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**Integrated Biobehavioral Analysis of Sex Differences in a Rat Model of
Schizophrenia**

A Dissertation Presented

by

Igor Feinstein

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Neuroscience

Stony Brook University

December 2011

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Abstract of the Dissertation

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Schizophrenia is a mental illness affecting roughly 1% of the population that is characterized by both positive/affective and negative/cognitive symptoms and is associated with abnormalities in multiple neurotransmitter systems and brain areas. Recently, reduced function of the N-methyl-D-aspartate receptor (NMDAR) has emerged as an etiologic hypothesis with explanatory power for this spectrum of disturbances. However, it is unknown how NMDAR hypofunction relates to the striking sex differences that are also seen in the incidence and severity of schizophrenia's positive (female > male) vs. negative (male > female) signs, in its associated neurochemical imbalances and in the efficacies of drugs commonly used to treat patients. To approach these questions, my dissertation explored whether a rodent model of NMDAR hypofunction recapitulates sex differences in behavior, drug response and neurochemistry that mirror those in schizophrenia. The first studies probed behavioral sequelae of NMDAR hypofunction in adult male and proestrus female rats and found that females had increased startle responses, locomotor activity, ataxia, and emitted more spontaneous vocalizations indicative of negative affect which are positive symptom correlates, while males had greater deficiencies in prepulse inhibition, rearing and grooming which are models for schizophrenia's negative signs. Further, the typical neuroleptic haloperidol was more effective in attenuating positive but not negative symptom correlates and was more effective in females while the atypical neuroleptic clozapine attenuated both positive and negative symptom correlates in both sexes but was slightly more effective on negative symptom correlates in males. The second set of studies used microdialysis and magnetic resonance spectroscopy (MRS) to explore

whether NMDAR antagonism produces sex-specific changes in amine and amino acid neurotransmitter levels in the prefrontal cortex *in-vivo*. Both studies had their caveats; locomotor activity confounded quantification of drug effects via microdialysis and while MRS showed promise in quantifying levels of both classes of neurotransmitters, paroxysmal apnea induced by NMDA antagonism also prevented accurate quantification. Though further investigation is required for neurochemistry, my findings suggest that NMDAR hypofunction model does recapitulate certain sex differences seen in schizophrenia and is a promising model for examining their biological bases and developing novel, effective treatments for overcoming this devastating disorder.

Dedication

The last several years as a student in the Graduate Program in Neuroscience have taken me on a journey unlike any other. In these few short years, I have not only undergone tremendous professional growth by learning the core fundamentals of being a scientist but also underwent enormous maturation as an individual, learning how to objectively think, evaluate ideas and solve problems. Together, the experiences of my tenure as a graduate student have empowered me to go out into the world not only as a promising young scientist but as a better person. I would like to acknowledge those people whose support and encouragement throughout the course of this endeavor made it all possible.

First, I would like to genuinely thank my advisor, Dr. Mary Kritzer. I don't believe that there are sufficient words to express the true level of my gratitude for her mentorship, encouragement, care, friendship and support from the first day of my dissertation work until its successful completion. I truly could not have asked for more from a mentor and genuinely hope to continue a long-lasting personal and professional relationship with her. I would also like to thank all members of the Kritzer lab, past and present, for their assistance and support throughout the long days and nights that went into this work. Further, I'd like to thank my thesis committee, Drs. Craig Evinger, Peter Thanos and John Krystal for their time and valuable comments and critiques of the work presented in this dissertation. Also, I would like to acknowledge Dr. Michael Frohman and Carron Kaufman in the Medical Scientist Training Program for their help and assistance.

Finally, I want to thank my loved ones for their support and encouragement. First, the constant support of my father Dima and the rest of my family- Ira, Zhenya and Sam- and their steadfast belief that I could accomplish anything I set out to do kept me going through each and every day. Also, to my closest friends, Michael, Vlad and Jane: your friendship and support has helped me get through some extremely tough times and I truly could not have asked for better friends than you. Lastly, I want to thank my beautiful and loving fiancée Lana. I would have never thought that I would be so fortunate as to meet someone who would become my most trusted confidant and my best friend. Her unwavering support of me through each and every day as well as her amazing ability to put a smile on my face no matter how difficult my day was is what kept me going at times when I otherwise wanted to give up. For this, it is to her that I dedicate this dissertation. Thank you from the bottom of my heart.

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GENERAL INTRODUCTION

Out of all neurobiological disorders, schizophrenia stands out as perhaps the most striking manifestation of brain dysfunction and its consequences. This mental illness, which predominantly starts in young adulthood, fundamentally disrupts perception, thought and cognition and as a result, imparts severe lifetime disability upon those affected. Sufferers of schizophrenia typically have significant difficulties integrating with and functioning in society and as a result, social problems such as unemployment, poverty and homelessness are endemic (Cohen, 1993; Folsom et al., 2005). Further, those with schizophrenia are often afflicted with other related comorbidities such as depression, anxiety and drug abuse (Goodwin et al., 2002; Westermeyer, 2006; Buckley et al., 2009). In fact, the average life expectancy of schizophrenia sufferers is approximately 15 years lower than in healthy counterparts; one major reason for this is a markedly increased rate of suicide in schizophrenics (Palmer et al., 2005; Saha et al., 2007). It thus becomes evident that the human suffering associated with this devastating disorder is immense and considering that the overall global prevalence of schizophrenia is approximately 1%, it is clear that this disease represents a significant public health issue (Jablensky, 1997). In fact, schizophrenia has been found to be the third most disabling medical condition after quadriplegia and dementia (Ustun et al., 1999).

Schizophrenia manifests with a broad range of symptoms; these can essentially be classified into several primary categories. Positive symptoms, by definition, are those that are brought on by the disease and are the most well known hallmarks of

schizophrenia. They include hallucinations, delusions, disorganized speech and bizarre behavior (Andreasen and Flaum, 1991; Arndt et al., 1991; Andreasen, 1995). Negative symptoms, on the other hand, are characterized by the absence of normal behaviors in the disease state. These include anhedonia, blunted affect, social withdrawal, alogia and avolition (Andreasen, 1982; Andreasen et al., 1995; Kirkpatrick et al., 2006) Further, in addition to these two main symptom categories, schizophrenia is further characterized by significant cognitive deficits which are a core component of the disease. These include behavioral inflexibility, memory impairment and defects in thought processes, attention and cognitive speed (Nuechterlein et al., 2004; Keefe, 2008) Interestingly, while positive symptoms are perhaps the most well-known, it is the negative and cognitive symptoms that are actually the most enduring and debilitating; in fact, their severity has been positively correlated to poor long term outcome (Green, 1996). Finally, schizophrenia is characterized by many affective disturbances including depression, anxiety, irritability and impulsive behavior (Escamilla, 2001; Buckley et al., 2009).

Schizophrenia is Sexually Dimorphic

Of keen interest to the questions posed in the dissertation is the well-established finding that striking sex differences are evident in nearly every aspect of schizophrenia. For example, epidemiological data has repeatedly shown that males are at higher risk for developing schizophrenia; the male:female prevalence of this disorder has been reported to be between 1.5:1 and 2:1 (McGrath et al., 2008). Men also develop

schizophrenia an average of 5 years earlier than women (Lewine, 1980; Lewine, 1981; Loranger, 1984; Faraone et al., 1994; Hafner et al., 1994) and males have been reported to have more frequent hospitalizations and an overall lower quality of life and poorer long-term outcomes than female patients (Angermeyer et al., 1989; Angermeyer et al., 1990). Of particular relevance, however, are the well-described sex differences in symptomatology and in response to pharmacologic treatment. Thus, female schizophrenia patients tend to have more pronounced positive and affective symptoms such as bizarre behavior, hostility and depression while males are predominantly afflicted with negative symptoms and cognitive deficits such as social withdrawal, blunted affect and deficits in cognitive performance (Goldstein, 1988; Goldstein and Link, 1988; McGlashan and Bardenstein, 1990; Seeman and Lang, 1990; Ring et al., 1991; Szymanski et al., 1995). Women are also much more responsive to treatment with neuroleptic drugs, showing a more rapid and a greater degree of amelioration of their symptoms upon starting neuroleptic pharmacotherapy (Hogarty et al., 1974; Seeman, 1986; Angermeyer et al., 1990; Andia et al., 1995). Men, on the other hand, are more refractory to treatment and despite requiring larger doses of medication, lag behind females in achieving symptom remission (Szymanski et al., 1995). While the underlying cause of these sexual dimorphisms is still unknown, a significant body of evidence implicates gonadal hormones in these findings. For example, epidemiological findings have shown that in women, the symptoms of schizophrenia wane in pregnancy when estrogen levels are high (Chang and Renshaw, 1986; Trixler et al., 1995) and that there is a spike of new-onset schizophrenia in women entering menopause (Hafner et al., 1998; Leung and Chue, 2000). In male patients, studies have identified

abnormalities in the levels of testicular hormones; specifically, decreased testosterone levels have repeatedly been positively correlated with increased severity of negative symptoms (Shirayama et al., 2002; Goyal et al., 2004; Huber et al., 2005; Ko et al., 2007). These findings have spurred interest in the use of sex steroids as adjunctive treatments of schizophrenia and some preliminary studies have reported encouraging results (Kulkarni et al., 2002; Ko et al., 2008; Kulkarni, 2009). Further, preclinical studies have indicated that in male rats, normal levels of testosterone are necessary for proper performance on cognitive tasks and that their removal severely disrupts cognitive performance (Kritzer et al., 2001; Kritzer et al., 2007; Aubele et al., 2008). However, despite this body of evidence, the biological bases of sexual dimorphisms in schizophrenia remain unknown, in a large part due to the lack of an animal model that reliably and accurately recapitulates these sex differences in symptomatology.

While several etiological hypotheses of schizophrenia and resultant animal models exist, recent developments have led to the well-accepted postulation that hypofunction of the glutamate N-methyl-D-aspartate (NMDA) receptor as the primary defect underlying schizophrenia (Olney et al., 1999). However, while this model has proven face, predictive, and construct validities in recapitulating the full schizophrenic phenotype in animals and humans alike, little is known about whether this also extends to the sex differences that are ubiquitous in schizophrenia. Thus, the experiments in this dissertation first sought to investigate whether NMDAR hypofunction produces sex-specific changes in rodent behaviors known to be correlated to positive, negative and affective symptoms of schizophrenia, whether they mirror the sex differences seen in

organic disease and whether pretreatment with two classes of widely used neuroleptic drugs can attenuate these symptom correlates in sex-specific ways (Chapters I-III). Further, since all behavior is driven by underlying mechanisms, the second set of experiments sought to examine whether NMDAR hypofunction produces sex-specific changes in the neurochemical milieu in the prefrontal cortex, a brain region closely linked to schizophrenia (Chapter IV). Lastly, cognizant of the fact that current techniques used for assaying *in-vivo* neurochemistry in the preclinical arena can never be applied to humans, the final experiments in this dissertation sought to extend magnetic resonance spectroscopy, a non-invasive imaging paradigm to the measurement of dopaminergic neurochemistry and to characterizing the neurochemical sequelae of NMDAR antagonism (Chapter V).

In the sections below, the complex nature of schizophrenia and its etiologic theories are first introduced. With a focus on human disease and its therapeutics, this is followed by a discussion of the evidence leading to the development of the NMDAR hypofunction model as a widely accepted and leading framework for investigating schizophrenia. Then, the goals of this dissertation will be further discussed and finally, a rationale for the progression of the studies contained in this work will be presented.

Current Limits of Schizophrenia Treatments Illustrate a Need for Innovation

The complex nature and likely multifactorial etiology of schizophrenia (discussed below) make the effective treatment of this disorder a serious challenge to this day. In

fact, while schizophrenia was first described over one hundred years ago, treatment options remained severely limited and largely ineffective until the 1950s when the serendipitous discovery of chlorpromazine revolutionized treatment of this disorder. This drug markedly improved severe symptoms in many sufferers of schizophrenia and heralded a new era of pharmacologic treatment of this disorder (Lopez-Munoz et al., 2005). Chlorpromazine and the related early antipsychotic drugs (subsequently classified as typical antipsychotics) were found to exhibit their effects via strong antagonism at the dopamine D₂ receptor; this pharmacological action was subsequently shown to be sufficient for amelioration of psychosis (Creese et al., 1976). However, due to their strong antidopaminergic effects, extrapyramidal side effects such as rigidity and akathisia are common with these medications and often require their discontinuance (Llorca et al., 2002). Furthermore, these drugs are mostly seen to be effective in ameliorating only positive symptoms of schizophrenia (Barnes and McPhillips, 1995; King, 1998). These typical antipsychotics were a mainstay of therapy until several decades later, the first “atypical” antipsychotic, clozapine was introduced. This drug was found to be effective in ameliorating both positive and negative symptoms of schizophrenia despite having very low affinity for the D₂ receptor unlike any other antipsychotic (Seeman et al., 1997). Its pharmacodynamic profile is complex and involves activity at a wide range of receptors in the brain including D₁, D₂, serotonergic, muscarinic, and adrenergic receptors (Lieberman, 1993; Gerlach and Peacock, 1995). Similar drugs followed, all exhibiting slightly differing pharmacology and subsequent evidence showed them to be at least equally effective as typical antipsychotics at ameliorating symptoms of schizophrenia and having a much improved side effect profile

(Leucht et al., 2009; Salimi et al., 2009). Thus, these two classes of drugs comprise the mainstay of pharmacotherapy in schizophrenia. However, these treatments are far from ideal as even atypical antipsychotics have serious side effects and many patients are refractory to treatment even with the most potent drugs. Moreover, the sex differences in schizophrenia and the strong evidence for involvement of gonadal hormones in this disorder provide strong impetus for investigating sex-specific treatments. Interestingly, while support for sex-specific therapeutics of schizophrenia has been recently gaining traction in clinical circles, there is a noticeable lack of empirical data from preclinical animal models that bolsters these suggestions. To that end, the questions and studies of this dissertation are framed with the ultimate goal of human therapeutics in mind and thus inquire not only into whether NMDAR hypofunction recapitulates salient sex differences in schizophrenia but also into whether currently used antipsychotic treatment regimens act differently in males and females in reversing deficits induced by NMDAR hypofunction.

Etiologic Theories and Biological Underpinnings of Schizophrenia

The etiology of schizophrenia remains unclear and is most likely multifactorial. A role for a genetic component is strongly implicated by epidemiological observations such as that monozygotic twins of schizophrenia sufferers have a 40% lifetime risk of being afflicted with the disorder themselves and that the lifetime risk of first-degree relatives in developing schizophrenia is increased to over 600% relative to the general population (Heston, 1970; Picchioni and Murray, 2007). Further studies have implicated

dozens of discrete genes in the pathogenesis of schizophrenia which are involved in a multitude of systems including dopaminergic, glutamatergic and GABAergic signaling, cellular signal transduction and immune response modulation (Lang et al., 2007). However, a lone defect in any of these putative genetic targets is neither necessary nor sufficient to cause schizophrenia, implying that genetics plays an incomplete etiologic role. Furthermore, there is evidence for a developmental etiology as well; for example, retrospective studies have established a positive correlation between perinatal complications such as hypoxia or infection and an increased risk of developing schizophrenia later in life (Dean and Murray, 2005).

Just as the underlying insult that triggers schizophrenia is most likely due to a confluence of factors, the underlying neurobiological aberrances in schizophrenia are similarly diverse with structural and functional alterations involving numerous brain structures and neurotransmitter systems. Firstly, anatomical studies, (both imaging and those conducted post-mortem) have demonstrated significant reductions in overall gray matter volume in schizophrenics compared to healthy controls (Fornito et al., 2009). Further, numerous structure-specific abnormalities have been reported; these involve a number of cortical and subcortical structures including the amygdala, hippocampus, prefrontal cortex, basal ganglia and striatum (Shenton et al., 2001; Antonova et al., 2004). While this large breadth of structural abnormalities in schizophrenia implicates widespread underlying pathology, anatomical studies have proven useful in establishing links between symptoms and specific brain areas. Specifically, subcortical structures such as the striatum and nucleus accumbens have been linked to positive symptoms;

studies have shown that size and metabolism in this structure are positively correlated to the intensity of positive symptoms (Epstein et al., 1999; Crespo-Facorro et al., 2007). Furthermore, the prefrontal cortex (PFC) has been repeatedly linked to negative and cognitive symptoms with schizophrenia patients having smaller frontal lobe volume and decreased metabolic activity in this structure with both measures being negatively correlated to negative symptom severity (Weinberger and Berman, 1988; Andreasen et al., 1994; Sanfilippo et al., 2000).

Pathophysiology of Schizophrenia: Initial Focus on Dopamine

The first theory that unified the diverse abnormalities in schizophrenia into a cohesive framework was the dopamine hypothesis of schizophrenia. This theory, which stemmed from evidence indicating that many early drugs that were effective in ameliorating psychotic symptoms had significant pharmacological activity as dopamine receptor antagonists, initially posited that overactivity of dopaminergic signaling accounts for the abnormalities seen in schizophrenia (van Rossum, 1966; Matthysse, 1973). This hypothesis was first supported by studies in human subjects showing that low doses of drugs that increase dopamine levels (e.g. amphetamine, cocaine) markedly exacerbate psychosis in schizophrenics while having little effect on healthy controls (for reviews, see Lieberman et al., 1987; Curran et al., 2004). Further, *in-vitro* assays established a positive correlation between the affinity of typical neuroleptic drugs for the dopamine D₂ receptor and their documented clinical potency (Creese et al., 1976; Seeman et al., 1976). However, this hypothesis has several notable drawbacks.

Firstly, it was observed that while neuroleptic drugs with D₂ antagonist activity are effective at ameliorating positive/psychotic symptoms, negative and cognitive symptoms persist despite treatment (Barnes and McPhillips, 1995; King, 1998). Furthermore, it was seen that while high-dose amphetamine challenge in healthy humans would produce a marked psychosis that was very similar to the positive symptoms of schizophrenia, it would not generally produce negative symptoms or cognitive impairments (Crow, 1980). Subsequent studies revealed that frontal lobe dysfunction is inexorably linked to these refractory negative and cognitive symptoms; functional imaging demonstrated decreased blood flow in the frontal cortex of schizophrenia patients compared to controls while engaging in the Wisconsin Card Sorting Test (WCST), a sensitive measure of frontal function (Weinberger et al., 1986). Dopamine was soon implicated in these findings as well; studies employing positron emission tomography (PET) revealed that schizophrenics (both medicated and drug-naïve) have lower levels of D₁ receptors than healthy controls and that the density of D₁ receptors is positively correlated to performance of the WCST (Okubo et al., 1997). Further, electrophysiological data from primates has shown that D₁ receptors modulate neurotransmission in neurons directly involved in cognitive function and that the modulation is facilitated when D₁ receptor occupancy is moderate and it was observed that this facilitation is impaired when D₁ occupancy is either low or high (for review, see Goldman-Rakic et al., 2000). From these observations, it was postulated that a relative deficit of dopaminergic activity in the prefrontal cortex is responsible for the negative symptoms of schizophrenia and the dopamine hypothesis was reworked to postulate that a overactivity of the subcortical mesolimbic dopamine system results in

positive/psychotic symptoms and hypoactivity of the mesocortical dopamine system results in prefrontal hypodopaminergia and resultant negative symptoms.

Focus Shifts onto Glutamate

At the same time that the dopamine hypothesis was being refined, focus was rapidly shifting onto the excitatory neurotransmitter glutamate. This paradigm shift was borne out of early anecdotal observations that about 10-30% of patients undergoing anesthesia with phencyclidine (PCP) would develop transient psychoses (Johnstone et al., 1959). These observations were followed by human studies which remarkably revealed that subanesthetic doses of PCP administered to healthy subjects reliably produced psychomimetic symptoms highly reminiscent of schizophrenia (Luby et al., 1959; Davies and Beech, 1960). As PCP was found to exert its pharmacologic effects by binding to and antagonizing the glutamate N-methyl-D-aspartate (NMDA) receptor, it was posited that glutamatergic dysfunction may play a role in schizophrenia. As PCP was found to be acutely neurotoxic, contemporary studies shifted to using ketamine (another NMDAR antagonist) and substantially extended these early findings; sensitive neuropsychological testing revealed that ketamine not only causes psychotic symptoms but also the avolition, social withdrawal, and marked cognitive deficits reminiscent of organic disease in healthy controls (Javitt and Zukin, 1991; Krystal et al., 1994; Lahti et al., 1995; Krystal et al., 1998) and exacerbates both psychotic and negative/cognitive symptoms when administered to stable schizophrenics (Malhotra et al., 1997). Interestingly, direct comparison of the administration of ketamine and amphetamine to

healthy subjects by Krystal and colleagues found that while both agents produced positive symptoms, only ketamine produced negative symptoms and cognitive deficits, indicating that glutamatergic dysfunction is a key component in the pathophysiology of negative/cognitive symptoms (Krystal et al., 2005). Combined with findings in rodents that NMDAR antagonists induce changes in other neurochemical systems that have been implicated in schizophrenia such as GABA and dopamine (Yonezawa et al., 1998; Lorrain et al., 2003; Stone et al., 2007), these studies gave rise to the contemporary NMDAR hypofunction hypothesis. Cognizant of findings that the effects of NMDAR hypofunction are markedly more pronounced in GABAergic interneurons than any other cell type (Grunze et al., 1996), this theory posits that the primary defect of schizophrenia is an aberrant decrease in NMDA reception that results in disinhibition of the GABAergic neurons that receive modulatory glutamate input via NMDA receptors. In turn, this results in excess glutamate release and overstimulation of postsynaptic neurons via other glutamate receptors and downstream dysregulation of other systems including dopamine, acetylcholine and serotonin which together produce the spectrum of abnormalities that are pathognomic for this disease. This hypothesis has the benefit that it is not mutually exclusive with any other etiologic hypothesis and dovetails the findings of dysregulation in varied circuits and systems into a cohesive theoretical framework. Finally, the validity of the NMDAR hypofunction hypothesis is further bolstered by the results of a recent Phase II clinical trial where a novel drug that solely modulates glutamatergic function by reducing the release of glutamate was found to be as effective as traditional antipsychotic drugs at ameliorating positive, negative, and cognitive symptoms of schizophrenia (Patil et al., 2007).

Etiologic Theories of Schizophrenia Drive the Creation of Animal Models

As animal models are crucial for not only studying the biological underpinnings of disease but also for development of novel therapeutics, the diverse neurobiological abnormalities known to exist schizophrenia have spurred the creation of no fewer than 50 animal models of this disorder (Marcotte et al., 2001; Carpenter and Koenig, 2008). However, out of all of these models, only a few stand out as capable of simultaneously modeling multiple parts of the full phenotype of organic disease. One of the first animal models that fit these criteria was the amphetamine model of psychosis which was borne out of the human evidence discussed above. Thus, when amphetamine is acutely administered to animals (i.e. rats), it causes a marked motor syndrome consisting of hyperlocomotion, ataxia and stereotyped movement; these are reminiscent of the bizarre behaviors that are a prominent component of the positive symptoms of schizophrenia (Creese and Iversen, 1974; Bardo et al., 1990a). Interestingly, these amphetamine-induced behaviors were found to be reversible by pretreatment with clinically used neuroleptics such as haloperidol (Jackson et al., 1994; Samaha et al., 2008). However, while these findings showed that the acute amphetamine administration in rats had good validity for modeling the positive symptoms of schizophrenia and their responsiveness to neuroleptic treatment, it was found that acute amphetamine is not very effective at inducing negative symptom correlates such as social withdrawal and avolition (Ellenbroek and Cools, 2000). Thus, while this model

effectively models a full range of positive symptoms, it is of limited use in studying the full phenotype of schizophrenia.

Two other animal models, however, are notable in that they induce not only positive, but also the negative and cognitive symptom correlates found in schizophrenia. The first is the neurodevelopmental neonatal ventral hippocampal lesion (NVHL) model. In this model, lesions of the ventral hippocampus early in life trigger the gradual emergence of abnormal behavioral, molecular and physiological changes relevant to schizophrenia. Notably, these lesioned animals exhibit the full phenotype of symptoms relevant to schizophrenia; in addition to locomotor hyperactivity and stereotypic behavior, they exhibit deficits in social interaction, inappropriate aggressive behavior and poor performance on sensitive cognitive tasks (Tseng et al., 2009). However, while this model is advantageous in that it recapitulates the full schizophrenic symptomatology and underlying neurochemical abnormalities and takes into account a developmental component in the etiology of this disorder, it has drawbacks in that the invasive lesioning process and resultant trauma and inflammation may serve as a confound. Thus, for this reason, the studies in this dissertation made use of the one remaining animal model capable of simultaneously recapitulating many of the diverse abnormalities found in schizophrenia: the acute NMDAR hypofunction model. In this pharmacological construct, acute administration of subanesthetic doses of NMDA receptor antagonists such as PCP, MK801 or ketamine results in psychomotor agitation similar to that seen after amphetamine but also induces social withdrawal, avolition, and cognitive impairments (Corbett et al., 1995; Jentsch et al., 1997; Rung et al., 2005).

These behavioral changes in animals closely mirror the effects of NMDAR antagonism in humans which have in turn been linked to the full symptom spectrum seen in schizophrenia, conferring validity of this model in studying this disorder. Further, NMDAR hypofunction in rodents causes profound changes in dopamine, glutamate, and GABA levels in various brain areas, all neurochemical systems at risk in schizophrenia. Thus, while this model has a drawback in that it does not account for the indolent and likely multifactorial onset of disease, the strong resemblance of the behavioral and neurochemical sequelae of NMDAR antagonism to those known to exist in schizophrenia provided the impetus for its selection for the studies in this body of work.

Substrates Affected by NMDA Receptor Hypofunction are Sensitive to Gonadal Hormones

Despite overwhelming evidence that NMDAR hypofunction holds great validity in investigating the biological bases of schizophrenia, little is known about whether NMDA receptor antagonism and resultant hypofunction affects males and females differently. Although this question has not been extensively addressed to date in either the human or preclinical arena, there is a considerable body of evidence suggesting that this may be the case. Thus, the main question of this dissertation which asks whether NMDAR hypofunction recapitulates the sex differences known to exist in schizophrenia's symptomatology was formulated by keeping in mind an extensive body of work which has established that many of the neural substrates affected by NMDAR hypofunction are sensitive to the actions of gonadal hormones. First, estrogen is known to exert

effects on NMDA receptors themselves. Specifically, estrogen directly modulates NMDA receptor expression in a region-specific manner in female rats and non-human primates; it has been shown that ovariectomy decreases NMDA receptor binding density in the hippocampus and that treatment with estrogen rapidly reverses this decrease (Cyr et al., 2000). This effect is reversed in the prefrontal cortex; ovariectomy marginally increases NMDA receptor density and treatment with estrogen reduces NMDA receptor density to levels below hormonally intact controls (Cyr et al., 2000). Furthermore, while these effects of estrogen in the hippocampus are limited to the NMDA receptor (Weiland, 1992), estrogen's action in the prefrontal cortex reduces both NMDA and AMPA receptor density (Cyr et al., 2001). Even more interestingly, these effects of estrogen on NMDA receptor binding and expression are directly linked to profound morphological, structural and physiological changes. Specifically, it has been shown that in the hippocampus, ovariectomy decreases dendritic spine density of pyramidal cells and that treatment with estrogen reverses this effect (Woolley and McEwen, 1994; Leranth et al., 2002). Fluctuations in spine density have also been found to occur throughout the estrus cycle with spine density being highest in proestrus when estrogen is highest (Woolley and McEwen, 1992). However, treatment with NMDAR antagonists blocks estrogen-induced spine formation, indicating that signaling via NMDA receptors is necessary for these effects (Woolley and McEwen, 1994). As hippocampal spine density has been linked to learning and memory (Moser et al., 1994; Trommald et al., 1996), this suggests that estrogen's effects on NMDA receptors may at least partially influence cognitive performance. Thus, when the NMDAR hypofunction hypothesis of schizophrenia is considered in light of these profound effects of estrogen

on NMDAR neurotransmission, it is well within the realm of possibility that reduced function of NMDA receptors has different effects in males and females which in turn establishes the importance of questions asked in this dissertation.

In addition to hormone effects directly on NMDA receptors, there is a large body of evidence indicating that dopamine systems are also sensitive to gonadal hormones. First, dopamine release from mesolimbic DAergic cells terminating in the nucleus accumbens in female rats has been shown to fluctuate across the estrus cycle and to be highest in proestrus when estrogen levels are highest (Becker, 1999). Furthermore, the mesocortical system is also sensitive to estrogen; ovariectomy has been shown to increase DA axon density in the prefrontal cortex and concurrently impair cognitive performance (Kritzer and Kohama, 1999; Frye and Walf, 2008). Likewise, in males, gonadectomy and the resultant decrease in testosterone increases DA axon density and extracellular DA in the PFC and impairs performance on a number of sensitive cognitive tasks (Kritzer, 2000; Aubele et al., 2008; Aubele and Kritzer, 2011b; Aubele and Kritzer, 2011a). Thus, as NMDAR hypofunction is known to dysregulate DA neurotransmission (Lorrain et al., 2003), it is possible that sex differences in this system may also result in sexually dimorphic sequelae of NMDA receptor hypofunction.

Studies of Sex Differences in NMDAR Hypofunction are Surprisingly Scarce

Although a large body of evidence suggests that NMDAR hypofunction has face, construct and predictive validity in modeling schizophrenia and that many substrates

affected by NMDAR hypofunction are sensitive to sex hormones, the question of sex differences in the behavioral and neurochemical sequelae of NMDAR hypofunction has not been extensively studied. Specifically, the vast majority of studies that utilize the NMDAR hypofunction model to investigate underlying mechanisms of disease or to evaluate novel treatment regimens include only male animals in their subject cohorts. While this is most likely stems from a desire to eliminate potential effects of fluctuating hormones/estrus cycle that exists in females, this approach has resulted in the fact that effects on females have simply not been studied; the current thinking implies that findings in males and simply be extended to apply to females as well. However, as it is known that sex differences are apparent in the metabolism of drugs in both rats and humans (Schenkman et al., 1967; Harris et al., 1995), it is imprudent to assume that the two sexes can be equated. Thus, to address these issues, the experiments in this dissertation studied both male and female rats under identical treatment conditions to probe for sex differences that can be masked by comparisons across different studies and protocols.

Questions Addressed in this Dissertation

As outlined above, the first goal of this dissertation was to rigorously evaluate the rodent NMDAR hypofunction model to determine whether it accurately recapitulates sex differences in behavior relevant to schizophrenia and its treatment. To that end, the first chapters of this dissertation made use of several behavioral tasks to investigate this question. The studies in Chapter I and II evaluated prepulse inhibition (PPI) of the

acoustic startle reflex (ASR) and a wide range of open field behaviors in adult male and proestrus female rats. As these behaviors have been linked to the positive and negative symptoms of schizophrenia, these paradigms stood out as a reasonable starting point for investigation of sex differences in behavior under NMDAR hypofunction. In these studies, NMDAR hypofunction induced higher startle responses and more locomotion and ataxia, all of which are positive symptom correlates in females over males while causing larger deficiencies in prepulse inhibition, rearing and grooming, all correlates of the negative symptoms of schizophrenia, in males over females. Further, pretreatment with haloperidol attenuated the positive but not negative symptom correlates in both sexes though it was more effective in females. Pretreatment with clozapine, on the other hand, attenuated both positive and negative symptom correlates in both sexes though it was more effective on positive symptoms. Taken together, this suggests that the NMDAR hypofunction model does recapitulate the prominent sex differences in symptomatology and treatment response of schizophrenia's positive and negative symptoms. Next, to investigate whether NMDAR hypofunction also models the sex differences in affective symptoms in schizophrenia, the studies in Chapter III evaluated the effects of MK801 and neuroleptic pretreatment on spontaneous ultrasonic vocalizations in male and female rats, behaviors that are a rich source of information regarding affect of the animal. These experiments found that while MK801 increased the number of vocalizations in both sexes, it increased low-frequency vocalizations indicative of negative affect to a much greater degree in females and that clozapine but not haloperidol reversed these increases, mirroring the clinical scenario wherein women suffer from more severe affective symptoms than men

and where only atypical neuroleptics are effective at treating these symptoms. Having established that the rodent NMDAR hypofunction model does indeed recapitulate a full range of sex differences in schizophrenia's symptomatology, the experiments in Chapter IV used *in-vivo* microdialysis in male and female rats to investigate the effects of NMDAR hypofunction on the levels of dopamine and its metabolites in the prefrontal cortex, a neurochemical system and brain region involved in high-order cognitive and executive tasks and implicated in the negative and cognitive symptoms of schizophrenia (Weinberger et al., 1986; Goldman-Rakic, 1987; Akil et al., 1999). The results showed that despite some confounding factors, prefrontal DA turnover was increased in both sexes and was greater in females. Finally, as the goal of the studies here is eventual translation to human therapeutics, it was evident that while study of *in-vivo* DAergic neurochemistry in human studies is invaluable for investigating bases of disease and investigating novel therapeutic strategies, current techniques for assessing DAergic neurochemistry in human subject are limited. To address this, the studies in Chapter V sought to investigate, on a proof-of-concept level, whether magnetic resonance spectroscopy, a non-invasive imaging method, can be extended to the study of DA and its metabolites. Pairing a novel data analysis approach with pharmacological manipulation of DA levels, the experiments showed that homovanillic acid (HVA), a major metabolite of DA can be reliably assessed with this method. Thus, the findings in this dissertation not only give strong impetus to further exploration of the neurobiological underpinnings of sex differences in behavior as seen in NMDAR hypofunction, but also promote further development of novel techniques that hold promise for eventual translation and use in human investigations and therapeutics.

CHAPTER I

Prepulse intensity dependent differences in sensorimotor gating of acoustic startle following MK801 challenge and neuroleptic pretreatment in male and female rats

Schizophrenia is a complex mental illness affecting approximately one percent of the population worldwide. This disorder is characterized by an array of diverse, debilitating symptoms that include so-called positive symptoms such as hallucinations, delusions and bizarre behavior, negative symptoms, which include anhedonia, social withdrawal and flattened affect and cognitive deficits such as behavioral inflexibility and impairments in memory and executive functions (Andreasen and Flaum, 1991; Andreasen, 1995; Nuechterlein et al., 2004). Since its original description as a thought disorder of young men (Kraepelin et al., 1919), many features of schizophrenia are recognized to be sexually dimorphic. Males, for example first present with schizophrenia on average some 5 years earlier than women, experience more and more frequent hospitalizations and overall have a lower quality of life and poorer long-term outcomes than female patients (Lewine, 1981; Angermeyer et al., 1990; Hafner et al., 1994). Of particular relevance to this study, however, are the sex differences that also differentiate schizophrenia's symptoms and their response to pharmacologically based treatment. Specifically, women tend to suffer more from schizophrenia's positive and affective symptoms and show better responses to treatment with neuroleptic drugs while men suffer more from negative and cognitive symptoms and are more refractory to neuroleptic treatment (Seeman, 1986; McGlashan and Bardenstein, 1990; Szymanski et al., 1995). These data combine with epidemiological findings showing that in women symptoms of schizophrenia wane during pregnancy (Chang and Renshaw, 1986; Trixler

et al., 1995) and worsen at menopause (Hafner, 2003) and that in males, low androgen levels are significantly correlated with severity of negative/cognitive symptoms (Goyal et al., 2004; Huber et al., 2005) to show that sex differences and the roles for gonadal steroids that they imply are an important part of the disease process. However, the relevance and the neurobiology that contributes to sex differences in schizophrenia and its effective treatment remain poorly understood, due in part to the lack of knowledge of whether preclinical animal models of schizophrenia in fact recapitulate its striking male/female differences. The studies presented here ask whether the N-methyl-D-aspartate receptor (NMDAR) hypofunction model in rats meets these criteria and accurately recapitulates the sex differences evident in this disease.

While the study of schizophrenia benefits from numerous animal models that capture specific elements of its pathology or subsets of its symptoms (e.g., amphetamine psychosis, neonatal ventral hippocampal lesions), the NMDAR hypofunction model stands out as uniquely able to model a spectrum of schizophrenia's positive, negative and cognitive signs (Javitt and Zukin, 1991; Enomoto et al., 2007; Stahl, 2007). Buoyed especially by evidence showing that phencyclidine (PCP, an NMDA antagonist) produces psychoses in healthy human subjects that are virtually indistinguishable from organic schizophrenia (Luby et al., 1959; Davies and Beech, 1960), this hypothesis also dovetails with dopamine (DA) hypotheses and other theories of the disorder that focus on dysregulation in multiple neurotransmitter systems (Jentsch and Roth, 1999). This hypothesis has also gained particular traction in animal models for its ability to evoke a range of behavioral abnormalities that mimic schizophrenia's

positive, negative and cognitive symptoms (Sams-Dodd, 1996; Jentsch and Roth, 1999; Stefani and Moghaddam, 2005; Darrah et al., 2008) and to stimulate neurochemical abnormalities that closely match those observed in human patients (Giovannini et al., 1994; Verma and Moghaddam, 1996; Moghaddam et al., 1997; Yonezawa et al., 1998). What has been unexplored until now, however, are whether the effects of NMDAR hypofunction differentially affect males and females. To begin to address this question, we chose to compare behavioral effects of acute NMDAR antagonism in male and female rats using treatment with MK801 paired with analyses of prepulse inhibition (PPI) of the acoustic startle reflex (ASR).

Prepulse inhibition of the ASR is a phenomenon wherein the reflexive startle response (e.g. twitch, blink) to a loud or unexpected auditory stimulus (pulse) is attenuated or inhibited when preceded by a softer auditory stimulus (prepulse) presented 20-500 ms before the pulse (Braff et al., 1978; Hoffman and Ison, 1980). While the ASR represents baseline excitability, PPI is viewed as an index of sensorimotor gating; i.e. the ability of the brain to filter out irrelevant stimuli to prevent overstimulation and resultant cognitive fragmentation (Braff and Geyer, 1990). Further, sex-specific abnormalities in ASR and PPI have been repeatedly shown in schizophrenia. Specifically, while ASR is increased and PPI decreased in both sexes, females have greater increases in ASR than males, and males have more impairments in PPI than females (Kumari et al., 2004; Braff et al., 2005). While other behaviors could have been used for these purposes, it is noteworthy that ASR and PPI and the ways in which they are measured are directly translational from humans to animal

models including rats (Swerdlow et al., 1999a). Thus, investigation of ASR and PPI in male and female rats in proestrus (when estrogen levels are highest) is a particularly strong starting point for assessment of sex differences in the behavioral consequences of NMDAR hypofunction. Further, while given the validity of ASR and PPI testing for predicting therapeutic efficacy of novel neuroleptic drugs, my study will ask whether sex differences in the NMDAR hypofunction model extends to the differential attenuation of MK801-induced deficits in ASR and/or PPI via haloperidol and/or clozapine treatment in a manner that recapitulates sex differences seen in human populations in neuroleptic drug response.

METHODS

Animal Subjects: A total of 39 male and 40 female adult Sprague-Dawley rats were used; animals were purchased from Taconic Farms (Hudson NY). Animals were housed in same-sex pairs in a temperature controlled room under a 12 hour light/dark cycle (lights on at 7 AM) with food and water available *ad libitum*. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Stony Brook University and were designed to minimize their discomfort and use.

Monitoring the Estrus Cycle in Females and Handling in Yoked Males: Prior to behavioral testing, female rats underwent daily vaginal lavage for at least 8 days to determine estrus cycle stage and regularity; rats that did not cycle regularly were excluded from the study. Lavage was performed between 11 AM and 2 PM, and was facilitated by gently wrapping the animals in a thick towel and briefly holding them in a

supine position. For each female subject, one male rat was handled similarly (wrapped and held supine) in parallel. After tracking one complete estrus cycle, females in proestrus and their companion males were randomly assigned to one of five experimental groups (Table 1) and tested as per the schedule below.

Drugs and Drug Treatment Groups: All drugs used in these studies were purchased from Sigma-Aldrich (St. Louis, MO). On the day of testing, rats first received an initial subcutaneous injection of either haloperidol (0.04 or 0.08 mg/kg) or clozapine (5 or 10 mg/kg) dissolved in saline acidified with 0.025% acetic acid or of acidified saline vehicle alone (volumes ranging from 0.4 to 0.8 mL). This injection was followed 45 minutes later by an intraperitoneal injection of either MK801 (0.05, 0.1 or 0.2 mg/kg) dissolved in saline, or saline vehicle alone (volumes ranging from 0.2 to 0.4 mL). Behavioral testing began 15 minutes after the second injection was given.

Control animals (VEH/VEH, 8 males and 8 females) received injections of acidified neuroleptic vehicle followed by MK801 vehicle prior to all three testing sessions. The MK801 dose response group (VEH/DR, 8 males and 8 females) received acidified neuroleptic vehicle followed by 0.05 mg/kg, 0.1 mg/kg and 0.2 mg/kg MK801 on testing days 1, 2 and 3 respectively. The MK801 control group (VEH/0.2 MK801, 7 males and 8 females) received neuroleptic vehicle followed by 0.2 mg/kg MK801 on all three days of testing. The haloperidol group (HDL, 8 males and 8 females) received neuroleptic vehicle followed by 0.2 mg/kg MK801 on testing day, and received 0.04 mg/kg or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 on testing days 2 and 3

respectively. Finally, the clozapine group (CLZ, 8 males and 8 females) received neuroleptic vehicle followed by 0.2 mg/kg MK801 on testing day 1 and received 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 on testing days 2 and 3 respectively.

Testing Apparatus: All testing was conducted using the SR-LAB startle response system (San Diego Instruments, San Diego, CA). Each of four testing units consisted of a ventilated inner Plexiglas cylinder (20 cm in length and 8.2 cm in diameter) mounted on a platform over a piezoelectric accelerometer that transduced the rats' motor responses to acoustic stimuli. Each testing unit was housed inside of a sound attenuated chamber equipped with a loudspeaker (RadioShack supertweeter) mounted 24 cm above the Plexiglas cylinder that delivered background noise and all acoustic stimuli. Before each testing session, the background noise in each cylinder was measured using a sound meter (RadioShack) and the response sensitivity of each accelerometer was calibrated to a standard value (SR-LAB Startle Calibration System).

Testing Paradigm/Sessions: On each testing day background noise (78 dB) was presented for a 5-minute acclimatization period. Background noise remained on for the remainder of the session, during which a total of 65 trials were presented separated by inter-trial intervals that varied pseudorandomly from 15 to 25 seconds. The first five trials were pulse-only trials (120 dB broadband burst, 40 ms) and the remaining trials were divided into four blocks of 15 trials each. Each of these blocks included 5 pulse-only trials, 8 prepulse-pulse trials, consisting of a 20-ms prepulse of 3, 6, 9, 12, 15, 18,

21 or 24 dB above background presented 100 ms before a 120 dB pulse and 2 prepulse-only trials that were all presented in pseudorandom order.

Testing Sequence: All animals underwent an initial testing session to acclimate them to the testing procedure; animals received no injections on this day. After a 7-day interval, females were lavaged (and paired males were handled) daily. Females found to be in proestrus along with yoked males immediately underwent the dual injection regimen appropriate for their assigned group (Table I.1) and were tested (testing day 1). After a 7-day interval, lavages again commenced and testing day 2 occurred upon females' re-entry into proestrus; a similar sequence dictated the timing of testing day 3. Any animals taking longer than 7 days to re-enter proestrus from the day of resumption of lavage were excluded from the study.

Data Analysis: All analyses were based on animals' motor responses measured per trial over a 100 ms time window that followed the onset of the acoustic stimulus. As a first step in the analyses, Grubbs' test for outliers was applied to identify outliers within the data. This resulted in the removal of a small number of data points from a subset of animals from each of the groups. Outliers occurred with an equal frequency in all groups and in both sexes; there was no obvious bias to any experimental condition. The acoustic startle reflex (ASR) was then calculated as the mean of the 20 within-block pulse-only trials; these values were normalized to/divided by animals' body weights to enable comparisons of the data between the heavier males and the significantly lighter

females. Percent PPI was also calculated for each prepulse intensity used within each testing block as per the formula:

$$[(\text{mean ASR} - \text{prepulse}) / \text{mean ASR}] * 100$$

The four values of PPI calculated per block for each of the eight prepulse intensities used were then averaged to obtain a final value of percent PPI for each prepulse intensity used.

