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Assessing a Two-Hit Model of Schizophrenia: Prenatal choline deficiency and induced hypofunction of NMDA receptors in male and female Long Evans rats

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Signature Page

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Abstract

Choline is an essential dietary nutrient essential to the development and function of the central nervous system. Prenatal choline deficiency alters hippocampal development as well as acetylcholine metabolism, leading to cognitive impairments and attentional and sensory processing deficits into adulthood. MK-801 is an NMDA receptor antagonist frequently used in rodent models of neuropsychiatric conditions, particularly schizophrenia. Acutely and sub-chronically, it causes hyperlocomotion and social withdrawal. One primary goal of the present study were to investigate prenatal choline deficiency induces a biological vulnerability to the motor deficits, anhedonia, and social impairment precipitated by low-dose sub-chronic MK-801 administration in adulthood. Another goal was to illustrate any sex differences that exist in response to this treatment. Using the saccharin preference task, we found that deficient-fed males administered MK-801 had a diminished preference during test 2, but the effects of drug and diet condition were inconsistent overall and across sex. In motor assessments immediately following injection with MK-801 or saline, males and females injected with MK-801 demonstrated motor impairment across three dimensions. Females sustained this level of impairment throughout the course of injections. Taken together, the results of this study suggest that prenatal choline deficiency may represent a vulnerability to MK-801 induced anhedonia in males. Females are also affected to a greater degree by this neurotoxic treatment, as indicated by motor responses to acute and sub-chronic administration. This research contributes to the existing body of research investigating the effect of prenatal choline availability and pharmaceutical treatment on the expression of symptoms relevant to schizophrenia.

1. Introduction

Schizophrenia is a debilitating mental disorder that affects approximately 1% of the global population (Jadi, 2016). This brain illness is characterized by positive and negative symptoms as well as cognitive impairments (Addington et al., 1991), though the combination of these can vary widely at presentation, making prognosis and treatment heterogenous and complex. Although positive symptoms are appropriately managed with available pharmaceutical treatments, antipsychotic treatment has proven less effective in the management of cognitive symptoms (Kirkpatrick et al., 2006; Barnes et al., 2014). Schizophrenia symptoms typically onset between the late teen years to early thirties in males and females, however, female incidence rates are higher when onset occurs later in life (Abel et al., 2010) . Cognitive decline occurs prior to first-episode psychosis manifesting as deficits in speed-of-processing, visual learning and social cognition (Corigliano et al., 2014). Cognitive deficits persist throughout the progression of the illness and have a substantial impact on daily function, maintenance of social relationships and the ability to sustain employment (Murueta-Goyena et al., 2019). In the acute phase and thereafter, patients with schizophrenia experience debilitating positive symptoms, manifesting as episodes of hallucinations and delusions and can also present with negative symptoms, including marked social withdrawal, anhedonia, and flattened affect. Underlying variable symptom profiles is a host of biological changes with widespread impacts on several brain structures and circuits. Although numerous studies have accumulated evidence pointing towards specific neuroanatomical targets and neurobiological changes implicated in schizophrenia pathology, the exact cause is not known. Investigations aimed at uncovering

causal relationships have yielded a number of potential risk factors for later expression of schizophrenia symptoms including genetic mutations and specific environmental events. To better understand the impacts of those factors on behavioral outcomes, animal models have been employed, mainly in mice and rat subjects (reviewed in Nestler & Hyman, 2010). The current project assesses the impact of prenatal choline deficiency on adult behavioral outcomes related to schizophrenia symptoms in rats that are exposed to biological features of this illness in adulthood. Another goal of this study is to investigate whether biological sex differences mediate symptom expression in this model.

Negative symptoms experienced in schizophrenia, i.e. avolition, anhedonia, social withdrawal, and blunted affect, can be modeled in animals but are also characteristic of other neuropsychiatric disorders including autism and depression. In schizophrenia, these negative symptoms are consistently present in the prodromal stage prior to expression of positive symptoms and they may or may not persist to this degree during the active-phase (American Psychiatric Association, 2013). Approximately 20% of those patients diagnosed with schizophrenia experience persistent negative symptoms and consequently, have a reduction in normal function and/or a decrease in quality of life (Kirkpatrick et al., 2006). A discussion of schizophrenia would be incomplete without acknowledgement of sex differences that exist in humans and rats being used to model the expression of these symptoms. Males have a higher rate of incidence and earlier age-of-onset compared to females. At presentation, females are more likely to have depressive symptoms and less likely to have negative symptoms. Males, however, present with more negative symptoms and earlier age-of-onset generally predicts worse negative and disorganizational symptoms and worse subsequent short and medium term outcomes (Abel et al., 2010).The results of a study exploring the relationship between symptom presentation at

first-episode hospitalization and quality-of-life at 2-year follow-up found that negative symptom severity positively correlate with later occupational impairment, financial dependence on others, impaired relationships with friends, impaired ability to enjoy recreational activities, and reduced global assessments of functioning (Ho et al., 1998). Less research has focused on the amelioration of negative symptoms than positive symptoms and fewer targeted interventions have been developed (Turner et al., 2018). The current animal model used in this study will be assessed primarily for expression of behavior related to negative symptoms observed in patients with schizophrenia.

Current animal models of schizophrenia fall into two related etiological frameworks, the neurodevelopmental and the two-hit. In both frameworks, it is posed that a perinatal event, whether genetic and/or environmental, mediates the development of abnormal neuronal morphological features. The neurodevelopmental hypothesis poses that this is the only essential etiological event, whereas the two-hit hypothesis includes a second “hit” that occurs during adolescence and is necessary for the later expression of behavioral symptoms (Rapoport et al., 2012; Feigenson et al., 2014). The present work assesses prenatal choline deficiency as a potential first “hit” in model of negative symptoms present in schizophrenia pathology.

