fear conditioning tasks, but not in delay fear conditioning or in unpaired controls, supporting the role that the mPFC plays in working memory/bridging the CS representation during empty trace intervals (Gilmartin and Helmstetter, 2010). Thus, it is possible that SSR administration enhances working memory through its ability to enhance the firing of Layer II/III pyramidal cells, shown previously to exhibit evidence of reduced excitability in rats exposed to KYNA in utero (Chapter 3).
Alpha 7/NMDAR Interactions

EKYN adults show significant decreases in NR1 and NR2A gene expression, which may explain the reduced cue-dependent freezing. How then might SSR enhance TFC in rats with decreased NMDAR function/alterations in NMDAR composition? Gating mechanisms mediated by α7nAChR control long-term potentiation/depression (LTP/LTD) balance in multiple brain regions that support behavior (Mistushima et al, 2013; Prestori et al, 2013). Specifically, LTD requires the activation of NR2B-containing NMDAR whereas LTP requires the activation of NR2A-containing NMDARs. NR2A-containing NMDARs also mediate de-potentiation (Massey et al., 2004). Furthermore, interneurons in the mPFC contain more NR2A-containing NMDARs than principal cells. Thus, alterations in NMDAR subunit composition may alter the excitability of local recurrent circuits in the mPFC. Alpha7 nAChR forms a protein complex with NMDARs through a direct protein–protein interaction (Li et al., 2013). These protein complexes may underlie learning and memory, as activation of α7nAChR with a specific agonist leads to significant increase in α7nAChR-NR2A interactions and corresponding increases in LTP, while disruption of the protein-complex prevents LTP and impairs novel object recognition and (Li et al., 2012, 2013). Taken together, the data collected in this aim further support that α7 activation may enhance cognition through interaction with NMDA cognitive circuits. Future experiments will more clearly elucidate mechanisms of action and the utility of α7nAChR agonists as early interventions.
Chapter 6: General Discussion

Kynurenic acid (KYNA) is an astrocyte-derived metabolite of tryptophan degradation that is suspected to contribute to the pathophysiology of schizophrenia through its ability to antagonize N-Methyl-D-Aspartate (NMDA) and α7 nicotinic acetylcholine (α7nACh) receptors. We have previously established that the administration of KYNA’s bioprecursor, L-kynurenine, to pregnant Wistar rats during the last week of gestation results in deficits in PFC- and hippocampus-mediated tasks in conjunction with elevated brain KYNA (Pocivavsek, 2014; Pershing et al., 2014). The experiments outlined in this dissertation were directed at expanding our initial investigations in order to elucidate: (1) the interaction of kynurenine pathway changes and adolescent brain maturation on the emergence of cognitive dysfunction; (2) whether prenatal KYNA exposure results in long-term changes in cholinergic/glutamatergic gene expression; and (3) the potential of α7nAChR modulation to improve cognition. Several novel findings can be reported. First, we have confirmed our previous reports that prenatal kynurenine results in elevated KYNA levels in adults, but notably have clarified that this is a distinct post-pubertal emergence. Second, in contrast to brain KYNA levels, age-dependent changes in cued-fear response and NMDAR expression were evidenced in both juveniles and adults. Third, administration of an α7nAChR partial agonist to adults
restores cued-fear response to control levels when administered prior to training, but not when administered prior to testing. The overall relevance of these findings and the utility of this method as a translationally valid method for studying SZ are discussed below.