Statistical Analyses: All statistical tests were conducted using SPSS software (IBM, version 19). Two-way (ASR) or three-way (PPI) analyses of variance (ANOVA) with repeated measures designs were used for within group comparisons of the data across sex and across testing day/drug treatment. One-way ANOVA (ASR) and two-way ANOVA (PPI) with repeated measures designs were also used to compare data within sex separately across group and testing day/drug treatment and to compare data from individual testing sessions within groups across sex. All repeated measures ANOVAs were preceded by Mauchly's sphericity test to ensure that observations obeyed required assumptions; a Greenhouse-Geisser correction was applied as necessary. One-way ANOVA were also used to compare data from individual testing sessions within sex and within prepulse intensity across testing groups and within sex and prepulse intensity across testing day/drug treatment. Bonferroni corrections were applied in cases of multiple pair-wise comparisons for all post-hoc testing. All observations were considered significant at a $p < 0.05$ level and near-significant at a $p < 0.075$ level.

RESULTS

Baseline Studies: Initial studies were carried out to establish baseline measures of ASR and PPI in drug-naïve and MK801-treated male and proestrus female rats over the course of three sessions of repeated testing. The drug-naïve (VEH/VEH) group received an injection of neuroleptic vehicle followed by MK801 vehicle prior to each testing session. The MK801 group (VEH/0.2 MK801) received injection of neuroleptic vehicle followed by injection of 0.2 mg/kg MK801 prior to each testing session.

VEH/VEH

ASR: In drug-naïve rats (VEH/VEH), normalized mean ASR values were slightly higher in males than in females, and in both sexes ASR values increased slightly with each testing session. Thus, in males, mean ASR values were approximately 0.30 on testing day 1, 0.36 on testing day 2 and 0.40 on testing day 3. In females corresponding mean ASR values were approximately 0.30 on day 1, 0.32 on day 2, and 0.38 on day 3 (Figure I.1). Neither the incremental increases in mean ASR observed across testing sessions nor their modest sex differences proved to be significant. Rather, one-way repeated measures ANOVAs identified no significant main effects of testing day on ASR in males or in females and when the data from males and females were combined into an overall two-way repeated measures ANOVA, no significant main effects of sex were found.

PPI: Analyses of PPI over a range of 8 prepulse intensities in males and proestrus females of the VEH/VEH group revealed clear sex differences in resultant prepulse sensitivity curves. Thus, in males, PPI ranged from 40-70% across the lowest four intensity prepulses used (+3-12 dB), and plateaued at between 75%-85% across the remaining middle to high intensity prepulses (+12-24 dB). This somewhat asymptotic sensitivity curve differed markedly from that observed in females where PPI began at markedly lower levels for the lowest intensity prepulse (20%) and increased more linearly (~10-15% with increasing prepulse intensity) to reach maximal levels that were similar to those in males (70-85%, Figure 1.3A,B). Although both sexes showed a modest improvement for the lower prepulse intensities used with testing experience, two-way repeated measures ANOVAs revealed no significant main effects of day, and no significant interactions between day and prepulse intensity in either sex. These analyses did, however, reveal expected significant main effects of prepulse intensity in both sexes (males: $[F(7,49)=18.08 \text{ } p<.001]$, females: $[F(7,49)=27.67 \text{ } p<.001]$). The sex differences observed in PPI profile were also statistically validated. First an overall three way repeated measures ANOVA in which data from males and females were combined identified significant main effects of sex $[F(1,14)=6.47 \text{ } p=.023]$ and a significant interaction between sex and prepulse intensity $[F(3.51,49.13)=4.41 \text{ } p=.006]$ on PPI. Further, evaluations of PPI by testing day (two-way, repeated measures ANOVAs) identified main effects of sex that were significant or near significant on all testing days (day 1: $[F(1,14)=6.57 \text{ } p=.023]$, day 2: $[F(1,14)=6.11 \text{ } p=.027]$, day 3: $[F(1,14)=4.207 \text{ } p=.059]$). And finally, a series of one-way ANOVAs that compared data

by day and by prepulse pinpointed significant sex differences in PPI as arising mainly from prepulses in the +3 to +18 dB range (Figure I.3A,B).

VEH/0.2MK801

ASR: Mean ASR values were noticeably higher in MK801 compared to drug naive rats. Thus, in VEH/0.2 MK801males, normalized mean ASR values were more than twice those observed in males of the VEH/VEH group (0.60-0.89) and; in females ASR values were nearly three times higher in the drug-treated compared to the drug-naïve cohort. (1.03-1.13, Figure I.2). Although, some session-to-session variance was observed, one-way repeated measures ANOVAs found no significant main effects of testing day in either sex. Surprisingly, when the data from males and females were combined, no significant main effects of sex on MK801-stimulated startle were found. However, comparisons with drug-naïve baselines did corroborate a proportionately greater sensitivity of ASR to drug treatment in females. Specifically, while effects of MK801 were significant in both sexes (two-way repeated measures ANOVAs, males: [F(1,13)=5.74 p=.032], females: [F(1,14)=24.10 p<.001]), subsequent one-way ANOVAs showed that in males, drug effects were only significant or near significant for testing days 1 and 2 (day 1: [F(1,13)=17.26 p=.001], day 2 [F(1,13)=4.63 p=.051]) and were not significant on day 3. In females, on the other hand, the effects of MK801 on ASR were significant across all testing sessions [F(1,13)=14.43 - 31.57, p<.001 - .002].

PPI: Prepulse sensitivity curves in VEH/0.2 MK801 animals were markedly lower than those observed in drug naïve subjects and were essentially overlapping for male and female subjects. Thus, in males and females alike, prepulses in the +3 to +9 dB range showed little to no ability to inhibit ASR while prepulses in the mid to upper range (+12-24 dB) produced PPI that rose incrementally from 2-30% to a peak of about 70% (Figure I.3C,D). However, when assessed with respect to the sex-specific baselines established in drug naïve animals, MK801 was found to reduce PPI by more than 50% across the lower prepulse intensities (+3 to +12 dB) and by more than 30% for the remaining prepulses in males. In females, however, MK801 decreased PPI more uniformly and only by some 20-30% (Figure I.3D). Within-group analyses of the drug-treated cohorts identified expected main effects of prepulse. However, these analyses revealed no main effects of testing day and no significant interactions between prepulse and testing day for males or for females (two-way repeated measures ANOVAs). Comparisons in which data from males and females were combined (three-way repeated measures ANOVA) likewise identified no significant main effects of sex and no significant interactions between sex and prepulse. Finally, although group comparisons (VEH/VEH vs. VEH/0.2MK801) identified significant main effects of MK801 treatment on PPI in both sexes (three-way repeated measures ANOVAs, males: [F(1,13)=107.98 p<.001], females: [F(1,14)=39.92 p<.001]) and on each individual day (two-way repeated measures ANOVAs, males: [F(1,13)=37.67-136.58 p<.001], females: [F(1,14)=12.80-33.26 p<.001-.003]), further analysis of drug effects by prepulse and by day (one-way ANOVAs) showed that while significant effects of MK801 on PPI were found across all prepulses in males, the effects of MK801 were only consistently

different from drug-naïve baseline in females for the higher intensity prepulses used (Figure I.3C,D).

Dose Response Study: Sex differences in the effects of 0.2 mg/kg MK801 on ASR and PPI were explored using a limited dose response paradigm. Specifically, rats in these studies (VEH/DR) received an injection of neuroleptic vehicle followed by 0.05 mg/kg MK801 prior to the first testing session, neuroleptic vehicle followed by 0.1 mg/kg MK801 prior to the second testing session, and neuroleptic vehicle followed by 0.2 mg/kg MK801 prior to the third testing session.

ASR: All three doses of MK801 increased normalized startle amplitudes relative to VEH/VEH rats. However, in males, 0.05 mg/kg MK801 increased ASR only slightly (by less than 50%) while the two higher doses (0.1 and 0.2 mg/kg) increased ASR by a higher but similar degree (60-80%). In contrast, the effects of MK801 in females were larger overall and were incremental with increasing dose (140-150%, Figure I.4). Not surprisingly, main effects of MK801 dose (day) were only significant in females (one-way repeated measures ANOVA, $[F(2,14)=11.68 \text{ } p=.001]$). However, comparisons where data from males and females within the VEH/DR were combined (two-way repeated measures ANOVA), failed to identify a significant effect of sex or a significant interaction between sex and dose (day) on ASR. Group comparisons of data from the dose-response and drug-naïve cohorts, on the other hand, did substantiate sex differences in dose-dependent effects of MK801 on ASR. Thus, while initial two-way repeated measures ANOVAs revealed significant main effects of MK801 in both sexes

overall, (males: [F(1,14)=6.11 p=.027], females: [F(1,14)=12.21 p=.004]) follow-up comparisons made by day (one-way ANOVAs) showed that in males, ASR was not significantly different from control in rats treated with 0.05 mg/kg MK801, was nearly significantly different from control in rats treated with 0.1 mg/kg MK801 [F(1,15)=4.47 p=.053], and was significantly different from control only in rats treated with 0.2 mg/kg MK801 [F(1,15)=6.40 p=.024]. In females, however, differences in mean ASR in the VEH/VEH compared to VEH/DR group were significant for all three doses of MK801 (0.05 mg/kg: [F(1,15)=7.77 p=.015], 0.1 mg/kg: [F(1,15)=9.26 p=.009], 0.2 mg/kg: [F(1,15)=14.38 p=.002]).

PPI: The lowest dose of MK801 used (0.05 mg/kg) had little effect (<5%) on PPI in either sex. At the 0.1 mg/kg dose, MK801 decreased PPI by ~15% elicited by the lower intensity prepulses and had marginal effects (~5%) on PPI elicited by the remaining prepulses used in males. However, it had more robust effects (15-30%) over all of the prepulse intensities used in females. Finally, the results obtained at the 0.2 mg/kg dose mirrored those obtained from the VEH/0.2MK801 group above, i.e., PPI was markedly reduced in both sexes to similar absolute levels, but to levels that, relative to drug-naïve baselines, were more impaired in males than in females (Figure 1.5A,B). While differences were clear, main effects of MK801 dose (day) were nonetheless significant in both sexes (two-way repeated measures ANOVAs, males: [F(2,14)=51.28 p<.001], females: [F(2,14)=16.91 p<.001]). Allowed post-hoc testing showed that in both sexes, these differences were driven by the effects of the 0.2 mg/kg dose compared to all others (0.2 mg/kg vs. 0.5 mg/kg: p< .001; 0.2 mg/kg vs. 0.1 mg/kg, p<.001-.035).

However, while within-group comparisons in which data from males and females were combined (three-way repeated measures ANOVA) also identified significant main effects of sex [$F(1,14)=7.08$ $p=.019$], these analyses further identified significant interactions between dose and sex [$F(2,28)=6.82$ $p=.004$]. The observed sex differences in MK801 sensitivity were also substantiated in within-sex comparisons of data from the VEH/DR and VEH/VEH groups. Thus, following initial three-way repeated measures ANOVAs that affirmed significant main effects of MK801 in both sexes (males: [$F(1,14)=20.96$ $p<.001$], females: [$F(1,14)=9.27$ $p=.009$]), subsequent comparisons by dose (day) found no significant effects of 0.05 mg/kg MK801 on PPI in either sex; main effects of 0.1 mg/kg MK801 that were significant in females [$F(1,14)=6.10$ $p=.027$] but near-significant in males [$F(1,14)=3.97$ $p=.066$] and main effects of 0.2 mg/kg MK801 that were significant in both sexes (males: [$F(1,14)=58.27$ $p<.001$], females: [$F(1,14)=20.66$ $p<.001$]). Finally, within-sex comparisons of group by dose (day) and by prepulse (one-way ANOVAs) revealed no significant differences from drug-naïve control for 0.05 mg/kg at any prepulse in either sex; revealed effects of 0.1 mg/kg that were significant or near-significant for the highest prepulse intensities used (+15 dB - +24 dB) in females, but only for the +6 dB prepulse in males; and effects of 0.2 mg/kg that, as in the baseline studies above were significant in males for all, and in females for all except the +3 dB prepulse (Figure 1.5A,B).

Neuroleptic Attenuation Studies: The abilities of haloperidol and clozapine to attenuate the effects of MK801 on ASR and its PPI were compared in male and proestrus female rats. Each drug was tested at two doses that represented equivalents to the low or high

therapeutic range used to treat human psychoses (Schotte et al., 1996; Kapur et al., 2003). Thus, animals in the HDL/MK group received neuroleptic vehicle followed by 0.2 MK801 on testing day 1, 0.04 mg/kg haloperidol followed by 0.2 mg/kg MK801 on testing day 2 and finally, 0.08 mg/kg clozapine followed by 0.2 mg/kg MK801 on testing day 3. Animals in the CLZ/MK group received neuroleptic vehicle followed by 0.2 MK801 on testing day 1, 5 mg/kg clozapine followed by 0.2 mg/kg MK801 on testing day 2 and finally, 10 mg/kg clozapine followed by 0.2 mg/kg MK801 on testing day 3.

Haloperidol

ASR: In the HDL/MK group, pretreatment with haloperidol at the low and high dose had no appreciable effect on MK801 induced increases in ASR in either sex. Thus, in males, haloperidol reduced ASR by at most only some 25% relative to MK801 alone, which was not significant (one-way repeated measures ANOVA). In females, corresponding doses of haloperidol appeared to increase ASR by 25-50% relative to MK801 alone (Figure I.6). However, it is important to note that ASR in this MK801-treated cohort was markedly lower than all others (Figures I.2, I.4). Thus while one-way repeated measures ANOVA identified a significant main effect of haloperidol on ASR in females [$F(2,14)=7.10$ $p=.007$], it most likely reflects a lack of drug effect due to the abnormally low ASR values obtained after treatment with MK801 alone.

PPI: Haloperidol demonstrated virtually no ability to attenuate the effects of MK801 on PPI in males or in females. At the lower dose (0.04 mg/kg), PPI was essentially

unchanged from levels seen after treatment with MK801 alone, and at the higher dose at best only marginal increases in PPI on the order of 5-10% were seen (Figure 1.7A,B). Not surprisingly, statistical analyses (two-way repeated measures ANOVAs) revealed no significant main effects of haloperidol on PPI in males or in females.

Comparisons between the HDL/MK and the drug-naïve groups further substantiated the inability of haloperidol to attenuate MK801's depression of PPI, i.e., there was no evidence that pretreatment brought PPI any closer to control values. Rather, within-sex comparisons of PPI after higher and lower haloperidol dose compared to drug-naïve control (two-way repeated measures ANOVAs) identified significant differences between the two in both sexes (males: [F(1,14)=29.30 p<.001], females: [F(1,14)=54.06 p<.001]) (males: [F(1,14)=30.99 p<.001], females: [F(1,14)=31.66 p<.001]). Finally, analyses by prepulse revealed that only one isolated instance in females where values of PPI in haloperidol pretreated cohort were not significantly different from control.

Clozapine

ASR: While pretreatment with clozapine attenuated the effects of MK801 on ASR, these effects were not clearly sex or dose-dependent. Thus, both the high and low doses of clozapine were effective in attenuating the effects of MK801 on ASR; however, for neither sex was the higher dose obviously more potent than the lower dose. Thus, in males, pretreatment with both 5 and 10 mg/kg clozapine decreased ASR relative to

MK801 alone to within 20-35% of drug naïve baseline. In females, clozapine also decreased ASR but only to within 50-70% of baseline (Figure I.8). This apparent edge observed in the males, was not statistically robust. Thus, while initial within-group analyses (one-way repeated measures ANOVAs) confirmed a significant overall effect of clozapine on ASR in both sexes (males: $[F(2,14)=8.26 \text{ } p=.004]$, females: $[F(2,14)=6.56 \text{ } p=.010]$), allowed post-hoc testing revealed that differences from MK801-stimulated levels were only significant in males for the 5 mg/kg dose ($p=.024$) and were only significant in females at the 10 mg/kg dose ($p=.045$). Statistical comparisons of the clozapine group to the drug-naïve cohort also substantiated the ability of clozapine to restore ASR and underscored the similarities in its actions across sex. Specifically, comparisons of animals treated with 5 and 10 mg/kg clozapine to their corresponding drug-naïve baselines (one-way ANOVAs) revealed that there were no differences in ASR in drug-naïve animals compared to those pretreated with both doses of clozapine in males or in females.

PPI: Clozapine attenuated the effects of MK801 on PPI in a dose-dependent manner in both sexes. However, clozapine pretreatment was noticeably more effective in restoring behavior in males. Thus, relative to MK801 treatment alone, pretreatment with 5 mg/kg and 10 mg/kg clozapine in males improved PPI for all prepulse intensities by 20-40% and by 35-50%, respectively (Figure I.9A). In females, however, these same pretreatments increased PPI relative to MK801 alone only by 15-30% and 20-35%, respectively (Figure I.9B). Comparisons with the sex-specific baselines established in drug-naïve controls, also indicated a greater effectiveness of clozapine pretreatment in

males. For example, while the 5 mg/kg dose, restored PPI to within 25% of control levels in both sexes for prepulses in the +3-12 dB range, in males PPI was restored to values that were within 10% of control for the remaining prepulses while in females, differences from control more on the order of 20% remained. Similarly, at the 10 mg/kg dose, PPI was largely restored to within 5-15% of drug-naïve baseline in males, but only to values that were 10-25% lower than baseline in females (Figure I.9A,B). These dose and sex-specific effects proved to be statistically robust. Thus, while initial analysis within the CLZ/MK group (two-way repeated measures ANOVAs) identified significant main effects of clozapine on PPI overall in both sexes (males: $[F(2,14)=13.70\ p=.001]$, females: $[F(2,14)=9.48\ p=.002]$), allowed post-hoc testing showed that in males, PPI was significantly higher than MK801 alone following pretreatment with both doses of clozapine (5 mg/kg: $p=.032$, 10 mg/kg: $p=.009$) whereas in females, this was only true for the 10 mg/kg dose ($p=.016$).

Similarly, while within-sex comparisons of PPI after 5 mg/kg clozapine compared to drug-naïve control (two-way repeated measures ANOVAs) identified significant main effects of group in both sexes (males: $[F(1,14)=6.42\ p=.024]$, females: $[F(1,14)=9.55\ p=.008]$) further analyses by prepulse (one-way ANOVAs) indicated that in males, PPI was restored to levels that were not significantly different from control for all but the +3 dB, +6 dB and +9 dB prepulses. However, in females, PPI was only restored to control levels at the +9 dB and +12 dB prepulses (Figure I.9A,B). Likewise, although after 10 mg/kg clozapine, significant or near-significant group differences compared to control were found for both sexes (males: $[F(1,14)=5.14\ p=.040]$, females: $[F(1,14)=3.89$

p=.069]), subsequent analyses by prepulse revealed that differences in males were entirely driven by group differences in PPI for a single (+3 dB) prepulse while in females, PPI at +6 dB, +9 dB and +24 dB were significantly or near-significantly lower than drug-naïve values (Figure 1.9A,B).

DISCUSSION

Sex differences in schizophrenia have been identified in many aspects of this complex and debilitating disorder, including those that have relevance for therapeutic approaches and clinical outcomes. For example, female patients have been repeatedly identified as being more vulnerable to the positive symptoms, e.g., hallucinations, bizarre behavior, which also tend to be more responsive to available neuroleptic treatments (Seeman, 1986; McGlashan and Bardenstein, 1990). Males on the other hand are more often and more severely affected by schizophrenia's negative and cognitive symptoms, such as social withdrawal and behavioral inflexibility which unfortunately are also those symptoms that are most refractory to available pharmacologic treatments (Ring et al., 1991; Szymanski et al., 1995). These findings along with others have spurred interest in the possibility for sex-specific pathologies in this disorder and have initiated the exploration and in some cases the adoption of sex-specific approaches in its treatment, including the use of gonadal steroids as adjunct treatments (Ko et al., 2008; Cosimo Melcangi and Garcia-Segura, 2010; Kulkarni et al., 2011). While the latter have produced some promising results, an understanding of the neurobiological and neuroendocrine systems that are either at risk or interact with disease processes that underlie the sex differences that mark schizophrenia and its

treatment has been hindered by in part by the lack of available animal models that recapitulate these male/female-specific characteristics. To address this issue, the studies presented here asked whether an established rat model of NMDAR hypofunction, which has an impressive track record in exploring the etiology neurochemistry and behavioral abnormalities associated with schizophrenia, also has face and construct validity for modeling the sex differences in its symptomatology and their sensitivity to neuroleptic drugs. Specifically, this study compared the behavioral effects of acute treatment with MK801, with and without pretreatment with the typical neuroleptic haloperidol and the atypical neuroleptic clozapine, on the ASR and its PPI in adult male rats and female rats in proestrus. These studies confirmed and extended findings from previous studies in drug naïve rats for similarities in ASR across the sexes and for greater PPI in males than in females. Further, it was found that MK801 exaggerated startle responses to a greater extent in females compared to males, while inducing larger deficits in PPI in males compared to females. Finally, while haloperidol pretreatment was confirmed to have almost no ability to attenuate MK801 effects on ASR or its PPI in either sex, clozapine was found to ameliorate both, albeit more so in males than in females. Discussions of these findings are found in the sections that follow. These include comparisons to previous behavioral and drug challenge studies in rodents, to findings from healthy human subjects and to analyses of human patient populations with an emphasis on diagnosed schizophrenics. Overall, these comparisons illustrate that the translational value of ASR and PPI indeed extends to sex differences in these behaviors and identifies the rodent NMDAR hypofunction model as

a potentially valid platform for studying the neurobiology of the sex differences that characterize schizophrenia.

Sex Differences in ASR and PPI in Drug-Naïve Rats: Comparisons to Previous Studies in Humans and Rodent Models

The present studies included analyses of ASR and PPI in drug naïve male and proestrus female rats. These analyses revealed that weight-corrected values of ASR were similar in males and females, but that PPI was consistently greater in males, especially for inhibition produced by prepulses in the lower intensity range, i.e., less than 15 dB above background. Both of these findings have precedent in the literature describing the ASR and its PPI in healthy human subjects. Thus, studies where ASR was assessed by measuring blink response via electromyography from the orbicularis oculi muscle have consistently identified values of ASR that differ by less than 20% among males and females and that are also highly consistent among females tested at follicular compared to luteal stages of the menstrual cycle i.e., when estrogen levels are lowest compared to highest (Swerdlow et al., 1993; Swerdlow et al., 1997; Kumari et al., 2008). Prepulse inhibition of the ASR, on the other hand, has been consistently shown to be both greater in males than in females and greater in females in follicular compared to luteal phases of the menstrual cycle (Swerdlow et al., 1997; Jovanovic et al., 2004). In fact, a meta-analysis that included some 779 subjects (360 men, 419 women) and evaluations of PPI using prepulse intensities that ranged from 2 to 16 dB above background revealed a clear consensus for male over female differences in PPI

(Swerdlow et al., 1999b). Interestingly, like the present study, the largest differences in PPI in males compared to females were also found to be for prepulses of lower intensity (Swerdlow et al., 1993).

While the translational value of assessing ASR and PPI in animal models for understanding human sensorimotor processes and their integration and gating has long been recognized, it is surprising that few studies have explored whether the sex differences that have been clearly identified in human subjects also characterize these metrics in rats or other laboratory animals. Further, even among the small numbers of studies that have investigated this question, there are some inconsistent findings. For example, of the three prior studies in rats that have examined sex differences in ASR, like the present investigation, two report nearly identical values in males and females (Swerdlow et al., 1993; Faraday et al., 1999). However, there is one report of greater startle responses in young adult males compared to females (Lehmann et al., 1999). It may be important to note, however, that these differences in ASR were paralleled by findings of significantly greater body weights in the male compared to female subjects. Given that experimental measurements of ASR in rats are influenced by body mass, this may well explain the discrepancy from this study-- where a weight correction was applied and from two other studies reporting similar findings-- where rats of both sexes had similar weights. It may also be important to note that the study by Lehmann and colleagues also differed in rat strain; their studies utilized Wistar rats while the present and the two matching studies cited above used Sprague-Dawley rats. As strain differences in ASR have been previously demonstrated (Bast et al., 2000; Hince and

Martin-Iverson, 2005; Swerdlow et al., 2008), it is possible that strain differences might also extend to sex differences in ASR.

Previous studies examining sex differences in PPI of rats are also few. Further, they have come to one of two conclusions. Thus, studies either agree with the present results, i.e., they conclude that PPI is greater in males than in females (Faraday et al., 1999; Lehmann et al., 1999), or they report no evidence of a sex difference in this sensorimotor gating process (Swerdlow et al., 1993). This disparity may be resolved, however, by considering the results of studies examining the effects of estrus cycle stage on PPI in female rats. Specifically it has been shown that PPI in female rats is significantly lower for rats in proestrus- when estrogen levels are high, compared to all other stages of the estrus cycle when circulating estrogen levels are low (Koch, 1998). In fact these differences were surprisingly large; PPI was more than 25% lower in proestrus while at all other days of the four day cycle PPI had similar, higher values. Given this, it may be noteworthy that in those studies where no sex differences were found, estrus cycle was not controlled for. As it can reasonably assumed that rats in proestrus compared to diestrus or estrus would be in the minority it is easy to envision how this could blur differences between the sexes. On the other hand, those studies including the present where comparisons were made specifically with proestrus female rats PPI has consistently been shown to be significantly lower in female cohorts compared to males (Koch, 1998). In sum, there seem to be clear explanations for inconsistencies in the extant literature and overall it seems reasonable to conclude that just as has been established in healthy humans, in rats the ASR is similar in males and

females and PPI is significantly greater in males than in females. This gives the assessment of these behavioral measures translational value in exploring the basis for their sex differences in healthy human populations. In the sections below, we discuss further whether the study of ASR and PPI under conditions of acute NMDAR hypofunction (MK801) also have translational value for investigating the biological basis for the complex sex differences that mark the dysfunction of ASR and PPI in human mental and neurological illness, including schizophrenia.

Response to MK801 in Male and Female Rats: Comparison to Previous Studies and to Human Clinical Populations

Acute MK801 treatment in animals is a powerful model for human psychoses, including that seen in schizophrenia. While other drugs and manipulations can produce deficits that model singular symptoms or symptom clusters of this disorder, the NMDAR hypofunction that MK801 induces is unique for its ability to produce a spectrum of behavioral deficits in animal subjects that map on to the positive, negative and cognitive symptoms of schizophrenia (Jentsch and Roth, 1999). What has never been explored, however, is whether there are sex differences in the behavioral anomalies produced by MK801 that match those observed in organic human mental illness. Here we addressed this question by comparing the effects of acute MK801 in male and proestrus female rats on ASR and PPI. While my study is the first to explore drug effects both across sex and over a 0.05-0.2 mg/kg range, overall, the findings here are concordant with those reported separately in previous studies. In male rats, for example, the present studies

identified a small, non-significant increase in ASR after 0.05 mg/kg MK801 and larger (~60-80%) more robust (significant to near-significant) increases in ASR produced by MK801 at doses of 0.1 and 0.2 mg/kg. These findings are consistent with those from previous studies using 0.05 mg/kg MK801 that found little to no effect on ASR (Bast et al., 2000; Bortolato et al., 2005), and with data from other studies where doses of 0.1, 0.15 and 0.2 mg/kg were used that reported increases of ASR that were similar in magnitude to those observed here, i.e, on the order of 60-100% (Varty et al., 1999; Pietraszek et al., 2005; Lombardo et al., 2009). The data presented in this study for PPI also provide a good match to the literature in male rats. For example, the present findings of a small, non significant decrease in PPI (roughly 10%) in male rats given 0.05 mg/kg MK801, fits with data from a previous study showing a similarly small, non-significant reduction of PPI (5-10%) by MK801 at 0.075 mg/kg (Varty et al., 1999). Further, findings that PPI was nearly significantly decreased by 20-30% by 0.1 mg/kg MK801 and was significantly decreased by 30-50% after 0.2 mg/kg MK801 are also consistent with the literature describing similarly robust disruption of PPI by MK801 doses of 0.1 to 0.2 mg/kg (Bakshi et al., 1994; al-Amin and Schwarzkopf, 1996; Depoortere et al., 1999; Levin et al., 2005; Pietraszek et al., 2005). It should be noted, however, that some discrepancies exist at lower doses with two studies reporting that MK801 at 0.05 and 0.075 mg/kg does significantly reduce PPI by around 25% (Bast et al., 2000; Bortolato et al., 2005). The source of these discrepancies is unclear, especially since experimental conditions, subjects and timing of drug administration were similar across these studies. However, a considerable number of other reports briefly state that only doses of 0.1 mg/kg and above were found to reliably and

consistently reduce PPI (Geyer et al., 1990; Bakshi and Geyer, 1995; Kesby et al., 2006). Thus, while important for characterization of a dose-response curve, it appears that the use of lower doses in investigating the full NMDAR hypofunction phenotype may be limited.

Fewer studies have examined the effects of MK801 on ASR and PPI in female rats and among these only MK801 doses of 0.15 mg/kg and lower have been used. Thus, while some available comparisons exist, they are limited and none controlled for estrus cycle stage in their subjects. However, the substantial increases in startle elicited at all three doses identified here have some precedent in the literature in results from a prior study showing that ASR is roughly doubled in magnitude by MK801 at 0.1 mg/kg (Gogos et al., 2011). Further supporting the results obtained here, another study has reported a significant increase in ASR after 0.05 and 0.1 mg/kg MK801 though the magnitudes of these increases were not shown (Levin et al., 2005). It should be noted, however, that there is one contradictory report of no increase in ASR after 0.15 mg/kg MK801 (Nespor and Tizabi, 2008). Interestingly, in that study, a significant rise in ASR after acute MK801 relative to vehicle was seen in a different experimental arm that received daily saline injections for 7 days prior to MK801. While the reason for this is unclear, taken together, these findings do indicate that MK801 does indeed increase ASR.

With regard to PPI, the present study was also able to show that MK801 had a dose-dependent effect on this measure, with a non-significant lowering of PPI (~10%) at

0.05 mg/kg and significant lowering of PPI (15-30%) at 0.1 mg/kg and 0.2 mg/kg. This is also mostly consistent with the literature; other than one published report showing that 0.05 mg/kg significantly lowers PPI in female rats by about 15% (Levin et al., 2005), all studies using MK801 at 0.05 to 0.15 mg/kg report effects on PPI that are quantitatively similar to those identified here (Nespor and Tizabi, 2008; Gogos et al., 2011). It remains unclear why this study is not in agreement with results obtained here as subjects and experimental conditions were similar across this and all studies cited here.

Sex Differences in Response to MK801: Comparison and Relevance to Schizophrenia

While the effects of MK801 have been separately studied in males and in female rats (above), the present study is the first to directly compare drug effects across the sexes in this animal model. The stimulus for making these comparisons comes from the sex differences in ASR and PPI that have been seen in human disorders such as schizophrenia that are believed to be modeled by acute MK801 administration in animals. More specifically, I wished to determine whether the face and predictive validity of the NMDAR hypofunction model in rats extends to sex differences in symptomatology of schizophrenia. The data obtained in this study that suggests that it does. First, published data indicate that women suffering from schizophrenia have more highly exaggerated startle responses than do men (Kumari et al., 2004). These patterns are consistent with findings from this study showing that MK801-induced NMDAR hypofunction has markedly greater effects on the ASR in female compared to

male rats. As ASR is closely linked to the affective state of the subject and increased startle responses have been linked to fear, anxiety and generally negative affective state (Cook et al., 1991; Bradley et al., 1993; Grillon and Baas, 2003), it stands out as an affective symptom correlate of schizophrenia and the greater increase in ASR in females induced by MK801 indeed recapitulates the especial vulnerability of females to the affective symptoms of this disorder. Furthermore, as increased ASR has also been linked to the positive symptoms of schizophrenia (Lang et al., 1990; Geyer et al., 2001; Castagne et al., 2009), the findings here may also recapitulate the findings of a predominance of positive symptoms such as irritability and bizarre behavior in female over male schizophrenics. Likewise, findings that MK801 impairs PPI in both male and female rats matches findings of significant deficits in PPI in both male and female schizophrenics compared to healthy controls (Braff et al., 1999; Braff et al., 2005; Castagne et al., 2009). More importantly, however, findings that MK801 was significantly more disruptive to PPI in male compared to female rats are also consistent with evidence of a greater reduction in PPI in male compared to female schizophrenics (Kumari et al., 2004). As an indicator of defective sensorimotor gating which is theorized to result in cognitive fragmentation (Swerdlow et al., 1994; Swerdlow and Geyer, 1998), disruptions in PPI have been linked to the negative/cognitive symptoms of schizophrenia (Karper et al., 1996; Powell et al., 2009). Accordingly, the results from this study showing significantly greater PPI deficits in male compared to female rats supports the NMDAR hypofunction model as also recapitulating the disproportionate representation of negative symptoms in male compared to female schizophrenics (Lewine, 1981; Szymanski et al., 1995; Leung and Chue, 2000). In the section below

we take the model a step further; while it is known to have validity in predicting treatment efficacy, here we asked whether it has validity in predicting sex-specific treatment efficacy-- an issue that is critical as sex-specific treatments and gonadal steroids as adjunct therapies are increasingly adopted in the treatment of mental illness.

Sex Differences in the Effects of Neuroleptic Pretreatment

Behavioral assessments of ASR and PPI in animal models have served as staple screens for predicting the therapeutic efficacy of neuroleptic and other antipsychotic agents (Varty and Higgins, 1995; Swerdlow and Geyer, 1998). While it is generally held that in schizophrenia male and female patients respond differentially to typical and atypical neuroleptics, to my knowledge the present studies are the first to use animal models to explore whether two currently used therapeutic drugs are differentially able to attenuate the effects of acute NMDAR hypofunction, i.e., of MK801 on ASR and/or PPI in male compared to female rats.

My first investigations focused on the typical neuroleptic haloperidol. A significant body of clinical literature shows that haloperidol is effective at ameliorating both positive and negative symptoms of schizophrenia in both men and women (Labarca et al., 1993; Palao et al., 1994; Awad et al., 1997) at high and low dose equivalents to those used here. Nonetheless, the findings from this study mirrored those previously obtained in rats, albeit to date only in males; that haloperidol failed to attenuate MK801-induced increases in ASR and deficits in PPI. While the present

studies extend these findings to females, they offer little insight as to how to reconcile the disparities between these outcomes from those predicted from the clinical literature. Given the pharmacological profile of haloperidol, the most parsimonious explanation is one that invokes limitations of acute MK801/NMDAR hypofunction to fully model the dopaminergic phenotypes associated with of the neurochemically complex disorder of schizophrenia.

In contrast to haloperidol, clozapine was found to effectively antagonize MK801-induced disruptions in both ASR and PPI. However, in the case of ASR, clozapine was found to have roughly equal efficacy in restoring ASR in males and females alike and roughly equal potency at both the higher and lower dose used. In contrast, clozapine effects on PPI were both more efficacious in males and more obviously dose-dependent. That is, the lower dose of clozapine aptly restored PPI in males but was minimally effective in females. Further, while the higher clozapine dose was more effective than the low in both sexes, it was again clearly more potent in rescuing PPI in males.

The findings in males with respect to ASR are in agreement with previous studies in rats showing attenuation of MK801-induced increases in startle that were more or less independent of clozapine dose (Bakshi et al., 1994; Varty and Higgins, 1995; Bubenikova et al., 2005). The findings here are also consistent with those showing that clozapine attenuates MK801-induced PPI deficits in a dose-dependent manner (Bakshi et al., 1994; Bortolato et al., 2005; Bubenikova et al., 2005). However, it should be

noted that some studies found clozapine to be ineffective in attenuating PPI disrupted by MK801 at doses similar to those used in this study and in others where clozapine rescue was clear (Bast et al., 2000; Lombardo et al., 2009). In considering the source for these differences it may be important to note that the study by Lombardo and colleagues was unique in that clozapine was administered orally whereas a parenteral route was used here and in the other cited studies. As drug bioavailability is markedly lower via the oral compared to any parenteral route (Kwan, 1997; Manjunath and Venkateswarlu, 2005), it is conceivable that the effective serum and brain levels of clozapine were much lower than those achieved in this and other studies, a fact which can reasonably explain the lack of clozapine effect seen in that experiment. Also, while Bast and colleagues reported no significant effect of parenteral clozapine on rescuing PPI, assessments were made with PPI merged into an overall value across four prepulse intensities spanning +4-16 dB. Thus, it is conceivable that by merging PPI across prepulses, a possible rescue effect at the higher prepulses was masked. Hence, while some discrepancies in the literature exist, the results here are most parsimonious with studies using similar experimental designs and analysis and in turn indicate that clozapine is indeed effective at restoring MK801-disrupted ASR and PPI and in turn, is more effective in males.

In explaining this greater effectiveness of clozapine that we found in restoring PPI in males several potentially causal factors should be considered. Sex differences in drug metabolism, for example is an important factor. However, in the case of clozapine, there are several reasons to suspect that this is not a primary cause for the sex

differences observed. First, drug effects on ASR were virtually identical in male and female subjects. Further, the typically slower metabolic rates found in females would favor a prolonged drug action in females compared to males. Thus, a more intriguing explanation is one that takes into account the fact that clozapine is pharmacologically active at a several receptor subtypes in the brain including 5HT_{1A}, 5HT_{2A}, 5HT_{2C}, α 1, α 2, and histamine H₁ receptors. Thus, it is possible that sex differences in its efficacy here relate to sex differences in the transmitter systems that it impacts. In pinpointing this further, it may be instructive to focus on those circuit elements that distinguish PPI from its essential output ASR. Thus, the serotonergic system and its innervation of target brain areas including frontal, cingulate and piriform cortices and nucleus accumbens (structures modulating PPI of startle but not part of the circuit producing ASR) stands out as a potential candidate with explanatory power for this difference in drug response. First, serotonergic circuits have been shown to be involved in the complex system modulating PPI (Koch, 1999). Further, animal studies have indicated that overactivity of serotonin at 5HT_{1A} (Sipes and Geyer, 1995a; Gogos and Van den Buuse, 2004) and 5HT_{2A} receptors (Sipes and Geyer, 1995b) can robustly disrupt PPI and that antagonist drugs acting selectively at these sites can restore PPI disrupted by MK801 (Varty et al., 1999; van den Buuse and Gogos, 2007; Bubenikova-Valesova et al., 2010). Finally, the density of 5HT_{2A} (but not 5HT_{1A}) binding sites has been shown to significantly increase in parallel with estrogen levels over the course of the estrus cycle in brain areas like the frontal cortices and nucleus accumbens, structures that are critically involved in modulating PPI (Sumner and Fink, 1997; Cyr et al., 1998; Sumner and Fink, 1998; Cyr et al., 2002). Taken together, these findings draw focus to the 5HT_{2A} receptor and

suggest that a relative excess of 5HT_{2A} sites in females that need to be blocked by clozapine in order for it to rescue PPI may put this sex at disadvantage in terms of its potency, particularly in proestrus. Also, it should be noted that other transmitter systems may also contribute to this sex difference. For example, as selective antagonism at the histamine H₁ receptor has also been shown to reverse MK801-disrupted PPI (Roegge et al., 2007), further investigation is required to ascertain whether the histamine and other systems are involved in this differential effect of clozapine on PPI.

Conclusion

In sum, the studies here identify that acute MK801 challenge has reasonable face and construct validity in modeling sex differences seen in schizophrenia's symptomatology and that it is suitable for investigating their underlying neurobiology. Furthermore, these studies also indicate that this model can also be applied to objectively screen putative treatment regimens for sex-specific efficacy. Finally, findings outlined here of sex-specific efficacy of clozapine present several intriguing targets that may hold relevance to the sex-specific treatment of schizophrenia and may spur the screening and development of sex-specific treatment regimens for this debilitating disease. With this data in hand, the studies in the next chapter of this dissertation expand the characterization of sex differences effects of NMDAR hypofunction and neuroleptic pretreatment by utilizing open field testing to explore a wide range of

behaviors also relevant to the positive, negative and affective symptoms of schizophrenia.

GROUP	TESTING DAY 1	TESTING DAY 2	TESTING DAY 3
VEH/VEH	VEH / VEH	VEH / VEH	VEH / VEH
VEH/0.2 MK801	VEH / 0.2 MK801	VEH / 0.2 MK801	VEH / 0.2 MK801
VEH/DR	VEH / 0.05 MK801	VEH / 0.1 MK801	VEH / 0.2 MK801
HDL/MK	VEH / 0.2 MK801	0.04 HDL / 0.2 MK801	0.08 HDL / 0.2 MK801
CLZ/MK	VEH / 0.2 MK801	5 CLZ / 0.2 MK801	10 CLZ / 0.2 MK801

Table I.1: Testing schedule of all five experimental groups following acclimatization on Day 0. All doses are in mg/kg.

VEH / VEH

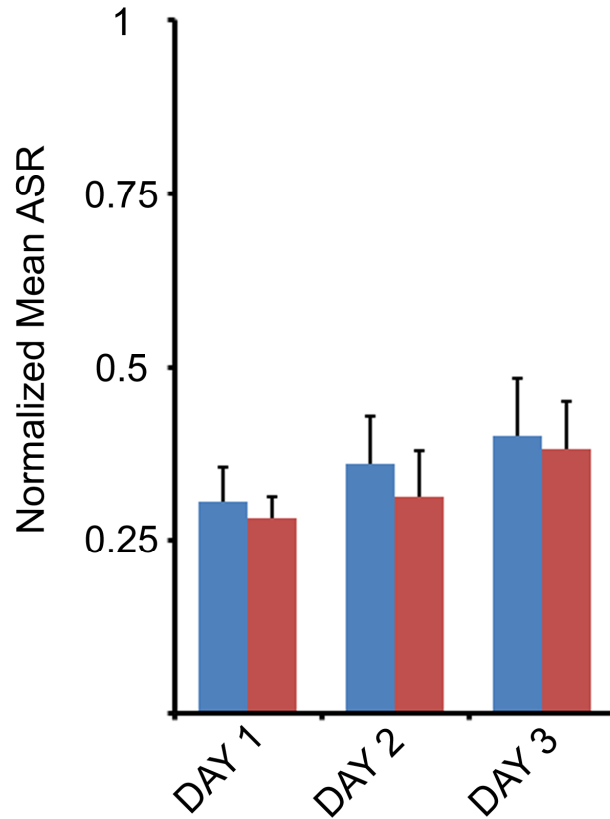


Figure I.1. Bar graph showing mean amplitudes of the acoustic startle reflex (ASR) normalized to body weight (\pm standard error of the mean) of male (blue bars) and female (red bars) rats that were vehicle-treated on three consecutive testing sessions. There were no significant effects of repeated testing in both males and females alike and there were no significant differences in ASR between males and females on any of the three testing days.