The present work was designed to contribute to a body of work on the detrimental effects of inadequate intake of dietary factors during sensitive periods, particularly during pregnancy and embryonic development (Zeisel, 2006; Meck et al., 2008). Choline is an essential dietary nutrient that is a critical precursor of components of biological membranes, intracellular messengers, a methyl donor betaine involved in gene expression and the neurotransmitter acetylcholine. Only a small amount of choline is produced endogenously in the liver, so it must be obtained from dietary sources (Zeisel & da Costa, 2009). Women with low choline intake

during pregnancy have a greater risk of having a child with a neural tube defect or cleft palate (Blusztajn & Mellot, 2013). During fetal and early postnatal life, adequate dietary choline intake is necessary for proper development of brain areas rich in cholinergic neurons including the hippocampus, basal forebrain, and cortical areas and is demonstrated to have a profound modulatory effect on adult behavior (reviewed in Meck & Williams, 2003; Zeisel, 2006; Meck et al., 2008; Zeisel & da Costa, 2009; Blusztajn & Mellott, 2013). In addition to its involvement in the synthesis of acetylcholine throughout the lifespan, choline is a selective agonist for the α7 nicotinic acetylcholine receptor (nAChR) which are found in the developing rat hippocampus before acetylcholine is available (Stevens et al., 2008). Cerebral inhibition is also mediated by α7 nAChR activation in the fetal brain by extracellular concentrations of choline. This activation switches GABA interneurons from excitatory to inhibitory by chloride transporter KCC2. When excitatory, GABAergic neurotransmission promotes neuronal growth and integration into circuits in the developing brain, but it is essential that its function is switched to inhibitory for proper sensorimotor gating and attention (Liu et al., 2006; Ross et al., 2013). Prenatal choline availability mediates fetal cholinergic neurotransmission and proper development of the hippocampus and cortical areas, neuroanatomical structures heavily implicated in the pathology of schizophrenia (Konradi et al., 2011; Carr & Sesack, 2002). Perinatal choline supplementation in rats enhances cognitive functioning throughout the lifespan and protects against a number of cognition-impairing neural insults (Guo-Ross et al., 2003; Blusztajn & Mellott, 2013). An important mechanism that has a direct impact learning and memory processes rely on throughout life is adult neurogenesis in the dentate gyrus. Following prenatal choline supplementation and environmental enrichment, adult hippocampal neurogenesis is enhanced and is accompanied by

increased concentrations of brain-derived neurotropic factor (BDNF), a marker of hippocampal plasticity (Glenn et al., 2007).

In contrast to prenatal choline supplementation, deficient prenatal choline availability results in selective changes in hippocampal development and function which manifest as marked behavioral deficits and neuroanatomical and neurochemical differences that persist into adulthood. Prenatal choline deficiency significantly decreased the rate of mitosis and birth of hippocampal progenitor (undifferentiated) cells and increased the number of apoptotic cells in the dentate gyrus (Allbright et al., 1999). In the hippocampus, cell generation and migration to regions associated with learning and memory processes in the adult brain are significantly impaired by prenatal choline deficiency (1999). Prenatal choline deficiency also impairs acetylcholine synthesis and release, wherein these animals display accelerated ACh turnover evidenced by elevated acetylcholinesterase and choline acetyltransferase activity, increased synthesis, degradation of ACh and reuptake of choline into the presynaptic terminal and an inability to sustain depolarization-evoked ACh release. This aberrant ACh metabolism compared to controls and prenatal choline supplemented animals is thought to be an adaptive response in order to maintain adequate ACh release despite a reduced ACh pool (Cermak et al., 1998). Normal enrichment-induced increases in hippocampal neurogenesis are impaired and BDNF concentration reduced in the hippocampus and cortex of rats with a prenatal choline deficient diet (Glenn et al., 2007). These neuroanatomical and neurochemical changes are accompanied by altered behavioral expression. Postnatal choline deficiency has been demonstrated to make male rats more vulnerable to the effects of a two-hit model of schizophrenia which included prenatal stress and adult neurotoxic treatment with an NMDA receptor antagonist, MK-801. Cognition was impaired in choline deficient rats that received either hit alone or both (Corriveau & Glenn,

2012). Expanding upon these findings, the current project is assessing if prenatal choline deficiency is a predisposing factor that is necessary to induce biological conditions that resemble those in schizophrenia. In order to do this, we will investigate if prenatal choline deficiency and later sub-chronic neurotoxic treatment with MK-801 will produce behavioral abnormalities outside of the cognitive realm; hyperlocomotion, anhedonia, and social inhibition. By using male and female subjects, we will also be able to investigate biological sex differences in response to this treatment.

Prenatal choline supplementation has been found to be neuroprotective against a number of cognition-impairing neural insults and exerts neuroprotective effects in animal models of schizophrenia (Stevens et al., 2008; Corriveau & Glenn, 2012). The neurotoxic effects of MK-801 administration are attenuated by prenatal choline supplementation (Guo-Ross, 2003; Nickerson et al., 2015); however, prenatal choline deficiency has not been investigated as a factor promoting vulnerability to later neurotoxic insults. A well-studied neurotoxic agents, MK-801, is a non-competitive NMDA receptor antagonist that blocks the activity of glutamate by binding to an allosteric site on the receptor and preventing glutamate binding. NMDA receptor antagonism and induced hypofunction of NMDA receptors has been used to reliably produce positive, negative, and cognitive symptoms of schizophrenia as well as associated neuroanatomical and neurochemical changes in animal models (reviewed in Mouri et al., 2013; see also Olney et al., 1999; Bubeníkova-Valešová et al., 2008; Neill et al., 2010; Balu, 2016). In rats, chronic or large acute doses of MK-801 induce hyperlocomotion, social withdrawal (Matsuoka et al., 2005, 2008; Ünal & Aricioglu, 2018) and ataxia (Nickerson et al., 2017) while small acute doses induce anhedonia (Vardigan et al., 2010), hyperlocomotion, and social inhibition (Rung et al., 2005). Excluding motor deficits, which are used as indices of positive