**Critical Periods of Vulnerability**

Acute elevations of KYNA have been shown by numerous investigators to impair multiple cognitive domains (sensory gating, working memory, cognitive flexibility). This is not surprising given KYNA’s ability to negatively modulate acetylcholine, dopamine, glutamate, and GABA (cf., Introduction). In the current studies, prenatal kynurenine exposure resulted in distinct pre- and post-pubertal changes in behavior and gene expression, while KYNA elevations were noted only in adulthood. Specifically, cued-fear response was increased in juveniles and decreased in adults, corresponding to decreased NR2A expression at PD32 and decreased NR2A, NR1, and dendritic spine density at PD70. Because KYNA elevations were determined to be a post-pubertal occurrence, the alterations in pre-pubertal TFC and NMDAR expression do not likely reflect a simple interaction of elevated KYNA and subsequent reductions in neurotransmission. Instead, these changes likely emerge as a consequence of altered brain development initiated by prenatal elevation in KYNA. The third week of gestation, when dietary manipulation occurred during these experiments, is a critical period of rodent brain development (Semple et al., 2013). Increased levels of forebrain KYNA during this period of rodent development could antagonize activity at both the α7nAChR (nM range; Albuquerque & Schwarcz, 2013; Hilmas et al., 2001) and the glycineB co-
agonist site of the NMDA receptor (μM range; Stone, 1993). The effects, together, may serve to alter excitatory/inhibitory balance in during development by altering neuronal migration, dendritic spine/synapse formation, etc. Prenatal α7nAChR antagonism has been shown to delay the early developmental GABA switch in conjunction with alterations in synaptic contacts and neurite outgrowth in the hippocampus (Liu and Berg, 2006). Similarly to rats administered MLA, preliminary collaborative studies with The Department of Cellular and Molecular Pharmacology, The Chicago Medical School at Rosalind Franklin University, North Chicago, IL, show that KYNA impairs GABAergic tone that regulates hippocampal control of excitatory transmission in prefrontal circuits (Thomases et al., 2014). Namely, α7nAChR tone in the PFC was required for prefrontal GABAergic response to ventral hippocampus stimulation, because infusion of KYNA disrupts LFP processing of afferent drive in a frequency-dependent-manner. This frequency-dependent suppression was mimicked by infusion of MLA but not by the NMDA glycineb site antagonist 7-Chloro-kynurenine, suggesting that the actions of KYNA are attributed to its actions at the α7nAChR. Thus, there may be considerable implications for α7nAChR antagonism during development.

We have previously shown that EKYN rats have significant reductions in mRNA levels of α7nAChR at PD2 (Pershing et al., 2014). There was a continued marginal decrease in adult rats, which was replicated in the current study. These findings may be particularly relevant given the interactions of α7nAChR and NMDAR and subsequent effects on neurotransmitter release and synaptic plasticity. NMDA receptor activity is centrally involved in early phases of brain development, including neuronal migration
(Behar et al., 1999), the formation and guidance of axonal branches (Rajan and Cline, 1998), the induction of dendritic spines (Ultanir et al., 2007), and the formation and maintenance of excitatory synaptic contacts (Colonnese et al., 2005; Sala and Segal, 2014). A characteristic finding in SZ is decreased dendritic spine density in Layer II/III of the PFC. Alpha 7 knock-out mice fail to develop glutamatergic synapses (Lozada et al., 2012); further, NMDAR subunit composition has a dramatic impact on spines. NMDAR subunit composition determines bidirectional synaptic plasticity during development such that reductions in NR2A result in the inappropriate strengthening of non-stimulated synapses and the elimination of which means that appropriate synapses are strengthened/maintained and inappropriate connections are pruned (Philpot and Bear, 2007). Ewald et al. (2008) utilized NR2A and NR2B knock-down methods to determine the role of NMDAR subunits on dendritic arbor formation and found that NR2A and NR2B have distinct, but overlapping, roles in neuronal development. Expression of both subtypes was required for dendritic arbor formation, while NR2A knock-downs exhibited decreased branch clustering. Thus, the observed alterations in NR2A:NR2B during development, possibly in conjunction with slight reductions in α7nAChR, may contribute to altered synaptic plasticity. These changes may be particularly exacerbated by the pubertal/adolescent process in which spines are eliminated. Similar age-dependent changes in synaptic plasticity and NMDAR expression were observed by Stone and colleagues, who reported that offspring born to mothers given a KMO inhibitor during gestation exhibited increased long-term potentiation (LTP) as juveniles and decreased LTP/delayed recovery from LTD as adults (Forrest et al., 2013a, 2013b; Khalil et al.,
These age-dependent alterations in synaptic plasticity occurred in conjunction with alterations in NMDAR subunit composition and decreased dendritic spine density.

Taken together, these studies indicate that manipulation of the kynurenine pathway through various methods results in age-dependent alterations in brain growth and development, behavior, and gene expression that are characteristic of those in the clinical population. Risk genes for schizophrenia have convergent effects on synaptic processes known to be required for associative learning (Hall et al., 2009), which may explain why patients show deficits in a range of cognitive tasks dependent on the formation of novel associations. When tested in a trace fear conditioning paradigm, EKYN rats exhibit significantly higher cued-fear response than ECON as juveniles (PD32), whereas adult EKYNs have lower cued-fear than their age-matched controls. This may relate specifically to the observed changes in NMDAR subunit composition, as EKYNs had significant reductions in NR2A at both PD32 and PD70, while reductions in NR1 were also observed at PD70. These reductions altered the ratios of NR2A:NR2B throughout development.