VEH / 0.2 MK801

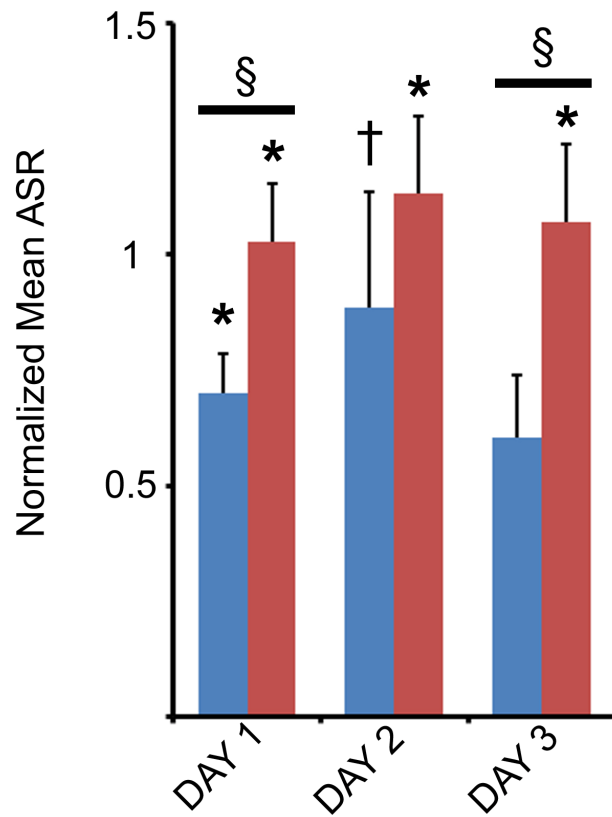


Figure I.2. Bar graph showing mean amplitudes of the acoustic startle reflex (ASR) normalized to body weight (\pm standard error of the mean) of male (blue bars) and female (red bars) rats that were treated with 0.2 mg/kg MK801 on three consecutive testing sessions. Asterisks (*) indicate a significant difference from values at corresponding testing sessions in drug-naïve controls (VEH/VEH animals), crosses (†) indicate a near-significant difference from values at corresponding testing sessions in drug-naïve controls and section signs (§) indicate a significant difference between males and females.

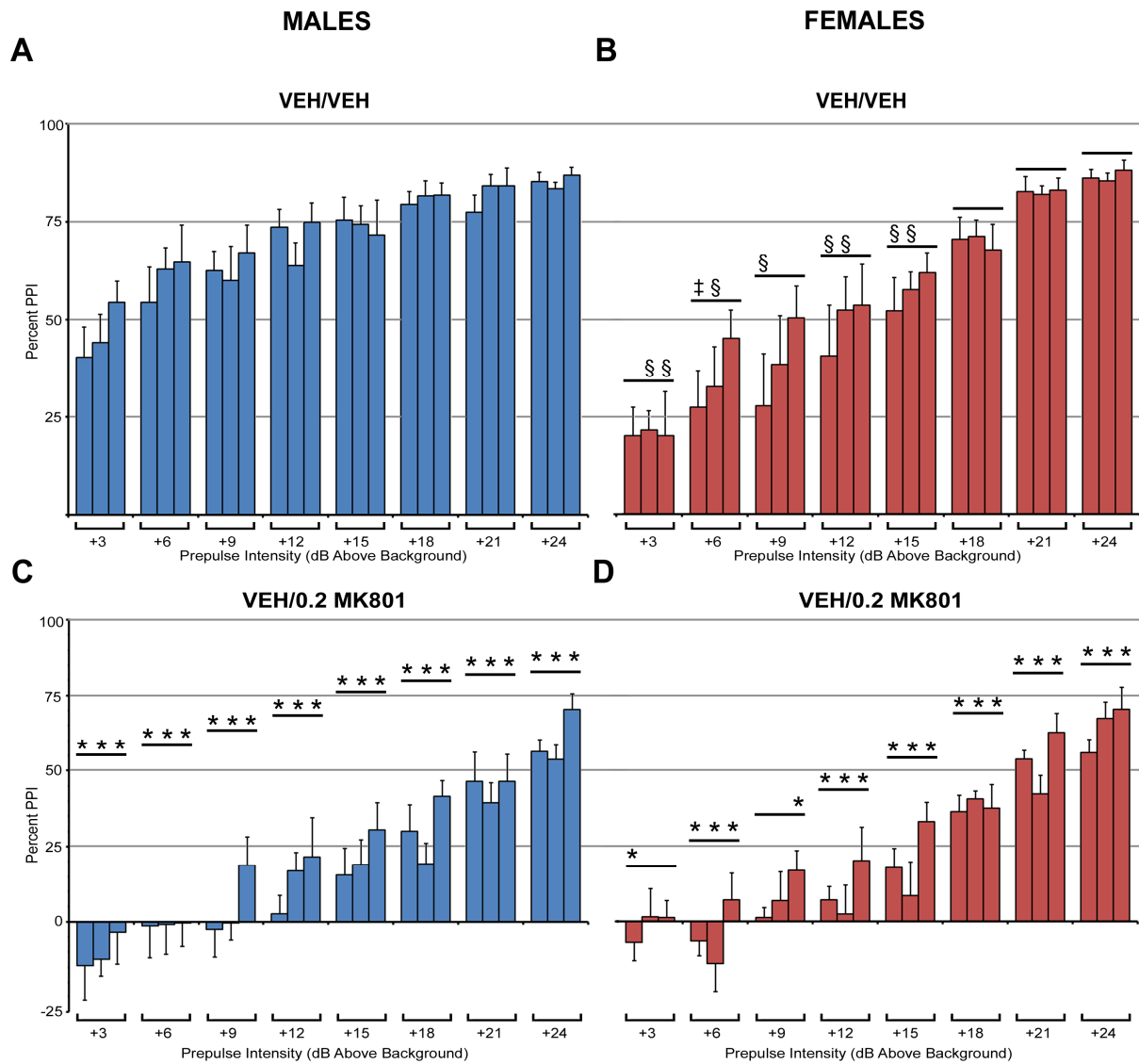


Figure I.3. A,B: Bar graphs showing average percent PPI at eight prepulse intensities of male (A) and female (B) rats that were vehicle-treated on three consecutive testing sessions. The left-most bars show PPI values from the first testing session, the middle bars show PPI values from the second testing session and the right-most bars show PPI values from the third testing session. Section signs (§) indicate a significant difference in PPI ($p < 0.05$) between males and females at individual prepulse intensities and double cross signs (‡) indicate a near-significant ($p < 0.075$) difference in PPI between males and females. C,D: Bar graphs showing average percent PPI at eight prepulse intensities of male (C) and female (D) rats that were treated with 0.2 mg/kg MK801 on three consecutive testing sessions; the right-most bars show PPI values from the first testing session. Horizontal black lines above the bars indicate the average PPI values in the vehicle-injected cohort for reference. Asterisks (*) indicate a significant difference from drug-naïve controls (VEH/VEH animals) at corresponding individual prepulse intensities. All data is presented as mean \pm SEM.

DOSE RESPONSE

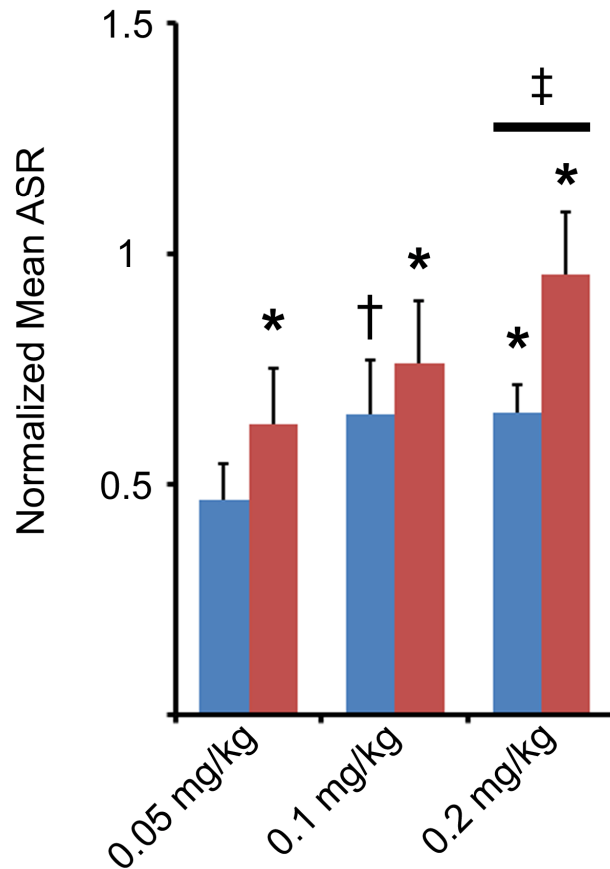


Figure I.4. Bar graph showing mean amplitudes of the acoustic startle reflex (ASR) normalized to body weight (\pm standard error of the mean) of male (blue bars) and female (red bars) rats that were in the MK801 dose response group. Rats were treated with 0.05 mg/kg MK801 on the first testing session, 0.1 mg/kg MK801 on the second testing session, and 0.2 mg/kg MK801 on the third testing session. Asterisks (*) indicate a significant difference from values at corresponding testing sessions in drug-naïve controls (VEH/VEH animals), crosses (†) indicate a near-significant difference from values at corresponding testing sessions in drug-naïve controls and double crosses (‡) indicate a near-significant difference between males and females.

DOSE RESPONSE

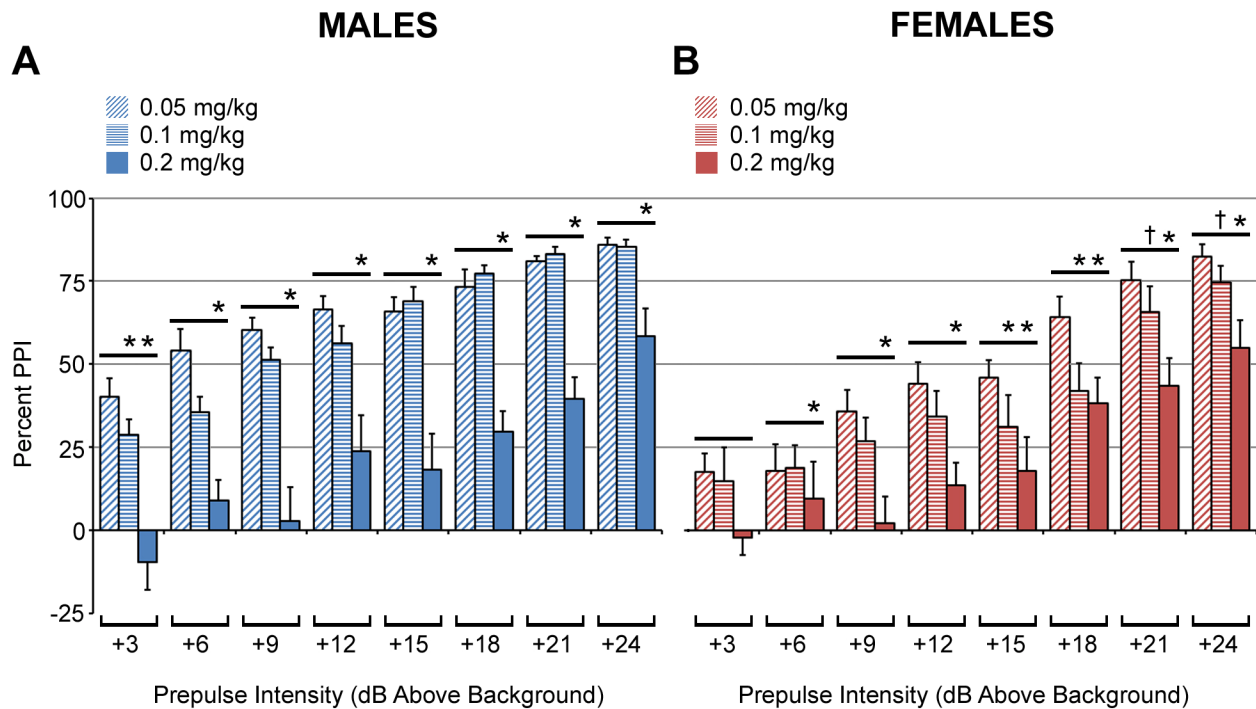


Figure I.5. Bar graphs showing average percent PPI at eight prepulse intensities of male (A) and female (B) rats in the MK801 dose response group. Rats were treated with 0.05 mg/kg MK801 on the first testing session (diagonal lines), 0.1 mg/kg MK801 on the second testing session (horizontal lines) and 0.2 mg/kg MK801 on the third testing session (solid fill). Asterisks (*) indicate a significant difference ($p < 0.05$) from drug-naïve controls (VEH/VEH animals) at corresponding prepulse intensities and crosses (†) indicate a near-significant difference from drug-naïve controls at corresponding prepulse intensities. All data is presented as mean \pm SEM.

HDL / MK801

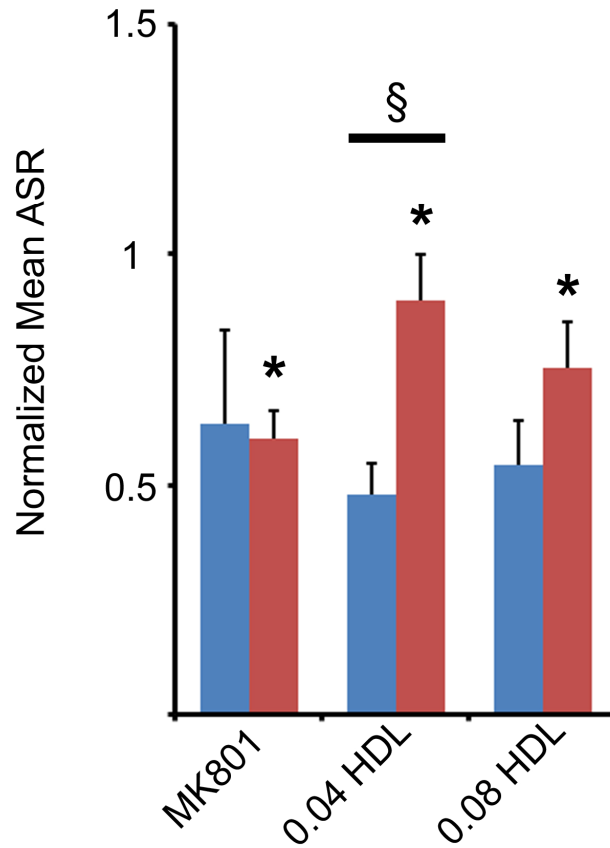


Figure I.6. Bar graph showing mean amplitudes of the acoustic startle reflex (ASR) normalized to body weight (\pm standard error of the mean) of male (blue bars) and female (red bars) rats that were in the haloperidol pretreatment group. Rats were treated with 0.2 mg/kg MK801 on the first testing session (MK801), with 0.04 mg/kg haloperidol followed by 0.2 mg/kg MK801 on the second testing session (0.04 HDL) and 0.08 mg/kg haloperidol on the third testing session (0.08 HDL). Asterisks (*) indicate a significant difference from values at corresponding testing sessions in drug-naïve controls (VEH/VEH animals), and section signs (§) indicate a significant difference between males and females.

HDL / MK801

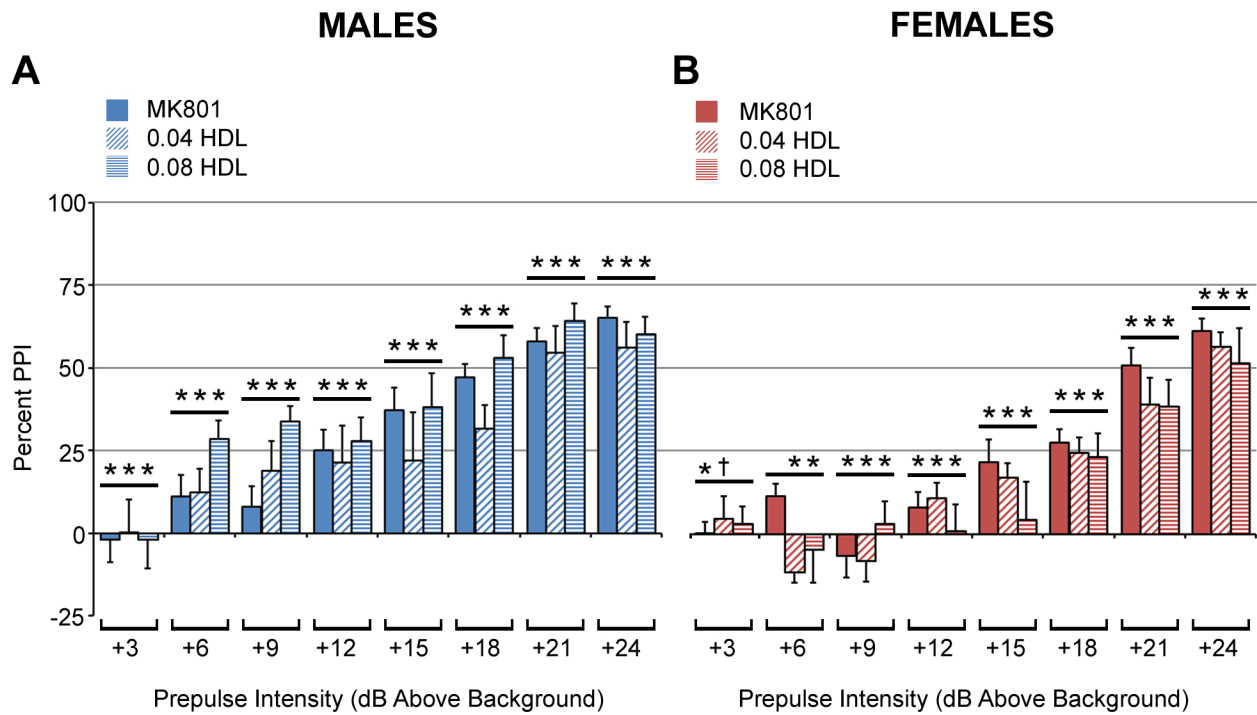


Figure I.7. Bar graphs showing average percent PPI at eight prepulse intensities of male (A) and female (B) rats in the haloperidol pretreatment group. Rats were treated with 0.2 mg/kg MK801 on the first testing session (solid fill), 0.04 mg/kg haloperidol followed by 0.2 mg/kg MK801 on the second testing session (diagonal lines) and 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 on the third testing session (horizontal lines). Asterisks (*) indicate a significant difference ($p < 0.05$) from drug-naïve controls (VEH/VEH animals) at corresponding prepulse intensities and crosses (†) indicate a near-significant difference from drug-naïve controls at corresponding prepulse intensities. All data is presented as mean \pm SEM.

CLZ / MK801

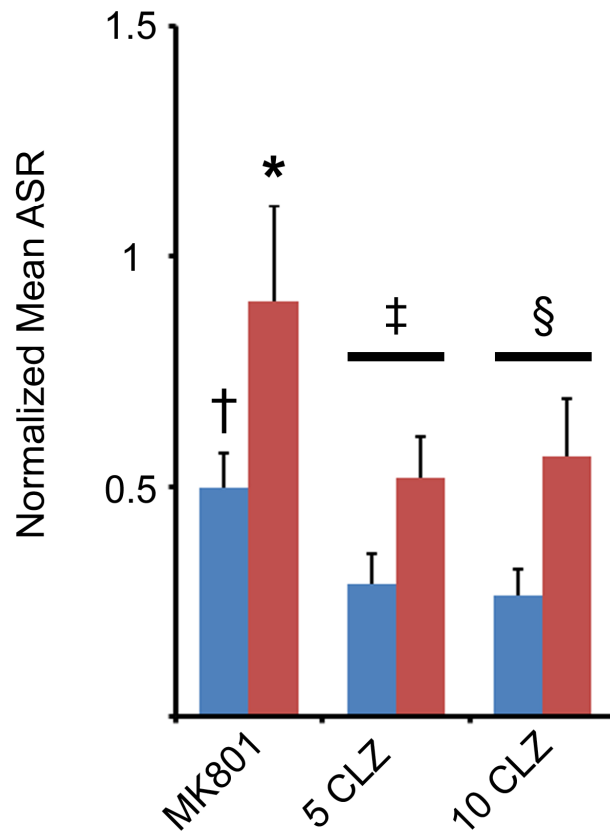


Figure I.8. Bar graph showing mean amplitudes of the acoustic startle reflex (ASR) normalized to body weight (\pm standard error of the mean) of male (blue bars) and female (red bars) rats that were in the clozapine pretreatment group. Rats were treated with 0.2 mg/kg MK801 on the first testing session (MK801), with 5 mg/kg clozapine followed by 0.2 mg/kg MK801 on the second testing session (5 CLZ) and 10 mg/kg clozapine on the third testing session (10 CLZ). Asterisks (*) indicate a significant difference from values at corresponding testing sessions in drug-naïve controls (VEH/VEH animals), and crosses (†) indicate a near-significant difference from values at corresponding testing sessions in drug-naïve controls. Section signs (§) indicate a significant difference between males and females and double crosses (‡) indicate a near-significant difference between males and females.

CLZ / MK801

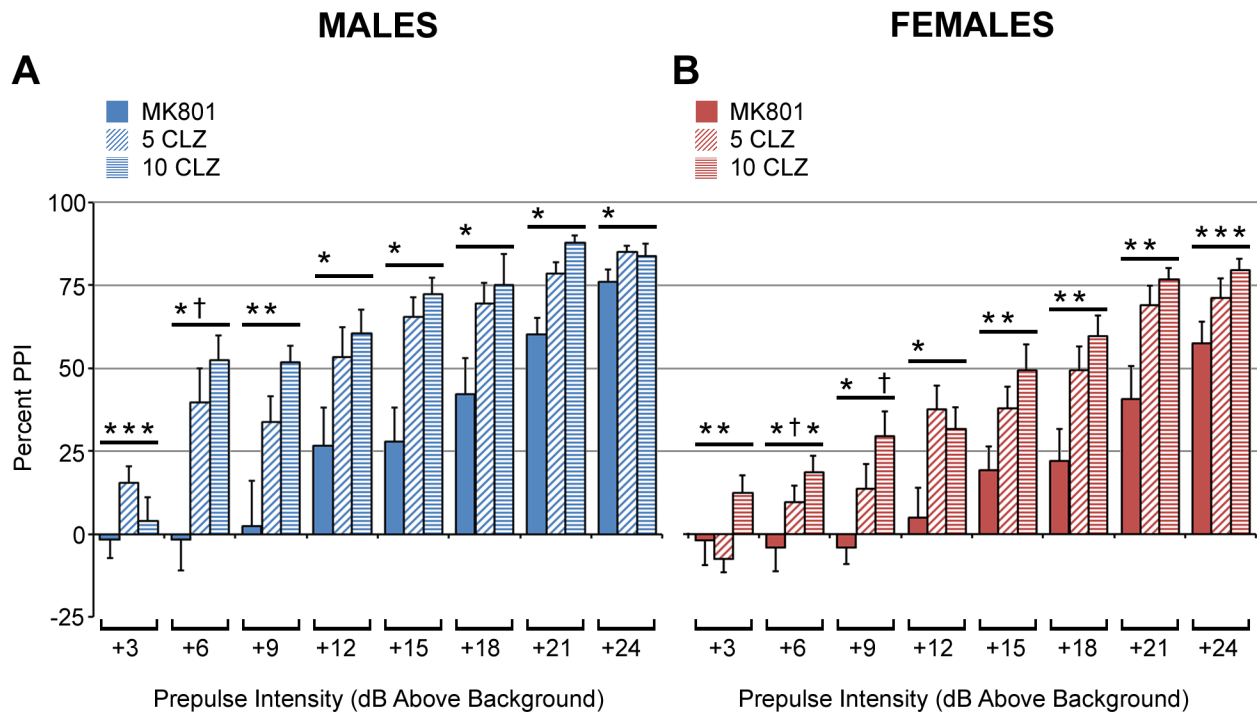


Figure I.9. Bar graphs showing average percent PPI at eight prepulse intensities of male (A) and female (B) rats in the clozapine pretreatment group. Rats were treated with 0.2 mg/kg MK801 on the first testing session (solid fill), 5 mg/kg clozapine followed by 0.2 mg/kg MK801 on the second testing session (diagonal lines) and 10 mg/kg clozapine followed by 0.2 mg/kg MK801 on the third testing session (horizontal lines). Asterisks (*) indicate a significant difference ($p < 0.05$) from drug-naïve controls (VEH/VEH animals) at corresponding prepulse intensities and crosses (†) indicate a near-significant difference from drug-naïve controls at corresponding prepulse intensities. All data is presented as mean \pm SEM.

CHAPTER II

NMDA receptor hypofunction evokes sex-specific changes in behavior and behavioral dynamics in open field testing in adult male and female rats

The behavioral sequelae of schizophrenia are complex and include a range of positive, negative and affective symptoms. That these symptoms often differentially affect and/or are differentially attenuated by treatment with neuroleptic drugs in males versus females suffering from this disorder is a primary stimulus for the studies of my dissertation. More specifically, it is the work of this dissertation to determine whether rodent models of this disorder have utility or validity in modeling these clinical sex differences. Whereas it is impossible to fully recapitulate fundamentally human symptoms such as hallucinations and delusions with any animal model, the core positive, negative and affective symptoms of schizophrenia do have recognized behavioral analogs in rodent models of this disorder. My work takes a systematic approach to determining whether these differ in male and female rats. In the previous chapter, measures of behavioral endpoints of PPI and ASR provided the first indications that the focus of my studies, the NMDAR hypofunction model in rats induced by acute MK801, indeed has validity for not only producing positive, affective and negative symptom correlates of schizophrenia, but for producing sex differences in these behavioral measures as well. Here I extend these analyses to additional behavioral metrics that also represent schizophrenia's diverse symptom clusters using an open field testing paradigm.

It has long been known that rats treated with psychomimetic drugs such as amphetamine, phencyclidine (PCP) and MK801 exhibit marked hyperactivity, ataxia and stereotypic movements (Castellani and Adams, 1981; Hoffman, 1992; Ouagazzal et al., 1994). It has also been recognized and accepted that these behaviors have certain face validity for modeling positive symptoms of schizophrenia such as bizarre behavior and motor over-activity that are commonly seen in patients— and especially in female patients (Goldstein and Link, 1988; Perry et al., 2010). These behaviors are also well known to be amenable to quantitative study in open field testing which is an untrained, unrewarded behavioral paradigm in which an animal is placed into an open arena (enclosed by walls) and allowed to freely explore for a fixed period of time. During exploration, spontaneous behaviors are assessed and measured. The advantages of this paradigm are that it allows observation of multiple behaviors in their innate state, i.e. unaffected by training and not driven by desire for reward (Walsh and Cummins, 1976). It is also regarded as an extremely sensitive task, capable of identifying even subtle perturbations in behavior, although not one that is amenable to correlating observed behavioral effects to specific brain systems or structures. Finally, it is a paradigm that has been successfully used to study behavioral responses of rats to various drug treatments including acute treatment with NMDA receptor antagonists such as PCP and MK801 (Hargreaves and Cain, 1992; Hoffman, 1992; Hargreaves and Cain, 1995). As has been stated previously, however, analyses of schizophrenic-like behaviors in animals including those carried out in open field testing have largely ignored the question of sex differences. Furthermore, of the very few studies that asked questions of sex differences, none have addressed the companion issue of sex

differences in the effectiveness of neuroleptic pretreatment to reverse behavioral changes induced by NMDAR antagonism. Both of these important issues are addressed in this and other chapters of this dissertation.

In approaching this study, it was appreciated that the full set of behaviors that are elicited and measurable in an open field setting extends far beyond the positive symptom correlates, e.g., stereotypy, ataxia, that have been the main foci of previous studies. Thus, in addition to locomotor behaviors, rats in an open field also exhibit several other innate behaviors, including some that also hold face validity for investigating the negative and affective symptom clusters of schizophrenia. For example, rats spontaneously engage in thigmotaxis, grooming, rearing, sniffing and other activities. While these have been less often studied specifically in the context of NMDAR hypofunction, there are some precedent-setting studies that do suggest their sensitivity to MK801 (Tang et al., 2006; Manahan-Vaughan et al., 2008). Here these behaviors and their drug-induced absence or exacerbation are considered alongside analyses of locomotion, stereotypy and ataxia as critical parts of a comprehensive, continued investigation into the basic questions of the ability of NMDAR hypofunction to model sex differences in the positive, negative and affective symptoms of schizophrenia and explore sex differences in the ability of typical and atypical neuroleptics to attenuate them. To achieve these objectives, the experiments presented in this chapter assessed open field testing at baseline, after acute treatment with MK801 and after pretreatment with haloperidol and clozapine prior to MK801 in male and proestrus female rats.

METHODS

Animal Subjects: A total of 64 adult male and 64 adult female Sprague-Dawley rats (Taconic Farms, Hudson, NY) were used. Throughout the testing period, animals were housed in same-sex pairs on a 12:12 hour light/dark cycle (lights on 7 AM) with food and water available *ad libitum*. All experimental procedures involving vertebrate animals were approved by the Institutional Animal Care and Use Committee of SUNY Stony Brook and were designed to minimize animal use and discomfort.

Determination of Estrus Cycle Stage in Females and Yoking in Males: Female rats underwent daily vaginal lavage for at least one week to determine the stage and regularity of the estrus cycle (Marcondes et al., 2002; Goldman et al., 2007). Only rats that cycled regularly were included in the study. Lavage was performed between 11 AM and 2 PM, and was facilitated by wrapping the animals gently in a thick towel and gently restraining them supine against the tabletop; this process was designed to minimize stress and animal motion during the lavage procedure. Each female was yoked to a male animal that was handled in an equivalent manner on the same days to minimize potential for handling bias. After tracking over at least one complete estrus cycle, females found to be in proestrus were randomly assigned to an experimental group (see below) and behaviorally tested on that day; the yoked male subject was assigned to the same drug treatment group and tested on the same day.

Drugs and Drug Treatments: All drugs used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO). Each animal received two injections on the day of

testing. Rats first received a subcutaneous injection of either haloperidol (0.04 or 0.08 mg/kg) or clozapine (5 or 10 mg/kg) dissolved in saline acidified with 0.025% acetic acid or acidified saline vehicle. Fifteen minutes later, rats were injected intraperitoneally with either MK801 (0.05, 0.1 or 0.2 mg/kg) dissolved in saline, or saline vehicle. Eight female rats in proestrus and eight yoked male rats were randomly assigned to one of the following eight groups: vehicle/vehicle; vehicle/0.05, 0.1 or 0.2 mg/kg MK801; 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801; or 5 or 10 mg/kg clozapine followed by 0.2 mg/kg MK801. All animals were behaviorally tested 15 minutes after the second injection.

Open Field Apparatus and Testing: The open field arena was located in a sound attenuated room and consisted of an opaque plastic enclosure, measuring 80 x 30 cm with walls 30 cm high; the floor was marked in 10 x 10 cm squares. The arena was surrounded by a white curtain to minimize extra-maze cues. A video camera (Apple iSight) suspended one meter above the arena floor was used to record the animals' activity in the arena on a computer (Apple Powerbook/Apple iMovie). On the day of testing, animals were brought into the testing room for the first time and allowed to acclimate in a holding cage for 5 minutes. They were then transferred to the start position in the center of the arena and allowed to freely explore for 15 minutes. During this time the investigator remained outside of the room. After testing, the animal was returned to its home cage and the arena floor and walls were cleaned with 70% ethanol. Individual animals were only tested once.

Behavioral Analysis: All analyses of behavior were performed by a single observer. Assessments were performed off-line from the archived video recordings and were scored using event-capture software (JWatcher 1.0). Each video was analyzed for the following behaviors:

- Locomotion: defined as sustained forward motion of front and hind paws (measured as amount of time spent and as number of line crosses of the rat's midsection)
- Stereotypic behaviors (measured as the sum of the amounts of time spent on the behaviors below):
 - Head-weaving (defined as rapid, repeated side-to-side motion of the proximal end of the rat; to be counted as head-weaving required that animals made two or more consecutive movements wherein the head deviated more than 45 degrees from the body axis).
 - Circling (defined as a closed loop of locomotion along a small diameter path that started and ended at the same point without detours)
 - Axial rotation (defined as repeatedly turning of the body around the haunches)
- Discrete ataxic behaviors (measured as of the total number of instances of the behaviors below):
 - Falling over; all four feet losing contact with the floor.
 - Failed rearing; an attempt to rear that resulted in animal falling or failing to elevate itself.

- Rearing: either in contact with the wall or not in contact with any surface (measured as instances and duration)
- Grooming (measured as duration)
- Thigmotaxis: defined as staying in contact with the wall during locomotion (measured as percent of time in locomotion)
- Time stationary: defined as the sum of time the rat spent sniffing and the time when no behaviors were evident (measured as duration)

Data Analysis and Statistics: Each behavioral metric was measured as above per 1 minute intervals. The data was then summed together and statistical analyses were carried out on these summed values. All statistical tests were conducted using SPSS software (IBM, version 19). For each behavior, two-way analyses of variance (ANOVA) were first used for comparisons of the data across sex and across drug treatment. Further, one-way ANOVA was also used to compare data within each treatment condition across sex. Finally, one-way ANOVA was also used to compare data within each sex across treatment conditions. All allowed post-hoc testing was conducted using Fisher's protected least significant difference (PLSD) test; all observations were considered significant at a $p < 0.05$ level and near-significant at a $p < 0.075$ level.

RESULTS

Behavioral metrics were divided into three broad categories. The first included behaviors that involved some sort of sustained forward motion and consisted of locomotion and thigmotaxis. The second category involved behaviors that were conducted with animals remained in a fixed place; these included rearing, grooming and stationary behavior. The third category was abnormal behavior, i.e., that not seen drug-naïve animals: these included stereotypy, and ataxia. These three categories are presented separately below.

Behaviors of Sustained Forward Motion:

Locomotion: At the start of the testing session, vehicle injected male and proestrus female rats both made between 35 and 45 line crossings/minute. However, with increased time in the arena, line crosses in both sexes progressively decreased and by sessions' end, only some 5-10 line crossings/minute were being made. Of the three doses of MK801 used, the lowest (0.05 mg/kg) had no discernible effect on locomotion in either males or females. The middle dose (0.1 mg/kg) was also without effect in males. However, in females 0.1 mg/kg MK801 produced line crossings that began at levels that were similar to control (~50 line crossings/minute), but that remained at roughly this rate for the duration of the trial, resulting in a roughly 150% increase in line crossings overall. Finally, the highest dose (0.2 mg/kg) of MK801 increased line crossings in both sexes, albeit more so in females. Thus, males made from 40-50 crossings/minute throughout the duration of the trial which was roughly a 2-fold increase

in total locomotion from control. However, females made approximately 60-65 line crossings/minute which was roughly a 240% increase in total line crossings from baseline (Figure II.1A,B).

Pretreatment with haloperidol and clozapine both showed some ability to reduce the hyperlocomotion induced by 0.2 mg/kg MK801. However, clear differences in potency were observed both between the two drugs and between the two sexes. Thus, haloperidol pretreatment in males had no discernible effect on MK801-induced hyperlocomotion at the lower dose used (0.04 mg/kg). However, at the higher dose (0.08 mg/kg) haloperidol did diminish MK801-induced hyperlocomotion by about 50% and also restored the habituation of this behavior with time spent in the arena (Figure II.1C). In contrast, in the females, both doses of haloperidol decreased MK801-induced hyperlocomotion (by some 25-40% overall), and both doses restored its habituation over time (Figure II.1D). Compared to haloperidol, however, clozapine pretreatment was more effective in attenuating MK801-induced effects on locomotion in both sexes. Thus, in males, pretreatment with either the high or low dose of clozapine produced an overall decrease in MK801-induced hyperlocomotion of around 70-75 percent, bringing it to within 5-15% of control. In females, the lower dose of clozapine (5 mg/kg) showed similar potency, i.e., it brought locomotion levels down to near that of drug naïve controls. However, at 10 mg/kg, clozapine much more markedly reduced locomotion to values that were well below baselines established in the drug-naïve control (Figure II.1C,D).

The drug and sex differences observed in locomotor behavior were also supported in statistical comparisons of total locomotor scores (total line crossings, see Figure II.1E). First, an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified significant main effects of drug treatment [$F(7,111)=34.50$ $p<.001$] and of sex [$F(1,111)=13.24$ $p<.001$] and also revealed a significant interaction between the two [$F(7,111)=8.69$ $p<.001$]. A subsequent series of one-way ANOVAs that compared data from each treatment group across sex confirmed that locomotion in male and female rats that were drug-naïve or treated with 0.05 mg/kg MK801 did not differ, but that after 0.1 or 0.2 mg/kg MK801, locomotion was significantly higher in female compared to male rats (0.1 mg/kg: $F(1,15)=29.77$ $p<.001$; 0.2 mg/kg: [$F(1,14)=5.10$ $p=.042$]). Finally, one-way ANOVAs that compared data within sex across all treatment groups identified significant main effects of treatment in males [$F(7,62)=15.70$ $p<.001$] and in females [$F(7,63)=27.52$ $p<.001$]. Allowed post-hoc testing further showed that in males, only 0.2 mg/kg MK801 significantly increased locomotion relative to drug-naïve levels ($p<.001$); that pretreatment with the high dose of haloperidol and both doses of clozapine significantly attenuated MK801-induced hyperlocomotion (all $p<.001$); and that all three of these pretreatments produced locomotor activity that was statistically indistinguishable from that of drug-naïve controls. For the females, post-hoc testing showed that both the 0.1 and 0.2 mg/kg doses of MK801 significantly increased locomotor activity relative to control (both $p<.001$); that locomotion produced by 0.2 mg/kg MK801 was significantly higher than that produced by the 0.1 mg/kg dose ($p=.006$); and that, the

hyperlocomotion produced by 0.2 mg/kg MK801 was significantly attenuated by both doses of haloperidol (0.04 mg/kg: $p < .001$, 0.08 mg/kg: $p = .017$) and both doses of clozapine (both $p < .001$). However, neither dose of haloperidol fully restored locomotion to baseline (drug naïve) (different from control, $p < .001$) and while 5 mg/kg clozapine did reduce locomotion to control levels, the 10 mg/kg clozapine pretreatment yielded locomotion that was significantly *lower* than control ($p = .009$).

Thigmotaxis: Expressed as percent of time spent on locomotion, vehicle injected male and female rats engaged in thigmotaxis 30% and 45% of the time during the first few minutes of testing, respectively. With time spent in the arena, however, thigmotaxis steadily decreased and accounted for only some 10% of locomotion time by sessions' end. Treatment with MK801 had no appreciable effect on thigmotaxis in females at any dose, and had no effect on males at the 0.05 mg/kg dose. However, at 0.1 and 0.2 mg/kg, MK801 nearly doubled the amount of time males engaged in thigmotaxis and virtually eliminated its habituation over time (Figure II.2A,B).

Thigmotaxis in female rats pretreated with either dose of haloperidol did not appreciably differ from that of control or rats given 0.2 mg/kg MK801 alone. In contrast, pretreatment with 0.04 and 0.08 mg/kg haloperidol in males attenuated the stimulatory effects of MK801 on thigmotaxis and restored its habituation to baseline levels (Figure II.2C). Finally, while the low (5 mg/kg) dose of clozapine virtually eliminated thigmotaxis in both sexes, pretreatment with the higher (10 mg/kg) dose restored thigmotaxis to baseline in males and had no effect on thigmotaxis in females (Figure II.2C,D).

The group differences in thigmotaxis observed above were also supported in statistical comparisons of total times spent in thigmotaxis (as percent of locomotion, see Figure II.2E). Thus, an initial two-way ANOVA encompassing data from both sexes and from all treatment groups identified a significant main effect of drug treatment [$F(7,111)=12.33$ $p<.001$] and a significant interaction between drug treatment and sex [$F(7,111)=3.67$ $p=.001$]. A follow-up series of one-way ANOVAs that compared data from each treatment group across sex showed that thigmotaxis did not differ between male and female rats that were drug-naïve or treated with 0.05 mg/kg MK801. However, there were near significant to significant male over female differences in the effects of MK801 at both the 0.1 mg/kg [$F(1,15)=3.90$ $p=.068$] and 0.2 mg/kg dose [$F(1,14)=14.90$ $p=.002$]. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified significant main effects of treatment in both sexes (male: [$F(7,62)=11.49$ $p<.001$]; females: [$F(7,63)=4.42$ $p=.001$]). Allowed post-hoc testing showed that in males treatment with 0.1 mg/kg and 0.2 mg/kg significantly increased thigmotaxis relative to control (both $p<.001$) and that pretreatment with both doses of haloperidol and both doses of clozapine significantly attenuated the MK801-induced increase in thigmotaxis (all $p<.001$). Surprisingly, while three of four of these pretreatments restored thigmotaxis to control levels, the *lower* dose of clozapine reduced thigmotaxis to levels that were significantly lower than control ($p=.005$). In females, on the other hand, MK801 had no significant effect on thigmotaxis at any dose; pretreatment with both doses of haloperidol and the higher dose of clozapine had no

impact on behavior; and as in the males, pretreatment with the *lower* dose of clozapine significantly reduced thigmotaxis relative to control ($p < .001$).

Behaviors Carried out in Place

Rearing: In vehicle injected animals, rearing was similar in both sexes with both males and females rearing 9-11 times per minute at the start of the testing session and progressively rearing less and less to end the with approximately 1-3 instances of rearing per minute. Of the three doses of MK801 used, the lowest (0.05 mg/kg) reduced rearing overall by about 60% in males and females; it also increased the rate of habituation (Figure II.3A,B). At the middle dose (0.1 mg/kg), rearing and the habituation of rearing was affected more in males; while both sexes started off rearing at the same rates, males quickly reduced activity to very low levels (about 70% reduction overall), while in females, rearing dropped off more gradually yielding an overall decrease of about 45%. After treatment with 0.2 mg/kg, rearing in males remained similar to that at the two lower doses but females now showed the more rapid habituation. It should be stated, however, that a relative basement effect due to near elimination of the behavior at this dose increased susceptibility to outlying data points, which were found to be driving the relatively elevated amount of rearing seen in males in this cohort.

Pretreatment with haloperidol at either dose did not restore MK801-disrupted rearing in males although in females, the low dose of haloperidol seemed to exert a partial rescue effect on MK801-disrupted rearing. Closer inspection, however, revealed

that the marginal increase in rearing observed in females was driven by scores from two of the eight animals that reared frequently over the entire testing interval. That, the high dose of haloperidol was ineffective in increasing rearing from MK801-depressed levels in females affirmed that these two points were outliers in the data from haloperidol-treated rats. Finally, pretreatment with both doses of clozapine were ineffective at reversing MK801-induced disruptions in rearing in males and females at either dose.

The group and sex differences in rearing that were observed were supported in statistical comparisons of total rearing instances (Figure II.3E). Thus, an initial two-way ANOVA encompassing data from both sexes and all treatment groups identified a significant main effect of treatment [$F(7,111)=37.18$ $p<.001$] and a significant interaction between treatment and sex [$F(7,111)=3.68$ $p=.001$]. A subsequent series of one-way ANOVAs comparing data from each treatment group across sex showed that rearing was not significantly different among males and females that were drug-naïve or treated with 0.05 mg/kg MK801 but that rearing was significantly reduced in males compared to females after 0.1 mg/kg [$F(1,15)=6.05$ $p=.028$] and was significantly reduced in females compared to males after 0.2 mg/kg [$F(1,14)=9.47$ $p=.009$]. Finally, one-way within-sex ANOVAs that compared data across all treatment groups identified significant main effects of drug treatment on rearing in both sexes (males: $F(7,62)=16.00$ $p<.001$; females [$F(7,63)=24.58$ $p<.001$]) and were followed by post-hoc testing that showed that in both sexes, all three doses of MK801 significantly reduced rearing relative to control (all $p<.001$).

Grooming: Vehicle injected rats of both sexes exhibited minimal grooming at the start of testing, but engaged in grooming progressively more as time went on with both males and females spending about 20% of time grooming by sessions' end. At the lowest dose used (0.05mg/kg) MK801 had no effect on grooming in either sex. The middle dose (0.1 mg/kg) had no effect in females, but reduced time spent grooming overall by roughly 60% and delayed the onset of the behavior to the mid portion of the trials in males (Figure II.4A,B). At the highest dose however, grooming was severely reduced to non-existent levels in both sexes.

Pretreatment with haloperidol at both doses was ineffective in restoring MK801-disrupted grooming in male rats. In females, however, treatment with the low dose (0.04 mg/kg) seemingly partially restored grooming. However, closer analysis of the data revealed that this rescue effect was driven by scores from two of eight animals that spent an abnormally large amount of time grooming during the testing session. Thus, combined with the finding that the high dose of haloperidol did not exert a similar rescue effect to suggest that these points were aberrations and that haloperidol is ineffective in females at either dose. Likewise, clozapine also had no effect on restoring grooming or its increase with time at either dose in either sex.