symptomology in animal models of schizophrenia, these behaviors are reflective of negative symptoms observed in patients with schizophrenia. MK-801 administration also produces impairments in recognition, spatial, and working memory (de Lima et al., 2005; Hill et al., 2015), cognitive flexibility (Stefani & Moghaddam, 2005), and sensorimotor gating (Nespor & Tizabi, 2008; Uehara et al., 2009). These are symptoms commonly observed in neuropsychiatric disorders for which diminished excitatory neurotransmission is implicated pathologically, like in schizophrenia and are also impaired by prenatal choline deficiency (Meck and Williams, 2003; Montoya, 2000; Stevens et al., 2008b). In a study comparing sensorimotor gating and hippocampal volume in male and female patients with schizophrenia, it was found that P50 gating ratio was reduced as hippocampal volume increased in males and followed the opposite trend in female subjects (Huang et al., 2019).

We will assess if prenatal choline deficiency leads to increased expression of these behaviors induced by MK-801 administration that reflect negative symptoms observed in patients with schizophrenia. The neuropathological basis of this model adheres exclusively to the glutamate hypothesis through its antagonism of NMDA receptors. Reductions in glutamatergic neurotransmission in the prefrontal cortex (PFC) differentially effects the activity of GABA-containing and dopamine-containing neurons in the ventral tegmental area (Carr & Sesack, 2000; Volk & Lewis, 2010). As these afferent PFC projections are excitatory, reduced activity results in inhibition of both of these VTA neuron populations, which results in disinhibition of ascending mesolimbic dopaminergic projections to the nucleus accumbens via GABA interneurons and inhibition of mesocortical dopaminergic projections that innervate the PFC. Increased dopamine release into the nucleus accumbens is thought to contribute to positive symptoms observed in schizophrenia (Gray, 1998), while the reduced dopaminergic signaling to

inhibitory and excitatory neurons in the PFC is thought to underly negative symptoms (Sesack & Carr, 2002). In studies utilizing a chronic MK-801 dosing regimen, reduced hippocampal parvalbumin gene expression and reduced expression of the NR1 subunit of the NMDA receptor in the hippocampus and prefrontal cortex have been demonstrated (Ünal & Aricioglu, 2018; Murueta-Goyena et al., 2019). Normal sexually dimorphic differences in the healthy brain are likely mediated by the same developmental factors that result in sex-specific brain abnormalities in schizophrenia. These differences include larger hippocampal and thalamic nuclei volume in healthy females and smaller hippocampus and thalamic nuclei in males with schizophrenia. The dlPFC is similarly effected in males and females with schizophrenia, however, it is larger in healthy females (Abel et al., 2010). Although not directly tested in this project, it is important to acknowledge that if prenatal choline deficiency increases behavioral expression of correlates of negative symptoms in schizophrenia, it will do so by inducing biological conditions relevant to schizophrenia in concert with those produced by NMDA receptor antagonism.

The behavioral and functional effects of MK-801 administration and subsequent NMDA receptor hypofunction are also convergent with results from studies in which cholinergic neurotransmission is altered. In animal models devoid of α7 nAChRs, the amount of paravalbumin-containing GABAergic interneurons in the prefrontal cortex and NMDA receptor expression in these cells is significantly reduced (Lin et al., 2014). The activation of α7 nAChRs, is known to increase glutamate and dopamine release and be pro-cognitive in nature (Balu, 2016). In female rats, but not males, an α7 nACh receptor agonist reversed sensorimotor deficits induced by exposure to a two-hit model of schizophrenia and also increased expression of hippocampal parvalbumin-containing interneurons (Monte et al., 2019). Commonalities in the behavioral profiles of these two perinatal manipulations include sensorimotor gating deficits, as

measured by PPI, as well as some aspects of cognition. In addition, both manipulations resulted in decreased BDNF levels in males, however, in choline deficient animals these levels were not restored with environmental enrichment. The current two-hit model of schizophrenia used in this study assesses correlates of negative symptomology expressed in response to prenatal choline deficiency and sub-chronic MK-801 administration in adulthood in male and female rats.

The effects of prenatal choline deficiency on behavior as a function of MK-801 administration in adulthood will be assessed by examining three key markers of behavior; anhedonia, social behavior and motor responses. A number of reviews have explored the efficacy of animal models of schizophrenia investigating negative symptomology relevant to its pathology (reviewed in Barnes et al., 2014; Millan et al., 2014; Neill et al., 2014; Wilson & Koenig, 2014). Anhedonia in schizophrenia is properly defined as the marked and consistent impairment in looking forward to a reward, recreational or other pleasurable experience (anticipatory anhedonia) rather than the appreciation of the experience itself (consummatory anhedonia) (Kirkpatrick, 2006). Although the general consensus is that pharmacological models, using acute and chronic NMDA receptor antagonism, that test anhedonia using sucrose preference observe changes in only consummatory anhedonia (Millan et al., 2014). Another consideration is that high doses of NMDA receptor antagonists producing an anhedonic profile do so without the presence of other effects relevant to modeling schizophrenia and may depend on a neural pathology distinct from that seen in schizophrenia (Neill et al., 2014). Saccharin preference was chosen as our measure over the more common sucrose preference, due to the fact that sucrose, in addition to being sweet, is also energy-rich. This is potentially confounding, given that NMDA receptor antagonism is known increase hyperlocomotion and subsequent energy expenditure. Social withdrawal or asociality in schizophrenia is defined as the diminished

interest in, motivation for, and appreciation of social interaction with others (Millan et al., 2014). In humans, social withdrawal emerges in the premorbid stage, worsens during the prodromal period, and generally persists throughout the illness. As rodents have a stable degree of social behavior, measuring differences in social interaction is relatively straightforward (Wilson & Koenig, 2014). Acute MK-801 administration reduces social interaction in male rats immediately following injection in a social interaction task (Rung et al., 2005; Gururajan et al., 2011). Implementation of the social interaction task including two unfamiliar conspecific following sub-chronic PCP treatment has been established as a model with face, construct, and predictive validity in female rats (Neill et al., 2014). Sub-chronic MK-801 treatment has also been observed to produce deficits in social interaction in male rats (Matsuoka et al., 2008). Taken together, NMDA receptor antagonism is a valid mechanism through which to study social withdrawal as it relates to schizophrenia pathology (Wilson & Koenig, 2014). NMDA antagonism has a number of effects paramount among them is induction of hyperlocomotion. This is used as a manipulation check and an index of positive symptom expression. In this study, we will be assessing the behavior of male and females rats across these three dimensions throughout a 10-day sub-chronic injection period. Over the course of this low-dose treatment, saccharin preference, motor behavior and social behavior will be assessed at a number of time points.