The Role of Puberty in the Emergence of SZ

Overt symptoms of SZ generally manifest following puberty, and occur earlier in men than in women suggesting a distinct role of pubertal processes or of factors that occur during puberty in SZ emergence. This premise expands initial neurodevelopmental hypotheses of SZ and suggests that the early alterations in CNS development, a consequence of biological and early environmental risk factors, produce long-term
vulnerability to additional insult (e.g., a second hit), which precipitates the onset of frank illness (Maynard et al., 2001). There are several insults that are most relevant for consideration in this second-hit model, including stress and inflammation, which may interact with pubertal changes in gonadal steroids. Factors that contribute to post-pubertal emergence of elevated KYNA levels and differences in pre- and post-pubertal behavior may relate, in part, to hormones. Testicular hormones organize anxiety related behaviors (Sisk, 2005; Zuloaga et al., 2011). For example, social interactions in males are reduced in novel environments, which are presumed as anxiolytic, an effect that emerges during puberty as a function of gonadal hormones. Zuloaga et al. (2011) showed that rats that were castrated as neonates showed increased adult levels of corticosterone and altered PPI, suggesting that testosterone, specifically, acts during development to increase adult-related anxiety and reduce activity. Acute stress has also been shown to result in enhanced trace eyeblink in males, an effect which emerges during puberty; thus, interactions of pubertal changes in glucocorticoids with brain development are certainly worth exploring. SZ patients show larger autonomic responses than controls to conditioned stimuli that are not reinforced (Holt et al., 2009). The failure to differentiate paired and unpaired stimuli and the larger response to CS- are consistent with heightened neural responsivity and elevated arousal. Indeed, SZ patients show hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, as evidenced by increased glucocorticoid levels as well as systemic inflammation and increased production of cytokines.

Stress and inflammation as precipitating factors in the emergence of SZ are supported by epidemiological data and animal models (Corcoran, et al., 2003; Miller et
Immune activation early in development, or exposure to cytokines directly, results in similar cognitive deficits to those described in this study. For instance, exposure to cytokines in utero primes the brain such that a second stressor during adolescence activates developmental vulnerabilities (Howard, 2013). Importantly, early immune activation stimulates KP metabolism and KYNA production (Holtze et al. 2008, Asp et al. 2010), providing a link to long-term immune activation and the kynurenine pathway. Maternal immune activation models, whereby pregnant rodents are administered poly I:C, also show post-pubertal emergence of cognitive deficits, reduced PPI, anxiety, and increased amphetamine-response (Nilsson et al., 2005; Ozawa et al., 2006). Peripubertal stress has been shown to exacerbate behavioral and neurochemical dysfunction induced by prenatal immune activation, further supporting the interaction of prenatal and postnatal events to the subsequent emergence of SZ (Giovanoli et al., 2012). An early priming of immune dysbalance (immune sensitization) has been proposed to account for the adult emergence of SZ by Müller and colleagues (2014).

The global increases in KYNA may be reflective of changes in upstream enzymes (IDO/TDO and/or KMO). SZ patients show genetic alterations in TDO/IDO and KMO, all of which are strongly influenced by immune activation or stress (Schwarcz et al., 2012; Müller, 2014), suggesting perhaps life-long changes in KP. This interpretation is supported in the current animal model by preliminary data showing decreased KMO expression EKYN rats (Pocivavsek et al., 2013). Reduced KMO would direct production of kynurenine to KYNA rather than quinolinic acid, which is consistent with postmortem brain tissue in the clinical population as well (Miller et al., 2004; Sathyasaikumar et al.,
IDO/TDO have not yet been evaluated in the current model, but changes in these enzymes may also contribute to elevated brain KYNA. TDO, which is one of the enzymes that converts tryptophan to kynurenine, is induced by tryptophan, glucocorticoids (including corticosteroids), and stress (Gibney et al., 2014; Mackay et al., 2006; Ruddick et al., 2006). There is also evidence, from the clinical population, that SZ individuals interpret stress more acutely than control subjects (Myin-Germeys et al., 2002; Collip et al., 2013). Perhaps, the process of puberty in and of itself, results in atypical production of glucocorticoids, which induce TDO activity and shunt kynurenine production to KYNA. This interpretation is particularly intriguing since kynurenine, but not tryptophan, is elevated in the clinical population.

**Cognitive Enhancement with SSR180711**

Administration of SSR prior to training, but not testing, restored cue-dependent freezing in adult EKYNs to control levels. This suggests that the deficits observed in adults are mediated by their ability to associate CS with US on Day 1 (attention/working memory) or ability to encode/consolidate these associations rather than an ability to recall these associations on Day 3. Decreased cued-fear response in adults also occurred in conjunction with decreased dendritic spine density; therefore, SSR may strengthen and weaken distinct synapses as a function of learning, due to α7nAChRs role in stabilizing glutamatergic synapses (Lozada et al., 2012; Yang et al., 2013). There are several mechanisms through which nicotinic and glutamatergic synapses converge. However,
administration of SSR180711 was systemic; therefore, claims to mechanism of action are speculative and require follow-up.