The drug and sex differences described above were supported in statistical comparisons of total time spent grooming (Figure II.4E). First, a two-way ANOVA encompassing data from male and female subjects from all treatment groups identified a significant main effect of treatment [$F(7,111)=35.50$ $p<.001$] and a significant

interaction between treatment and sex [$F(7,111)=2.74$ $p=.012$]. Follow-up one-way ANOVAs comparing data from each treatment group across sex confirmed that total time spent grooming did not significantly differ between males and females that were drug-naïve or treated with 0.05 or 0.2 mg/kg MK801 but showed that grooming was significantly lower in males compared to females treated with 0.1 mg/kg MK801 [$F(1,15)=6.05$ $p=.028$]. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified significant main effects of treatment on grooming time in both sexes (males: [$F(7,62)=25.76$ $p<.001$]; females [$F(7,63)=15.53$ $p<.001$]). Allowed post-hoc testing showed that in males, 0.05 mg/kg MK801 had no effect on grooming, that both 0.1 and 0.2 mg/kg significantly reduced grooming time relative to control (both $p<.001$) and that none of the neuroleptic pretreatments rescued grooming. In females, the lowest dose of MK801 slightly but significantly increased grooming relative to drug-naïve control ($p=.018$), the 0.1 mg/kg dose had no effect on grooming, and the 0.2 mg/kg dose significantly reduced grooming relative to control ($p<.001$). Finally, pretreatment with haloperidol at the low dose yielded a slight but significant increase in grooming relative to MK801 alone ($p=.039$). This group difference was driven by aberrant scores from the two subjects described above. None of the other neuroleptic pretreatments had any effects on MK801-depressed levels of grooming in females.

Stationary Behavior: Vehicle injected rats of both sexes spent similar amounts of time stationary overall and both showed a progressive increase in stationary behavior over the course of the testing session. Thus, both sexes spent 20-30% of time stationary at

the start of the session that increased to 55-65% of time by the end of the session. The lowest and middle dose of MK801 used (0.05 and 0.1 mg/kg) had little effect on time spent stationary in either sex. However, treatment with 0.2 mg/kg MK801 markedly reduced stationary behavior in both sexes, diminishing it to values of about 15-25% of time at the start of testing that did not increase as the testing time went on (Figure II.5A,B).

Pretreatment with the lower dose of haloperidol had no impact on MK801-induced effects on stationary behavior in males or females. However, the high dose robustly antagonized the effects of MK801 in both sexes. Thus, males pretreated with 0.08 mg/kg spent roughly the same amount of time stationary at the start and end of testing as the controls. Clozapine was even more potent in reversing MK801-induced decreases in stationary activity. Thus, pretreatment with the low dose of clozapine reversed MK801-induced decreases in stationary activity to the same extent as the high dose of haloperidol while the high dose of clozapine nearly doubled the time both sexes spent stationary relative to drug-naïve controls (Figure II.5C,D).

The drug and sex differences described above in animals' stationary behavior proved to be statistically robust in comparisons of total time spent stationary (Figure II.5E). First, an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified significant main effects of treatment [$F(7,111)=25.54$ $p<.001$] and of sex [$F(1,111)=6.36$ $p=.013$] as well as a significant interaction between the two [$F(7,111)=2.41$ $p=.024$]. Subsequent one-way ANOVAs

that compared data from each treatment group across sex showed that time spent stationary was not significantly different between males and females that were vehicle injected but was significantly lower in females compared to males following treatment with 0.05 mg/kg ($p=.018$) and 0.1 mg/kg MK801 ($p=.009$) but that after treatment with 0.2 mg/kg MK801, time spent stationary was reduced to similar levels in both sexes and did not significantly differ between males and females. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified a significant main effect of treatment in both sexes (males: $F(7,62)=11.88$ $p<.001$; females [$F(7,63)=16.19$ $p<.001$]). Allowed post-hoc testing showed that for both sexes only the highest dose of MK801 significantly decreased total time spent stationary (both $p=.020$), that the low dose of haloperidol failed to significantly reverse this MK801-induced decrease in both males and females but that the high dose of haloperidol and the low dose of clozapine were effective at reversing this decrease (males: both treatments $p<.044$, females: both treatments $p<.004$) to values were statistically indistinguishable from drug-naïve control levels. Finally, the high dose of clozapine reversed the MK801-induced decrease in both males and females but increased time spent stationary to levels that were significantly higher than those in drug naïve controls (both $p<.001$).

Abnormal Behaviors:

Stereotypy: Stereotypy was non-existent in vehicle-injected males and females. However, treatment with 0.05 mg/kg MK801 produced small amounts of stereotyped behavior in both sexes; each spent approximately 7% of time engaged in stereotypic

behaviors. Treatment with 0.1 mg/kg, however, increased the time males spent in stereotypy to about 20% , while having roughly the same effect in females as the lower dose (~7% of time engaged in stereotypic behavior). Finally, after treatment with 0.2 mg/kg MK801, time spent in stereotypy remained on the order of about 20% in the male, and increased in females to similar levels, i.e., accounting for roughly 20% of all testing time (Figure II.6A,B,E).

Pretreatment with haloperidol showed no ability to diminish stereotypic behavior in either sex. Clozapine was also ineffective in reducing stereotypy at the low (5 mg/kg) dose. However, after pretreatment with 10 mg/kg clozapine, stereotypy was evenly reduced across the whole testing session by 35-45% relative to MK801 alone in both sexes yet still well above the non-existent levels in controls (Figure II.6C,D).

The results described above proved to be robust in statistical comparisons of total times spent in stereotypy during the trial (Figure II.6E). First, a two-way ANOVA encompassing all data from both sexes and all treatment groups identified a significant main effect of treatment [$F(7,111)=14.64$ $p<.001$]. A subsequent series of one-way ANOVAs that compared data from each treatment group across sex confirmed that total time spent in stereotypy was not significantly different between males and females that were drug-naïve or treated with 0.05 mg/kg MK801, that after treatment with 0.1 mg/kg, stereotypy was significantly more often observed in males compared to females [$F(1,15)=22.75$ $p<.001$] and that at the 0.2 mg/kg dose, increased stereotypy to similar extent in males and females. Finally, one-way ANOVAs that compared data within sex

across all treatment groups identified significant main effects of drug treatment overall on time spent in stereotypy in both sexes (males: $[F(7,62)=10.40 \text{ } p<.001]$; females $[F(7,63)=6.04 \text{ } p<.001]$). Allowed post-hoc testing showed that in males the middle (0.1 mg/kg) and high (0.2 mg/kg) dose of MK801 significantly increased time spent in stereotypy relative to control (both $p<.001$), that haloperidol was not effective in diminishing MK801-induced stereotypy, that pretreatment with the low dose of clozapine did not significantly reduce time spent in stereotypy and that pretreatment with the high dose of clozapine, did significantly reduce time spent in stereotypy relative to MK801 alone ($p=.038$). In females, only the high dose of MK801 significantly increased time spent in stereotypy relative to control ($p<.001$) and none of the neuroleptic pretreatments were effective in reversing these effects.

Ataxia: As for stereotypy, instances of ataxia were non-existent in vehicle-injected males and females. Treatment with 0.05 and 0.1 mg/kg MK801 also produced very little ataxia in either sex. In contrast, however, treatment with 0.2 mg/kg MK801 produced a substantial amount of ataxia in both sexes. Specifically, males showed approximately 2-3 instances of ataxia per minute during the testing session while females proved were somewhat more sensitive and showed about 4-5 instances of ataxia per minute (Figure II.7A,B)

Pretreatment with the low dose of haloperidol had no impact on ataxia in males but reduced MK801-induced ataxia by roughly 45% in females. The high dose of haloperidol, however, reduced MK801-induced ataxia by 70-75% in both sexes. In

comparison to haloperidol, pretreatment with clozapine at both doses was more effective in reducing ataxia in both males and females. Hence, after pretreatment with both doses of clozapine, ataxia was reduced by 85-95% relative to MK801 alone in males and by 90-95% in females (Figure II.7C,D).

The differences observed in ataxia were also supported in statistical comparisons of total ataxic instances during the testing session (Figure II.7E). First, an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified a significant main effect of treatment [$F(7,111)=29.65$ $p<.001$] on total ataxia. A subsequent series of one-way ANOVAs that compared data from each treatment group across sex confirmed that ataxia was not significantly different between males and females under any drug treatment condition. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified a significant main effect of treatment in both sexes (males: [$F(7,62)=19.66$ $p<.001$]; females [$F(7,63)=14.99$ $p<.001$]). Allowed post hoc testing further revealed that in males and females alike, 0.05 and 0.1 mg/kg MK801 had no significant effect on ataxia, that 0.2 mg/kg MK801 significantly increased ataxia (both sexes $p<.001$) and both doses of clozapine significantly reduced MK801-induced ataxia (all $p<.001$). Finally, the effects of haloperidol pretreatment were sex-specific; while both doses of haloperidol significantly reduced ataxia relative to MK801 alone in females (both $p<.001$), only the higher dose was effective in males ($p<.001$).

DISCUSSION

While sex differences have been identified in several aspects of schizophrenia, some of the most striking disparities are in the very way this disorder affects its sufferers. For example, women are most often afflicted predominantly by positive and affective symptoms such as paranoia, bizarre behavior and depression, whereas men tend to suffer more often and more severely from schizophrenia's negative and cognitive symptoms which include social withdrawal, behavioral inflexibility and cognitive deficits (Andreasen, 1982; Andreasen, 1995; Szymanski et al., 1995). Furthermore, while the positive symptoms in females usually respond favorably to pharmacological treatment, the negative symptoms in males tend to be refractory to both typical and atypical neuroleptic drug therapies (Seeman, 1986; Szymanski et al., 1995). Interestingly, although these observations have spurred the development of sex-specific therapeutic efforts in the clinic, attempts to understand the neurobiological bases of these sex differences have been minimal. In one respect, these efforts are perhaps hampered by the lack of an animal model that recapitulates the sex-differences that are characteristics of the disorder. This dissertation tackles this issue head on in exploring the validity of the rodent model of NMDA receptor hypofunction, a leading etiologic hypothesis of schizophrenia, to also model the striking sex differences seen in this disorder and in its treatment. In this chapter, open field testing was used to further explore these central questions at a behavioral level.

Open field testing stands out as a deceptively simple but powerful tool for evaluating a range of spontaneous animal behaviors in an untrained, unrewarded state;

from observation alone it allows quantification of discrete, diverse behaviors including those that have relevance for schizophrenia (Jentsch and Roth, 1999; Rung et al., 2005). Furthermore, there is a limited literature in which open field testing has been previously used to study the behavioral consequences of NMDA receptor hypofunction and acute treatment with MK801 (or analogues). While limited to motor symptoms including hyperlocomotion, stereotypy and ataxia and most often to one sex, these nonetheless show utility of this behavioral paradigm for exploration of schizophrenia's symptom correlates. Here, however, open field testing has been exploited to its fuller potential to determine whether the behavioral changes in rats induced by NMDA antagonism are consistent not only with the sex-specific behavioral manifestations of schizophrenia's positive signs, but also its negative, and affective symptoms as well. Specifically, I explored a range of behaviors, their sensitivity to MK801, and their attenuation by neuroleptic pretreatment in male rats and female rats in proestrus. These studies confirmed and extended previous findings that MK801 induces marked hyperlocomotion, stereotypy and ataxia in a dose-dependent manner and that it is more pronounced in females than in males. Further, this study also found that haloperidol and clozapine are both effective at attenuating these MK801 effects in a dose dependent manner with clozapine being more effective in both sexes. Finally, new results are reported here showing sex-specific effects of MK801 on grooming, rearing and thigmotaxis, all of which were more pronounced in males and that on the whole were less sensitive to pretreatment with haloperidol or clozapine. In the sections that follow, these results are discussed in further detail, are compared to previous behavioral and pharmacological studies in rodents and are considered in relation to

human patient populations to demonstrate the point that not only do MK801-stimulated open field behaviors provide a good match to schizophrenia's complex spectrum of symptoms, but that sex differences in these drug-induced behaviors in large part recapitulate sex difference found in schizophrenia's symptomatology and effective treatment.

Effects of MK801 on Locomotion, Stereotypy and Ataxia: Comparison to Previous Studies in Males

Although previous studies have examined behavioral consequences of acute MK801 treatment by using open field testing, it is important to emphasize at the outset that direct comparisons across studies are often made difficult by differences in the precise definitions and criteria used for measuring discrete behaviors even as seemingly simple as locomotion. Nonetheless, there is clear agreement that MK801 treatment induces a robust motor activation that emerges within minutes of drug treatment. Specifically, all studies agree that within 10-15 minutes, rats show increased locomotion relative to baseline (hyperlocomotion) and begin to demonstrate abnormal stereotypic movements including head-weaving and circling as well as gait disturbances or ataxia. The extant literature also includes evidence that the extent of these motor abnormalities differs between MK801-treated males and females that largely agree with the present results. For example, like the present findings, prior studies have shown that males given 0.05 mg/kg or 0.1 mg/kg MK801 show little increase in locomotion or ataxia (Danysz et al., 1994; Carey et al., 1998; Andine et al., 1999; Rung et al., 2005;

Scorza et al., 2008) but that at 0.2 mg/kg both of these motor signs are markedly increased. Again, while studies often differ in definition and measurement tactics, it may be interesting to note that the degree of increase in locomotion observed in male rats in this study corresponded closely to the 100-250% increases that has been reported previously (Danysz et al., 1994; Carey et al., 1998; Andine et al., 1999; Scorza et al., 2008).

The concordance across studies with stereotypy was slightly less consistent. Specifically, while all studies agree that 0.05 mg/kg MK801 fails to induce stereotypy and that 0.2 mg/kg MK801 does (Andine et al., 1999; Jackson et al., 2004), the present results of stereotypy also being induced in males at the 0.1 mg/kg dose is at odds with previous results (Andine et al., 1999). However, this could be due the fact that Andine and colleagues based measurements solely on stereotypic sniffing, while quantification in this study encompassed the sum of stereotypic behaviors that also included turning, circling and head-weaving. In fact, other studies utilizing a similar definition of stereotypy as in the study presented here did find that 0.1 mg/kg MK801 increased stereotypic behaviors in male rats (Jackson et al., 2004).

Effects of MK801 on Locomotion, Stereotypy and Ataxia: Comparison to Previous Studies in Females

Few studies have examined the effects of MK801 in female rats on open field behavior and even fewer examined MK801 using doses that were comparable to the

ranges evaluated here. Nonetheless, there does seem to be some concordance with the literature. For example, the present results of a dose-dependent effect of MK801 on locomotion, stereotypy and ataxia are wholly consistent with prior findings (Loscher and Honack, 1992; Honack and Loscher, 1993; Andine et al., 1999; Frantz and Van Hartesveldt, 1999). That the Andine study reported proportionally greater responses at all three doses than the present and Franz studies could be due in part to the fact that Andine and colleagues used a different strategy for quantifying locomotion which, at its heart, is more sensitive to smaller changes in behavior (Sturgeon et al., 1979). More specifically, this quantification strategy involves rating locomotor activity from 1 (movements in a localized area) to 5 (continuous movement in the entire cage). However, even mild motor activation could cause the animal to move within a large part of the cage, resulting in increased behavior scores, without greatly affecting the distance traveled. Thus, the increases in locomotion observed in this study might have possibly been proportionally exaggerated by such an analytical approach. However, it is important to note that qualitatively, the data here are in agreement with all previous reports, with differences in methodology likely resulting in small variations between studies.

With respect to the remaining two behaviors involving forward motion, results from this study showed that in females 0.05 and 0.1 mg/kg MK801 produced small amounts of stereotypy and ataxia and that 0.2 mg/kg MK801 was much more potent in stimulating these abnormal behaviors. As a measure dependent in part on movement, it may not be surprising that the same study that that showed greater locomotion

responses (Andine et al., 1999) also reported greater drug induced increases in stereotypy at all drug doses; other studies in females have also reported the presence of stereotypy after 0.1 mg/kg (Loscher and Honack, 1992; Honack and Loscher, 1993). Again, these are small differences which are very likely due in part to definition of behaviors and in analytical approach; it should be noted that overall agreement is good. This gets further traction from ataxia, where all studies took a similar approach to behavioral definition and measurement and all studies show good agreement that ataxia only emerges with 0.1 mg/kg and is more severe following administration of the 0.2 mg/kg dose (Honack and Loscher, 1993; Andine et al., 1999).

Effects of MK801 on Rearing, Grooming and Thigmotaxis

In contrast to changes in motor behaviors in response to NMDAR antagonism which have received the bulk of prior attention, almost no information exists regarding the effects of NMDAR antagonism on other open field behaviors. To that end, the second objective of this study was to evaluate the effect of MK801 with and without neuroleptic pretreatment on rearing, grooming and thigmotaxis. The rationale for examining these behaviors stems from the fact that rearing is a normal exploratory adaptive response to a novel environment and that a reduction in this behavior corresponds to avolition, a prominent negative symptom of schizophrenia (Bardo et al., 1990a; Bardo et al., 1990b; Scorza et al., 2008). Likewise, a lack of grooming has also been portrayed as a manifestation of avolition (Dubiel et al., 2011). Finally, thigmotaxis has been established as a manifestation of anxiety and/or fear which

represent a negative affective state (Simon et al., 1994). Furthermore, thigmotaxis in rodents is attenuated by clinically used anxiolytic drugs, conferring predictive validity on this behavior (Treit and Fundytus, 1988). As negative symptoms and affective disturbances are key components in schizophrenia's symptomatology and both are sexually dimorphic in their prevalence and severity, I sought to evaluate whether acute NMDAR antagonism causes changes in rearing, grooming and thigmotaxis and whether these putative changes are consistent with the sex differences seen in the negative and affective symptoms of schizophrenia.

My data indicated that all three doses of MK801 diminished rearing in males. The 70% decrease observed at the middle dose used is also in good agreement with previous studies that also observed 60-90% reduction in rearing after 0.1 mg/kg MK801 (Scorza et al., 2008; Dubiela et al., 2011). My study also showed that in females, rearing was also inhibited at all doses of MK801 and largely to similar extents as in males. However, there was a trend indicative of a higher sensitivity to MK801 in males as the middle dose decreased rearing in males to a greater extent than females. Next, grooming was also affected in a sex specific manner. Thus, in males, grooming was unaffected at 0.05 mg/kg, decreased by roughly 65% at 0.1 mg/kg and was almost non-existent after 0.2 mg/kg MK801; this dose-dependent effect of MK801 is consistent with that previously reported (Dall'Olio et al., 1996; Dubiela et al., 2011). Grooming in females, on the other hand, was not affected by MK801 until the highest dose where it was also virtually non-existent. Finally, my results showed that while MK801 increased thigmotaxis in a dose-dependent manner in males, it had no effect in females at any

dose. Thus, perhaps even more than motor activity, these ancillary behaviors show sex differences in drug effect that may be qualitative as well as quantitative.

Effects of Neuroleptic Drugs: Comparison to Prior Studies in Males

While the effects of haloperidol and clozapine on behaviors elicited by MK801 have been studied, existing data are sparse and address only motor sequelae of hyperlocomotion and stereotypy. Furthermore, there have been no direct comparisons of the efficacy of these antipsychotic drugs between males and females at doses relevant to human therapeutics (Kapur et al., 2003). Focusing on the effect of neuroleptics on motor activity first, results here indicate that while both drugs have some ability to ameliorate the psychomotor effects of MK801, both sex and drug differences in their efficacy exist. First, in keeping with previous studies in males, although no studies exist using the same combination of doses used here, the findings here have some precedent in the literature. For example, haloperidol at doses of 0.1 mg/kg and higher is effective in partially reducing hyperlocomotion induced by 0.1-0.3 mg/kg of MK801 in male rats (Hoffman, 1992; Bubenikova et al., 2004). The consensus regarding haloperidol's effect on stereotypy is less clear. Thus, while the findings here of a lack of haloperidol effect on stereotypy are in agreement with a report by Tiedtke and colleagues where 0.1 mg/kg haloperidol was ineffective in reversing stereotypy induced by 0.16 mg/kg MK801 (Tiedtke et al., 1990), it is also in contrast with several reports indicating that haloperidol doses of 0.1 mg/kg or higher decrease stereotypy in male rats induced by 0.1-0.3 mg/kg MK801 (Behrens and Gattaz, 1992; Hoffman,

1992). It is unclear why this discrepancy exists, though the fact that all cited studies used a higher dose of haloperidol indicates that there may be an antagonistic effect at higher doses, which in turn falls into a suprathreshold, and possibly sedating, range (Kapur et al., 2003). Surprisingly, no previously reported data exists that evaluated the effects of haloperidol on MK801-induced ataxia in males, making this the first report showing that this drug is effective in reducing ataxia in males. In contrast to the inter-study variability seen with haloperidol, the results here indicating a robust rescue effect by clozapine of these three MK801-stimulated behaviors are wholly concordant with the extant literature; findings here that both doses of clozapine completely eliminated hyperlocomotion and ataxia to baseline levels and that the high dose reduced (but did not eliminate) stereotypy are agreeable with findings reported previously (Hoffman, 1992; Scorza et al., 2008).

Effects of Neuroleptic Drugs: Comparison to Prior Studies in Females

While the effects of haloperidol on MK801-induced behaviors have been studied far less in females, the results here are also in agreement with a majority of the extant literature. Thus, doses of 0.1-0.25 mg/kg haloperidol were previously reported to be effective in reversing hyperlocomotion and ataxia induced by 0.1-0.3 mg/kg MK801 (Loscher and Honack, 1992). Interestingly, these doses of haloperidol also reduced stereotypy, though this was not observed in the present study; this may suggest that stereotypy may be sensitive to the dosage differences used here versus in the cited studies. Finally, it should be noted that another report showed the failure of 0.1 mg/kg

haloperidol to antagonize all three behaviors induced by 0.2 mg/kg MK801 (Andine et al., 1999). However, this discrepancy can be explained by the fact that the behavior scoring strategy in that study is much less sensitive to small reductions in behavior, which may preclude the characterization of the partial reductions in behavior seen here. Furthermore, findings here that clozapine robustly and dose-dependently reverses MK801 effects on all three behaviors are in agreement with the only study probing the effects of clozapine on MK801-stimulated open field behaviors (Andine et al., 1999). However, it should be noted that the high dose of clozapine increased stationary behavior in both sexes above that in controls, indicating that some sedative effects may drive the drug effects at this dose, a caveat that should be taken into mind when interpreting this result. In the sections that follow, I discuss how all measured constructs map on to sex differences in schizophrenia and how observed differences in the abilities of neuroleptic drugs to attenuate various behaviors represent well-characterized sex and drug differences in schizophrenia's treatment.

Sex Differences in the Effects of MK801: Relevance to Schizophrenia

Before embarking on complex comparisons, it is important to re-emphasize that while the sex differences below could reflect sexually dimorphic pharmacokinetics and metabolism, these effects are likely to be minimal here and indeed in all chapters of this dissertation as a keystone part of all experimental strategy was to conduct and conclude testing within an hour of drug administration- when sex differences in metabolism have been shown to be minimal (Andine et al., 1999).

Motor Behaviors/ Positive Symptoms: While the effects of MK801 on open field motor behaviors have been previously studied separately in male and female rats, the present study directly compared behavioral drug effects across sex. The impetus for this was two-pronged, stemming from the findings of sexually dimorphic symptom expression in schizophrenia and the established finding that the motor syndrome induced by MK801 is representative of the bizarre behavior that is a component of the positive symptoms of this disorder (Rung et al., 2005). Thus, as women suffering from schizophrenia tend to exhibit increased severity of positive symptoms (Franzek and Beckmann, 1992), the major question driving this study was whether the intensity of motor symptoms elicited by MK801 in rats mirrored the increased severity of the positive symptoms of schizophrenia they model in female patients. Our results indicate that they do. Specifically, at the 0.2 mg/kg dose, MK801 induced significantly greater locomotion and a trend toward more ataxia in females. Thus, open field testing adds additional support to the conclusion that the NMDAR hypofunction model indeed recapitulates the disproportionate representation of positive symptoms in female compared to male schizophrenics. Stereotypy was a possible exception as overall it appears similarly increased by MK801 in males and females alike. A potential explanation for this may be that a number of different stereotypic behaviors comprised the combined measure. It remains possible that more specific analysis of these discrete measures may uncover that some stereotypic behaviors do follow expected sex-specific patterns in response to MK801.

In addition to recapitulating the sex differences known to exist in the positive symptomatology of schizophrenia, the increased psychomotor activation seen in females relative to males may also provide insight into underlying mechanisms that are relevant to schizophrenia. Thus, as the motor activation induced by NMDAR antagonism is thought to be a consequence of increased DA release in subcortical structures such as nucleus accumbens and striatum (Silbersweig et al., 1995; O'Donnell and Grace, 1998; Epstein et al., 1999; Yang and Chen, 2005), the behavioral findings here indicate that there may be increased DA release in females over males in these structures. To that end, there may be several mechanisms that may cause this. To that end, it is important to note that all female rats used in this study were in proestrus when hormone levels are highest which is relevant as estrogen has been shown to directly and indirectly affect subcortical dopamine release. First, as estrogen acts directly on mesolimbic DAergic neurons to cause DA release (Becker, 1999); the high estrogen levels in the females and resultant high basal DA levels in subcortical structures may reduce the threshold for behavioral activation due to additive DA-releasing effects of MK801 relative to males. A second, more intriguing view takes into account that estrogen also modulates the expression of NMDA receptors in the prefrontal cortex. Thus, as estrogen reduces NMDA receptor density in this brain region (Cyr et al., 2000), it is possible that the high levels of estrogen in the females in this study may increase their sensitivity to NMDAR hypofunction by reducing the absolute levels of NMDA receptors in this brain region. In turn, this would result in increased activation of corticofugal glutamatergic pyramidal cells and subsequent disinhibition of mesolimbic and mesostriatal DAergic cells relative to males (Yang and Chen, 2005). However, as

there are no studies to date directly comparing NMDA receptor expression in the prefrontal cortex of males and females, this explanation remains within the realm of conjecture until effects of sex and gonadal hormones on this factor are further investigated.

Rearing and Grooming: Negative Symptoms: Given how rearing and grooming have been interpreted and especially their decrease in response to MK801, these two behaviors may be valid animal correlates of schizophrenia's negative symptoms. As such, the underlying hypothesis is that they will be more vulnerable to MK801 in males. My results suggest that to a large extent they are. In sum, MK801 in males was more potent in diminishing grooming and reducing rearing which mirrors male vulnerability to negative symptoms. This gives a further validation that NMDAR hypofunction in rodents induced by MK801 may also hold face validity in modeling the sex differences in severity of negative symptoms of this disease. Furthermore, it also opens the door for further evaluating the emergence of negative symptoms in NMDAR hypofunction with other behavioral tasks sensitive to other components of the negative symptom cluster. For example, anhedonia in rats can be evaluated by measuring the intake of freely available sucrose solution (Papp et al., 1991) and social deficits can be quantified by measuring interactions with other animals (Rung et al., 2005). Thus, while the results here give the first indication that the NMDAR hypofunction model recapitulates the overwhelming male susceptibility to the negative symptoms of schizophrenia further investigation is warranted to further characterize these differences.

Thigmotaxis: Affective Disturbance: The third objective of this study was to evaluate the effects of MK801 and neuroleptic pretreatment on an animal model of affective disturbance which in this case corresponded to thigmotaxis. While MK801 was found to increase this behavior in males but not females may be contrary to expected findings based on epidemiological findings from patients with schizophrenia where females are more susceptible to affective disturbances. However, there may be another valid way of looking at these data. Specifically, it may be that the exaggerated fearful/anxiety response in males in response to a bolus of psychomimetic drug is a normal response, and the failure of females to alter behavior may reflect an affective behavioral abnormality. If it is taken that the results here indicate that while males exhibit appropriate behavior in response to the drug by increasing thigmotaxis, females show inappropriate, perhaps impulsive or reckless behavior by continuing to venture out away from the relative safety of the walls, then these findings would in fact be commensurate with sex differences seen in organic disease. Thus, while these results clearly do not indicate increased anxiety in females, they could signify that females exhibit more impulsivity and hence improper affect after MK801 than males. I thus take the guarded position that the NMDAR hypofunction model may also emulate sex differences in affective symptoms that are seen in schizophrenia. These more uncertain conclusions aside, it is evidence that the analyses of open field have clearly extended and reinforced findings from the prior chapter that the NMDAR hypofunction model in rats has predictive validity in modeling sex differences in the positive and negative symptoms of schizophrenia. The final discussion section below further considers the possibility that this model can also recapitulate and/or define sex-specific pharmacological effects that

might lead to improved treatment approaches to these diverse sets of symptoms in male and female patient populations.

Sex Differences in Effects of Neuroleptic Drugs on the MK801-Induced Behaviors:
Relevance to Schizophrenia

Neuroleptic pretreatment results provided several salient findings. First, the results identified that haloperidol is effective at ameliorating MK801-induced hyperlocomotion and ataxia in females at lower doses while clozapine was similarly efficacious in both sexes. As these motor symptoms have been linked to the positive symptoms of schizophrenia, particularly the bizarre behavior that is a core component of the positive symptom cluster (Powell et al., 2009; van den Buuse, 2010), the results here recapitulate evidence from human therapeutics which indicates that females require lower doses of neuroleptics than males to achieve a similar antipsychotic effect (Andia et al., 1995). Furthermore, the superior efficacy of clozapine versus haloperidol in both sexes is also validated by data indicating that atypical neuroleptics are generally more effective than typical neuroleptics (Gallhofer et al., 1996; Lieberman, 1996; Kapur and Remington, 2001). Finally, the efficacy of atypical neuroleptics was equal in male and female rats; this is also concordant with reports of equal efficacy of atypical drugs in male and female schizophrenia patients. Taken together, this indicates that the NMDAR hypofunction model also recapitulates at least some of the sex and drug differences in neuroleptic attenuation of positive symptoms seen in schizophrenia.

Assessments of the abilities of haloperidol and clozapine to reverse the MK801 induced deficits in rearing and grooming were without any precedent in the literature; these surprisingly revealed that neither dose of either drug was able to restore rearing or grooming in males and females alike. While a paradoxical rescue of both behaviors was observed in females with the low dose of haloperidol, closer analysis of the data revealed that this effect was driven by two animals that showed abnormally high behavior relative to the others. Thus, combined with the lack of effect of the high dose of haloperidol, it seems unlikely that this drug can effectively antagonize MK801 induced disruptions in these behaviors. Interestingly, this lack of neuroleptic efficacy correlates to human therapeutics as negative symptoms are typically more resistant to treatment with both typical and atypical neuroleptic drugs (Kapur and Remington, 2001; Murphy et al., 2006). These results indicate that the effects of NMDAR antagonism on rearing and grooming are modulated by a separate mechanism than the motor behaviors discussed above. However, it should be noted that while rearing and grooming are open field behaviors with established relevance to certain negative symptoms of schizophrenia such as avolition, there are other behavioral paradigms that may be employed to further quantify the emergence of negative symptoms in NMDAR hypofunction. Finally, this signifies that analysis of grooming and rearing, their impairment by MK801 and reversal by pharmacologic agents may have utility as a screening tool in evaluating novel treatments effective on negative symptoms of schizophrenia.

Finally, with regard to the affective measure of thigmotaxis, results were less definitive. Thus, treatment with haloperidol reduced the MK801 induced increase in

thigmotaxis in males down to drug-naïve levels while not affecting females. This indicates that this drug plays a role in altering affect in a positive way, which is consistent with several reports showing that haloperidol may have anxiolytic and mood-improving effects in both rats and humans (Rickels et al., 1971; Budden, 1979; Pich and Samanin, 1986). Interestingly, treatment with the low dose of clozapine almost eliminated thigmotaxis completely in both sexes. This superior activity of clozapine relative to haloperidol may be due to the polypharmacological profile of clozapine which includes potent action on the serotonin system which is believed to at least partially modulate affect (Millan, 2000; Dayan and Huys, 2009). However, the high dose of clozapine had the same effects in both sexes as either dose of haloperidol. This may be due to a sedating effect of the high dose of clozapine as with this pretreatment both males and females spent a majority of time engaged in stationary behavior (significantly more than control) and may have been precluded from exploring the arena, falsely elevating thigmotaxis. Thus, since the relevance of thigmotaxis to the affective disturbances in schizophrenia is not totally clear, more study is needed to further evaluate this behavior and its response to neuroleptic drugs.

Conclusion

While open field motor behaviors in a state of acute NMDA receptor antagonism have been previously used as a model for positive symptoms of schizophrenia, little was known about whether these drug-induced behaviors were sexually dimorphic and similar to the sex differences in schizophrenia. Furthermore, while other open field

behaviors such as rearing, grooming and thigmotaxis have potential to model negative and affective symptoms of this disorder, they has never been fully explored, rigorously compared across sex and never combined with drug attenuation studies. Results obtained here revealed that acute NMDAR antagonism in fact may have much more far reaching value as a behavioral model of the full spectrum of sex-specific behavioral disturbances that characterize schizophrenia. Thus, it produces more severe positive symptom correlates such as hyperlocomotion and ataxia in females and more severe negative symptom correlates such as greater disturbances in rearing and grooming in males. Furthermore, sex and drug differences in behavior sensitivity to neuroleptics were also identified with typical neuroleptics being more effective in females at lower doses at reversing the positive symptom correlates while atypical neuroleptics were equally effective in both sexes. However, neither class of neuroleptic drugs was effective at attenuating negative symptom correlates. Combined with the results in the previous chapter which showed sex-specific effects of MK801 and neuroleptic pretreatment on ASR and PPI, there is now very compelling evidence that the rodent NMDAR hypofunction model recapitulates the major salient sex differences in the expression and treatment of positive and negative symptoms of schizophrenia. However, despite the results here indicating the utility of thigmotaxis in evaluating some aspects of affect, it is still uncertain whether the NMDAR hypofunction model also recapitulates the sex differences in affective symptoms. To address this uncertainty, the next chapter uses a more established measure of affect- analysis of spontaneous ultrasonic vocalizations emitted by male and female rats- to complete the

characterization of the NMDAR hypofunction model's ability to recapitulate sex differences in the full behavioral phenotype of schizophrenia and its treatment.

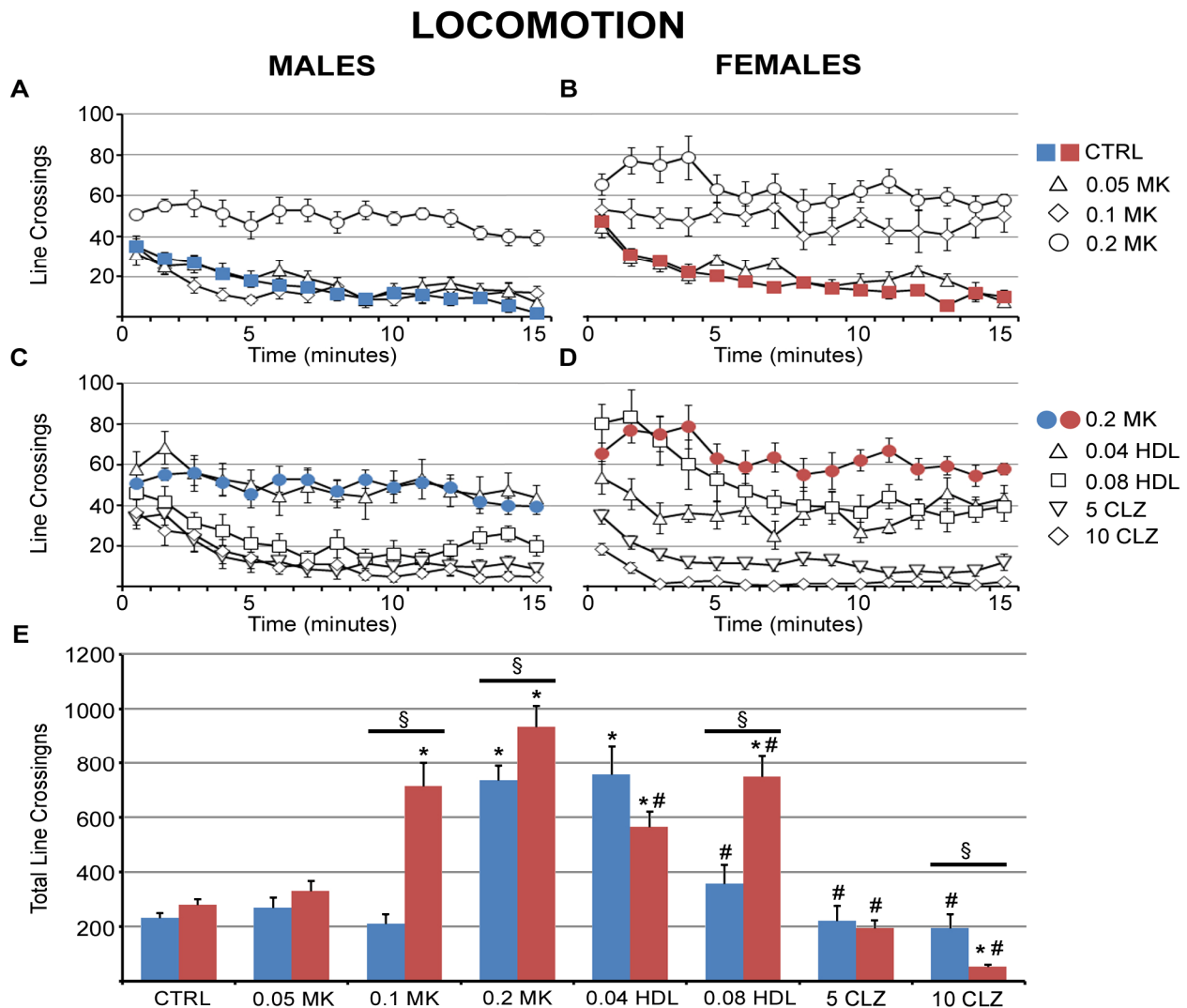


Figure II.1. Effects of MK801 and neuroleptic pretreatment on locomotion. A,B: Line graphs showing the number of lines crossed per minute during the 15 minute testing session by male (A) and female (B) rats in the MK801 dose response treatment groups. Rats were either vehicle-injected (CTRL), treated with 0.05 mg/kg MK801 (0.05 MK), treated with 0.1 mg/kg MK801 (0.1 MK) or treated with 0.2 mg/kg MK801 (0.2 MK). C,D: Line graphs showing the mean number of lines crossed per minute during the 15 minute testing session by male (C) and female (D) rats in the neuroleptic pretreatment groups. Rats were either treated with 0.2 mg/kg MK801 (0.2 MK), 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 (0.04 HDL and 0.08 HDL), or 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 (5 CLZ and 10 CLZ). E: Bar graph showing the total number of lines crossed over the entire 15 minute testing session by male (blue bars) and female (red bars) rats in all treatment groups. All data is expressed as mean±SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, pound signs (#) indicate a significant difference compared to MK801 and section signs (§) indicate a significant difference between males and females.

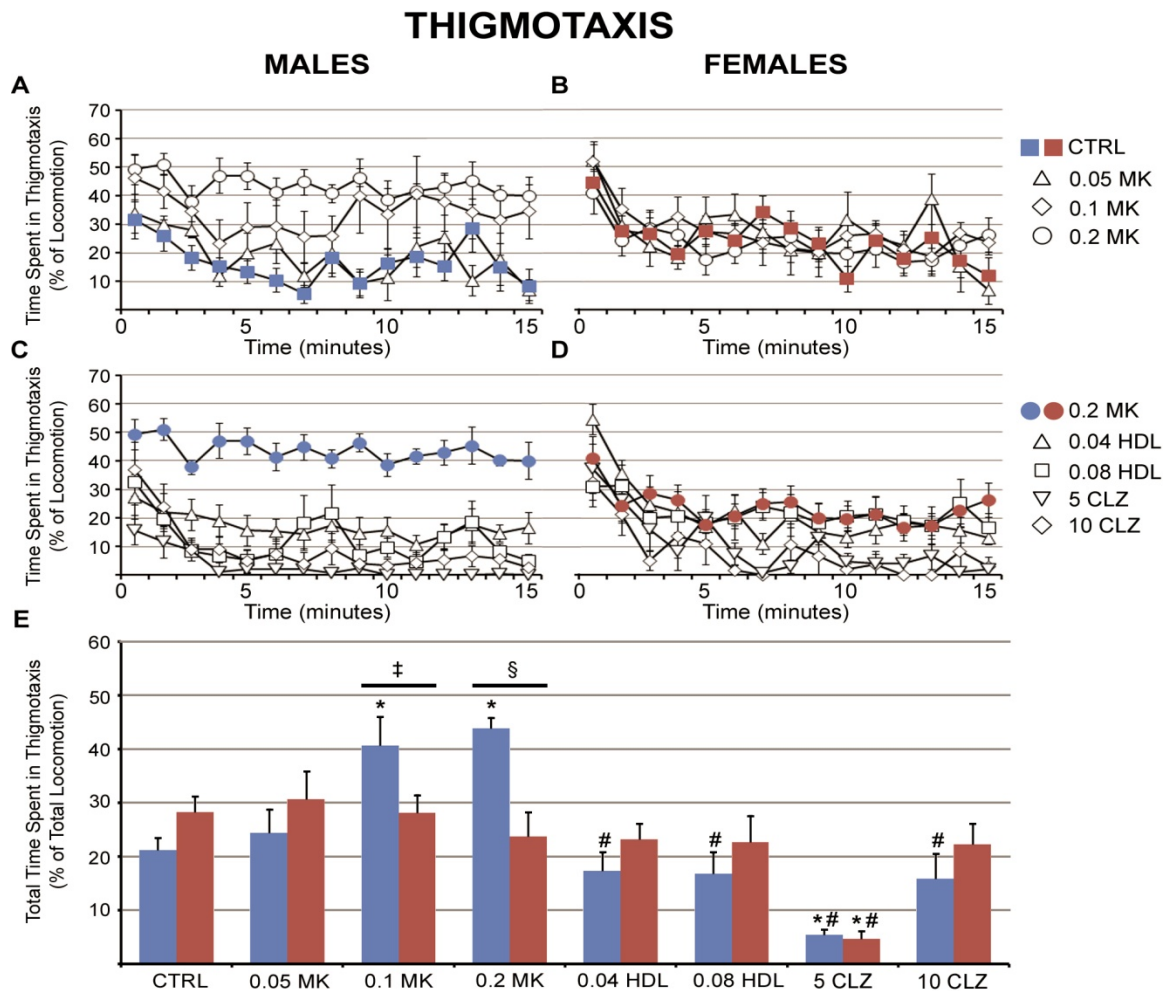


Figure II.2. Effects of MK801 and neuroleptic pretreatment on thigmotaxis. A,B: Line graphs showing percentages of locomotor time spent in thigmotaxis per minute during the 15 minute testing session by male (A) and female (B) rats in the MK801 dose response treatment groups. Rats were either vehicle-injected (CTRL), treated with 0.05 mg/kg MK801 (0.05 MK), treated with 0.1 mg/kg MK801 (0.1 MK) or treated with 0.2 mg/kg MK801 (0.2 MK). C,D: Line graphs showing percentages of locomotor time spent in thigmotaxis per minute during the 15 minute testing session by male (C) and female (D) rats in the neuroleptic pretreatment groups. Rats were either treated with 0.2 mg/kg MK801 (0.2 MK), 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 (0.04 HDL and 0.08 HDL), or 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 (5 CLZ and 10 CLZ). E: Bar graph showing percentages of total locomotor time spent in thigmotaxis over the entire 15 minute testing session by male (blue bars) and female (red bars) rats in all treatment groups. All data is expressed as mean \pm SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control and pound signs (#) indicate a significant difference compared to MK801. Section signs (§) indicate a significant difference between males and females and double crosses (‡) indicate a near-significant difference ($p < 0.075$) between males and females.