Based on the two-hit model, it was hypothesized that the most considerable expression of negative symptoms and hyperlocomotion will be observed in prenatal choline deficient rats that are administered sub-chronic MK-801 in adulthood compared to rats with sufficient prenatal choline diets that receive the MK-801 treatment. Choline deficiency alone should not result in the expression of negative symptoms or hyperlocomotion. Over the course of the sub-chronic injection period, we hypothesize that saccharine preference will increase in animals that do not

receive MK-801, will decrease in choline-sufficient animals that receive MK-801, but to a lesser degree than in choline-deficient animals that receive MK-801. In addition, we hypothesize that hyperlocomotion, as well as stereotypy and ataxia, will differ across conditions, but will remain stable throughout the injection period and that females will exhibit more pronounced hyperlocomotion and ataxia, but not stereotypy, than in males (Hill, 2016). Finally, we expect that social interaction with cage mates will be reduced in animals that receive both hits, and to a lesser degree in those animals that only receive MK-801. We expect to see more social withdrawal in male rats compared to females. Assessing these behavioral outcomes over the course of the injection period will shed light on when differences between acute and sub-chronic MK-801 treatments may occur. Comparing sex differences may also illuminate future routes of inquiry to be explored using pharmaceutical models of schizophrenia as well as differences in the face validity of this model across sex.

2. Methods

2.1 Colony Conditions

All rats were housed in clear polycarbonate cages (30.5 x 30.5 x 18.5 cm), which were individually ventilated (Thoren Caging Systems, Inc., Hazelton, PA). Pregnant dams and dams with litters were housed individually; after weaning, pups were housed in same-sex pairs. All cages contained a thin layer of corncob bedding. The colony room was maintained at 20-23 °C with 10-50% humidity and was kept on a 12-h light/12-h dark cycle, with the lights on at 0800 h daily; every procedure was conducted in the light phase of the cycle. All rats had *ad libitum* access to food and water throughout the experiment. Food and water intake were recorded for the dams, dams with litters and for 3 weeks postnatal, and body weights were recorded once a week

for the duration of the study. All testing procedures were approved by the Colby College Institutional Animal Care and Use Committee and performed in accordance with federal standards.

2.2 Prenatal Choline Manipulation, Cross Fostering, and Experimental Groups

Sixteen timed-pregnant female Long Evans rats arrived at the lab from Charles River Breeders (*) on gestational day (GD) 8. Pregnant dams, housed as described above, were fed commercially available rat chow (Harlan Teklad, Madison, WI) until GD 10. On GD 10, pregnant dams were switched to a synthetic diet prepared based on formulations by the American Institute of Nutrition (AIN76A; Dyets, Inc., Bethlehem, PA). Half of the pregnant dams (n = 8) were placed on a standard choline diet (STD; AIN76A with 1.1 g/kg choline chloride substituted for choline bitartrate) while the remaining half (n = 8) were placed on a choline-deficient diet (DEF; AIN76A with 0 g/kg choline chloride). Following birth, on postnatal day (PD) 2, male and female pups from standard-fed and choline-deficient mothers were gathered, toe-clipped to indicate diet condition, and cross-fostered among all dams so that each litter contained 8-10 pups with a mix of males and females, both standard-fed and choline-deficient. Cross-fostering of pups ensures minimal influence of litter effects as all conditions are represented in each of the balanced litters. Also on PD 25, all dams were switched to rat chow (Harlan). On PD 23 (or 25), rat pups were weaned into same-sex, same prenatal condition pairs and switched to the regular, commercially available rat chow. (WEANING)

In accordance with the national imperative to use sex as a biological variable in animal studies, we chose to include males and females. Of the male subjects in the current study (n = 40), 20 were the offspring of dams fed the standard choline diet (STD), while 20 were the

offspring of dams fed the choline deficient diet (DEF). Of the female subjects (n = 28), 14 were the offspring of dams fed the STD choline diet, while 14 were the offspring of dams fed the DEF diet. Within each diet condition, half were randomly selected to receive MK-801 injections (MK), and the remaining half received 0.9% saline (SAL). Therefore, there were eight total experimental groups: females; STD-MK (n = 7), STD-SAL (n = 7), DEF-MK (n = 7), and DEF-SAL (n = 7), males; STD-MK (n = 8), STD-SAL (n = 8), DEF-MK (n = 8), and DEF-SAL (n = 8).