Aside from mechanism of action, it is intriguing that a single dose of SSR is sufficient to restore cue-dependent freezing in a neurodevelopmental model. SZ neuropathology is initiated early in life, and likely reflects failures of early cell development and migration processes that lead to macro- and micro-circuit dysfunction across a distributed neural system. The emergence of frank symptoms (psychosis) reflects these early developmental changes and post-pubertal maturation changes. Pharmacotherapy based on single receptors, or single doses, likely will not be effective in the clinical population. This may explain the lack of efficacy in clinical trials.

**Limitations and Future Directions**

These experiments were designed as initial investigations to the emergence of cognitive deficits and underlying cellular/molecular mechanisms of those deficits. Future experiments can be designed to further elucidate these interactions; indeed there are several additional lines of research that can be pursued based on the differential age-dependent effects in this study alone. One particular important question is the neural circuit underlying TFC in the current paradigm. The addition of the trace interval, presumably, recruits additional brain structures such as the mPFC and the hippocampus. However, the dependence of the task on the hippocampus relies on both the trace interval length and the shock intensity. We selected a relatively high shock intensity (1 mA) because, as mentioned in Chapter 3, preliminary experiments in intact rats showed that a
higher shock intensity was required for juveniles and adults to freeze similarly; a requirement in these experiments to elucidate effects of prenatal manipulations versus age effects. Given the premise that SZ patients (and perhaps KYNA-exposed rats) may interpret stressors differently than controls, it would be of merit to conduct experiments at different shock intensities and/or different trace intervals to see if the magnitude of cued-fear deficit changes. Further, an examination of extinction in fear conditioning can be assessed. Extinction is the gradual decrease in conditioned response when a conditioned stimulus loses salience. For instance, percentage freezing to an auditory tone should decrease over time when the tone is presented in the absence of reinforcement. Extinction is a form of new learning, dependent upon BLA-infralimbic cortex interactions, and results in dendritic spine changes that are distinct from those observed in TFC acquisition (Lai, Franke, and Gan, 2012). SZ patients show impairments in context-dependent extinction, particularly when fear extinction memories are recalled following a delay (Holt et al., 2012). Thus, future experiments can examine extinction, but can also examine dendritic spine changes in performing animals providing a cellular substrate of learning in performing animals. In that regard, future experiments are planned to elucidate changes in dendritic spines over the course of development. The experiments described herein did not include an examination of dendritic spines at PD32, nor were there examinations of spine morphology. Given the link between immune activation, stress and the emergence of cognitive dysfunction, pro-inflammatory cytokines may also be examined in order to elucidate factors that result in post-adolescent KP changes. Finally, investigations thus far have evaluated the expression of mRNA levels of
α7nAChR and NMDAR. While informative, subsequent experiments with additional methods (autoradiography; Western blots) will be necessary to evaluate protein levels in order to fully appreciate changes in cholinergic/glutamatergic receptor expression.

Conclusions

The experiments described herein further demonstrate the utility of early kynurenine exposure as a translationally valid method to assess pathophysiology of schizophrenia. These experiments expand the knowledge base regarding cellular/molecular changes in the prodromal period, providing an important link to the KP, sensitive periods of CNS development, and the emergence of cognitive deficits.
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Appendix A

Pilot Fear Conditioning Data in Intact Rats
Figure 20. Pilot Trace Fear Conditioning Data in Intact Rats. Juvenile and adult intact, Wistar rats were assessed for contextual and cued-fear response following trace fear conditioning at three different shock intensities (ten pairings of a 15-second CS, 10-second trace; 240 ± 30 second ITI). The graphs are plots of contextual fear against cued fear. First, juveniles require a higher shock intensity to show subsequent freezing levels that are comparable to adults. That is, juveniles trained at 0.6 and 0.8 mA exhibited low cue-dependent freezing (less than 40%). At a shock intensity of 1 mA, both juveniles and adults exhibited greater than 40% freezing to the tone. Secondly, increased shock intensity generally resulted in cue-dependent freezing over contextual freezing, while lower shock intensity resulted in greater contextual freezing.
Appendix B

Food Consumption Data
### Embryonic Day

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Table 6. Food Consumption during Gestation. The amount fed each day was based upon the consumption the previous day in order to ensure that EKYN rats consumed all kynurenine.