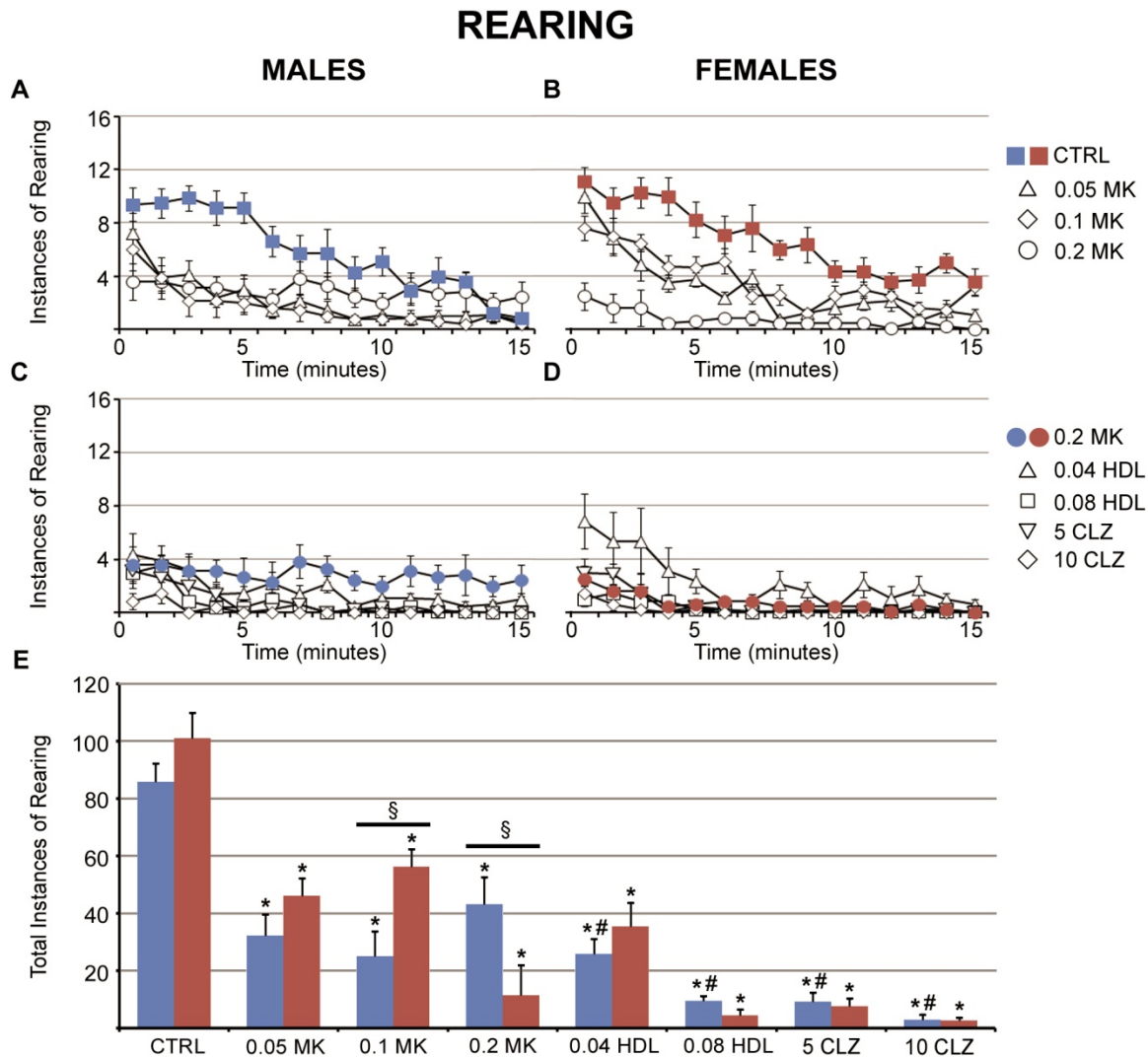


Figure II.3. Effects of MK801 and neuroleptic pretreatment on rearing. A,B: Line graphs showing instances of rearing per minute during the 15 minute testing session by male (A) and female (B) rats in the MK801 dose response treatment groups. Rats were either vehicle-injected (CTRL), treated with 0.05 mg/kg MK801 (0.05 MK), treated with 0.1 mg/kg MK801 (0.1 MK) or treated with 0.2 mg/kg MK801 (0.2 MK). C,D: Line graphs showing instances of rearing per minute during the 15 minute testing session by male (C) and female (D) rats in the neuroleptic pretreatment groups. Rats were either treated with 0.2 mg/kg MK801 (0.2 MK), 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 (0.04 HDL and 0.08 HDL), or 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 (5 CLZ and 10 CLZ). E: Bar graph showing the total instances of rearing over the entire 15 minute testing session by male (blue bars) and female (red bars) rats in all treatment groups. All data is expressed as mean±SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, pound signs (#) indicate a significant difference compared to MK801 and section signs (§) indicate a significant difference between males and females.

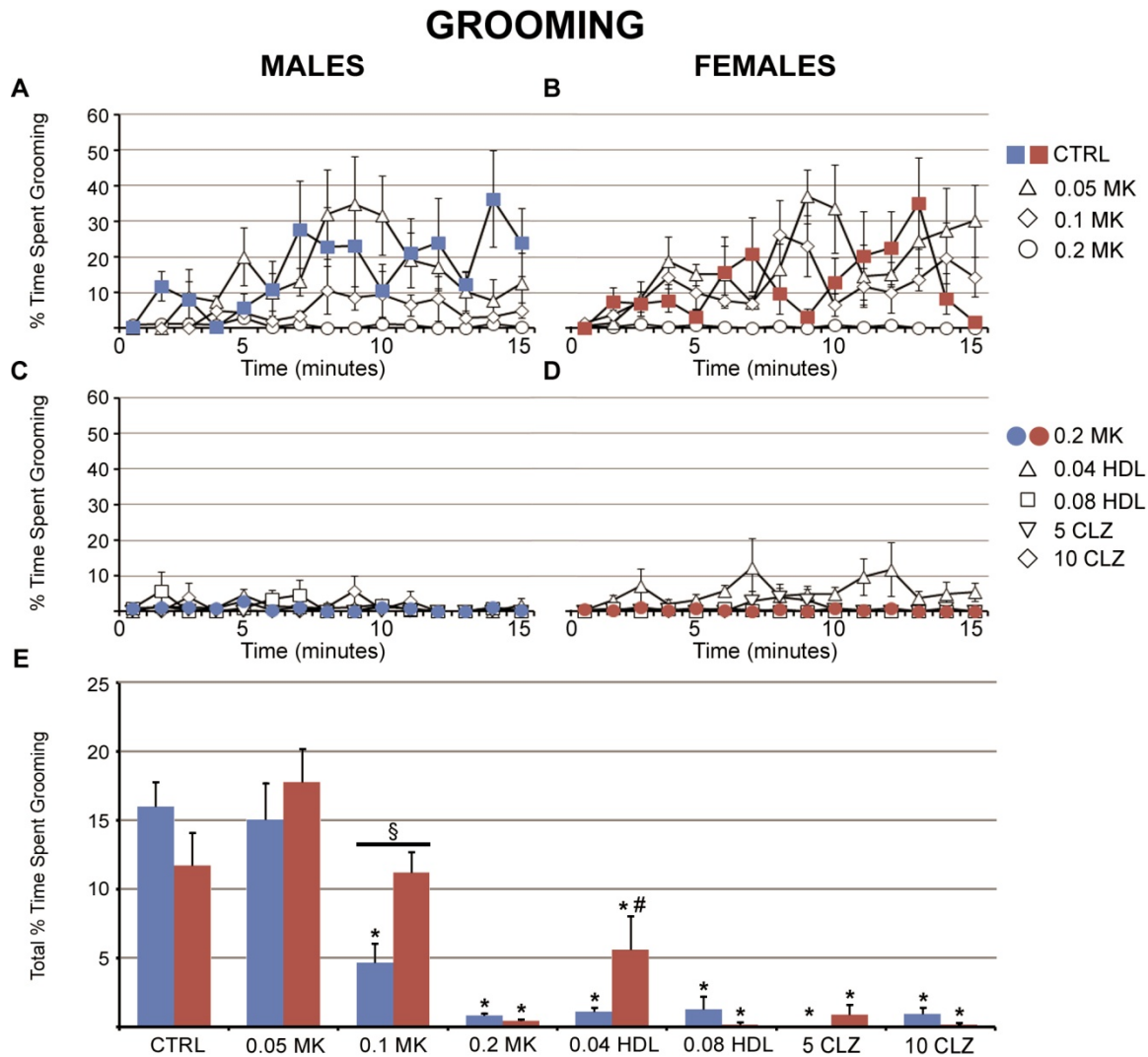


Figure II.4. Effects of MK801 and neuroleptic pretreatment on grooming. A,B: Line graphs showing percentages of time spent grooming in each minute during the 15 minute testing session by male (A) and female (B) rats in the MK801 dose response treatment groups. Rats were either vehicle-injected (CTRL), treated with 0.05 mg/kg MK801 (0.05 MK), treated with 0.1 mg/kg MK801 (0.1 MK) or treated with 0.2 mg/kg MK801 (0.2 MK). C,D: Line graphs showing percentages of time spent grooming in each minute during the 15 minute testing session by male (C) and female (D) rats in the neuroleptic pretreatment groups. Rats were either treated with 0.2 mg/kg MK801 (0.2 MK), 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 (0.04 HDL and 0.08 HDL), or 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 (5 CLZ and 10 CLZ). E: Bar graph showing total percentages of time spent grooming over the entire 15 minute testing session by male (blue bars) and female (red bars) rats in all treatment groups. All data is expressed as mean \pm SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, pound signs (#) indicate a significant difference compared to MK801 and section signs (§) indicate a significant difference between males and females.

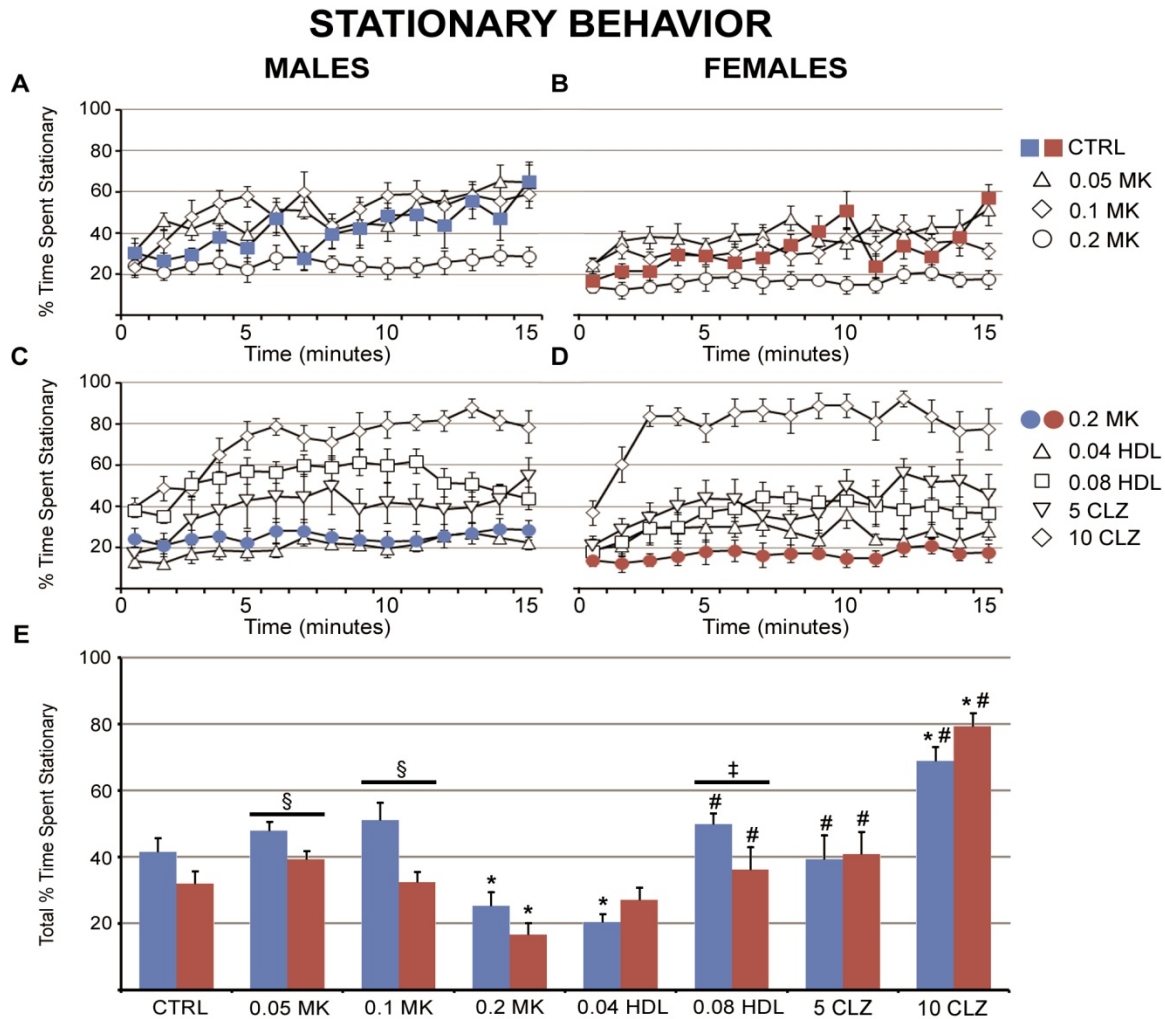


Figure II.5. Effects of MK801 and neuroleptic pretreatment on stationary behavior. A,B: Line graphs showing percentages of time spent stationary in each minute during the 15 minute testing session by male (A) and female (B) rats in the MK801 dose response treatment groups. Rats were either vehicle-injected (CTRL), treated with 0.05 mg/kg MK801 (0.05 MK), treated with 0.1 mg/kg MK801 (0.1 MK) or treated with 0.2 mg/kg MK801 (0.2 MK). C,D: Line graphs showing percentages of time spent stationary in each minute during the 15 minute testing session by male (C) and female (D) rats in the neuroleptic pretreatment groups. Rats were either treated with 0.2 mg/kg MK801 (0.2 MK), 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 (0.04 HDL and 0.08 HDL), or 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 (5 CLZ and 10 CLZ). E: Bar graph showing percentages of time spent stationary over the entire 15 minute testing session by male (blue bars) and female (red bars) rats in all treatment groups. All data is expressed as mean \pm SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control and pound signs (#) indicate a significant difference compared to MK801. Section signs (§) indicate a significant difference between males and females and double crosses (‡) indicate a near-significant difference ($p < 0.075$) between males and females.

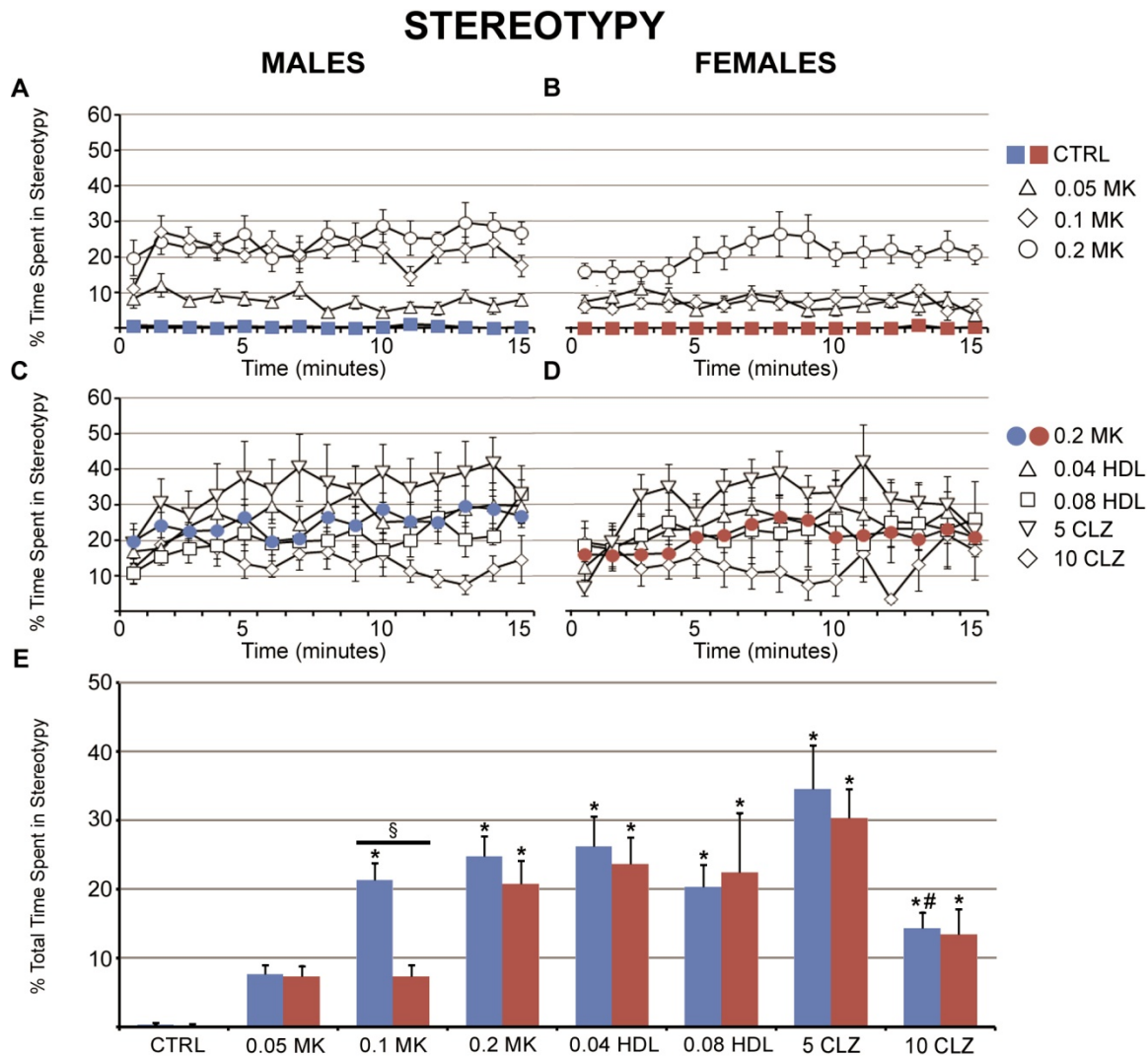


Figure II.6. Effects of MK801 and neuroleptic pretreatment on stereotypic behavior. A,B: Line graphs showing percentages of time spent in stereotypy in each minute during the 15 minute testing session by male (A) and female (B) rats in the MK801 dose response groups. Rats were either vehicle-injected (CTRL), treated with 0.05 mg/kg MK801 (0.05 MK), treated with 0.1 mg/kg MK801 (0.1 MK) or treated with 0.2 mg/kg MK801 (0.2 MK). C,D: Line graphs showing percentages of time spent in stereotypy in each minute during the 15 minute testing session by male (C) and female (D) rats in the neuroleptic pretreatment groups. Rats were either treated with 0.2 mg/kg MK801 (0.2 MK), 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 (0.04 HDL and 0.08 HDL), or 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 (5 CLZ and 10 CLZ). E: Bar graph showing total percentages of time spent in stereotypy over the entire 15 minute testing session by male (blue bars) and female (red bars) rats in all treatment groups. All data is expressed as mean±SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, pound signs (#) indicate a significant difference compared to MK801 and section signs (§) indicate a significant difference between males and females.

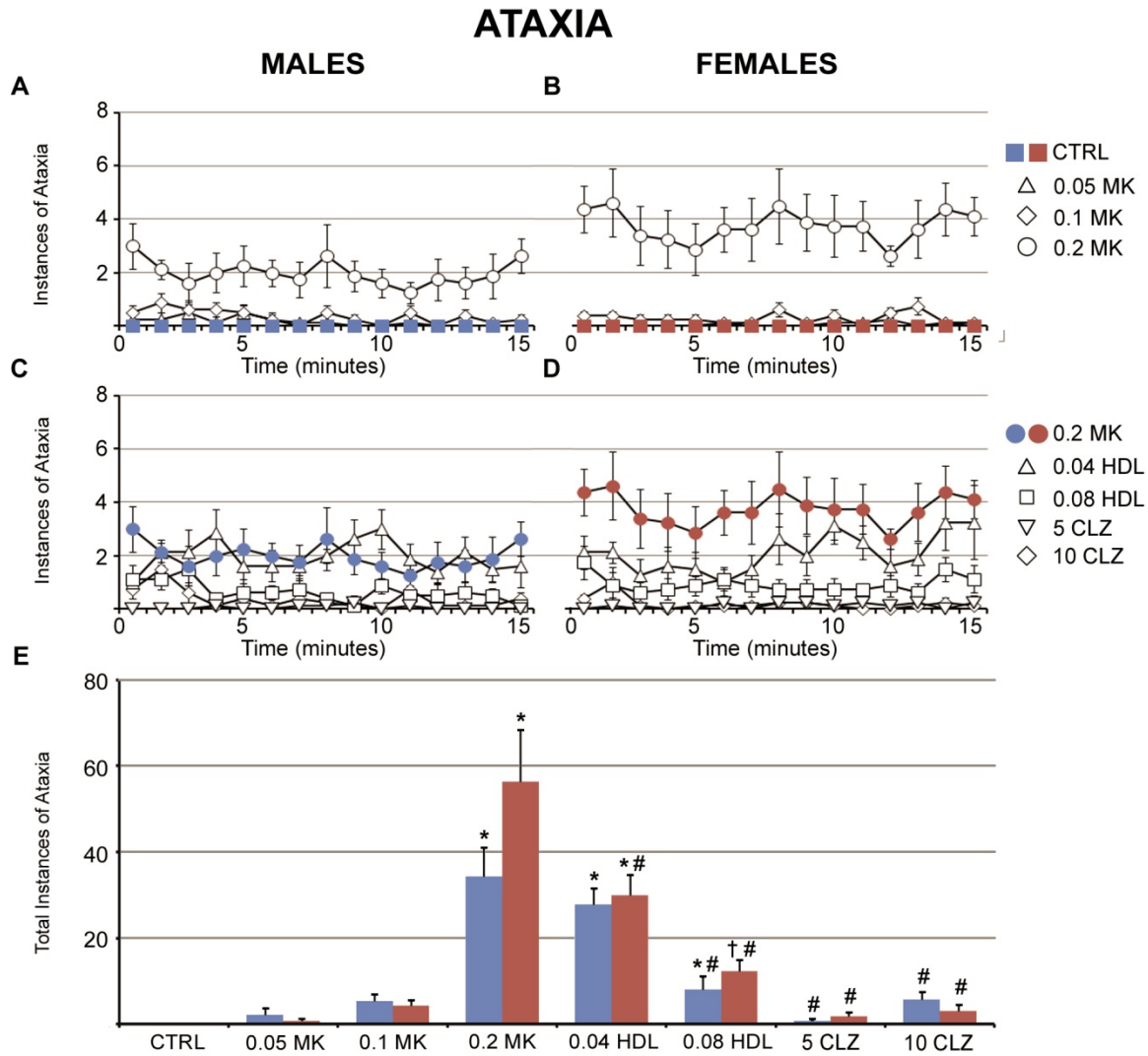


Figure II.7. Effects of MK801 and neuroleptic pretreatment on ataxia. A,B: Line graphs showing instances of ataxia per minute during the 15 minute testing session by male (A) and female (B) rats in the MK801 dose response treatment groups. Rats were either vehicle-injected (CTRL), treated with 0.05 mg/kg MK801 (0.05 MK), treated with 0.1 mg/kg MK801 (0.1 MK) or treated with 0.2 mg/kg MK801 (0.2 MK). C,D: Line graphs showing instances of ataxia per minute during the 15 minute testing session by male (C) and female (D) rats in the neuroleptic pretreatment groups. Rats were either treated with 0.2 mg/kg MK801 (0.2 MK), 0.04 or 0.08 mg/kg haloperidol followed by 0.2 mg/kg MK801 (0.04 HDL and 0.08 HDL), or 5 mg/kg or 10 mg/kg clozapine followed by 0.2 mg/kg MK801 (5 CLZ and 10 CLZ). E: Bar graph showing the total instances of ataxia over the entire 15 minute testing session by male (blue bars) and female (red bars) rats in all treatment groups. All data is expressed as mean±SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, cross signs (†) indicate a near-significant difference compared to control ($p < 0.075$) and pound signs (#) indicate a significant difference compared to MK801.

CHAPTER III

NMDA receptor hypofunction causes sex-specific changes in the number of low and high frequency spontaneous ultrasonic vocalizations emitted by adult male and female rats

As discussed in the previous chapters of this dissertation, schizophrenia is a prevalent mental disorder that is characterized by an array of symptoms. These have traditionally been divided into positive, negative and affective symptoms. Of particular interest to this dissertation is that these also differentially affect men and women diagnosed with this disorder. Previous chapters using quantification of the PPI of the ASR and open field testing have established that a rodent NMDA receptor hypofunction model indeed recapitulates sex differences observed in positive and negative symptoms of schizophrenia. However, findings related to affective symptom correlates were more equivocal. The experiments presented here sought to clarify the question of whether the rodent NMDAR hypofunction model recapitulates sexual dimorphisms in schizophrenia's affective symptom cluster and if so, whether pretreatment with haloperidol and/or clozapine can attenuate these in sex-specific ways.

While positive symptoms of schizophrenia such as delusions and bizarre behavior often preferentially affect women and negative symptoms such as anhedonia, social withdrawal and cognitive deficits tend to predominantly affect men (Ring et al., 1991; Szymanski et al., 1995; Schultz et al., 1997), the clinical picture of schizophrenia also includes the presence of affective disturbances. These can include depression, impulsivity, irritability and anxiety and have been recognized as a separate symptom

cluster (Leung and Chue, 2000; Lysaker and Salyers, 2007). The debilitating nature of affective symptoms and the importance of their successful treatment is highlighted by their significant contribution to overall morbidity and mortality in sufferers of schizophrenia as the sequelae of depression and anxiety have been linked to alarmingly increased rates of suicide in schizophrenic patients (Prasad and Kumar, 1988; Palmer et al., 2005). Further, this symptom cluster causes significant suffering and schizophrenics report a high desire to reduce these symptoms; a study found that the majority of patients view alleviation of affective symptoms as a major goal of pharmacologic therapy (Ginsberg et al., 2005). Unfortunately, affective symptoms are undertreated; one study found that affective symptoms tend to persist in approximately 50% of patients even after long-term treatment with antipsychotic drugs (Harrow et al., 1994) while another reported that around 75% of patients report that their affective symptoms are inadequately controlled on their medication regimen (Ginsberg et al., 2005). More relevant to the questions posed in this dissertation, however, is that similar to positive and negative signs, the predominance of this symptom cluster is also sexually dimorphic. Specifically, numerous studies have shown that female patients typically exhibit more and more severe affective symptoms than males (Lewine, 1981; Goldstein and Link, 1988; McGlashan and Bardenstein, 1990; Franzek and Beckmann, 1992).

While means for objective measurement of affect have been developed for use with rodent models (e.g. elevated plus maze, tail suspension test, forced swim test), the use of these particular paradigms in the context of NMDAR hypofunction poses unique

challenges. For example, the elevated plus maze and the forced swim test are dependent on stable baseline locomotor activity; that NMDAR hypofunction in general and acute MK801 challenge in particular are well known to induce hyperlocomotion, there can be real difficulties in the interpretation of drug effects (Cryan et al., 2005; Petit-Demouliere et al., 2005). Further, these particular tasks also require balance and coordinated motor activity. Accordingly, the marked ataxia that is also induced by MK801 is a further factor that makes reliable application and interpretation of these paradigms difficult for sensitively and reliably evaluating affect. In sum, these tasks are not well-suited for studying affective state induced by acute NMDAR hypofunction. For those reasons, this study shifted focus onto a motor-independent alternative rat behavior with proven validity in evaluating affect: spontaneous ultrasonic vocalizations (USVs).

My rationale for studying spontaneous rat USVs is that these vocalizations are a rich source of information about the affective state of the animal (Knutson et al., 2002; Brudzynski, 2005; Brudzynski, 2007). More specifically, rats spontaneously vocalize in the ultrasonic frequency range with the majority of the resultant calls falling into frequency ranges centered around approximately 22 kHz or around a mean of roughly 50 kHz (Brudzynski and Holland, 2005; Portfors, 2007). While the purpose of these different classes of vocalizations is incompletely understood, a sizable body of evidence suggests that the relative number of 22 and 50 kHz USVs emitted reflect certain aspects of the rat's affective state. For example, rats emit proportionately more 22 kHz ultrasonic calls in response to exposure to predators, during prolonged social isolation,

after receiving foot shocks and in drug withdrawal, all of which can be reasonably assumed to be aversive situations or stimuli (Francis, 1977; Blanchard et al., 1991; Barros and Miczek, 1996; Jelen et al., 2003). On the other hand, rats tend to emit more calls in the 50 kHz range in appetitive scenarios such as play, mating, and when receiving addictive drugs (Knutson et al., 1998; Knutson et al., 1999; Bialy et al., 2000). Further, overall affective state can also be surmised from the relative proportions of these calls. Presentation of aversive stimuli, for example, can reduce emissions of 50 kHz vocalizations in otherwise appetitive scenarios (Burgdorf et al., 2000; Knutson et al., 2002). Also, rats spontaneously vocalize in scenarios which are neither overtly aversive nor appetitive such as upon transfer to a novel cage (Wohr et al., 2008) suggesting that spontaneous vocalizations can represent baseline affective state. Finally, as this behavior is not dependent on locomotor activity it stands out as uniquely suitable for examining affective state under acute NMDAR hypofunction. Accordingly, I compared the effects of acute NMDAR antagonism in male and female rats using treatment with MK801 paired with analyses of spontaneous USVs emitted during time spent in a novel, open field environment. This assessment was conducted only at the highest dose of 0.2 mg/kg which was previously established to be the most relevant in the preceding chapters for inducing a full range of psychomimetic symptoms in both sexes. Further, as in the preceding sections, I also explored whether any MK801-induced changes in USVs are differentially attenuated in males in females after pretreatment with haloperidol and/or clozapine in order to determine whether this affective symptom correlate is sensitive to neuroleptic attenuation in sex-specific ways.

METHODS

Animal Subjects: A total of 34 male and 32 female adult Sprague-Dawley rats were used; animals were purchased from Taconic Farms (Hudson NY). Animals were housed in same-sex pairs in a temperature controlled room under a 12 hour light/dark cycle (lights on at 7 AM) with food and water available *ad libitum*. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Stony Brook University and were designed to minimize their discomfort and use.

Drugs and Drug Treatments: All drugs used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO). Each animal received two injections on the day of testing. Rats first received a subcutaneous injection of either haloperidol (0.04 mg/kg) or clozapine (5 mg/kg) dissolved in saline acidified with 0.025% acetic acid or acidified saline vehicle. Fifteen minutes later, rats were injected intraperitoneally with either 0.2 mg/kg MK801 dissolved in saline or saline vehicle. Rats were randomly assigned to one of the following groups: vehicle/vehicle (8 males and 8 females); vehicle/0.2 mg/kg MK801 (10 males and 8 females); 0.04 mg/kg haloperidol followed by 0.2 mg/kg MK801 (8 males and 8 females); or 5 mg/kg clozapine followed by 0.2 mg/kg MK801 (8 males and 8 females). All animals were behaviorally tested 15 minutes after the second injection.

Open Field Apparatus: The open field arena was located in a sound attenuated room and consisted of an opaque plastic enclosure, measuring 80 x 30 cm with walls 30 cm high; the floor was marked in 10 x 10 cm squares. The arena was surrounded by a

white curtain to minimize extra-maze cues. On the day of testing, animals were brought into the testing room for the first time and were immediately transferred to the start position in the center of the arena and allowed to freely explore for 15 minutes, during this time the investigator remained outside of the room. After testing, the animal was returned to its home cage and the arena floor and walls were cleaned with 70% ethanol. Individual animals were only tested once.

Ultrasonic Vocalization Recording: All recordings were done with a CM16 condenser microphone interfaced to an UltraSoundGate 116 recording system (Avisoft Bioacoustics, Berlin, Germany) which was in turn interfaced to a computer. The microphone was suspended 60 centimeters above the center of the open field arena. Acoustic data were recorded continuously for the entire 15 minute testing period with Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany) at a sampling rate of 250 kHz in 16 bit format; all data was saved to the computer for offline analysis.

Ultrasonic Vocalization Characterization and Analysis: For analysis, each recording was transferred to Avisoft SAS-Lab Pro (Version 4.52, Avisoft Bioacoustics, Berlin, Germany) and manually analyzed for USVs as per the method of Barker and colleagues (Barker et al., 2010). Specifically, a fast Fourier transform was applied to the time-domain audio data (length: 256, frame size: 100%, flat top window, 75% overlap); resultant spectrograms has a frequency resolution of 977 Hz and a temporal resolution of 0.512 ms. The spectrograms were the visually scanned for patterns resembling USVs (Brudzynski et al., 1993; Wohr et al., 2008). Once a putative USV was identified,

it was morphed to a lower frequency within the human audible range (5% of normal speed) for a separate confirmation of a “whistle”-like quality (Brudzynski and Holland, 2005; Ciucci et al., 2007). Once the identity of a USV was confirmed, median frequency was calculated by averaging the highest and lowest frequency values observed in the vocalization tracing. USVs that were close together temporally were designated as separate calls only if 50 ms or more separated them. For each identified USV, duration was determined by measuring the time from the beginning to the end of the USV as visualized on the spectrogram and bandwidth was computed as the difference between the highest and lowest frequencies observed in the USV.

Statistical Analyses: USVs from each animal were separated into three categories: 22 kHz, mid-range and 50 kHz. The 22 kHz category consisted of all USVs having a median frequency in the range of 18-32.99 kHz, the mid-range category consisted of all USVs having a median frequency in the range of 33-37.99 kHz and the 50 kHz category consisted of all USVs having a median frequency of 38-80.99 kHz; all USVs falling above or below these ranges were excluded from analysis. All statistical tests were conducted using SPSS software (Version 19, IBM). For analyses of USV counts, two-way analyses of variance (ANOVAs) were first used for comparisons of the data across sex and across drug treatment. Further, one-way ANOVAs were also used to compare data within each treatment condition across sex. Finally, one-way ANOVA were used to compare data within each sex across treatment conditions. All allowed post-hoc testing in analysis of call number was conducted using Fisher’s protected least significant difference (PLSD) test. For analyses of USV duration and bandwidth, all USVs in each

frequency range emitted by all animals in each cohort were combined together and statistical analyses were carried out on these large groups. First, two-way analyses of variance (ANOVAs) were used for comparisons of the data across sex and across drug treatment. Further, one-way ANOVAs were used to compare data within each treatment condition across sex. Finally, one-way ANOVA were also used to compare data within each sex across treatment conditions. Due to unequal sample sizes, i.e, differences in the number of calls emitted in each cohort, all allowed post-hoc testing for these analyses was conducted using the Tukey-Kramer method. All observations were considered significant at a $p < 0.05$ level and near-significant at a $p < 0.075$ level.

RESULTS

22 kHz USVs

Number of Vocalizations: Vehicle injected male and female rats emitted few 22 kHz ultrasonic vocalizations. Thus, over the 15 minute testing period, males emitted an average of approximately 1 vocalization at 22 kHz while females vocalized approximately 3 times in the same 15 minute interval (Figure III.1A). After treatment with MK801, 22 kHz vocalizations increased in both sexes, although females showed a much larger increase than males. Specifically, males vocalized an average of approximately 6 times in the 22 kHz frequency range over the testing period after treatment with 0.2 mg/kg MK801 but females emitted on average roughly 25 vocalizations in this frequency range over the same time period. Next, while pretreatment with haloperidol did not appreciably decrease the number of 22 kHz USVs

relative to MK801 alone in both males and females and pretreatment with clozapine also did not have an appreciable effect on the number of 22 kHz USVs emitted by males, clozapine pretreatment in females drastically reduced the number of 22 kHz vocalizations to levels comparable to those at drug-naïve baseline.

The drug and sex differences observed in numbers of emitted 22 kHz USVs were also supported in statistical comparisons. Thus, initial analysis with a two-way ANOVA encompassing data from all treatment groups and both sexes identified a significant main effect of treatment [$F(3,58)=6.02$ $p=.001$] and sex [$F(1,58)=11.00$ $p=.002$] on the number of emitted 22 kHz USVs as well as a significant interaction between treatment and sex [$F(3,58)=4.85$ $p=.004$]. Subsequent one-way ANOVAs that compared data from each treatment group across sex showed no significant differences in the number of 22 kHz USVs between vehicle-injected males and females, that females vocalized significantly more than males after treatment with MK801 [$F(1,14)=10.94$ $p=.005$] and after pretreatment with haloperidol [$F(1,17)=5.62$ $p=.031$] but that after pretreatment with clozapine, the number of USVs emitted by females was significantly lower than in males [$F(1,15)=6.92$ $p=.020$]. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified a significant main effect of treatment on the number of emitted 22 kHz USVs in females [$F(3,28)=5.33$ $p=.005$], but no corresponding significant main effect of treatment in males. Allowed post-hoc analyses in females showed that MK801 significantly increased the number of emitted 22 kHz USVs relative to control ($p=.012$) and that while haloperidol was ineffective in reducing the number of these MK801 induced vocalizations, clozapine significantly reduced the

number of 22 kHz USVs relative to MK801 alone ($p=.007$) to levels statistically indistinguishable from those seen in drug-naïve controls.

Duration: Mean durations of low frequency vocalizations ranged from approximately 16 to 29 ms across all treatment groups in male and female rats. However, within this narrow range, some differences were observed. First, in vehicle-injected animals, mean low-frequency USV duration was approximately 17 ms in males while the corresponding value in females was about 28 ms (Figure III.1B). Next, treatment with MK801 only marginally increased mean USV duration in males but decreased it in females to approximately 20 ms. Pretreatment with haloperidol had no appreciable effect on mean low-frequency USV duration in males but slightly increased it in females to about 24 ms. Finally, pretreatment with clozapine had no effect on mean USV duration in females relative to MK801 alone but did increase it somewhat in males to approximately 28 ms.

These small sex and drug differences in USV duration proved to be statistically robust. First, an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified no main effect of treatment or sex on mean low-frequency USV duration but did identify a significant interaction between treatment and sex [$F(3,598)=4.48$ $p=.004$]. Subsequent one-way ANOVAs that compared data from each treatment group across sex showed that vehicle injected females had a significantly longer USV duration than males [$F(1,31)=4.98$ $p=.033$], that the duration of USVs males and females were not significantly different after treatment with MK801 and after pretreatment with haloperidol and that mean USV duration was

significantly longer in males over females in the clozapine pretreatment group [$F(1,80)=7.80$ $p=.007$]. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified significant effects of treatment on mean USV duration in both sexes (males: [$F(3,139)=3.74$ $p=.013$]; females: [$F(3,459)=4.90$ $p=.002$]). Allowed post-hoc testing showed that in males, no drug treatment significantly affected mean USV duration relative to drug-naïve control values but in females, MK801 significantly decreased USV duration ($p=.027$) and haloperidol but not clozapine significantly raised it relative to MK801 ($p=.013$).

Bandwidth: Mean 22 kHz USV bandwidth varied very little across sex and treatment group. Thus, in vehicle-treated animals, average bandwidth of a low-frequency USV was approximately 7-8 kHz in males and females alike (Figure III.1C). Mean bandwidth was largely unaffected by drug treatment in both males and females and remained squarely within a narrow range of 7-10 kHz. Any minor fluctuations between drug treatment and sex were not statistically robust; analysis with a two-way ANOVA inclusive of data from all treatment groups in both males and females identified no significant main effects of treatment, sex and no significant interaction between treatment and sex.

33-38 kHz (mid-range) USVs

Number of Vocalizations: Both males and females emitted mid-range USVs infrequently in all testing groups, though some group and sex differences were evident.

First, vehicle injected animals rarely vocalized in this range; both males and females emitted only about one mid-range USV over the 15 minute testing session (Figure III.2A). Next, treatment with MK801 slightly increased the number of emitted mid-range USVs by a similar amount in both sexes. Thus, males vocalized approximately 3 times after MK801 while females vocalized approximately 5 times. Further, while pretreatment with haloperidol only marginally reduced the number of mid-range USVs emitted by females, it was more effective in males where it reduced the number of mid-range USVs to drug-naïve levels. Pretreatment with clozapine, on the other hand, had no effect in reducing the MK801-induced increase in mid-range USVs in males but did effectively reverse it in females to levels similar to those seen in drug-naïve controls.

These drug and sex differences in the number of emitted mid-range USV were validated statistically. First, an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified a significant effect of treatment [$F(3,58)=3.43$ $p=.023$] on the number of emitted mid-range USVs as well as a significant interaction between treatment and sex [$F(3,58)=3.47$ $p=.022$]. A subsequent series of one-way ANOVAs that compared data from each treatment group across sex confirmed that the number of mid-range USVs emitted by males and females within the control and MK801 groups did not differ but that after haloperidol pretreatment females vocalized slightly but significantly more than males [$F(1,16)=4.91$ $p=.041$] and that after clozapine pretreatment males vocalized significantly more than females [$F(1,14)=6.06$ $p=.027$]. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified a significant effect of treatment on the number

of emitted mid-range USVs in both sexes (males: $[F(3,30)=4.27 \ p=.013]$; females $[F(3,28)=2.97 \ p=.049]$). Allowed post-hoc testing showed that MK801 significantly to near-significantly increased the number of mid-range USVs emitted by both males and females (males: $p=.054$; females: $p=.023$), that pretreatment with haloperidol near-significantly reduced the number of mid-range USVs relative to MK801 alone in males ($p=.068$) and brought it down to levels statistically indistinguishable from drug-naïve controls but it did not significantly reverse the MK801 induced increase in females. Further, while clozapine had no effect in males, it significantly reduced the MK801-induced increase in females ($p=.013$) to levels similar to control.

Duration: Average durations of mid-range USVs were comparable across all treatment groups in both sexes. Thus, in vehicle-injected males, the average duration of a mid-range USV was approximately 18 ms while in vehicle-injected females, the corresponding value was 24 ms (Figure III.2B). After MK801, average duration changed only slightly in both sexes; in males, it increased to about 27 ms while in females, it decreased to approximately 20 ms. Pretreatment with haloperidol did not affect average mid-range call duration in either sex relative to MK801 and pretreatment with clozapine slightly increased mean USV call duration to about 32 ms in males but did not have an effect in females. However, since mean USV durations across sex and treatment were relatively similar and since relatively few mid-range calls were emitted, statistical analysis with a two-way ANOVA encompassing data from all treatment groups in both sexes failed to identify a significant main effect of treatment or sex on mean mid-

range USV duration, nor did it identify a significant interaction between treatment and sex.

Bandwidth: Mean USV bandwidth varied very little across sex and treatment group. Thus, in vehicle-treated animals, average bandwidth of mid-range USVs was 8-9 kHz in males and females alike (Figure III.2C). After treatment with MK801 and after pretreatment with haloperidol and clozapine, mean bandwidth was largely unchanged in all cases, remaining in the range of 7.5-10.5 kHz. These minor fluctuations between drug treatment and sex were not statistically robust; analysis with a two-way ANOVA inclusive of data from all treatment groups in both males and females identified no significant main effects of treatment, sex and no significant interaction between treatment and sex.

50 kHz USVs

Number of Vocalizations: Similarly to what was seen for low frequency USVs, vehicle-injected male and female rats also vocalized infrequently in the 50 kHz range. Thus, males emitted approximately 6 high-frequency USVs in this frequency range over the 15 minute testing period while females vocalized about 4 times in the same period (Figure III.3A). Next, treatment with MK801 marginally increased the number of 50 kHz USVs emitted by males to approximately 9 calls throughout the session while the increase was more substantial in females who vocalized approximately 13 times. Next, pretreatment with haloperidol marginally attenuated the increase in 50 kHz USVs

emitted by males after MK801 back to drug-naïve levels but did not have an appreciable effect in females. Clozapine, on the other hand, was not effective in males but attenuated the MK801-stimulated increase in 50 kHz USVs in females to levels comparable to those in vehicle-injected controls.