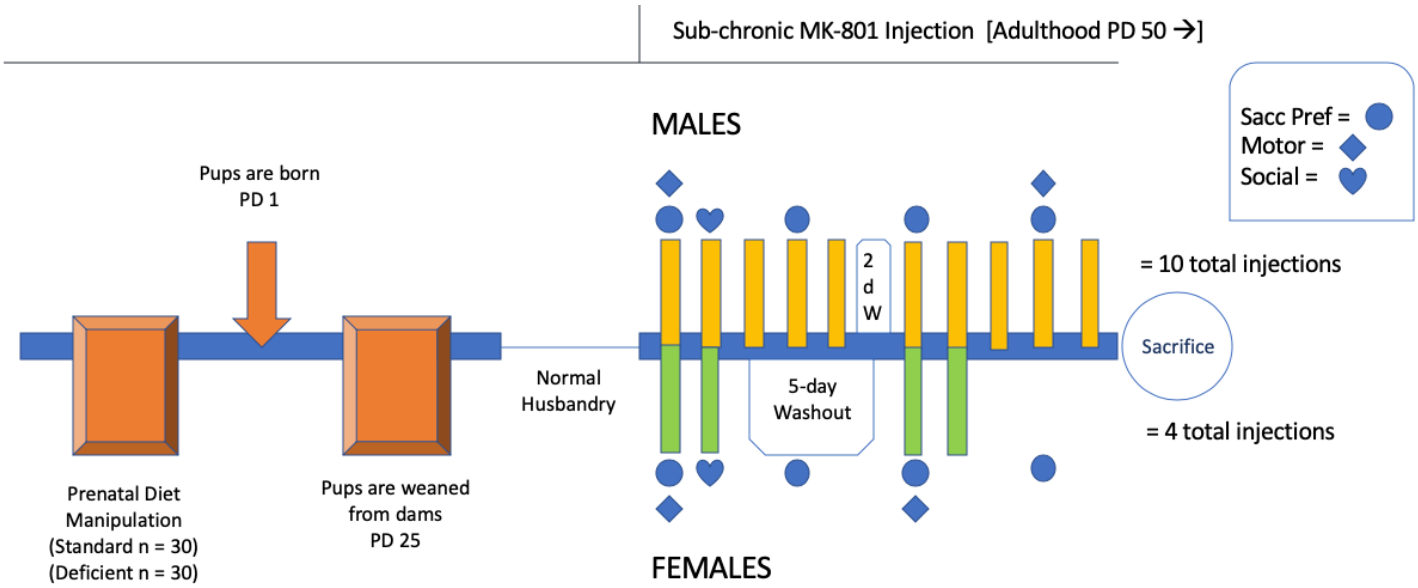
2.3 MK-801 Administration

In order to induce NMDA hypofunction implicated in the pathology of schizophrenia, a potent NMDA receptor antagonist was administered intraperitoneally. MK-801 (5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cycloheptene-5, 10-imine maleate; Sigma-Aldrich, St. Louis, MO) exists in two enantionmers. (+)-MK-801, as opposed to (-)-MK-801, is the more active enantiomer, binding to NMDA receptors with a slightly increased affinity. The present study utilized (+)-MK-801 as this is the most bioactive form of the drug, and we wanted to ensure that a full spectrum of symptoms would be incurred.

The (+) enantiomer of MK-801 hydrogen maleate (MK-801) was used in all behavioral tests (Sigma-Aldrich, St. Louis, MO). Testing began when the rats reached adulthood (PD) and continued until sacrifice (PD). MK-801 was administered sub-chronically for a 10-day period prior to testing once daily in males, with a two-day washout period between two 5-day periods of consecutive injections. Females were adversely effected by the low dose administered, so they only received injections on days 1, 2, 6, and 7 of the injection period, with a 5-day washout period between each set of two consecutive days of injections. The dose of 0.15 mg/kg (i.p.) was

chosen because we were using the stronger enantiomer and female Long Evans were known to be extremely sensitive to treatment with MK-801.

Experimental Timeline



2.4 Experimental Overview

Rats were put through the study in smaller groups denoted squads in order to make data collection more manageable. Three squads were created and each contained rats from all experimental conditions. Squad 1 contained 12 males and 10 females, squad 2 contained 12 males and 10 females, and squad 3 contained 8 males and 8 females.

2.4.1 Saccharin Preference

Each squad of rats was tested for saccharine preference following a 12h water deprivation period. A baseline test was conducted in the morning of day 1 of injections and three tests were

conducted throughout the injection period (see Experimental Timeline). Each test took place from 9:00 to 10:00.

For each test, following 12h water deprivation overnight, two small bottles fitted with droppers were placed in each cage. The position of the droppers were opposite to one another, so their relative position in the cage was balanced across conditions and were switched between each test. These bottles remained in the cages for 1 hour and were then taken out and weighed. A difference score was calculated for both bottles and saccharine preference was calculated by dividing the saccharine difference score by the total liquid consumed during that hour. Using 50% as a test value, we calculated whether or not each rat demonstrated a saccharine preference.

2.4.2 Locomotor Behavior

In order to analyze locomotor behavior immediately following injection, rats were placed in a clear polycarbonate cage lined with corncob bedding and covered with a metal cage top in a quiet room. Their behavior was recorded via a mounted web camera (Lorex *) connected to a computer in the same room for a total of 2 hours. These videos were saved onto an external hard drive and were analyzed by an experimenter blinded to the experimental conditions. Each individual was scored for 15 seconds at 15 total time points during each video (including $t = 120$ but not $t = 0$). Behavioral observations were made every 5 minutes for the first 30 minutes following injection and every 10 minutes for the remainder of the 2 hour duration. Each rat's behavior was coded for locomotion, stereotypy, and ataxia at each time point adapted from published scales specifically designed to assess motor effects of drugs like MK-801 (Corriveau & Glenn, 2012; Nickerson et al., 2017).

Behavioral ratings on the locomotor scale were used to gauge overall activity levels in the rats, immediately following MK-801 injection (i.p.). The scale is designed to take into account a range of locomotor features, including the use of the entire enclosed area, the frequency of movements, and the full use of limbs in the movement. Scores on this scale ranged from 0 to 5: 0 = stationary, 1 = movements with forelimbs only, including rearing on cage walls, 2 = intermittent movements within half of the cage area, 3 = continuous movement within half of the cage area, 4 = intermittent movements within the whole cage area, 5 = continuous movement within the whole cage area. Behavioral ratings on the stereotypy scale were used to detect expression of stereotyped movements in the rats. This behavioral feature is indicated by the repetitive engagement of motor behavior that is absent of any goal-directed locomotion. In this case, sniffing and head-weaving were stereotypical behaviors assessed. Scores on this scale ranged from 0 to 3: 0 = no sniffing or normal behavior (motionless or sleeping) 1 = normal exploration, sniffing, grooming, 2 = some stereotypical behavior, 3 = continuous stereotypy. Behavioral ratings on the ataxia scale were used to detect the presence of ataxia: the ability of rats to maintain adequate postural control to remain upright. Scores on this scale ranged from 0 to 3: 0 = normal body control, 1 = some falling, poor coordination, 2 = dragging hind legs while moving, 3 = almost unable to move (incapacitated). Rats receiving saline injection will display normal body control and will thereby consistently score a 0 on the ataxia scale. Accordingly, the analyses included are only on drug-treated rats (see Statistical Analyses).

2.4.3 Social Behavior

Each squad of rats was assessed for their social behavior with their cage-mate pairs the night following the second day of injections. Following injection, rats were placed back into their

home cage on a rack separate from the ventilated caging system and in view of a mounted web camera (Lorex *). The camera was mounted on a tripod and connected to a computer in an adjacent room and was infrared so that behavior could be assessed at night. Social behavior was assessed every 5 minutes from 24:00 to 2:00 the night following this injection by an experimenter blind to conditions. Rat pairs, denoted dyads, were scored as asleep together, asleep apart, awake together, awake apart, or not synced. Rats tend to sleep together, so this behavior was considered social, as was being awake together. The other three categories were considered asocial for our analyses.

2.5 Statistical Analyses

Means and standard errors of the means, displayed in the figures, were calculated for all behavioral results including the saccharin preference tests, motor response tests, and social behavior tests. As previously described, saccharin preference was calculated by dividing the saccharin difference score by the total liquid consumed over the hour. One-sample t-tests were conducted at each level of Diet (STD and DEF), Drug (SAL and MK), and Sex, using a test value of 50 to determine if the preference was significantly greater than chance. For all three motor behaviors assessed, 3-way ANOVAs were conducted comparing the effects of Diet, Drug, and Sex on the mean motor behavior scores from day 1. For all three, average scores were collapsed over the 15 time points. Using these average scores, a 2x2x2 repeated measures ANOVA was conducted in males and females independently, comparing the effects of Diet and Drug on motor behavior following the first and second-to-last injection (9^{th} and 4^{th}, respectively). For social behavior, independent samples t-tests were conducted at each level of Diet and Drug

in males and females. For all analyses, post hoc pairwise t-tests were conducted as appropriate. The significance level was 0.05 for all tests.

3. Results

3.1 Saccharin preference

In order to assess saccharin preference across the injection period, one-sample t-tests were conducted for each of the 4 tests at each level of the four experimental conditions (see Fig. 1A-D). This included a baseline saccharin preference prior to injection and 3 saccharin preference tests. Across these 4 measures, the pattern of one-sample t-tests that were not significant and, thus, indicate a lack of saccharin preference, varied between sex and over the course of the injection period. At baseline, prior to injection, DEF-MK males did not show a saccharin preference (t (7) = .679, $p > 0.05$) and DEF-MK females also lacked a preference (t (6) = 2.173, $p > 0.05$). All other groups had a saccharin preference in this baseline assessment (p's < 0.05). In test 1, STD-SAL males (t (7) = 1.808, $p > 0.05$), as well as STD-MK, DEF-MK, and DEF-SAL females showed a diminished saccharin preference (t (5) = 1.727, $p > 0.05$; t (7) = 1.458, $p > 0.05$; t (5) = .762, $p > 0.05$). In test two, only DEF-MK males showed a reduced saccharin preference (t (7) = 2.058, $p > 0.05$) and only STD-MK females demonstrated this reduced preference (t (5) = 1.617, $p > 0.05$). Finally, in test 3, all males exhibited a significant saccharin preference (p's < 0.01) and all females displayed a diminished saccharin preference (p's > 0.05).

3.2 Motor responses following acute MK-801 administration

A 2x2x2 ANOVA on mean locomotion scores obtained over the observation period following a single dose of MK-801 revealed main effects of both Sex (F (1, 2) = 25.539, $p <$

0.001) and Drug (F (1, 2) = 69.776, $p < 0.001$), but no main effect on Diet (F (1, 2) = 1.936, $p > 0.05$). There were no significant interactions between Sex and Diet ($F < 1$) and Diet and Drug ($F < 1$), in addition, there was no three-way interaction observed between Sex, Diet, and Drug; however, there was a significant interaction between Sex and Drug (F (1, 2) = 17.049, $p < 0.001$). To pursue the significant Sex by Drug interaction, pairwise comparisons were conducted first comparing SAL and MK males and then comparing SAL and MK females. Male and female MK rats had significantly higher mean locomotion scores compared with SAL rats (t (28) = -2.763, $p = 0.01$; t (26) = -9.626, $p < 0.001$) (see Figure 2). SAL males and females did not have significantly different locomotion scores (t (26) = -.706, $p > 0.05$), but MK females had significantly higher locomotion scores than did MK male rats (t (28) = -5.969, $p < 0.001$) (see Figure 2).