The drug and sex differences in the number of emitted 50 kHz USVs described above proved to be statistically robust. First, an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified a near-significant main effect of treatment [$F(3,58)=2.73$ $p=.052$] on the number of emitted 50 kHz USVs and a significant interaction between treatment and sex [$F(3,58)=2.90$ $p=.042$]. Subsequent one-way ANOVAs that compared data from each treatment group across sex showed that there were no significant differences in the number of 50 kHz USVs between males and females in the vehicle-injected, MK801-treated and haloperidol pretreated groups but that in the clozapine pretreatment group males emitted significantly more 50 kHz USVs than females [$F(1,14)=14.90$ $p=.002$]. Finally, one-way ANOVAs that compared data within sex across all treatment groups identified a significant main effect of drug treatment on the number of 50 kHz USVs in females [$F(3,28)=3.11$ $p=.042$] but not in males. Allowed post-hoc testing revealed that in females, MK801 significantly increased 50 kHz vocalizations ($p=.049$), that pretreatment with haloperidol did not significantly reverse these MK801-induced increases but that clozapine did significantly reduce the number of 50 kHz USVs relative to MK801 alone ($p=.019$) to levels statistically indistinguishable from vehicle-injected controls.

Duration: As with USVs in lower frequency ranges, average durations of 50 kHz USVs were largely similar across both sexes and all treatment groups. In vehicle-injected controls, mean 50 kHz USV duration was approximately 27-30 ms in males and females alike (Figure III.3B). Next, treatment with MK801 marginally reduced mean USV duration in both sexes to about 23-24 ms. Finally, pretreatment with haloperidol or clozapine had virtually no effect on mean USV duration in both males and females. These small variations across sex and drug treatment were not statistically significant. Thus, although an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified a near-significant main effect of treatment [$F(3,477)=2.29$ $p=.077$] on mean 50 kHz USV duration, subsequent one-way ANOVAs that compared data from each treatment group across sex showed that there were no significant differences in mean USV duration between males and females in any drug treatment group. Finally, one-way ANOVAs that compared data within sex across all treatment groups did not identify a significant main effect of treatment in males and females alike.

Bandwidth: Similar to USVs in lower frequency ranges, the mean bandwidth of high-frequency calls was also constrained to a relatively narrow range across all treatment groups in both sexes although mean bandwidths of 50 kHz USVs were slightly higher than those of lower frequency vocalizations. Thus, in vehicle injected controls mean bandwidth was approximately 9-10 kHz in males and females alike (Figure III.3C). Next, treatment with MK801 only marginally increased mean 50 kHz USV bandwidth in

both sexes. Finally, pretreatment with haloperidol and clozapine increased average bandwidth even further in both sexes, to values in a range of 12.5-15.5 kHz. However, these drug and sex differences were only somewhat statistically robust. Thus, while an initial two-way ANOVA comparing data from male and female subjects from across all eight experimental groups identified a significant main effect of treatment [$F(3,477)=4.18$ $p=.006$] on mean 50 kHz USV bandwidth, a series of follow-up one-way ANOVAs within each treatment group identified no significant differences in mean 50 kHz USV bandwidth between males and females within any treatment group. Furthermore, one-way ANOVAs that compared data within sex across all treatment groups identified a near-significant main effect of treatment in males [$F(3,239)=2.55$ $p=.057$] but not in females and subsequent allowed post-hoc testing in males showed that no treatment significantly changed average bandwidth relative to drug-naïve controls.

DISCUSSION

In addition to positive and negative/cognitive symptoms, affective symptoms also contribute significantly to the debilitating clinical picture of schizophrenia. Thus, sufferers of schizophrenia often exhibit signs of depression and anxiety which is thought to be a major causative factor in the extremely high rate of suicide associated with this mental illness (Johns et al., 1986; Palmer et al., 2005). However, clinical evidence suggests that these affective symptoms may be currently under-treated, especially as other, more prominent symptoms of this disorder can easily mask the more subtle anomalies in affective state. More relevant to this study, however, is the finding that like many other aspects of schizophrenia, the manifestation and prevalence of affective

symptoms is extensively sexually dimorphic. More specifically, women exhibit a greater degree of affective symptoms than men; numerous studies have found a higher prevalence of dysphoria, depression, anxiety and inappropriate affect in females (Szymanski et al., 1995; Leung and Chue, 2000). Furthermore, there is very little data studying sex differences in the alleviation of affective symptoms in response to pharmacological treatment. As with other symptom clusters of schizophrenia discussed in the preceding chapters of this dissertation, investigation into the underpinnings of sex differences in affective symptoms has been impeded by a lack of an animal model that can accurately recapitulate their differential prevalence and characteristics. Thus, having established that the NMDAR hypofunction model largely recapitulates sex differences in the prevalence and response to treatment of positive and negative symptoms, the experiments in this chapter paired treatment with MK801 with or without pretreatment with haloperidol and clozapine paired together with analysis of spontaneous ultrasonic vocalizations (USVs) emitted by male and female rats in order to examine whether the ability of NMDAR hypofunction in recapitulating sex differences in schizophrenia also extends to the third and final affective symptom cluster and if so, whether treatment with typical and atypical neuroleptics attenuates these symptoms in a sex-specific manner.

While several behavioral tasks could have been selected for studying affective changes, analysis of spontaneous USVs stands out as a deceptively simple method to assess complex changes in affective state. This behavioral task is driven by the fact that rats spontaneously vocalize in two main frequency ranges of 22 and 50 kHz, with

low frequency vocalizations being indicative of a negative affective state while high frequency vocalizations are in turn indicative of satiety, pleasure and generally positive affect (Knutson et al., 2002; Brudzynski, 2007). Surprisingly, however, spontaneous USVs have never been studied under a state of acute NMDAR hypofunction to assess affective state and sex differences within it in response to this class of drugs. Incidentally, USVs stand out as especially attractive for studying affect in this study due to the fact that their emission is not increased by excessive psychomotor activation which occurs in states of acute NMDAR hypofunction (Burgdorf et al., 2000). As discussed further below, the major finding of the experiments here were that while both sexes generally vocalized more after MK801, the increase was much more pronounced in females and consisted of a disproportionate increase of 22 kHz vocalizations than in males. Further, while haloperidol was generally ineffective in reducing the number of emitted USVs in both males and females, clozapine did effectively attenuate MK801-induced increases in vocalization in both sexes. In the sections that follow, these results are discussed in detail and related to previous studies in rodents and humans in order to illustrate that the sex-specific changes in spontaneous USVs emitted after acute MK801 with and without neuroleptic pretreatment accurately model the sex differences in affective symptoms seen in schizophrenia and its treatment. Finally, these findings are discussed in the context of studies investigating the anatomical and neurochemical underpinnings of ultrasonic vocalizations to explore some potential theories of these differences, their relevance to schizophrenia, and their implications for further studies.

Effects of MK801 and Neuroleptic Pretreatment on USVs: Relevance to Sex Differences in Affective Symptoms of Schizophrenia

Despite the utility and validity of spontaneous ultrasonic vocalizations in studying affect, it is surprising that no studies to date have attempted to study this endpoint in relation to acute NMDAR hypofunction. Thus, this study is the first to report that acute NMDAR hypofunction and neuroleptic pretreatment causes sex-specific changes in specific characteristics of spontaneous USVs. While USV bandwidth and duration were evaluated these were largely unaffected, drug treatment profoundly affected the number of vocalizations in sex-specific ways.

Duration and Bandwidth: The analyses in this study included assessment of mean duration and bandwidth of USVs in all frequency ranges in order to probe for a drug effect on these parameters. However, neither of these measures showed clear differences across sex or drug responsiveness for USVs in any frequency range. For example, mean duration of 22 kHz, mid-range and 50 kHz USVs all fell within ranges that have been reported previously in drug naïve rats (Brudzynski, 2005; Brudzynski, 2007). It should be noted that all 22 kHz calls observed in this study were in the subcategory of short (<300 ms) calls, which are structurally different from long alarm calls that are exclusively emitted by rats in response to a predator or extremely aversive stimuli. Nonetheless, short 22 kHz calls are emitted in response to generally unpleasant stimuli such as handling and foot shock (Brudzynski et al., 1991). Mean bandwidth of all calls was also noted to be within or near ranges reported previously

(Brudzynski, 2005) and showed very little difference in drug-treated compared to drug naïve animals. Thus, while, some significant group differences in bandwidth and duration were identified statistically, these differences were quantitatively small and likely an artifact of the large numbers of observations (calls) that were compared.

Number of USVs: As all previous assessments of USVs in an open field have been done in males, baseline measures of relatively few and predominantly 50 kHz vocalizations emitted by vehicle-injected males agree with previous reports examining USV emissions in the open field (Wohr et al., 2008). The baseline measures in females are new findings that corresponded closely to the infrequent and predominant high-frequency vocalizations emitted by the vehicle-injected male cohort. After MK801, however, vocalizations in all frequency ranges increased in both sexes. However, there were some notable differences between them. For example, in males, MK801 modestly and similarly increased the emission of low, mid-range and high frequency USVs. In contrast, while MK801 treatment had similar effects on USVs in the mid and high frequency ranges in females, it increased their 22 kHz vocalizations to a much higher degree. Thus, while MK801 generally increased all types of vocalizations in both sexes, it produced a higher ratio of 22 kHz to 50 kHz USVs in these animals, indicating a more negative affective state compared to the males. This is in turn consistent with a large body of evidence in the clinical literature which shows that schizophrenic women suffer from more affective disturbances than men (Goldstein, 1988; McGlashan and Bardenstein, 1990; Leung and Chue, 2000) and indicates that the rodent NMDAR

hypofunction model also extends to modeling the sex differences seen in affective symptoms of schizophrenia.

Further, while pretreatment with haloperidol was not effective in reversing MK801-induced vocalization increases at 22 or 50 kHz in either sex, it was able to reduce the mid-range vocalization increase in males. However, the MK801-induced increases in mid-range USVs were small and against a very low baseline to begin with, making it difficult to assess the reliability or validity of these seemingly isolated effects. Further, since the behavioral relevance of mid-range USVs is also unclear, I would not use these data as evidence arguing against the wealth of clinical findings which indicate that affective symptoms of schizophrenia are refractory to treatment with typical neuroleptics (Azorin, 1995; Beasley et al., 1996; Tollefson et al., 1998; Siris, 2000).

In contrast, clozapine was effective at attenuating MK801-induced increases in 22 kHz, mid-range and 50 kHz vocalizations in both sexes, although this effect was much more robust in females. Again, the relatively low numbers of baseline vocalizations and their increases induced by MK801 in the male cohort made it difficult to rigorously characterize the degree of clozapine efficacy in this sex. Regardless, the effectiveness of clozapine on reducing these vocalizations in females- specifically, at normalizing the number of 22 kHz calls- was quantitatively robust and dovetails with clinical data indicating that atypical antipsychotics are effective at ameliorating affective disturbances in schizophrenia (Buchanan, 1995; Masan, 2004; Burton, 2006). Thus, having established that the NMDAR hypofunction model along with neuroleptic

pretreatment recapitulates salient sex and drug differences in affective symptoms and their treatment seen in schizophrenia, the following sections consider these results along with the neurobiological substrates of USV generation to provide new insights on potential underpinnings of the sex differences seen in schizophrenia.

Biological Substrates of USV Generation: New Insights into Schizophrenia's Pathophysiology and into Novel Targets for Investigating Sex Differences in Disease

The findings discussed above demonstrate clear sex differences in the numbers of different classes of ultrasonic vocalizations that are emitted by rats after treatment with MK801. They also suggest sex differences in the degree to which these drug-induced changes are attenuated by typical and atypical neuroleptic drugs. While I take this as evidence of a recapitulation of behavioral sex differences in schizophrenia, arguments for this may be strengthened by considering that the circuits responsible for generation of spontaneous USVs in rats are in many cases homologues to structures and neurochemical systems at risk in humans in schizophrenia. For example, pharmacological studies using local infusion of amphetamine into the nucleus accumbens (NAc) suggest that the generation of 50 kHz USVs that follows is dependent on dopamine release in this limbic brain area from its DA afferents arising from the mesolimbic dopamine system originating in the ventral tegmental area (VTA, Burgdorf et al., 2001; Thompson et al., 2006). Considering that activation of the mesolimbic DA system and resultant dopamine release in the NAc have been linked to motivation, salience and reward in rodents and humans alike (for reviews, see Koob, 1996; Ikemoto

and Panksepp, 1999), it may not be surprising that activation of this structure is linked to the class of vocalizations indicative of pleasure and positive affect. However, in addition to these normal behaviors, the mesolimbic dopamine system and its dysregulation are also associated with key pathological processes in schizophrenia. Specifically, increased activity and dopamine levels in the NAc have been linked to the positive symptom cluster of schizophrenia which is known to be more severe in women (Silbersweig et al., 1995; O'Donnell and Grace, 1998; Epstein et al., 1999). Potential significance of this circuit in the pathophysiology of the sex differences in the positive symptoms of schizophrenia is supported by findings that mesolimbic DAergic connections from the VTA to NAc are known to be sensitive to gonadal hormones through both intracellular and membrane receptors (Di Paolo et al., 1985; Di Paolo, 1994; Becker, 1999; Creutz and Kritzer, 2004). Combined with the finding that acute NMDAR antagonism increases DA levels in the NAc (Lecourtier et al., 2007), the results here of a female over male increase 50 kHz USVs after MK801 suggests that NMDAR hypofunction elevates NAc DA levels to a greater extent in females over males. However, the sex difference in MK801-stimulated USVs was relatively modest and is overshadowed by a much more robust difference in 22 kHz USVs emitted by males and females after MK801. This substantial sex difference shifts focus onto the underpinnings of this class of vocalizations as indicative of potential sexual dimorphisms that may be relevant to schizophrenia.

The generation of low-frequency (22 kHz) calls indicative of negative affect has been shown to be dependent on activation of cholinergic neurons arising in the

laterodorsal tegmental nucleus (LDT) in the brainstem (Brudzynski, 2007; Brudzynski et al., 2011). Cholinergic projections from the LDT innervate a wide range of midbrain and forebrain structures, including the thalamus, substantia nigra, VTA and all divisions of the medial prefrontal cortex (Satoh and Fibiger, 1986; Cornwall et al., 1990); the LDT also receives collateral inputs from many of the areas it innervates (Satoh and Fibiger, 1986). Interestingly, this structure has been associated with schizophrenia in that it modulates rapid eye movement (REM) sleep, which not only is profoundly disrupted in schizophrenia sufferers (Shiromani et al., 1987), but the severity of these disruptions is positively correlated to the severity of positive and negative symptoms of the disease (Tandon et al., 1992; Zarcone and Benson, 1997; Poulin et al., 2003; Benson, 2006). These epidemiological findings tie in with evidence indicating that REM sleep is initiated by the activation of cholinergic cells in the LDT (Yeomans, 1995) to suggest that the cholinergic system arising in the LDT is overactive in schizophrenia, a theory that is supported by evidence from post-mortem and imaging studies showing that muscarinic receptor density and availability is significantly reduced in schizophrenics versus healthy controls, findings consistent with chronically elevated acetylcholine levels and subsequent sensitization (Dean et al., 1996; Crook et al., 1999; Crook et al., 2000; Crook et al., 2001; Raedler et al., 2003). Furthermore, the use of direct muscarinic receptor agonists in schizophrenia has been associated with alleviation of both positive and negative symptoms, further lending support to the theory that chronically increased cholinergic tone leads to receptor downregulation, sensitization and reduced cholinergic signaling (Mirza et al., 2003); this finding has been confirmed in a clinical trial which

reported that monotherapy with xanomeline, an M₁/M₄ receptor agonist is effective at ameliorating positive and negative symptoms in schizophrenia (Shekhar et al., 2008).

Thus, it is clear that cholinergic signaling and tone plays a key role in schizophrenia's pathology and may be an attractive target for novel treatment strategies. However, it is still unclear what mechanism lies behind these aberrations in cholinergic signaling and even less is known about whether there are sex differences in the neuroanatomical and neurochemical substrates modulating these phenomena. Interestingly, the NMDAR hypofunction model is known to produce aberrations in the cholinergic system closely reminiscent of those seen in schizophrenia. For example, NMDAR hypofunction induces increased cholinergic tone which is known to exist in schizophrenia and behavioral abnormalities induced by NMDAR hypofunction are reversible by treatment with muscarinic antagonists (Kikuchi et al., 1997; Jentsch et al., 1998; Barak and Weiner, 2011). However, it is still unknown whether NMDAR hypofunction affects cholinergic tone in the LDT. The results of this study suggest that not only that NMDAR hypofunction affects the activity of the LDT but that there are sex differences in these loci. Specifically, the finding that NMDAR hypofunction induced a much larger increase in 22 kHz vocalizations in females than males dovetails with the previously discussed data showing that activation of cholinergic efferents of the LDT is required for 22 kHz USV generation to suggest that acute NMDAR hypofunction activates cholinergic neurotransmission arising in the LDT to a greater extent in females than males. Interestingly, almost nothing is known about sex differences and hormone sensitivity of this nucleus or its efferent connections which is particularly intriguing,

considering the far-reaching sequelae of LDT overactivation. In turn, these include increased acetylcholine release in the VTA which causes increased activity of the mesolimbic and mesocortical DA systems (Omelchenko and Sesack, 2006; Mena-Segovia et al., 2008), leading to aberrations in the circuits and brain areas that are intimately linked with schizophrenia's positive, negative/cognitive and affective symptoms. Further, while electrophysiological studies have confirmed that systemic administration of NMDAR antagonists increases burst firing of mesolimbic DA cells and increases the basal firing rate of mesocortical DA projections (French et al., 1993; Murase et al., 1993; Svensson, 2000), it is still unknown whether these effects are due to activation of cholinergic signaling arising from the LDT. Taken together, this evidence suggests that further study of the effects of NMDAR hypofunction on LDT activity and of gonadal hormone sensitivity of this neurochemical system may provide clues of sexually dimorphic substrates involved in regulation of a wide range of behaviors at risk in schizophrenia. To that end, measurement of spontaneous USVs may be a useful tool in assessing activity of this neurochemical circuit and warrants further study.

Conclusion

Although spontaneous rodent ultrasonic vocalizations have previously been studied and characterized in detail as powerful indicators of affective state, this is the first time that measurement of this behavior has been studied under a state of acute NMDAR hypofunction. As affective symptoms are a major component of schizophrenia

and are known to be sexually dimorphic in their incidence and severity, the experiments here compared USV characteristics in males and females in response to MK801 with and without neuroleptic pretreatment to see if the NMDAR hypofunction model extends to recapitulating sex differences in affective symptomatology seen in disease. The results obtained here indicate that it does. Specifically, while acute MK801 increased the number of spontaneous vocalizations in both sexes, the number of low-frequency 22 kHz vocalizations indicative of a negative affective state was increased to a much greater degree in females, mirroring a higher degree of affective symptom severity in female versus male schizophrenia patients. Furthermore, pretreatment with typical and atypical neuroleptics also mirrored differences in affective symptom treatment. Whereas haloperidol failed to reverse the effects of MK801, clozapine was generally effective, recapitulating clinical data finding that only atypical neuroleptics are effective at ameliorating affective symptoms. Thus, combined with the data from the previous two chapters which has shown that NMDAR hypofunction with and without neuroleptic pretreatment causes sex-specific behavioral changes in acoustic startle, prepulse inhibition and a range of open field behaviors with correlations to the positive and negative/cognitive symptoms of schizophrenia, the findings here show that NMDAR hypofunction also recapitulates sex differences in the third major symptom cluster of affective symptoms. This combination of findings makes the rodent NMDAR hypofunction model stand out as a suitable framework for investigating the underlying neurobiology of a wide range of sex differences evident in disease. To address this logical next question, the following chapters use two neurochemical assay techniques- microdialysis and magnetic resonance spectroscopy- to begin to probe brain areas and

transmitter systems at risk in schizophrenia for underlying sex differences that could translate to the sexually dimorphic behavior seen in NMDAR hypofunction and in disease.

22 kHz

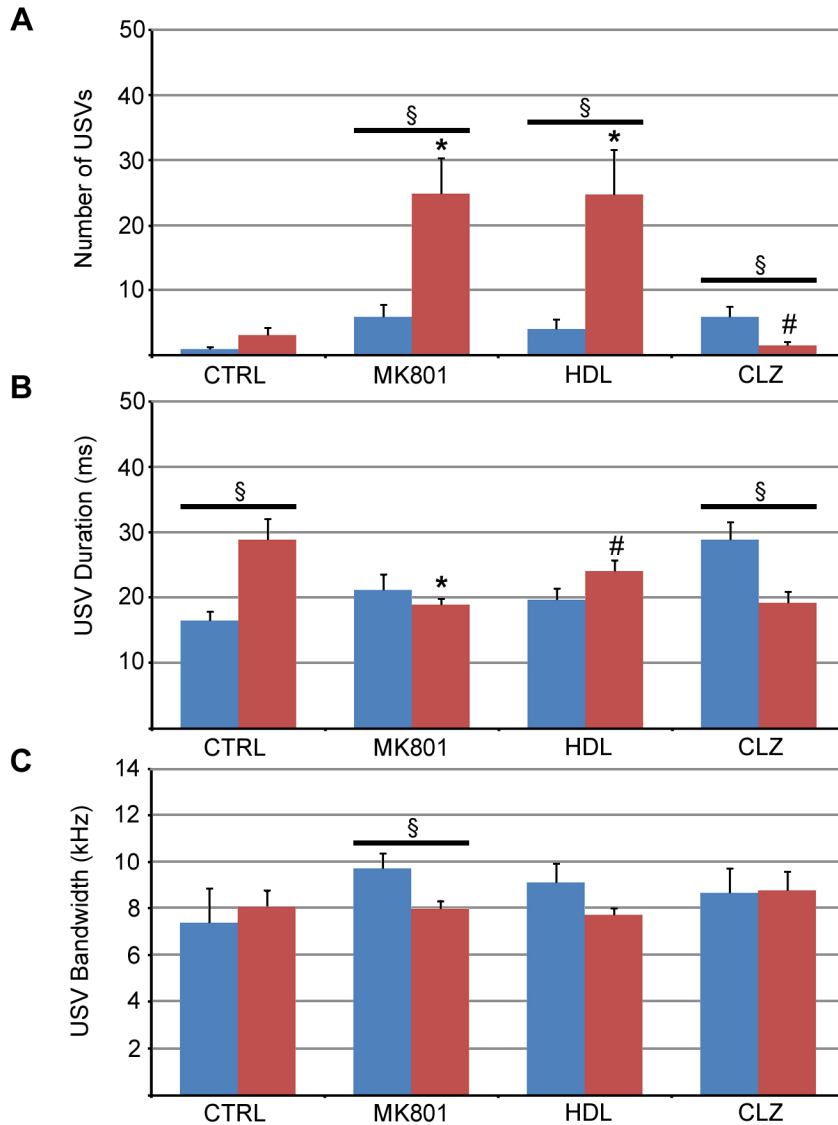


Figure III.1. Bar graphs showing the number (A), duration in milliseconds (B) and bandwidth in kilohertz (C) of low frequency (22 kilohertz) spontaneous ultrasonic vocalizations indicative of negative affect emitted by male (blue bars) and female (red bars) rats. Rats were either vehicle-injected (CTRL), treated with 0.2 mg/kg MK801 (MK801), treated with 0.04 mg/kg haloperidol followed by 0.2 mg/kg MK801 (HDL) or treated with 5 mg/kg clozapine followed by 0.2 mg/kg MK801. All data are expressed as mean±SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, pound signs (#) indicate a significant difference compared to MK801 and section signs (§) indicate a significant difference between males and females.

MID-RANGE (33-38 kHz)

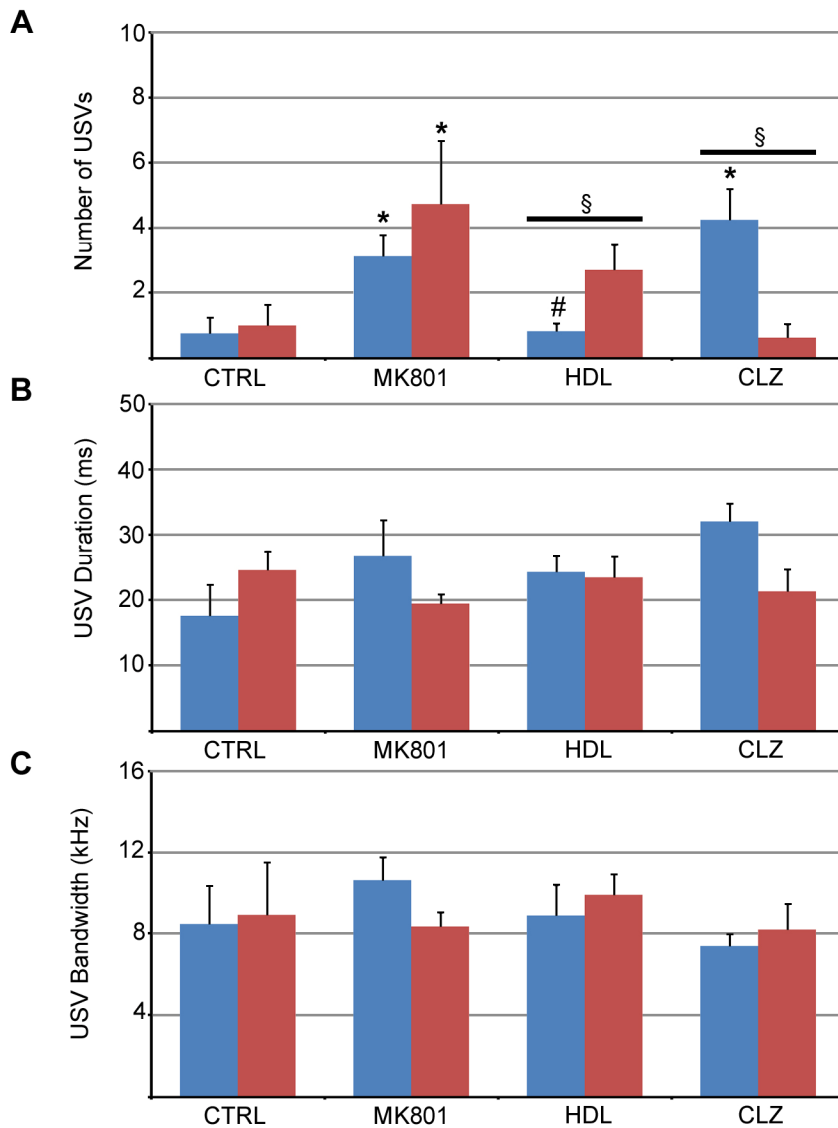


Figure III.2. Bar graphs showing the number (A), duration in milliseconds (B) and bandwidth in kilohertz (C) of mid-range (33-38 kilohertz) spontaneous ultrasonic vocalizations emitted by male (blue bars) and female (red bars) rats. Rats were either vehicle-injected (CTRL), treated with 0.2 mg/kg MK801 (MK801), treated with 0.04 mg/kg haloperidol followed by 0.2 mg/kg MK801 (HDL) or treated with 5 mg/kg clozapine followed by 0.2 mg/kg MK801. All data are expressed as mean \pm SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, pound signs (#) indicate a significant difference compared to MK801 and section signs (§) indicate a significant difference between males and females.

50 kHz

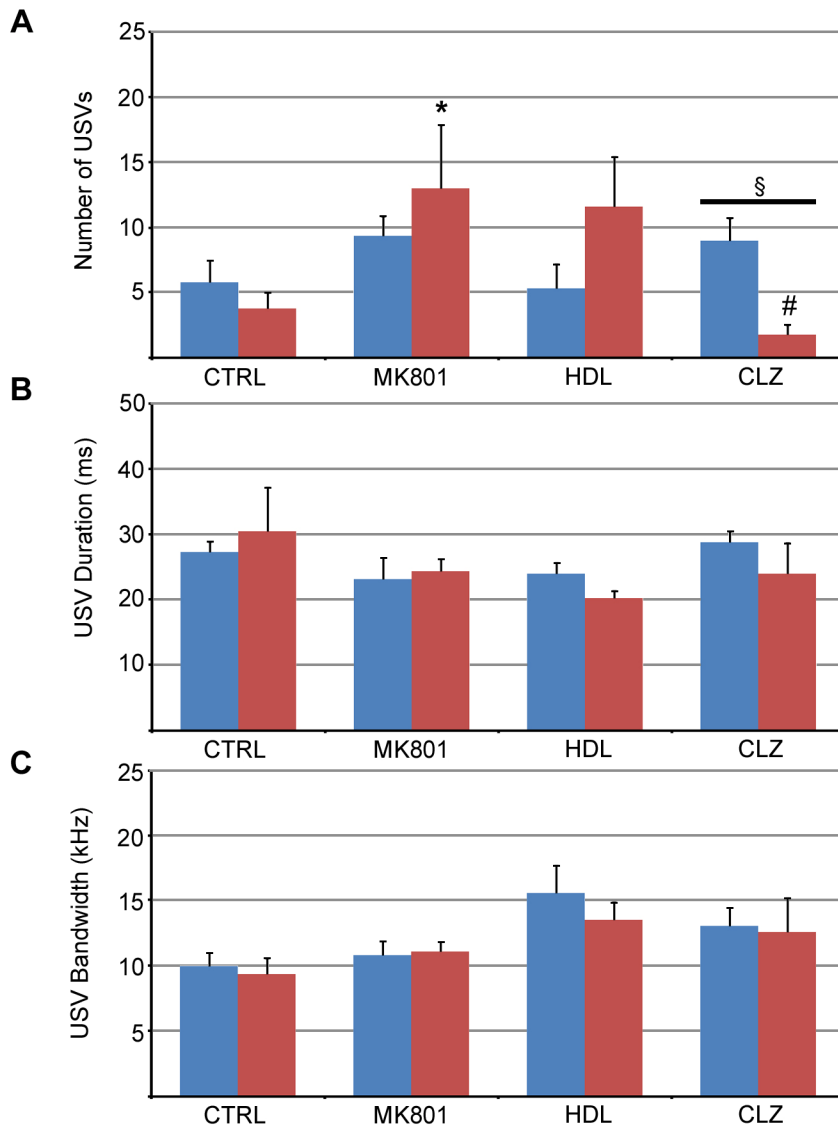


Figure III.3. Bar graphs showing the number (A), duration in milliseconds (B) and bandwidth in kilohertz (C) of high frequency (50 kilohertz) spontaneous ultrasonic vocalizations indicative of positive affect emitted by male (blue bars) and female (red bars) rats. Rats were either vehicle-injected (CTRL), treated with 0.2 mg/kg MK801 (MK801), treated with 0.04 mg/kg haloperidol followed by 0.2 mg/kg MK801 (HDL) or treated with 5 mg/kg clozapine followed by 0.2 mg/kg MK801. All data are expressed as mean±SEM. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to control, pound signs (#) indicate a significant difference compared to MK801 and section signs (§) indicate a significant difference between males and females.

CHAPTER IV

Effects of acute NMDA receptor hypofunction of the levels of dopamine and its metabolites in the prefrontal cortex of adult male and female rats

As discussed in the previous chapters of this dissertation, acute NMDAR antagonism produces a wide range of positive, negative and cognitive symptoms reminiscent of schizophrenia in humans and corresponding symptom correlates in animals. Further, the preceding studies have established that these behaviors are extensively sexually dimorphic in rats with females showing a greater degree of positive symptom correlates and males showing greater negative/cognitive symptom correlates, a pattern consistent with that seen in sufferers of schizophrenia. As all behavior is a final output of underlying neurobiological systems and circuits, a parallel endeavor of the behavioral studies of this dissertation asked whether the sex differences observed in behavior translate to sex differences in neurotransmitter systems and brain areas known to modulate these behaviors. While the structural and neurochemical bases of numerous behaviors could be studied, the underlying mechanisms of the negative/cognitive symptom cluster stand out as especially important for investigation. The rationale for this stems from the fact that while positive symptoms are responsive to medication, negative and cognitive symptoms are much more resistant to treatment and as a result, cause disproportionate disability, morbidity, and lower quality of life when compared to other symptoms of schizophrenia (Breier et al., 1991; Mayerhoff et al., 1994).

While a number of brain structures and transmitter systems could be studied with arguable relevance to the negative and cognitive symptoms of schizophrenia, a significant body of work has correlated this symptom cluster to abnormalities within the prefrontal cortices (PFC, Wolkin et al., 1992; Okubo et al., 1997; Wible et al., 2001). In humans and animals alike, the PFC is a series of forebrain regions that have been established to be involved in executive planning, information processing, and other high-order functions such as working memory and behavioral flexibility (Goldman-Rakic, 1987; Dalley et al., 2004). Interestingly, these functions are dependent on the proper functioning of the mesocortical dopamine (DA) system which arises in the midbrain ventral tegmental area (VTA) and projects to the PFC. For example, studies from human populations have shown that cognitive dysfunction is strongly correlated to depressed PFC DA levels in the PFC not only in schizophrenia but also in normal aging (Akil et al., 1999; Weinberger et al., 2001; Mattay et al., 2002). Furthermore, experiments in rats have demonstrated that lesions of PFC DA afferents and administration of dopamine D₁ agonists strongly impair performance on cognitive tasks that are frontal-lobe dependent such as in open field testing, delayed alternation paradigms and novel object recognition (Tassin et al., 1978; Kalsbeek et al., 1989; Zahrt et al., 1997). This evidence illustrates that PFC function is critically dependent on proper DA tone and that too much or too little DA can have profound effects on proper cognitive functioning. Of more interest to this study, however, are the findings that DA levels in the PFC and resultant behaviors are strongly affected by the manipulations of the glutamate system. For example, administration of systemic NMDA receptor antagonists causes a profound increase in DA levels in the PFC along with concomitant

deficits in sensitive PFC-dependent behavioral tasks (Verma and Moghaddam, 1996; Stefani and Moghaddam, 2005). Taken together, this indicates that PFC DA is a critical regulator of behavior and that its levels are markedly affected by a primary dysfunction in the glutamatergic system which is posited to be the primary defect in schizophrenia.

In addition to the above, further impetus for studying DA levels in the prefrontal cortex comes from a significant body of evidence showing that this system, which serves as a critical modulator of high-order executive and cognitive functioning, is highly sensitive to the actions of gonadal hormones in both sexes, proper levels of which are vital for proper cognitive performance. For example, in men, decreased testosterone titers have been positively correlated to the severity of cognitive deficits in aging (Janowsky, 2006), androgen deprivation therapy for prostate cancer (Nelson et al., 2007) and in schizophrenia (Shirayama et al., 2002; Goyal et al., 2004). This evidence from human studies is linked to prefrontal DA signaling through animal studies that have found that gonadectomy in adult male rats and resultant elimination of testosterone selectively increases DA axon density and extracellular DA in the PFC and concomitantly impairs performance on PFC-sensitive tasks (Kritzer, 2000; Aubele et al., 2008; Aubele and Kritzer, 2011b; Aubele and Kritzer, 2011a). In females, gonadal hormone levels have also been correlated with cognitive function; women perform better on perceptual, visual, and verbal memory tasks in the luteal compared to the follicular phase of the menstrual cycle when progesterone is high (Broverman et al., 1981; Hampson, 1990; Berman et al., 1997) and cognitive performance is decreased in menopause when estrogen and progesterone are low (Greendale et al., 2009).

Furthermore, experiments in animals also revealed that ovariectomy profoundly increases DA axon density in PFC and concurrently impairs cognitive performance (Kritzer and Kohama, 1999; Frye and Walf, 2008).

With these facts in hand, it becomes evident that the prefrontal dopamine system sits at a critical junction of being intimately involved in modulating cognitive behavior as well as being strongly sensitive to gonadal hormones and the effects of NMDAR antagonism, making it an ideal target in the initial study of the underpinnings of the sex differences in behavior in a state of NMDAR antagonism and their relevance to schizophrenia. Thus, the experiments presented in this chapter of this dissertation used *in-vivo* microdialysis to ask whether acute NMDAR hypofunction also induces sex-specific changes in the extracellular levels of dopamine and its major metabolites in the prefrontal cortex of adult male and female rats; levels of these moieties were measured at baseline and for two hours after acute injection of MK801.

METHODS

Animal Subjects: A total of 6 male and 6 female adult Sprague-Dawley rats were used; animals were purchased from Taconic Farms (Hudson NY). Animals were housed in same-sex pairs in a temperature controlled room under a 12 hour light/dark cycle (lights on at 7 AM) with food and water available *ad libitum*. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Stony Brook University and were designed to minimize their discomfort and use.

Surgery and Placement of Guide Cannulae: All surgeries were carried out under aseptic conditions. 48-72 hours prior to microdialysis, animals were anesthetized with intraperitoneal injections of ketamine (0.9 mg/kg) and xylazine (0.5 mg/kg). Upon verification of the absence of paw withdrawal and corneal reflexes, rats were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and incisions were made to expose the skull. A burr hole was drilled over the left medial prefrontal cortex (mPFC) and guide cannulae (CMA12, CMA Microdialysis, North Chelmsford, MA) were slowly inserted; coordinates for cannula insertion, relative to bregma, were: anteroposterior (AP), +3.2, mediolateral (ML), +0.3, and dorsoventral (DV), -2.2 (Paxinos and Watson, 1986). Cannulae were secured to the skull with jeweler's screws and acrylic dental cement. Following surgery, animals were given 0.03 mg/kg buprenorphine to manage post-operative discomfort.

Microdialysis: Two or three days after surgery, awake and freely moving animals were placed in Ratum rodent bowls (BioAnalytical Systems) and allowed to acclimatize for 15 minutes; all experiments were carried out during the animals' subjective night. Then, stylets from the guide cannulae were removed and microdialysis probes (CMA12, 100,000 dalton cutoff, 3 mm polyethersulfone membrane tip, CMA Microdialysis) were slowly lowered into place. Over the next two hours, probes were continuously perfused with artificial cerebrospinal fluid (145 mM NaCl, 2.8 mM KCl; 1.2 mM MgCl₂; 0.25 mM ascorbic acid; 5.4 mM D-Glucose, 1.2 mM CaCl₂ in 1L H₂O, pH 6.8) at a flow rate of 2 μ L per minute. Following this equilibration period, dialysate samples were collected every 15 minutes and directly injected into a PM-92E HPLC system (BioAnalytical

Systems) via an autoinjector (Pollen-8, BioAnalytical Systems). All analyses used a microbore analytical column (UniJet, 1.0 mm inner diameter, 150mm length, 3 μ m ODS particles; BioAnalytical Systems) and an Epsilon electrochemical detector (BioAnalytical Systems) with the applied potential set to 0.75 V versus the Ag/AgCl reference electrode. The mobile phase consisted of 14.5 mM NaH₂PO₄; 30 mM Sodium citrate; 10 mM diethylamine HCl; 2.2 mM 1-octanesulfonic acid; .027 mM EDTA; 7.2% acetonitrile (v/v), 1% tetrahydrofuran (v/v), pH 3.4. Baseline dialysates were collected until metabolite levels were seen to be steady for three successive samples at which point the animal received an intraperitoneal injection of 0.2 mg/kg MK801 dissolved in a volume of 2 mL/kg of normal saline. Following injection, dialysates were collected for two hours. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Locomotor Activity Rating: During the experiments, a video camera (Apple iSight) suspended half a meter above the bowl floor was used to record the animals' activity. Recordings were made for one minute immediately after collection of each dialysate began and were saved to a computer (Apple PowerBook) and analyzed off-line. Assessments were performed off-line from the archived video recordings and were scored by a single trained observer. Animal locomotor activity during collection of each dialysate was rated on a scale from 0-10 where 0 corresponded to no activity or the animal being asleep and 10 corresponded to extreme hyperlocomotion.

Determination of Estrus Cycle Stage in Female Rats: After conclusion of microdialysis, female rats underwent vaginal lavage. This was facilitated by wrapping the animals

gently in a thick towel and gently restraining them supine against the tabletop; this process was designed to minimize stress and animal motion during the lavage procedure. Estrus cycle stage was determined by light microscopy of a wet-mount preparation by observing the relative ratios of leukocytes, nucleated epithelial cells and cornified epithelial cells (Marcondes et al., 2002; Goldman et al., 2007).

Euthanasia and Histology: Immediately after the experiment or after lavage, rats were euthanized via rapid decapitation. The brains were removed and immersed in a solution of 30% sucrose and 10% formaldehyde for fixation. After the brains sank in solution, the frontal cortex was rapidly frozen and sectioned in the coronal plane on a freezing microtome; section thickness was 40 μm . For each animal, a 1 in 4 series of section taken from the mid olfactory bulb through the genu of the corpus callosum was collected, slide mounted, stained with 0.5% cresyl violet and examined to verify the location of the microdialysis probe. In order to be included in the analyses, probes had to have been placed in the left pregenual medial PFC, extend dorsoventrally through prelimbic and infralimbic areas but terminate prior to entering the dorsopeduncular cortex.

Data Analysis: DA, DOPAC and HVA peak identities were confirmed and quantified in relation to standard peaks of known concentrations (2 and 10 ng/mL) that were run through the HPLC system immediately prior to the microdialysis experiment; values of peak area were measured using ChromGraph 2.51 (BAS, West Lafayette, IN, USA). After quantification of peak area, these values were converted to concentrations

(fmol/ μ L) using values from the standard chromatograms. All subsequent statistical testing was conducted using SPSS software (IBM, version 19). Basal DA, DOPAC and HVA levels were averaged per subject from 3 separate stable measurements per subject before injection with MK801. These baseline values from individuals were first compiled and assessed with one-way analyses of variance (ANOVAs) with sex as the across-group factor to examine sex differences in basal metabolite levels. Drug-induced changes in DA, DOPAC and HVA were assessed as percents of these average baselines while drug-induced changes in behavior scores were assessed based on the raw values. These changes were first analyzed using two-way ANOVAs with repeated measures designs to probe for significant effects of time (drug treatment) and sex on the levels of each moiety. One-way ANOVAs with repeated measures designs were also used to compare data within each sex separately across time. All repeated measures ANOVAs were preceded by Mauchly's sphericity test to ensure that observations obeyed required assumptions; a Greenhouse-Geisser correction was applied as necessary. One-way ANOVAs were used to compare data at individual time points across sex. Bonferroni corrections were applied in cases of multiple pair-wise comparisons for all post-hoc testing. To assess the relationship between DOPAC and HVA levels and locomotor behavior, Pearson's product-moment correlation coefficients were computed between all values of each metabolite and all behavior score values across the entire experiment in each sex; these were followed by linear regression analyses. All observations were considered significant at a $p < 0.05$ level and near-significant at a $p < 0.080$ level.

RESULTS

Basal Levels: Basal levels of extracellular DA, DOPAC and HVA were assessed. For DA, levels in male rats were similar to values previously reported in *in-vivo* microdialysis studies of DA in the mPFC in this laboratory and others (Moghaddam and Jackson, 2004; Stefani and Moghaddam, 2005; Aubele and Kritzer, 2011b; Aubele and Kritzer, 2011a). Thus, mean basal DA values in males were approximately 0.15 fmol/ μ L. In females, however, mean basal DA levels were approximately three-fold higher and were approximately 0.27 fmol/ μ L (Figure IV.1). Next, levels of extracellular DOPAC were seen to be larger than those of DA in both sexes. Thus, in males, mean extracellular basal DOPAC levels were approximately 0.95 fmol/mL and in females, the corresponding value was 1.45 fmol/mL. Finally, levels of extracellular HVA fell between those of DA and DOPAC. Thus, in males, basal extracellular HVA had an average value of approximately 0.60 fmol/mL and the corresponding value in females was about 1.00 fmol/mL. However, despite a clear trend toward a higher level of all three moieties in females, these sex differences were not validated statistically; one-way ANOVAs comparing levels of DA, DOPAC and HVA between the sexes failed to identify a significant sex difference in the basal levels of any of the three metabolites.