A 2x2x2 ANOVA revealed no main effect of Diet on mean stereotypy score ($F < 1$), but there were main effects of Sex (F (1, 2) = 7.879, $p = 0.007$) and Drug (F (1, 2) = 44.197, $p < 0.001$). There was no significant interaction between Diet and Drug ($F < 1$), but the interactions between Sex and Diet (F (1, 2) = 2.851, $p = 0.098$) and Sex and Drug (F (1, 2) = 3.294, $p = 0.076$) approached statistical significance. There was also a significant three-way interaction among Sex, Diet, and Drug (F (1, 2) = 4.191, $p = 0.046$). To explore this three-way interaction, pairwise comparisons were made at each level of the three IVs. First, STD and DEF males and females were compared across Drug conditions. This analysis revealed that male DEF-MK rats had significantly higher mean stereotypy scores than did DEF-SAL males (t (12) = -4.133, $p = 0.001$) and also that in STD and DEF females, MK-801 increased mean stereotypy scores compared to saline treated rats (t (12) = -4.458, $p = 0.001$; t (12) = -2.659, $p < 0.05$). Following this, MK and SAL males and females were compared across Diet conditions. This analysis

yielded one significant difference wherein DEF-MK males had higher mean stereotypy scores than STD-MK males (t (14) = -2.170, $p < 0.05$). Finally, we compared males and females across both the Drug and Diet condition. Pairwise comparisons revealed that female MK rats had higher mean stereotypy scores than male MK rats (t (28) = -2.542, $p < 0.05$) (see Figure 2).

This same analysis revealed a main effect for Sex (F (1, 2) = 20.869, $p < 0.001$) and Drug (F (1, 2) = 63.298, $p < 0.001$) on mean ataxia scores, but no such effect for Diet ($F < 1$). There was neither a Sex and Diet interaction ($F < 1$) nor a Diet and Drug interaction ($F < 1$); however, there was a significant Sex and Drug interaction (F (1, 2) = 21.579, $p < 0.001$). The analysis yielded no three-way interaction (F (1, 2) = 1.054, $p > 0.05$). To explore the Sex by Drug interaction, pairwise comparisons were conducted and revealed that male and female rats injected with saline did not have significant difference in ataxia scores (t (26) = 1.000, $p < 0.05$) and only one male rat had an average score above 0. Female MK rats had a significantly higher ataxia score compared to male MK rats (t (28) = -4.835, $p < 0.001$) and both male and female MK rats had significantly higher mean ataxia scores compared with SAL rats (t (28) = -3.251, $p = 0.003$; t (26) = -7.338, $p < 0.001$) (see Figure 2).

3.3 Motor responses following sub-chronic MK-801 administration in males and females

In males, a 2x2x2 repeated measures ANOVA was conducted in order to assess motor responses over the course of the treatment period in comparing responses following the first and ninth injection of either MK-801 or saline. This analysis revealed a significant difference in mean locomotion score following the first and ninth injection (F (1, 2) = 11.171, $p = 0.003$). Pairwise comparisons confirmed that across conditions, mean locomotion score was lower following the ninth injection compared to those following the first (t (29) = 3.279, $p = 0.003$)

(see Figure 3). There were no main effects for Drug (F (1, 2) = 1.272, $p > 0.05$) or Diet ($F > 1$), and there was no two-way interaction between Drug and Diet (F (1, 2) = 1.581, $p > 0.05$). A 2x2x2 repeated measures ANOVA revealed a significant difference in mean stereotypy score between the two time points (F (1, 2) =12.967, $p = 0.001$) and this was confirmed to be a significant decrease between the two time points by pairwise comparison (t (29) = 3.282, $p = 0.003$). There was no main effect for Diet ($F < 1$) and no two-way interaction between Diet and Drug (F (1, 2) = 1.773, $p > 0.05$); however, a main effect for Drug approached significance (F (1, 2) = 4.187, $p = 0.051$). There were no differences observed between the two time points for mean ataxia score (all $F's > 1$).

In females, the aforementioned analysis was also conducted and revealed a main effect for Drug on mean locomotion score (F (1, 2) = 8.130, $p = 0.003$) but neither a main effect for Diet ($F < 1$) nor a interaction between Drug and Diet ($F < 1$). The analysis also revealed a significant difference between the locomotion score following the first and third injection (F (1, 2) = 5.189, $p = 0.032$) and pairwise comparisons confirmed that across conditions, mean locomotion score was lower following the third injection compared to those following the first (t (27) = 2.085, $p = 0.047$). To explore the main effect for Drug, a pairwise comparison was conducted and revealed that at each time point, MK females had a significantly higher mean locomotion score compared to SAL females (t (27) = -9.262, $p < 0.001$; t (27) = -12.777, $p < 0.001$). We then compared MK and SAL females across the two timepoints and found that there was no difference between MK females at the two time points (t (27) = -.403, $p > 0.05$) but that SAL females had significantly lower locomotion scores following the final injection compared to those following the first (t (27) = 5.243, $p < 0.001$) (see Figure 3). A repeated measures ANOVA revealed a difference in mean stereotypy score between the two time points (F (1, 2) = 18.246, p

< 0.001) and pairwise comparisons confirmed that this was an overall decrease in mean stereotypy score between the first and third injection (t (27) = 4.004, $p < 0.001$). There was no main effect for Diet ($F < 1$) and no two-way interaction between Drug and Diet ($F < 1$); however, a main effect for Drug approached significance (F (1, 2) = 3.211, $p = 0.086$). Finally, the analysis revealed a difference in mean ataxia score between the two time points (F (1, 2) = 6.361, $p = 0.019$) and pairwise comparison found that this was an overall decrease in mean ataxia score between the first and third injection (t (27) = 2.340, $p = 0.027$). There was no main effect for Diet ($F < 1$) and there was no two-way interaction between Drug and Diet ($F < 1$); however there was a significant main effect for Drug (F (1, 2) = 6.361, $p = 0.019$). To pursue this significant main effect, pairwise comparisons were conducted and revealed that MK females had significantly lower mean ataxia scores following the third injection compared to scores following the first (t (27) = 2.572, $p = 0.023$). All SAL females scored a 0 following both injections, so there was no significant difference between trials, and mean ataxia scores of MK females at both time points were found to be significantly higher than 0 (t (26) = -6.627, $p < 0.001$; t (26) = -7.338, $p < 0.001$).

3.4 Social Behavior

In males, independent samples t-tests revealed that in STD rats, those treated with MK-801 were significantly more likely to sleep together than those treated with saline, indicated by significantly higher sleep together ($p = 0.023$) and lower sleep apart ($p = 0.024$) scores. In DEF rats, those treated with MK-801 were also more likely to sleep together than those treated with saline ($p = 0.011$). There were no significant differences across Diet conditions in SAL or MK treated rats (all p's > 0.05).