Response to MK801

DA: After treatment with MK801, average extracellular PFC DA levels in both males and females remained around baseline for approximately 40 minutes and then slowly began to decline. Males showed a marginally larger rate of decline than females and at

the end of the experiment 2 hours after drug administration, mean DA levels in males were approximately 25% below baseline while DA levels in females were only about 10% lower than baseline (Figure IV.2A). These findings were supported statistically; initial analysis with a two-way repeated measures ANOVA encompassing data from both sexes across the entire experiment identified a near-significant main effect of time on DA levels [$F(2.74,27.40)=2.80$ $p=.063$] but did not identify a significant main effect of sex or a significant interaction between time and sex.

DOPAC: In contrast to DA, mean extracellular DOPAC levels started rising from baseline levels immediately after MK801 treatment in both sexes and proceeded to rise until the end of the experiment. Interestingly, while in males DOPAC levels rose slowly and linearly with little variance, in females, DOPAC rose much faster and greater variance was observed. Thus, DOPAC levels in males one and two hours after MK801 treatment were 75 and 100% above baseline, respectively; corresponding values in females were 175% and 250% (Figure IV.2B). These effects of MK801 and sex differences in drug response were confirmed statistically. Thus, initial analysis with a two-way ANOVA inclusive of data from all time points in both sexes identified a significant main effect of time on mean DOPAC levels [$F(1.60,15.97)=9.38$ $p=.003$], a near-significant main effect of sex [$F(1,10)=3.47$ $p=.062$] and a significant interaction between time and sex [$F(1.60,15.97)=2.22$ $p=.048$]. Subsequent within-sex analyses (one-way ANOVAs) further identified a significant main effect of time on extracellular DOPAC levels in both sexes (males: [$F(3.27,16.37)=37.55$ $p<.001$]; females: [$F(1.57,7.86)=5.24$ $p=.041$]) and allowed post-hoc testing revealed that in males, mean

DOPAC levels were significantly or near-significantly higher than pre-drug baseline at all time points after MK801 was given. In females, however, post-hoc testing surprisingly revealed that mean DOPAC levels were not significantly higher than baseline at any point after MK801 treatment. This could be due to the large variance observed in the female cohort. Final analyses comparing DOPAC levels in males and females at each time point (one-way ANOVAs) further supported this as these analyses also failed to identify a significant sex difference at any time point after MK801 treatment despite clear differences in average group values.

HVA: Mean extracellular HVA levels were seen to gradually increase with time after treatment with MK801 in both sexes, although the increases in both males and females were smaller and slower than those seen for DOPAC; data after MK801 treatment also showed significant variance in both sexes. Thus, one hour after MK801 injection, mean extracellular HVA was approximately 30% higher than baseline in both sexes and at the conclusion of the experiment two hours after MK801, HVA was 40-60% higher than baseline (Figure IV.2C). Despite this increase, large variance in both sexes precluded these observations from being statistically validated; an initial two-way ANOVA failed to identify a significant main effect of time, sex or a significant interaction between time and sex.

Behavioral Analyses: The second chapter of this dissertation showed that the same drug application used here induces significant increases in locomotor behavior that are greater in males than in females. This finding was reproduced here using a less

detailed rating scale designed to assess relative levels of locomotor activity. Thus, during the acquisition of baseline samples, both male and female rats were either asleep and immobile or awake but moving only minimally; behavior scores were in the range of 0-1. After injection of MK801, all animals remained awake and increases in locomotor behavior were seen immediately in both sexes. While males and females showed a similar increase in behavior at 15 minutes after MK801 (score of approximately 2), the drug induced increases in activity diverged across sex thereafter (Figure IV.3). Thus, in males, behavioral scores increased more or less linearly to a peak score of 4 about an hour after treatment and plateaued at this level for the remainder of the experiment. Females, however, showed more locomotor activation on average; behavior scores rose much faster and peaked at around 6.5 about an hour and a half after injection. These drug effects and sex differences were statistically robust. First, a two-way repeated measures ANOVA encompassing all behavioral data from both males and females identified a significant main effect of time on behavior score [$F(1.91,19.11)=91.82$ $p<.001$], a significant main effect of sex [$F(1,10)=5.32$ $p=.044$] and a significant interaction between time and sex [$F(1.91,19.11)=4.22$ $p=.032$]. Further analyses in males and females (one-way repeated measures ANOVAs) further confirmed a significant main effect of time in both sexes (males: [$F(3.45,17.26)=99.00$ $p<.001$]; females: [$F(1.55,7.72)=39.34$ $p<.001$]) and allowed post-hoc analyses showed that in males, behavioral scores at all time points after MK801 were significantly or near-significantly higher than at baseline. In females, however, behavior at the first time point after MK801 was not significantly higher than baseline, but was so at the remaining 7 post-drug time-points (Figure IV.3). Finally, analyses comparing behavior

in males and females at each time point (one-way ANOVAs) showed that behavior scores were not significantly different between males and females at baseline but were significantly or near-significantly higher in females over males at 6 out of 8 post-drug time points.

Correlation of Behavior to Metabolite Levels: Statistical correlations of between behavior and metabolite levels were driven by several factors, the first being that locomotor behavior increased after MK801 treatment in a similar manner as observed in metabolite levels in both males and females. Furthermore, the female cohort showed larger variance in behavior as well as a larger variance in metabolite levels. Thus, Pearson's product-moment correlations were computed and followed by regression analyses in order to assess the relationship between locomotor behavior and DOPAC and HVA levels in both males and females across the entire experiment. Results showed that in both sexes, DOPAC levels were strongly and significantly positively correlated with locomotor activity (males: $r=.860$, $p<.001$; females: $r=.801$ $p<.001$, Figure IV.4A,B). However, the female cohort had multiple outlying instances of substantially increased behavior and corresponding increased DOPAC, which enlarged the variance of the mean DOPAC increases observed after MK801 (Figure IV.4B). HVA levels also had a significant positive correlation with behavior in both sexes, although the correlations were weaker than for DOPAC (males: $r=.485$ $p<.001$; females: $r=.620$ $p<.001$). Here, increased behavior scores in females were not correlated with large increases in HVA levels, resulting in similar relationships between behavior and HVA levels in males and females alike (Figure IV.4C,D).

DISCUSSION

While sex differences are evident in nearly every aspect of schizophrenia, the overwhelming prevalence of negative symptoms and cognitive deficits in males stands out as extraordinarily relevant to disease outcome and therapeutics. These symptoms, which include anhedonia, avolition, behavioral inflexibility and memory problems, are notoriously difficult to treat and contribute disproportionately to the morbidity and disability seen in this disorder (Breier et al., 1991; Mayerhoff et al., 1994). Thus, identifying the neurobiological bases of this striking sex difference could provide insights into novel therapeutic approaches of these debilitating symptoms. However, this was previously hampered by the lack of an animal model that could accurately recapitulate the sex differences in symptomatology that occur in schizophrenia. In the past several chapters of this dissertation, it was established that the rodent NMDAR hypofunction model robustly and accurately models many of the sex differences seen in schizophrenia, including the predominance of negative symptoms in males. To begin to examine the biological underpinnings of this key behavioral sex difference, the studies here paired acute MK801 with microdialysis and HPLC in order to examine effects of NMDAR antagonism on the levels of dopamine (DA) and its metabolites in the prefrontal cortex (PFC) of male and female rats.

In selecting neural substrates with key relevance to the negative symptoms of schizophrenia, the dopamine system of the PFC stood out as especially appropriate for initial analyses as the PFC is inexorably linked to high-level executive and cognitive

functions such as goal-directed behavior, working memory and behavioral flexibility and its dysfunction has repeatedly been implicated in debilitating negative symptoms and cognitive impairments seen in a number of illnesses, including schizophrenia (Goldman-Rakic, 1987; Akil et al., 1999; Goldman-Rakic et al., 2000; Weinberger et al., 2001). Interestingly, despite the fact that NMDAR hypofunction stands out as an excellent model for the full spectrum of behavioral abnormalities in schizophrenia, only a handful of studies have evaluated the effects of NMDAR hypofunction on DA in the prefrontal cortex and none have looked at this in females. To that end, the studies here investigated whether there are sex differences in levels of DA and its metabolites in the mPFC in male and female rats after systemic treatment with MK801. As discussed further below, the major finding of this study was that although MK801 had almost no effect on DA levels in males and females alike, it caused a robust increase in DOPAC levels in both sexes which is indicative of increased DA turnover. Furthermore, though DOPAC levels increased in both sexes in response to MK801, the increase was larger in females. Although, the female cohort was found to have substantial variance in behavior and metabolite levels, the trend of a larger increase in DOPAC in females over males persisted after controlling for these differences. In the sections that follow, data from baseline and in response to MK801 is discussed and related to prior studies and while firm conclusions regarding the underpinnings of behavioral sex differences cannot be drawn from these results due to several identified confounds, the contributions of this study are discussed together with technical considerations for future investigations into this question.

Sex Differences in the Basal Levels of DA and its Metabolites: Comparison to Prior Studies

The first analyses in this study focused on sex differences in basal levels of dopamine and its metabolites in the PFC. This was driven by the fact that while numerous studies have found evidence for sex differences or estrus cycle variance in DA in the rat brain, the vast majority of these studies have focused on mesostriatal or mesolimbic DA systems (Shimizu and Bray, 1993; Petitclerc et al., 1995; Becker, 1999; Ohtani et al., 2001), while relatively little work has examined these factors in the context of mesocortical DAergic signaling. In this study we found that basal levels of dopamine in males was approximately 0.1 fmol/ μ L, a level consistent with those previously found in this laboratory and others (Moghaddam and Jackson, 2004; Del Arco et al., 2007; Aubele and Kritzer, 2011a; Aubele and Kritzer, 2011b). In females, DA levels were approximately three times higher, though a larger variance was observed which most likely precluded this difference in reaching statistical significance. However, this was in line with prior findings reporting similar levels of basal DA in females (Dazzi et al., 2007). Interestingly, the large amount of variance observed in the female cohort is most likely due to the fact that estrus cycle was not controlled for in this study as DA levels in the PFC of female rats have been shown to fluctuate across the estrus cycle, being highest in estrus and lowest in proestrus (Dazzi et al., 2007). Unfortunately, while the cohort in this study was not large enough to fully examine the effect of estrus cycle stage on DA levels, the results obtained here indicate the importance of taking into account estrus cycle stage in neurochemical studies involving female animals. Next,

DOPAC and HVA were seen to be much more present in greater quantities, with levels that were several-fold higher than those of DA; DOPAC was also seen to be marginally more predominant than HVA. Furthermore, there was also a non-significant trend toward higher levels of both metabolites in females over males. These results are also consistent with results from prior microdialysis studies indicating that DOPAC and HVA are present in much greater quantities than DA itself in both sexes; this is due to the fact that released DA is rapidly catabolized (Hernandez and Hoebel, 1989; Santiago et al., 1993; Marshall et al., 1997; Shoblock et al., 2004).

Results obtained here also showed a trend of greater levels of DA and its metabolites in females than males, which is supported by prior findings; in fact, a recent study showed that basal levels of DA in the ventromedial prefrontal cortex were approximately five-fold greater in females over males (Staiti et al., 2011). It should be noted, however, that the female cohort used in that study was ovariectomized and supplemented with a fixed amount of estrogen. Considering that DA levels in the PFC and in related subcortical areas have been reported to fluctuate with the estrogen variations of the estrus cycle (Shimizu and Bray, 1993; Dazzi et al., 2007), these findings may not accurately represent the sex difference in prefrontal DA levels but do establish an approximate basis for comparison with males. Interestingly, another prior study also reported that prefrontal DA is approximately two-fold higher in females than males, but that DOPAC is about two-fold higher in males; findings which are partly in contrast to what was found here (Duchesne et al., 2009). However, Duchesne and colleagues studied post-mortem brain homogenates and not *in-vivo* extracellular levels

as was done here, which could account for this discrepancy. In all, this study was the first to attempt to directly compare prefrontal cortical basal levels of dopamine and its two major metabolites between males and females and while a trend toward higher values of all three moieties in females was identified, further investigation is necessary to characterize sex differences in basal prefrontal dopamine neurochemistry.

Effects of Acute MK801 on DA and its Metabolites: Comparison to Prior Studies

Considering that the NMDAR hypofunction stands out as a well-accepted model for the behavioral abnormalities seen in schizophrenia and that a subset of these aberrations have been linked to improper prefrontal DA function, it is perhaps surprising that relatively few studies have characterized the effects of systemic administration of NMDA antagonists on DA levels in the PFC and of those, none have evaluated sex differences in this measurement. However, this limited body of work has established that systemic administration of 0.1 mg/kg MK801 in male rats causes a two to three-fold increase in prefrontal DA levels within approximately one hour after injection (Mathe et al., 1999; Homayoun et al., 2004). Thus, with these prior findings serving as guidance for this study, it was surprising that results obtained here found no change in DA levels after injection of MK801 in either sex. However, results obtained here did show increases in DOPAC and HVA after MK801 administration and while the HVA increases did not reach statistical significance, it is nonetheless evident that this metabolite did increase after MK801. This could indicate that DA turnover was increased in both sexes after MK801 but that enzymatic catabolism was able to maintain a relatively

steady level of DA. While it is unclear why these results are not completely consistent with prior studies, there is a reason why this may be the case. First, all prior studies used only 0.1 mg/kg MK801 and only used male animals. This is significant due to the fact that a dose of 0.1 mg/kg does not cause locomotor activation in males as demonstrated in Chapter II of this dissertation as well as in other published reports (Honack and Loscher, 1993; Carey et al., 1998). Thus, the higher dose used in the present study and resultant motor activation could conceivably alter the level and rate of DA metabolism. This is supported by several post-mortem studies where animals receiving doses of MK801 in the 0.2-0.5 mg/kg range showed a slight decrease in PFC DA but a significant increase in DOPAC and HVA levels one to two hours after drug administration (Hiramatsu et al., 1989; Loscher et al., 1991). While these cited studies involved whole-brain rather than extracellular assays of DA, DOPAC and HVA as in this experiment, a related study using varying doses of PCP paired with whole-brain assays of DA and DOPAC showed that at low doses of PCP, DA stays constant and DOPAC rises only slightly while after moderate and high doses of PCP, DA decreases and DOPAC markedly rises, suggesting that the rate of DA turnover is tied to the dose of NMDAR antagonist (Umino et al., 1998). Thus, although it is impossible to ascertain whether this difference is specifically due to increased locomotor activity caused by the higher dosage of drug, the results here are congruent with prior findings that higher doses of NMDAR antagonists differentially affect DA tone and metabolism in the PFC.

Sex Differences in Effects of Acute MK801 on DA and its Metabolites: All About Behavior?

Even though MK801 treatment caused increased DA turnover in the PFC of males and females alike, there were sex differences that were observed in this process. Specifically, the MK801-induced increase in DOPAC was seen to be sexually dimorphic with females having a greater increase in DOPAC levels in response to MK801 compared to males. Similarly, behavior also increased in both sexes after MK801, with females also having greater increases in locomotion than their male counterparts. Interestingly, while increases in DOPAC and locomotor behavior in response to MK801 were very consistent in males, the female cohort had much more variability in behavioral activation and in DOPAC increases. From this observation, subsequent analyses established that a strong and significant positive correlation exists between intensity of locomotor activity and DOPAC levels induced by MK801 in both sexes. However, while behavior scores and DOPAC levels were highly consistent in males, the female cohort had numerous outlying instances of extreme hyperlocomotion that were accompanied by very high DOPAC levels. Thus, the question arose as to why females exhibited such varied behavior. Focus immediately fell onto estrus cycle stage and while the female cohort in this study was too small to properly ascertain an effect of estrus cycle on locomotor behavior and resultant rises in DOPAC levels, there is evidence indicating that gonadal hormones do play a role in this process. Firstly, basal locomotor activity and basal DA levels in PFC and linked subcortical structures have been shown to fluctuate across the estrus cycle (Becker and Cha, 1989; Scimonelli et al., 1999; Dazzi et al., 2007) being highest in estrus, when hormone levels are highest (Goldman et al., 2007). More interestingly, the behavioral response to psychomimetic

drugs is also known to be similarly hormone-sensitive with amphetamine-induced rotation also being the most intense in estrus (Becker et al., 1982). While this has previously been thought to be due to hormone effects on the mesolimbic system (Becker 1999), a recent study using PCP argued that the PFC is a principal site for regulation of NMDAR antagonist induced hyperlocomotion (Takahata and Moghaddam, 2003). This line of evidence dovetails with findings that estrogen reduces the expression of NMDA receptors in the PFC of females to suggest that rats in estrus cycle stages when estrogen is high may be more vulnerable to the effects of NMDAR antagonists in this brain region. As NMDAR hypofunction in the PFC causes increased activation of corticofugal glutamatergic signaling which subsequently increases mesolimbic and mesocortical DAergic cell firing and DA release in the several subcortical areas (Yang and Chen, 2005), resulting in increased behavioral activation. Thus, gonadal hormones may affect the actions of NMDAR antagonists in the PFC to modulate locomotor activity which in turn may cause such a diverse range of behavior across the estrus cycle. Taken together, this indicates that estrus cycle stage significantly affects DA levels in the PFC in female rats and that future studies are needed to better understand the effects of sex hormones on behaviors and neurochemical changes induced by NMDAR antagonism.

However, despite an established strong influence of estrus cycle on locomotor activity and DA turnover in females, the results in this study indicate that these two factors alone may not account for the entire sex difference in DOPAC levels seen here. Specifically, when the females that exhibited outlying behavior and were in any other

estrus cycle stage but diestrus were removed from analysis, mean increases in DOPAC were still higher than in the male cohort. While the small number of female subjects made statistical analysis impractical, this result nonetheless implies that DA turnover in the PFC of females is increased relative to that in males. Intriguingly, this dovetails with behavioral evidence from Chapter III which showed that acute MK801 preferentially increased low-frequency ultrasonic vocalizations in females which in turn implies that the laterodorsal tegmental nucleus (LDT) is activated more in females over males. Since cholinergic activity arising in the LDT activates burst firing of mesocortical DAergic cells (Lodge and Grace, 2006), the increased DA turnover in females seen here is consistent with the premise that NMDAR hypofunction increases LDT activity and subsequent DA cell burst firing to a greater degree in females versus males, suggesting a hormone sensitivity of this area and/or circuit. While further work needs to be done to confirm this result, it is nonetheless an intriguing finding that provides evidence of sexual dimorphisms in a brain region intimately involved in regulation of many processes at risk in schizophrenia that has to date received little attention with regard to investigations in its sensitivity to gonadal steroids. Another intriguing explanation for this finding takes into account previously discussed evidence for hormone sensitivity of NMDA receptor density in the prefrontal cortex. Thus, evidence that NMDA receptor density in the female PFC is negatively correlated with estrogen levels (Cyr et al., 2000) combines with findings here to suggest that females may have lower levels of NMDA receptors compared to their male counterparts and may thus be more sensitive to the effects of drugs that antagonize the NMDA receptor. To that end, the increased DA release in the PFC may be due to increased disinhibition of

corticothalamic glutamatergic pyramidal cells which in turn activate mesocortical DAergic cells. However, as differences in NMDA receptor density in males and females have not, to my knowledge, yet been quantified this hypothesis is speculative and further study is necessary to examine this explanation of the results found here.

Conclusion

In this study, it was first found that basal levels of dopamine and its metabolites were somewhat higher in females. Further, it was shown that systemic treatment with 0.2 mg/kg MK801 does not alter levels of DA in either sex but does increase DOPAC and HVA in the PFC in both sexes which is indicative of increased DA turnover. However, the results in this study were significantly affected by large variances in the female cohort which were partly due to increased locomotor activity and variability in estrus cycle stage. Nonetheless, the female over male increase in DOPAC persisted after controlling for estrus cycle and removing overactive animals from analysis, indicating that MK801 causes higher DA turnover in females over males. When this result is considered in light of behavioral findings in the prior chapter, it implies that increased DA turnover in females may be related to increased LDT activation and resultant stimulation of burst firing in mesocortical DA cells relative to males. In turn, this provides impetus for investigation of the hormone sensitivity and/or sex differences in the architecture and function of this structure. Further, the results here also revealed important methodological considerations for future studies. First, the data here support prior reports indicating that locomotor activity is intimately tied to increased DA turnover

and suggest that assessment of drug effects when motor activity is increased makes it impossible to isolate the effect of the drug on neurochemistry. Further, the data here also illustrate that estrus cycle control in females is a necessity as it is linked to fluctuations in DA and behavior. In sum, while this study showed an intriguing finding which implicates sex differences in a brain circuit that has been previously understudied, there were several important methodological considerations that need to be addressed in future comparative quantifications of dopaminergic neurochemistry between males and females.

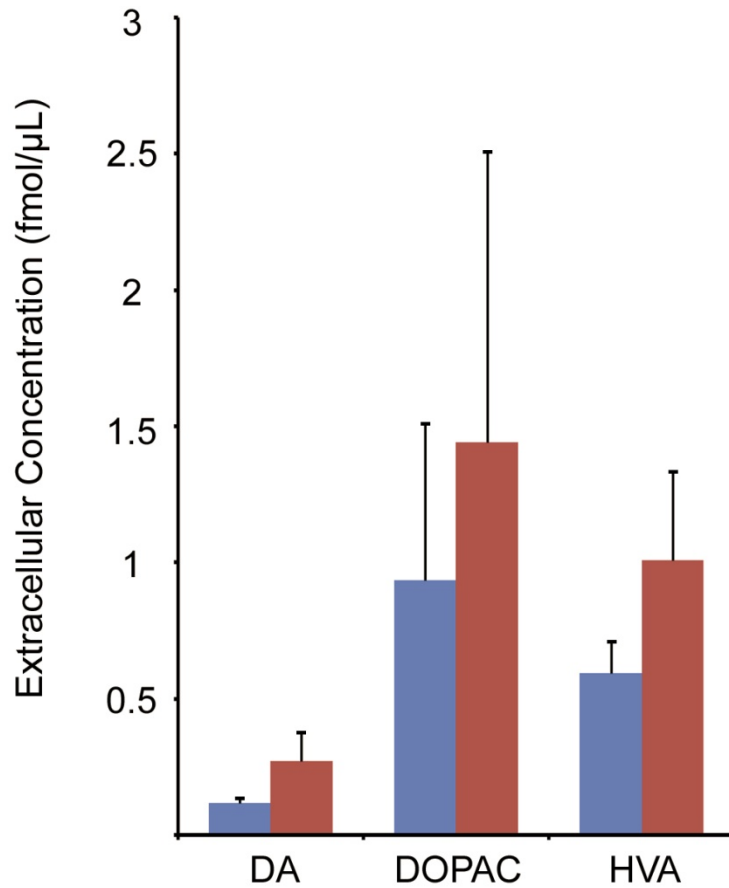


Figure IV.1. Bar graph showing extracellular basal dopamine (DA), DOPAC, and HVA levels measured in the medial prefrontal cortex of male (blue bars) and female (red bars) rats. All data is presented as mean levels (fmol/μL) ± standard error of the mean. There were no significant or near-significant differences in levels of any of these three metabolites between male and female animals.

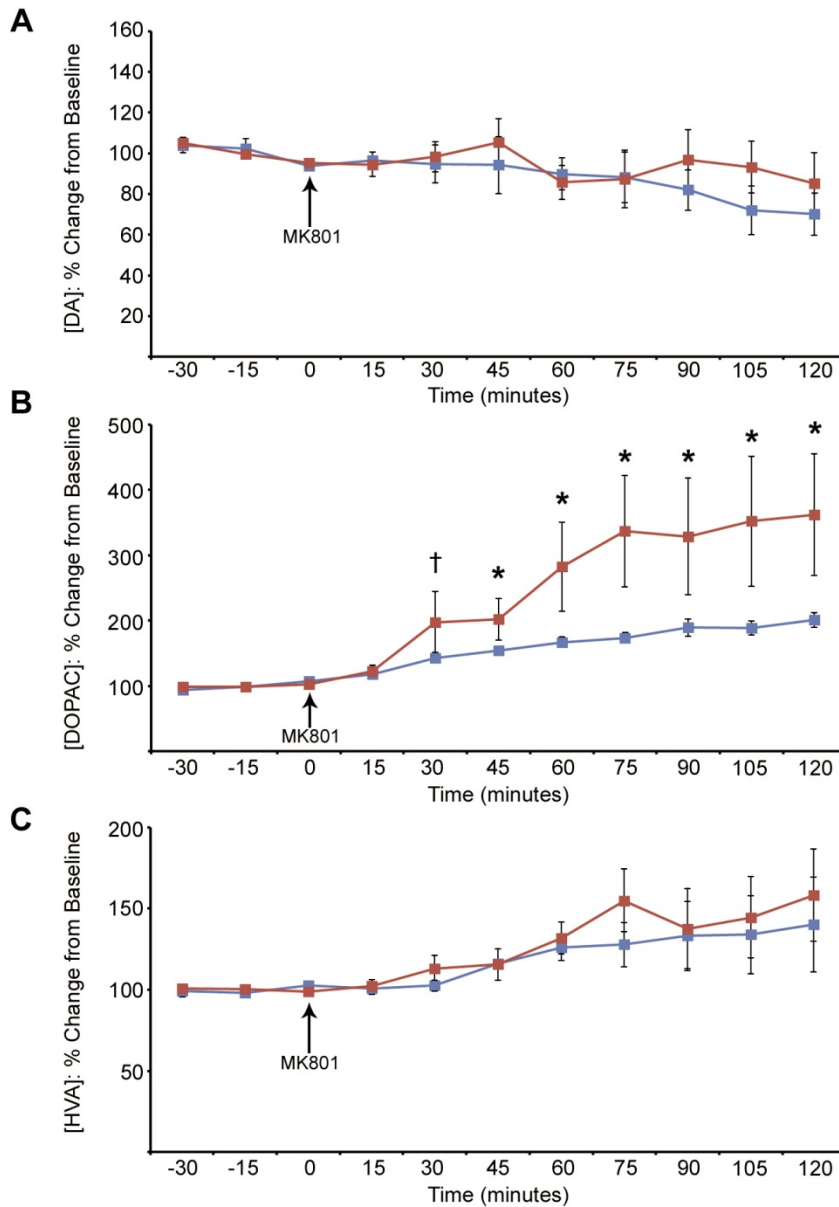


Figure IV.2. Line graphs showing the effect of an intraperitoneal injection of 0.2 mg/kg MK801 on dopamine (DA, A), DOPAC (B), and HVA (C) levels measured in the prefrontal cortex of male (blue) and female (red) rats. Data is expressed as mean percent change from baseline \pm standard error of the mean. MK801 was administered immediately after the data point at time 0 was acquired. Asterisks (*) indicate timepoints where drug effects on metabolite levels in male animals are significantly different from baseline ($p < 0.05$) and cross signs (†) indicate the timepoint where drug effects on metabolite levels in male animals are near-significantly different from control ($p < 0.08$).

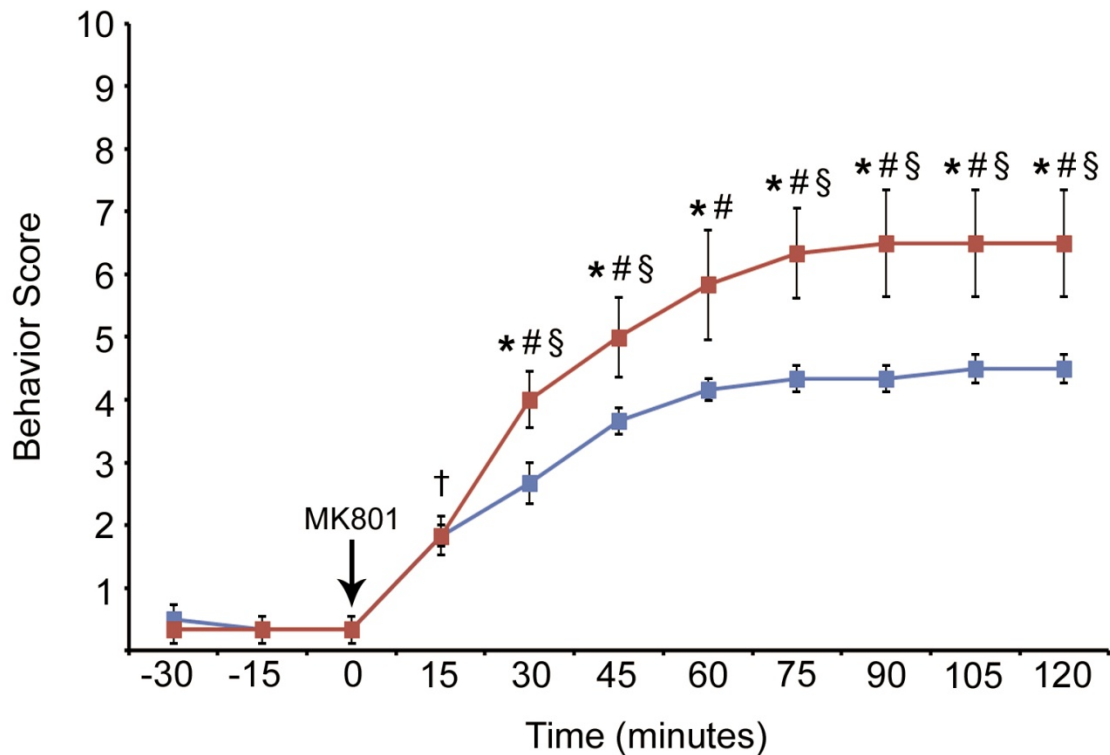


Figure IV.3. Line graph showing the effect of an intraperitoneal injection of 0.2 mg/kg MK801 on mean behavior scores (\pm standard error of the mean) in male (blue) and female (red) rats. Behavior was rated on a scale of 0 to 10 where 0 corresponded to no activity and 10 reflected extreme hyperlocomotion. MK801 was administered immediately after the data point at time 0 was acquired. Asterisks (*) indicate timepoints where drug effects on behavior score in males are significantly different from baseline ($p < 0.05$) and pound signs (#) indicate times where drug effects on behavior score in females are significantly different from baseline. A cross sign (†) indicates the timepoint where drug effects on behavior score in male animals are near-significantly different from control ($p < 0.08$) and section signs (§) indicate timepoints where behavior scores are significantly different between males and females.

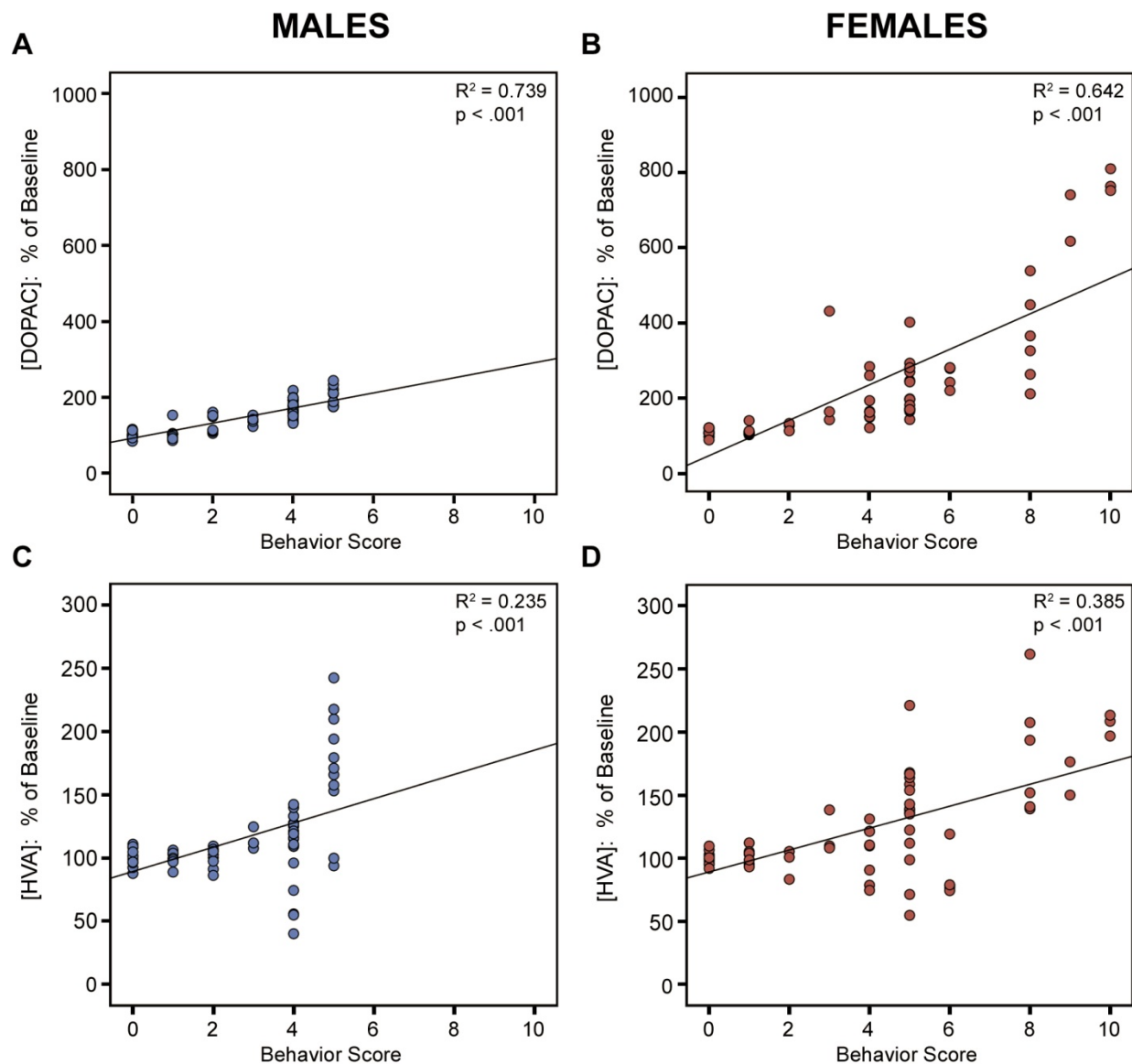


Figure IV.4. A, B: Regression plots that relate 15-minute bin measurements of individual male (blue, A) and female (red, B) rats' medial prefrontal cortex DOPAC levels to their behavior scores measured at the same time as each DOPAC measurement. R^2 and p values in the upper left hand corners reveal that DOPAC levels are positively and significantly correlated to behavior in both males and females alike, though there are more outlying points in the female group. C, D: Regression plots that relate 15-minute bin measurements of individual male (blue, C) and female (red, D) rats' mPFC HVA levels to their behavior scores measured at the same time as each HVA measurement. R^2 and p values in the upper left hand corners reveal that HVA levels are significantly and positively correlated to behavior in both sexes and that the relationship between HVA levels and behavior scores are similar in males and females alike.

CHAPTER V

Extending magnetic resonance spectroscopy to the non-invasive study of *in-vivo* dopaminergic neurochemistry and to the effects of NMDA receptor hypofunction

Neurochemical abnormalities are known to exist in numerous neurotransmitter systems and brain areas in schizophrenia. Thus, in addition to changes in dopamine (DA), glutamate and acetylcholine that have been discussed in prior chapters of this dissertation, neurochemical disruptions in schizophrenia extend to GABA, serotonin (5HT) and other systems (Dean, 2000; Reynolds and Harte, 2007; Shin et al., 2011). As all current pharmacological treatments of schizophrenia exert their effects on a multitude of receptor targets and transmitter systems and while preclinical animal models have contributed greatly to the knowledge of the mechanisms of schizophrenia and its therapeutics, the understanding of these systems and their interactions in human populations stands out as critically important in identifying novel therapeutic targets and strategies. However, techniques for assessing *in-vivo* neurochemistry remain limited; this is especially true for techniques that can be applied to human studies. For example, while microdialysis has been extensively used to study effects of drugs on neurochemistry, this method is hampered by a relatively poor temporal resolution and more importantly, its invasive nature relegates it only to preclinical applications. While human studies of *in-vivo* neurochemistry are made possible by the use of positron emission tomography, this method also has limits; the use of radioactive ligands contraindicates its use in certain patient populations and precludes its use in longitudinal studies. In fact, the only non-invasive method for assessing *in-vivo* neurochemistry that can be applied to human studies is magnetic resonance

spectroscopy (MRS). However, even though MRS provides a wealth of information about neurochemistry and at least 18 metabolites are widely believed to be detectable with this technique (Pfeuffer et al., 1999; Tkac et al., 2004), many molecular targets relevant to schizophrenia such as DA have not been studied with this method. Thus, the experiments presented in this final chapter evaluated the feasibility of utilizing proton MRS for the measurement and quantification of DAergic neurochemistry *in-vivo*.

While the appeal of MRS is mostly due to its non-invasive nature that readily allows for translation to the human arena, this method has several other important advantages. First, the assortment of molecules that can be detected are structurally and functionally diverse and range from markers of metabolism and neuronal health to the neurotransmitters glutamate (Glu) and GABA; no other available method allows the simultaneous quantification of such a wide variety of molecules in the living brain. Furthermore, it provides the ability to acquire data from very small volumes of interest with good temporal resolution, allowing multiple areas to be studied in a single experiment. However, even though proton MRS provides much information about localized neurochemistry, it was previously widely thought that this technique was limited to the detection and analysis of molecules present in relatively large concentrations and that moieties present at low concentrations cannot be quantified by this technique. Thus, the studies in this chapter are driven by recent findings indicating that MRS, paired with a novel data analysis algorithm, is a sensitive and specific method for *in-vivo* detection of ascorbate (vitamin C), a molecule present in extremely small quantities in both rats and humans (Terpstra et al., 2006; Terpstra et al., 2010).

Encouraged by this finding, the pilot studies presented here combined proton MRS with the Linear Combination of Model Spectra (LCModel) analysis method to investigate the feasibility of studying DA and its metabolites as well as GABA and glutamate in the rat medial prefrontal cortex (mPFC). The LCModel analysis is a cornerstone to this approach; this technique relies on prior knowledge information describing the identity of the signal from each molecule that purportedly contributes a non-negligible signal to the total spectrum and with a comprehensive prior knowledge library, efficient fitting can be performed on in-vivo data and an accurate quantification of each signal's contribution can be elucidated (Provencher, 1993; Provencher, 2001). Compared to traditional methods of MR spectral analysis such as basic Fourier Transform (FT) based techniques which are based on manually identifying individual peaks in the spectrum, LCModel utilizes the full spectral signature of each compound during fitting, providing a more accurate, non-interactive result. Most importantly, this method allows for the incorporation of novel metabolites into the prior knowledge library, allowing the quantification of moieties that were thought to be out of the reach of conventional analysis techniques. Thus, LCModel analysis is directly applicable to the objective of extending MRS to the study DA and its metabolites. To that end, the first set of studies in this chapter sought to establish the feasibility of using MRS combined with LCModel in studying the DAergic system by attempting to quantify the levels of DA and its metabolites *in-vivo* at baseline and in response to drugs that selectively manipulate levels of these moieties. Subsequently, the second set of experiments sought to simultaneously quantify levels of glutamate, GABA and DA and its metabolites

in the rat brain after acute treatment with MK801 in order to assess the effects of NMDAR hypofunction on the entire neurochemical composition of the mPFC.

METHODS

Animal Subjects: A total of 9 male adult Sprague-Dawley rats were used; animals were purchased from Taconic Farms (Hudson, NY). Animals were housed in same-sex pairs in a temperature controlled room under a 12 hour light/dark cycle (lights on at 7 AM) with food and water available *ad libitum*. All procedures involving animals were approved by the Institutional Animal Care and Use Committees of Stony Brook University and Brookhaven National Laboratory and were designed to minimize their discomfort and use.

Animal Preparation, Anesthesia and Monitoring: On the morning of imaging, rats were anesthetized with an intraperitoneal injection of 125 mg/kg methohexital. An intraperitoneal catheter for drug delivery was placed in the animal and sutured to the skin after which the animal was transferred to the magnet. 2% isoflurane in 4 L/min oxygen was subsequently given by nasal cannula to maintain sufficient anesthesia to prevent animal motion. The animal's respiratory rate, heart rate, oxygen saturation and body temperature were monitored throughout the experiment (Model 1025, SA Instruments, Stony Brook, NY USA) and anesthesia was be adjusted as needed to maintain stable vital signs and eliminate animal motion.

Drug Treatments and Experimental Groups: Three separate experiments were carried out involving acute administration of pargyline, levodopa and MK801. Thus, after undergoing baseline spectrum acquisition, animals were given either 75 mg/kg pargyline (n=2), 50 mg benserazide with 75 mg levodopa (n=5) or 0.1 mg/kg MK801 (n=2) through the intraperitoneal catheter and 1-2 post-drug spectra were subsequently acquired. All drugs were obtained from Sigma-Aldrich (St. Louis, MO USA).

Magnetic Resonance Spectroscopy: All imaging was performed on a 9.4T/31 cm horizontal bore microMRI magnet interfaced to a Bruker AVANCE console and controlled by Paravision 5.0 software (Bruker BioSpin, Billerica, MA, USA). The animal was positioned supine in the magnet with the head directly resting on the receiver radiofrequency coil. Care was taken to position the medial PFC (mPFC) directly over the center of the coil for maximal signal at which point the animal was securely padded and secured to the cradle to prevent motion. Subsequently, the animal was inserted into the magnet bore and positioning was checked with a short scout pulse sequence. Positioning was then adjusted until the mPFC was located as close as possible to the isocenter of the magnetic field. High resolution anatomical images were obtained in the axial, coronal and sagittal planes with a rapid acquisition refocusing echoes (RARE) sequence (repetition time (TR)=2500 ms, echo time (TE)=40 ms, number of slices=25, in plane resolution=0.117 mm/pixel, slice thickness=0.8 mm, slice gap=0.1 mm). Using these images, automated shimming using the FASTMAP method was performed on a 4.5 mm³ voxel centered over the mPFC. Spectra were subsequently acquired from a ~20 μ L voxel within the mPFC with a point resolved

spectroscopy (PRESS) pulse sequence (TE=20 ms, TR=2000 ms, 1796 averages, 1 hour acquisition time).

Data Analysis: Analysis of MR spectra was performed using linear combination modeling (LCModel, Stephen Provencher Inc., Toronto, Canada); this method fits spectra from individual metabolites to the acquired in-vivo spectrum, allowing the determination of each metabolite's contribution to the total signal and subsequent quantification. For this, individual raw spectra were first corrected for frequency drift and subsequently summed; these summed spectra were used for LCModel analysis. No other manipulations were applied to the data before LCModel analysis. The LCModel basis data set was composed from spectra of 21 metabolites acquired *in-vitro* under identical conditions as this experiment; it included spectra from alanine, aspartate, creatine (Cr), phosphocreatine, γ -aminobutyric acid (GABA), glucose, glutamine, glutamate, glycerophosphorylcholine (GPC), phosphorylcholine (PCh), glycine, myoinositol, scyllo-inositol, lactate, N-acetylaspartate, N-acetylaspartylglutamate, taurine, dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT). In addition, simulated spectra of lipids and macromolecules were included. The LCModel analysis was performed in the chemical shift range of 0.0 to 4.0 ppm. The Cramer–Rao lower bounds (CRLBs) provided by LCModel analyses were used to eliminate statistically unreliable values; all data with CRLB values greater than 35% was eliminated. All values from LCModel analysis were normalized to creatine in order to control for experimental variation in spectral quality and magnetic field stability. All data was then

averaged and analyzed with descriptive statistics; due to the pilot nature of this study and resultant small sample sizes, further statistical analyses were not conducted.

RESULTS

Extending MRS to the Quantification of DAergic Neurochemistry: The first part of this pilot study attempted to extend the ability of MRS coupled with LCModel analysis to the quantification of levels of DA and its metabolites. Thus, the initial step applied the novel prior knowledge basis set to spectra acquired from animals in a drug-naïve state in order to investigate which, if any compounds related to DA could be detected and quantified. This basis set contained spectral data from DA, DOPAC, HVA and 3MT along with those from the other major moieties that contribute significantly to the overall *in-vivo* spectrum (see Methods). When analyses were run on *in-vivo* spectra acquired from the rat PFC, it was found that only HVA was consistently and reliably detectable with CRLB confidence values under 30%. Upon closer analysis of the spectral signatures of DA and its metabolites, it was revealed that the major resonances of DA, DOPAC and 3MT are located in areas of the spectrum where they are overwhelmed by contributions from metabolites present in much larger concentrations. For example, the predominant resonance from DA is at approximately 3.2 ppm and is overwhelmed by the very large resonance from choline (Figure V.1). Similarly, the main resonance of DOPAC is a single peak at 3.4 ppm where it is overwhelmed by a large resonance from taurine. Further, the major resonance of 3MT falls close to a large contribution from creatine; this combined with the fact that 3MT is a very minor metabolite makes its reliable detection unlikely. The spectral signature of HVA, however, does not suffer

from the same problems as its major resonance lies at 3.8 ppm which is a relatively empty area of the spectrum between two major peaks. Thus, while DA, DOPAC and 3MT seem outside the range of detection with MRS, HVA is reliably quantifiable. Combined with the fact that HVA is one of the predominant metabolites in the rat PFC (Roffler-Tarlov et al., 1971) and is a direct measure of DA metabolism and turnover, it is clear that the addition of HVA to the realm of MRS-detectable moieties is of significant usefulness.

Having established that only HVA is reliably detectable at baseline, the next part of this pilot study sought to validate this finding; this was done by pairing pharmacological manipulations of DA metabolism with MRS and subsequent LCModel quantification of HVA levels. Thus, a set of animals (n=5) were given levodopa along with the peripheral dopa decarboxylase inhibitor benserazide; this treatment regimen is known to robustly increase brain DA, DOPAC and HVA within an hour (Cooper et al., 2007). Further, a second set of animals (n=3) were given pargyline, which markedly reduces the formation of HVA by blocking catabolism of DA via monoamine oxidase (Vidal et al., 2005). Before drug administration, normalized baseline concentrations of HVA were stable, with a value of approximately 24 μM . LCModel analyses of post-drug spectra showed that after benserazide/levodopa, normalized HVA concentrations increased by about 40% to an average of approximately 33 μM . The opposite effect was seen after pargyline; analyses of post-drug spectra showed that normalized HVA concentrations decreased by about 35% to values of approximately 16 μM (Figure V.2B). In all of these analyses, Cramer-Rao lower bounds were 30% or lower,

indicating that HVA was being detected and quantified with a high level of statistical certainty. Taken together, these results confirmed that levels of HVA can be reliably quantified in the prefrontal cortex using MRS paired with LCModel analysis and that changes in HVA levels can also be reliably assessed using this technique.

Applying MRS to Study Acute NMDAR Hypofunction: Having established that DAergic neurochemistry can be assessed by MRS through quantification of HVA levels, the final pilot experiments in this chapter evaluated the neurochemical sequelae of acute NMDAR hypofunction induced by treatment with MK801 with the goal of simultaneously assessing levels of glutamate, GABA and HVA. While the initial objective was to acquire serial spectra for 3-4 hours after drug treatment in order to track changes in neurochemistry, it was noted that MK801 administration resulted in profound physiological instability in the animal approximately an hour after injection which manifested itself as prolonged periods of apnea. This required the experiments to be terminated after the second spectrum was acquired, limiting analyses of drug effects. Nonetheless, LCModel analysis of the post-drug data revealed several salient results. For example, it was found that acute MK801 challenge reduced levels of glutamate and increased levels of glutamine. Correspondingly, the total pool (glutamate plus glutamine) was unaffected by MK801 but the glutamine/glutamate ratio was increased (Figure V.3). Furthermore, levels of GABA were seen to marginally decline after MK801 and levels of HVA were seen to remain relatively stable (data not shown). In sum, this proof-of-concept experiment indicates that there are notable MRS-detectable changes in the neurochemical milieu but due to the observed physiological instability,

experimental methodology needs to be modified in order to reliably study these changes further.

DISCUSSION

As schizophrenia is known to elicit profound neurochemical disturbances in a wide variety of transmitter systems and brain areas and since all pharmacological treatments of this disorder act by modulating the activity of these systems, the study of *in-vivo* neurochemistry is uniquely important. Specifically, as established in the previous chapters of this dissertation, dopamine and glutamate neurotransmission stand out as especially important targets in the study of the pathophysiology of this devastating disorder. Thus, it is surprising that techniques for evaluating these systems *in-vivo* are currently limited to microdialysis and positron emission tomography (PET). While these techniques are acceptable for use in preclinical research, microdialysis cannot be used in human studies and the use of PET is heavily restricted due to use of radioactive isotopes. Thus, it is clear that novel techniques are needed in order to examine human neurochemistry in health and disease. While magnetic resonance spectroscopy stands out with proven efficacy of being able to non-invasively evaluate neurochemistry in both animal models and humans alike and has been used to study the glutamate system (Bustillo et al., 2011; Lindquist et al., 2011; Marsman et al., 2011), it was previously thought that this technique was limited to the detection and analysis of molecules present in relatively large concentrations (van der Graaf, 2010) which would make it unsuitable for investigating DAergic neurochemistry, a key system closely linked to the pathogenesis of schizophrenia. However, bolstered by recent findings that MRS combined with LCModel analysis can reliably detect ascorbate *in-vivo* (Terpstra et al.,

2006; Terpstra et al., 2010), the first set of experiments in this final chapter of this dissertation applied this analytical technique to establish, as a proof-of-concept, that the capability of MRS can be extended into the unprecedented realm of studying the DAergic system directly. Thus, by applying a custom prior-knowledge basis set to the LCModel analysis algorithm, it was shown that HVA is reliably detectable via MRS. As HVA is a direct metabolite of DA and is thus closely correlated to total DA levels, it stands out as a valuable measure of DAergic activity and would be an unprecedentedly important addition to the repertoire of MRS-detectable molecules. Further, the studies here applied this discovery to the study of the neurochemical sequelae of NMDAR hypofunction in the mPFC. Even though initial data were intriguing, these experiments were complicated by the finding that MK801 induces paroxysmal apnea and resultant physiological instability. In the sections below, these findings are discussed in detail and outline further avenues for investigation.

Extending MRS to the Study of Dopaminergic Neurochemistry

When the custom prior knowledge data set was applied to the analyses of spectra acquired from the rat mPFC, it was noted that only HVA was reliably and consistently detectable. Closer analysis of the *in-vivo* data and the *in-vitro* spectra revealed that DA, DOPAC and 3MT are outside the realm of detection due to the fact that their major resonances are overwhelmed by those from other moieties present in larger concentrations. However, the major resonance peak of HVA was located at a point along the overall *in-vivo* spectrum that is fortuitously lacking in significant signal

contributions from other metabolites, allowing for its detection and quantification. However, as the complete spectrum is a complex amalgam of dozens of various signals, some of which may not be included in the basis set, it was paramount to ensure that the resonances identified as HVA during analysis truly corresponded to this particular moiety. Assessments of HVA using neuropharmacological manipulation provided a proven template for validating the identity of this compound; treatment with a combination of levodopa and benserazide increased HVA levels while treatment with pargyline decreased it. These observed pharmacological effects were consistent, yet less robust than those in corresponding studies using microdialysis (Vidal et al., 2005; Cooper et al., 2007). However, it should be noted that microdialysis only evaluates extracellular neurochemistry while MRS takes into account everything present in both intracellular and extracellular spaces within the volume of interest, which may account for this difference. Hence, the results of these prior microdialysis studies, while not completely ideal for comparison, provided general guidance for expected outcomes and ultimately were largely recapitulated by the results obtained here. In sum, while this data is extremely preliminary and only based on a small cohort of subjects, the results obtained here indicate that MRS can indeed be extended into the realm of directly studying DAergic neurochemistry.

Using MRS to Study the Neurochemical Effects of NMDA Receptor Hypofunction

With this data in hand, the second set of pilot experiments applied MRS to the study of the neurochemical sequelae of acute MK801 treatment. These experiments

sought to take advantage of the capability of MRS to simultaneously quantify a wide range of molecular moieties that are relevant to NMDAR hypofunction and schizophrenia, specifically glutamate, glutamine, GABA and HVA, the newest addition to the MRS-detectable repertoire of molecules. Results from these experiments showed that acute administration of MK801 causes a decrease in glutamate, an increase in glutamine and an increase in the glutamine/glutamate ratio. Furthermore, the results here suggested that GABA slightly decreases after MK801, though this effect was marginal. Most notably, the results obtained here are in complete agreement with those from a recent report that was the first to utilize MRS to study the neurochemical sequelae in the PFC after treatment with acute phencyclidine (Iltis et al., 2009). The results obtained here and by Iltis and colleagues are in agreement with numerous findings showing that acute injection of NMDA receptor antagonists causes an increase in glutamate release in the PFC while decreasing GABAergic neurotransmission. Although here, glutamate was seen to paradoxically decrease, it should be noted that since MRS measures whole-brain concentrations, analysis becomes more complicated. In this case, the decrease in glutamate was accompanied by a rise in glutamine. This can be explained by increased glutamate turnover; as glutamate is released from the presynaptic cell, a major mechanism in its subsequent clearance involves uptake by neighboring glial cells and conversion to glutamine after which glutamine is shuttled back to the presynaptic neuron and converted back to glutamate (Sanacora et al., 2008). Thus, since MK801 is known to increase glutamate release and accumulation in the extracellular space (Zuo et al., 2006; Lopez-Gil et al., 2009), the metabolic mechanisms coping with this efflux of glutamate would produce increased amounts of

glutamine, somewhat depleting the glutamate pool in the process. This is supported by the finding that the total glutamate+glutamine pool is unchanged, while the ratio of glutamine to glutamate is markedly increased. Interestingly, another prior study attempted to characterize the effects of a high dose of MK801 (0.5 mg/kg) on glutamate turnover with MRS, but reported no change after MK801 (Loubinoux et al., 1994). However, in that study, spectral resolution was poor and the authors were not able to resolve glutamate from glutamine which naturally resonate close together, indicating that their measurements were that of the total glutamate+glutamine pool, an outcome consistent with the findings reported here. Intriguingly, similar changes in the glutamine/glutamate ratio have been observed in the prefrontal cortex of schizophrenia (Bartha et al., 1997).

Aside from glutamate, the results here indicated a marginal decrease in GABA after MK801, though this result was less robust than that observed by Iltis and colleagues, who found that GABA decreased to under the threshold of detection after PCP. This discrepancy could be due to the fact that the present study used a relatively low dose of MK801, while the PCP dose used in the comparative study is of a larger equivalent. Thus, while the marginal decrease in GABA noted as well as the more drastic decrease reported previously are both in agreement with findings of a general decrease in GABAergic neurotransmission in the PFC that has been shown via electrophysiology (Homayoun and Moghaddam, 2007), the pilot nature and low power of this study necessitates further investigation on NMDAR antagonism induced changes in GABA as detected via MRS. Finally, this study was unique in that it also evaluated

MK801 effects on HVA. Interestingly, HVA was not seen to appreciably change after MK801 administration. This could be due to the fact that HVA increases relatively slowly after treatment and only by a modest amount (about 50%) after treatment with comparable doses of MK801 or PCP (Hertel et al., 1995; Kashiwa et al., 1995); it is possible that MRS may not be sensitive to these increases, or more likely, that the physiological instability in these animals precluded its accurate quantification although concrete conclusions cannot be drawn from the limited amount of data acquired here.

While these results indicate promise for the non-invasive study of neurochemistry relevant to schizophrenia and the conclusions drawn above stand, caution should be taken in interpreting this data for several reasons. As mentioned previously, this was only a pilot study and thus the number of subjects was low; replication is necessary to validate these results. Furthermore, the studies examining metabolomic effects of MK801 were confounded by physiological instability during imaging with rats developing prolonged periods of apnea approximately an hour after MK801. As apnea and resultant lack of oxygenation undoubtedly changes the brain metabolomic profile, it is a significant confounding factor. Thus, further experiments would need to switch anesthesia techniques to incorporate intubation of the subjects in order to be able to provide ventilation in order to overcome this problem.

Conclusion

In sum, the results presented here indicate that MRS stands out as a good technique for the non-invasive, localized and simultaneous investigation of diverse *in-vivo* neurochemistry relevant to schizophrenia. More specifically, the novel findings that changes in HVA levels are readily detectable by MRS in rodents indicate that this method holds much promise for eventual translation to human studies where the ability to study localized DAergic neurochemistry could provide insight into many poorly understood processes that play a prominent role in many mental illnesses. Thus, while the results here only served as proof-of-concept and further work is clearly required in the preclinical arena in order to refine and perfect this method before translation can be considered, this important avenue of research should undoubtedly be pursued further.

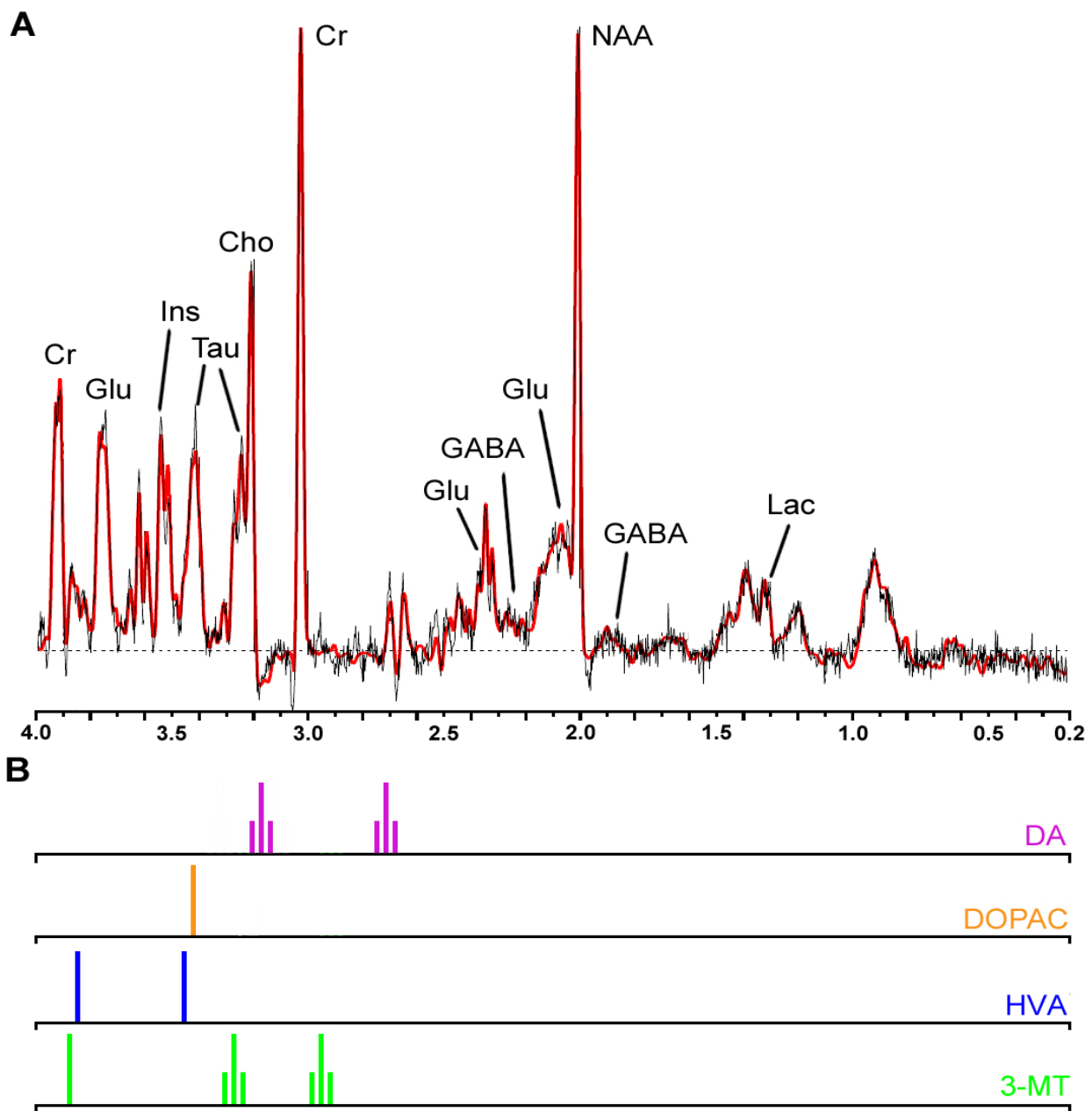


Figure V.1. A: Representative *in-vivo* MR spectrum (gray) in the range of 0.2 to 4.0 ppm acquired from the rat medial prefrontal cortex with an LCMoDel generated fit to the data (red) overlaid on top. Key contributions to the spectrum by various moieties are indicated. Abbreviations: Lac: lactate, Glu: glutamate, NAA: n-acetylaspartate, Cho: choline, Tau: taurine, Ins: inositol, Cr: creatine. B: Color-coded schematic representations of spectral signatures acquired from dopamine (DA), DOPAC, HVA and 3-MT *in-vitro* under identical conditions as the *in-vivo* rodent studies.

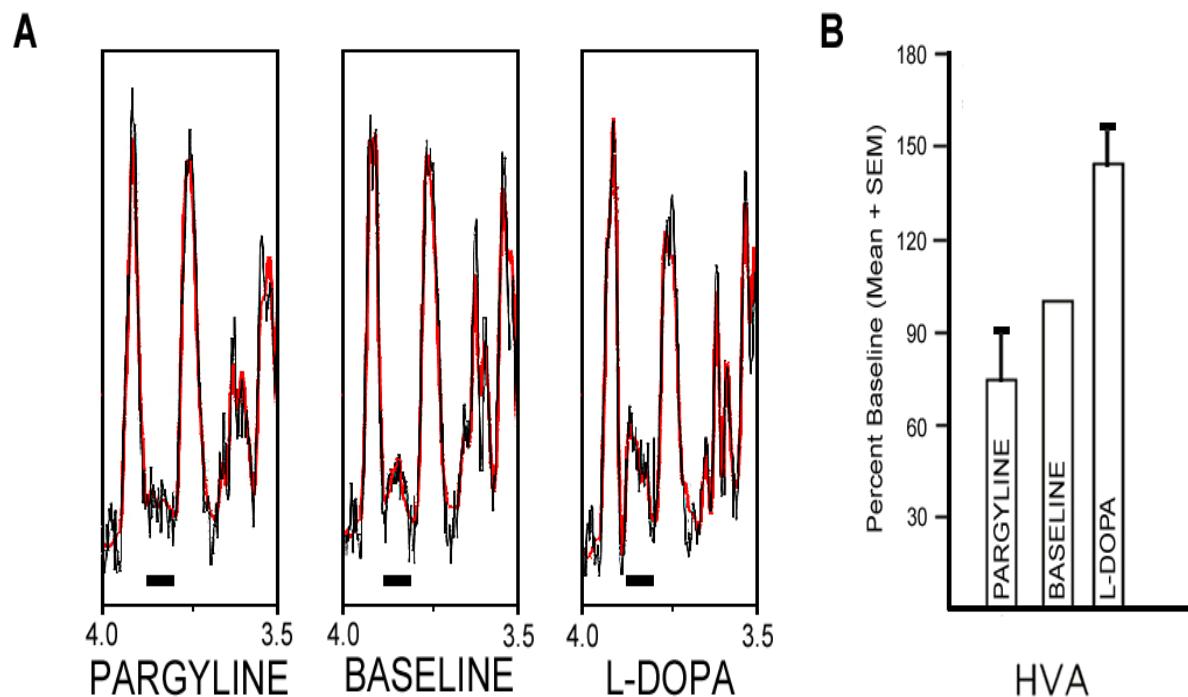


Figure V.2. A: Representative *in-vivo* MR spectra in the range of 3.5-4.0 ppm acquired from the medial prefrontal cortex of rats at baseline, after receiving 75 mg/kg pargyline or after 50 mg benserazide with 75 mg levodopa. The LCModel fit (red) is overlaid on top. Thick black lines indicate the area of the HVA resonance which is seen to decrease after pargyline administration and increase after levodopa. B: Bar graph showing HVA concentration as a percentage of baseline values in animals treated with pargyline or benserazide/levodopa.

PREFRONTAL CORTEX:

MK-801: 0.1 mg/kg

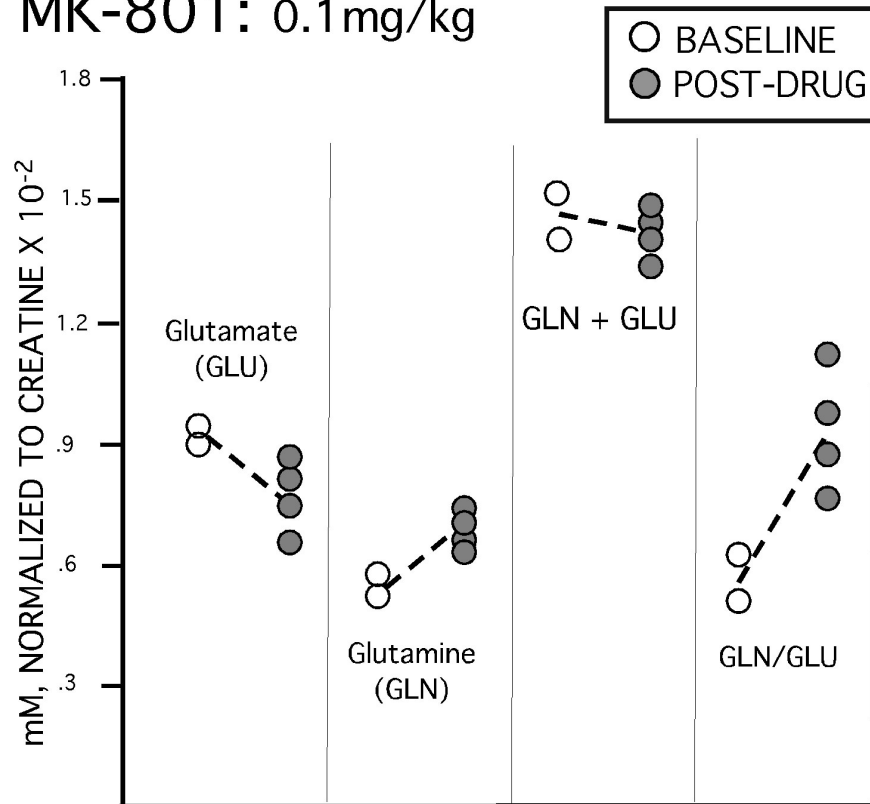


Figure V.3. Scatter plots showing the LCMoDel-derived concentrations of glutamate, glutamine, total glutamine and glutamate and the glutamine/glutamate ratio (all normalized to creatine concentrations) before and after treatment with 0.1 mg/kg MK801.

GENERAL DISCUSSION

Summary of Major Findings

The studies presented in this dissertation investigated sex differences in behavior and neurochemistry within the rodent NMDA receptor hypofunction model of schizophrenia. More specifically, since NMDAR hypofunction is a relatively recent theoretical framework for modeling schizophrenia that accurately recapitulates many behavioral and neurochemical abnormalities known to occur in organic disease, the main question driving the studies here was whether NMDAR hypofunction also recapitulates the striking differences in male versus female severity of positive, negative and affective symptoms and in response to treatment with typical and atypical neuroleptic drugs that characterize the clinical picture of schizophrenia and its treatment. Thus, in the initial three chapters of this dissertation, I first sought to thoroughly characterize behavior in adult male and female rats in an acute state of NMDAR hypofunction by using three behavioral paradigms that measure behaviors with high relevance to the positive, negative/cognitive and affective symptoms of schizophrenia. Furthermore, these studies also investigated whether treatment with typical and atypical antipsychotic drugs widely used in clinical practice can attenuate behavioral changes induced by NMDAR hypofunction in sex-specific ways. In answering this question, I found that the rodent NMDAR hypofunction model does indeed largely recapitulate the sex-specific differences seen in schizophrenia's symptomatology and its treatment. This finding led to a subsequent question, whose surface was only scratched in Chapter IV - what are the neurochemical substrates

responsible for these sexual dimorphisms in symptomatology and treatment response? Concurrently, realizing the fact that current techniques of quantifying *in-vivo* neurochemistry are limited and either cannot be ethically translated to human investigation or if they can, have not been shown to be able to study dopaminergic neurochemistry, Chapter V of this dissertation sought to evaluate, on a proof-of-concept level, whether magnetic resonance spectroscopy paired with a novel data analysis algorithm can be extended into the realm of studying dopaminergic neurotransmission.

In addressing the first major question that was the driving force behind the studies in this dissertation, behavioral paradigms were selected sequentially for a variety of reasons. Prepulse inhibition of the acoustic startle reflex was evaluated first due to the fact that these behaviors are highly conserved between humans and animals (Swerdlow et al., 1999a), that they are sexually dimorphic in healthy humans (Swerdlow et al., 1993; Swerdlow et al., 1999b) and that they are disrupted in schizophrenia in sex-specific ways which in turn mirror the sex differences seen in the severity and expression of positive and negative symptoms of schizophrenia (Kumari et al., 2004). Together, these advantages made the assessment of these behaviors an ideal first step in evaluating whether NMDAR hypofunction in the rodent recapitulates the sex differences seen in ASR and PPI in healthy humans, in their disruption in schizophrenia and in response to neuroleptic treatment. The results contained in Chapter I provided the first indication that the rodent NMDAR hypofunction model indeed mirrors these sex differences. Specifically, drug-naïve females exhibited higher ASR than males whereas drug naïve males showed higher levels of PPI than females, a pattern completely

consistent with that seen in the human population. Furthermore, while NMDAR antagonism induced by MK801 raised ASR and decreased PPI in both sexes, the increases in ASR were more substantial in females and the decreases in PPI were more pronounced in males, corresponding to higher ASR and more severe positive and affective symptoms in female schizophrenia patients and lower PPI and more severe negative symptoms in male schizophrenia patients. Finally, these results demonstrated that attenuation of MK801-induced changes in ASR and PPI by pretreatment with typical and atypical neuroleptics also mirrors salient drug and sex differences observed in treatment of schizophrenia. Thus, clozapine was more effective than haloperidol at reversing MK801-induced changes in ASR and PPI in both sexes and was somewhat more effective in males, reflecting the clinical evidence establishing the superior efficacy of atypical neuroleptics in reversing both positive and negative symptoms of schizophrenia relative to older, typical neuroleptics (Gallhofer et al., 1996; Lieberman, 1996; Kapur and Remington, 2001).

Given this initial indication that the NMDAR hypofunction model does indeed model the sex differences in symptomatology and in treatment response, the studies of the next chapter focused on expanding the characterization of these sex differences to a wide range of spontaneous behaviors by using open field testing combined with the same drug treatment regimens as in Chapter I. Open field testing is an extremely powerful behavioral paradigm that allows the observation and quantification of a wide range of spontaneous behaviors in a natural, unconditioned and unrewarded state; it is also extremely sensitive to subtle behavioral abnormalities. As many rodent behaviors

that are assessable in the open field have been directly linked to positive, negative and affective symptoms of schizophrenia (Simon et al., 1994; Jentsch and Roth, 1999; Rung et al., 2005), this behavioral paradigm stood out as ideal to extend and confirm the findings in the preceding chapter of sex differences in the sequelae of NMDAR hypofunction on various symptom correlates and in response to treatment with typical and atypical neuroleptic drugs. Interestingly, though open field testing has previously been used to evaluate behavior under acute NMDAR hypofunction, these analyses have mostly been limited to males and focused only on motor symptoms solely associated with the positive symptoms of schizophrenia (Danysz et al., 1994; Carey et al., 1998; Scorza et al., 2008) whereas the experiments in Chapter II of this dissertation, in addition to evaluating these motor behaviors, also extended the use of open field testing to evaluation of rearing, grooming and thigmotaxis, behaviors with proven relevance to negative and affective symptoms of schizophrenia (Treit and Fundytus, 1988; Bardo et al., 1990a; Dubiela et al., 2011). First, this experiment showed that while NMDAR hypofunction increased locomotion, ataxia, and stereotypy and simultaneously decreased rearing and grooming in both sexes, it caused a larger increase in hyperlocomotion and ataxia (both positive symptom correlates) in females than males and decreased rearing and grooming (both negative symptom correlates) to a greater degree in males than females. Further, while haloperidol was effective at reversing NMDAR-induced motor symptoms of hyperlocomotion, ataxia and stereotypy in both sexes and was more effective in females, it did not restore grooming and rearing in either sex. Clozapine, on the other hand, was equally effective in reversing positive symptom correlates in both sexes but was also not effective in ameliorating negative

symptom correlates in either sex. These findings extend those from Chapter I and confirm that NMDAR hypofunction accurately recapitulates the female over male severity of positive symptoms (particularly bizarre behavior) and the male over female severity of negative symptoms seen in schizophrenia. Further, these two studies also indicate that the rodent NMDAR hypofunction model mirrors sex and drug-specific responses in the treatment of schizophrenia's positive and negative symptoms wherein typical neuroleptics are mostly effective only in treating positive symptoms and are more effective in females, atypical neuroleptics are equally effective in both sexes and while somewhat effective at treating negative symptoms, are unable to fully ameliorate them. Additionally, open field testing provided some insight into the ability of NMDAR hypofunction to model the sex differences in affective disturbances in schizophrenia which are more predominant and severe in female patients. Thus, NMDAR hypofunction increased the affective symptom correlate of thigmotaxis in males but did not affect it in females, indicating that females show more impulsivity and improper affect than males, again mirroring the clinical condition (Lewine, 1981; Goldstein and Link, 1988; Leung and Chue, 2000). Together, these studies provide compelling evidence that the rodent NMDAR hypofunction model accurately recapitulates the sex differences in the severity of the positive and negative symptoms of schizophrenia and their response to neuroleptic treatment.

However, despite the insight provided into affective state by measurement of thigmotaxis, the ability of NMDAR hypofunction to model sex differences in affective symptoms remained unclear. To that end, the experiments in Chapter III quantified

spontaneous ultrasonic vocalizations (USVs), behaviors that have been identified as a rich source of information about the affective state of the animal. These experiments found that while NMDAR hypofunction increased vocalizations in both sexes, it increased vocalizations indicative of negative affect to a much greater degree in females than males and while haloperidol was ineffective in attenuating these affective sequelae of NMDAR hypofunction, clozapine robustly reversed them. These results indicate that the rodent NMDAR hypofunction model extends to modeling the sex differences evident in the affective symptom cluster of schizophrenia as well as modeling drug differences in its treatment. Additionally, since USVs indicative of negative affect are generated through activation of cholinergic signaling originating from the laterodorsal tegmental nucleus (LDT) which in turn causes increased dopaminergic signaling to midbrain and forebrain structures, these behavioral findings indicate that there may be sexually dimorphic substrates in that particular neural circuit.

Taken together, the results from the first three chapters of this dissertation provided a detailed behavioral characterization of the behavioral sequelae of NMDAR hypofunction in male and female rats and firmly established that this model does indeed recapitulate a wide variety of sex differences in schizophrenia's major symptom clusters and in their treatment and opens up the use of this model to further characterize sex differences in behavior with other behavioral paradigms. However, given that all behavior is driven by underlying biological substrates, the general question arising from these results asked which neuroanatomical and neurochemical underpinnings are responsible for these sex differences. The studies in Chapter IV started to address this

question by using *in-vivo* microdialysis to quantify the levels of dopamine and its metabolites in the prefrontal cortex of male and female rats during a state of NMDAR hypofunction. Given that DA activity in the PFC modulates a number of cognitive processes that are at risk in schizophrenia and that this system has been shown to be sensitive to gonadal steroids (Goldman-Rakic et al., 2000; Kritzer, 2000; Dalley et al., 2004; Aubele et al., 2008; Aubele and Kritzer, 2011a) made it a reasonable initial target for investigation. What was discovered is that while acute NMDAR hypofunction causes increased DA turnover in both sexes, turnover was increased more in females than males, even when locomotor activity and estrus cycle stage was controlled for which indicates that females have increased mesocortical DAergic activity when they are in a state of NMDAR hypofunction. Interestingly, these microdialysis results dovetail with the behavioral findings to suggest that increased activity of the LDT in females compared to males may be contributing to increased activation of the mesocortical system and increased DAergic activity in the PFC. Concurrently, this finding also suggests that females are more susceptible to the effects of NMDAR antagonism. One putative explanation for this is that females may have a relative reduction in NMDA receptor density in the prefrontal cortex relative to their male counterparts; this speculative theory is based on findings that high levels of estrogen have been found to reduce NMDA receptor expression in the PFC of females (Cyr et al., 2000; Cyr et al., 2001).

In parallel with neurochemical investigations using microdialysis, the studies in Chapter V of this dissertation studied whether magnetic resonance spectroscopy

(MRS), a non-invasive imaging method, can be extended to studying dopamine and its metabolites. While MRS has been used to study glutamate and GABA in humans and animals, it was previously thought that the relatively low concentrations of DA and its metabolites placed it outside the realm of detection with MRS. However, driven by studies showing that a novel data analysis algorithm was able to bring MRS into the realm of reliably detecting other low-concentration molecules (Terpstra et al., 2006; Terpstra et al., 2010), the experiments in Chapter V showed that MRS can reliably quantify the levels of homovanillic acid, (HVA), a major metabolite of dopamine, at baseline and in response to pharmacologic manipulation. This finding, while preliminary, opens up the potential of the non-invasive study of dopaminergic neurochemistry in multiple brain areas and holds potential for eventual translation to human studies, where non-invasive study of dopaminergic neurochemistry would undoubtedly be a powerful tool for evaluating many poorly understood processes that are implicated in many mental illnesses.

In sum, the results presented here established that the NMDAR hypofunction model, the leading etiologic hypothesis of schizophrenia which accurately models many of the complex behavioral and neurochemical abnormalities prevalent in this disorder, also extends to modeling sex differences seen in the severity of positive, negative and affective symptoms of schizophrenia. Further, the sex and drug differences in treatment of these various symptom clusters is also recapitulated by this model. Further, these sex-specific behavioral changes are accompanied by higher DA turnover the PFC in females than males, providing some indication of sexually dimorphic neurochemistry

that could be driving the differences seen in behavior. However the studies presented in this dissertation are only a small first step in exploring the sex differences inherent to many facets of schizophrenia and many questions remain about their underlying neurobiological underpinnings. Thus, the following sections will outline some of my thoughts on the findings raised by the studies presented here and discuss the implications of these findings for future preclinical and clinical studies alike.

Future Directions

The studies in this dissertation have provided compelling evidence that the rodent NMDAR hypofunction model accurately replicates sex differences in the expression of positive, negative and affective symptoms of schizophrenia and in their treatment. Naturally, this important finding stimulates further interest into exploring which neurobiological substrates are responsible for these sexually dimorphic behaviors as a previous lack of an animal model capable of recapitulating these differences has hampered these efforts. Thus, although this dissertation has focused more on a detailed behavioral characterization of these sex differences in a state of NMDAR hypofunction, these behavioral studies yielded intriguing results that went beyond just behavioral characterization. Specifically, by considering the substrates driving the sexually dimorphic behaviors that were assessed, several neural targets stood out as candidates for further study in investigating the neurobiology of sex differences in NMDAR hypofunction and schizophrenia which have interestingly received relatively

little attention despite being critically involved in modulating activity on well-studied structures and systems at risk in disease.

While behavioral studies are invaluable in characterizing even subtle phenotypic differences, most behaviors involve multiple brain areas which poses a challenge in using behavior to evaluate differences in specific systems. For example, PPI/ASR and open field behaviors that were assessed in the first two chapters, while critically important to the questions driving this body of work, are innately complex and modulated by a sophisticated interaction of brain areas, circuits and neurotransmitter systems (Swerdlow and Geyer, 1998; Koch, 1999). Conversely, the vocalizations assessed in Chapter III were unique in that these behaviors are thought to be modulated by specific structures. Thus, high-frequency vocalizations are emitted upon dopamine release in the nucleus accumbens resulting from activation of the mesolimbic DA system (Burgdorf et al., 2001; Thompson et al., 2006) and low frequency vocalizations are produced upon activation of cholinergic cells in the laterodorsal tegmental nucleus (Brudzynski, 2007; Brudzynski et al., 2011). Thus, due to this seemingly precise localization of the substrates responsible for these behaviors, my results showing that females vocalize more at both frequencies suggest that there is both increased DA release in the nucleus accumbens and increased activation of the LDT. Interestingly, overactivity of the DAergic projections to the NAc has been linked to the positive symptoms of schizophrenia, which are in turn more prevalent in females. This circuit is known to be sensitive to the actions of gonadal hormones; mesolimbic DAergic neurons contain a large amount of membrane and intracellular hormone

receptors (Creutz and Kritzer, 2004). Despite it being known that both androgen and estrogen actions potentiate the release of DA in the NAc (Di Paolo et al., 1985; Di Paolo, 1994; Becker, 1999), it is still relatively uncertain how these interactions relate to sexual dimorphisms in NMDAR hypofunction and schizophrenia. Thus, since evidence suggests that high-frequency USVs may be a measure of DAergic activity in the mesolimbic DA system projecting to NAc, measurement of this behavior along with hormone manipulation could shed light onto hormone-sensitive processes critical to normal function and disease.

However, while it was significant, the female over male predominance of 50 kHz vocalizations was quite small, which makes it less likely that robust sex differences in underlying substrates will be found. Conversely, the female over male increase in low-frequency vocalizations was much more robust. Since generation of these vocalizations is wholly dependent on activation of the LDT, this finding suggests that NMDAR hypofunction activates the cholinergic signaling arising in the LDT to a greater extent in females than males. This result is particularly intriguing considering that cholinergic projections from the LDT innervate a wide range of midbrain and forebrain structures at risk in schizophrenia including the VTA and prefrontal cortex (Sato and Fibiger, 1986). However, the function of the connection to the VTA is particularly relevant with regard to NMDAR hypofunction and schizophrenia. Specifically, cholinergic activity from the LDT is required to initiate burst firing activity of mesolimbic and mesocortical DA cells in the VTA (Lodge and Grace, 2006). In turn, burst firing of these cells causes DA release in the NAc and prefrontal cortex, which is functionally required for the appropriate focus of

the organism onto salient stimuli and for proper goal directed behavior and performance on cognitive tasks (Goto and Grace, 2005; Goto et al., 2007). However, overstimulation of burst firing leads to excessive dopamine release and subsequent inability of uptake and catabolic processes to clear these elevated dopamine levels, which leads to disrupted cognitive and executive functions which are extremely sensitive to proper levels of DA (Tassin et al., 1978; Kalsbeek et al., 1989; Zahrt et al., 1997; Goldman-Rakic et al., 2000). Thus, the LDT is at least partially involved in regulating processes that are at the heart of the neural circuits and neurochemical systems known to be disrupted in sex-specific ways in NMDAR hypofunction and schizophrenia. Despite this, however, very little is known whether the LDT or its projections are hormone sensitive or functionally different in males and females at baseline or under a state of NMDAR hypofunction. The behavioral evidence here suggests that it is. Thus, the predominant increase of 22 kHz vocalizations in females over males suggests that the NMDAR hypofunction activates cholinergic efferents in the LDT to a greater extent in females, which may in turn cause increased burst firing in the VTA. Interestingly, the results of the neurochemical assessments in Chapter IV support this suggestion. Thus, even though these assessments were limited, they showed evidence of a higher increase of DA turnover in the PFC of females over males in response to NMDAR hypofunction, a finding which is consistent with an increase in burst firing activity in mesocortical DAergic cells. Thus, these behavioral and neurochemical findings, in conjunction with evidence showing a critical role of the LDT in modulating mesocortical and mesolimbic DAergic activity strongly imply that there may be sexual dimorphisms in the architecture or function of this structure or its efferent projections which may in turn contribute to

sexual dimorphisms in normal behavior and in the complex behavioral aberrations in schizophrenia. Further investigation of hormone sensitivity of this system is clearly warranted as it may lead to novel findings with relevance to the treatment of schizophrenia.

Along with this potential avenue for further exploration, the results of the magnetic resonance spectroscopy studies in Chapter V also provide promising results that have much relevance for translational investigation. Thus, the experiments in Chapter V showed that MRS can be extended to the reliable detection of the DA metabolite HVA *in-vivo*. Although the results are preliminary and need to be further explored, they strongly imply that this moiety, which is directly representative of DA levels, can be quantified with this method and that pharmacological manipulations of the *in-vivo* levels of this molecule can be tracked with this method. The importance of this finding is highlighted by the fact that MRS is already capable of simultaneously detecting glutamate and GABA (Pfeuffer et al., 1999; Tkac et al., 2004); this addition makes MRS able to probe an additional dimension in the non-invasive study of the neurochemistry of many poorly understood processes at risk in disease. To that end, this newly expanded method was applied to probing the consequences of acute NMDAR hypofunction on the neurochemical milieu in the PFC. Unfortunately, these experiments were subverted by the finding that NMDAR hypofunction induces a profound paroxysmal apnea, making it impossible to fully evaluate effects of NMDAR hypofunction over the course of several hours. However, technical improvements that can eliminate this problem should be explored as the use of MRS to simultaneously

measure glutamate, GABA and HVA in a variety of brain areas after NMDAR hypofunction would undoubtedly prove useful in better understanding the interactions between these systems. Furthermore, while much work in perfecting this method needs to be conducted on the preclinical level, the fact that MRS can be safely and non-invasively applied to both animals and humans suggests great potential for eventual translation of this method to study dopaminergic neurochemistry in human health and disease.

Conclusion

The major findings of this dissertation were that the rodent NMDAR hypofunction model accurately recapitulates the sex differences in the severity of positive, negative and affective symptoms seen in schizophrenia. This finding extends the utility of this model to that of probing the neurobiological underpinnings of these sex differences and in fact, the studies here also provided some insight into neuroanatomical and neurochemical targets that are attractive for further investigation. Thus, while the studies in this dissertation were driven primarily by evidence of sexual dimorphisms in forebrain and midbrain structures in both health and disease, behavioral and neurochemical data obtained in the experiments here also suggest that cholinergic system arising from the LDT nucleus in the brainstem may be sexually dimorphic. These targets are neurobiologically and clinically appealing due to the fact that this system is closely involved in regulating activity in midbrain and cortical areas and neurotransmitter systems linked to schizophrenia (Yeomans, 1995; Forster and Blaha,

2000; Lodge and Grace, 2006) as well as because pharmacological manipulations of the cholinergic system have shown promise in the treatment of schizophrenia. However, despite a large body of circumstantial evidence implicating this system in the neurochemical aberrations in both schizophrenia and NMDAR hypofunction, these targets have received little attention and it remains unknown whether NMDAR hypofunction affects the functional properties of this system or whether it is sexually dimorphic or sensitive to gonadal hormones. Thus, while the underpinnings of the sexual dimorphisms seen in schizophrenia are undoubtedly diverse and most span multiple brain regions and systems, preliminary findings here provide impetus for the investigation of putative sexual dimorphisms in this system and their functional effects. Finally, it should be noted that in addition to these findings which spur further questions, the establishment of a model that recapitulates sex differences in schizophrenia is of great importance for human therapeutics. As several behavioral assays used in the studies of this dissertation have been established to have predictive validity in screening for novel neuroleptic drugs, there is now potential for preclinical screening of sex-specific treatment strategies. As evidence increasingly suggests that treatment of schizophrenia may benefit from a number of adjunctive treatment including hormone supplementation (Kulkarni et al., 2002; Akhondzadeh et al., 2003; Ko et al., 2008; Kulkarni, 2009) and drugs acting on serotonin and acetylcholine receptors (Shekhar et al., 2008; Biedermann and Fleischhacker, 2011; Meltzer and Massey, 2011), the findings outlined here will undoubtedly be of use in the efforts to develop novel and effective treatment regimens that address the sexually dimorphic symptomatology of this devastating disorder.

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