In females, independent samples t-tests revealed that in DEF rats, those treated with MK-801 were more likely than saline-rats to sleep together ($p = 0.46$) and less likely to be awake apart from one another ($p = 0.005$), however, they were less likely awake together than were SAL females ($p = 0.019$). In SAL rats, it was found that those in the DEF condition were more likely to be awake together than were STD rats ($p = 0.019$) . There were no other significant differences observed in the analyses conducted.

4. Discussion

The purpose of this project was to assess if prenatal choline deficiency constituted a first etiological hit when followed by neurotoxic treatment with MK-801 in adulthood. Prenatal choline deficiency was hypothesized to increase vulnerability to this later treatment as indicated by more robust expression of anhedonia, elevated levels of hyperlocomotion, and reductions in social interaction. Additionally, we hypothesized that females that received MK-801 alone and both hits would exhibit more hyperlocomotion and ataxia than males receiving the same treatment and that male rats would demonstrated increased social withdrawal compared to females that receive MK-801 or both hits. Although prenatal choline deficiency was not found to consistently impair responses to MK-801 treatment across behavioral measures and throughout the injection period in line with our predictions, the patterns that emerged reveal sex differences that will inform pharmaceutical models of schizophrenia in future studies. This research contributes to the growing body of research investigating pharmaceutical models of negative symptoms observed in schizophrenia and assessing the validity of these models, in this case face validity only.

4.1 Prenatal choline deficiency enhances MK-801-induced anhedonia in males but not females

Although there has been no prior investigation of the effect of prenatal choline deficiency on anhedonia as assessed by sucrose or saccharin preference, small acute doses of MK-801 have been demonstrated to induce anhedonia in rats (Vardigan et al., 2010). Our saccharin preference tests occurred in the morning following afternoon injection, so although we assessed this at four time points over the injection period, none of these tests occurred directly after acute administration of MK-801. Thus, we expected that anhedonic behaviors would emerge later in the injection period, as a result of cumulative effects of induced NMDA hypofunction. Both DEF-MK females and males demonstrated a lack of saccharin preference during the baseline test, prior to injection. This is curious result, however, if it was purely an effect of prenatal choline deficiency, we would have expected the DEF-SAL animals to also demonstrate a reduced preference. Prenatal choline deficiency and MK-801 treatment exerted their influences equally in females during test 1, wherein STD-SAL females were the only group with a significant saccharin preference. However, only STD-MK females displayed a diminished preference in test 2, following a 5-day washout period and only 2 total injections of MK-801, and all female groups had a diminished saccharin preference in test 4. These results are inconsistent and point towards no significant effect of either the drug or diet manipulation on expression of anhedonia in females. In males, however, during test 2, following 5 total injections and a 2-day washout period, only those rats that received both hits demonstrated a diminished saccharin preference. It is possible that this effect is mediated by prenatal choline deficiency resulting in greater deficits in GABAergic interneurons in the PFC and NMDA receptor expression in males compared to females. It has been demonstrated that an α7 nACh receptor agonist reversed

sensorimotor deficits induced by a two-hit model of schizophrenia in females but not males. In addition, the female dlPFC is larger in healthy females, so it may be more resistant to biological changes induced by neurotoxic treatment. Finally, females with schizophrenia are less likely to present with negative symptoms compared to males, so this sex difference is reflected in the results of the current study. Building off of the results of Nickerson et al. (2017), where prenatal choline supplementation was demonstrated to attenuate MK-801-induced cognitive and motor deficits, in males, prenatal choline deficiency was demonstrated to increase vulnerability to sub-chronic MK-801 treatment, resulting in expression of anhedonia.

4.2.1 Motor responses following acute MK-801 administration were mediated by sex differences

Chronic or large acute doses of MK-801 have been demonstrated to induce hyperlocomotion (Matsuoka et al., 2005, 2008) and ataxia (Nickerson et al., 2017), and small acute doses of MK-801 induce hyperlocomotion (Rung et al., 2005). This was supported in the current results: MK-801-treated males and females exhibited increased locomotion, stereotypy, and ataxia compared with saline-treated rats. We did not hypothesize that there would be an effect of diet on locomotor activity and our results revealed that DEF-MK males had higher mean stereotypy scores than did STD-MK males. Prenatal choline availability did not have an effect on any other locomotor behavior in response to acute-MK-801 treatment, however, this effect may represent an increased vulnerability to this treatment in males, especially given that the acute dose administered was rather small. In accordance with our hypotheses, females administered MK-801 had significantly higher mean locomotion and mean ataxia scores than MK males, replicating results reviewed by Hill (2016). MK-801-treated females also

demonstrated a significantly higher mean stereotypy score compared to MK males, in contrast to our hypothesis. This sex difference illustrates an increased sensitivity to low-dose MK-801 treatment in females. This sensitivity was also reflected in qualitative assessments and resulted in a number of amendments to the original experimental design.

4.2.2 Motor responses varied in response to acute and sub-chronic MK-801 administration in males and females

In males, locomotor behavior was compared following the first and ninth injection of MK-801, representing an acute and sub-chronic dose of the NMDA receptor antagonist, respectively. Over the injection period, it was demonstrated that mean locomotion and stereotypy scores decreased, and thus, that mean locomotion and stereotypy scores were lower in response to sub-chronic MK-801 administration compared to acute administration. Mean ataxia scores were not significantly different between the two time point. This reflects a habituation to the induced NMDA hypofunction induced by MK-801 treatment that is not impacted by prenatal diet.

In females, locomotor behavior was compared following the first and third injection of MK-801, also representing an acute and sub-chronic dose of the NMDA receptor antagonist. Over the injection period, it was demonstrated that mean locomotion, stereotypy, and ataxia score decreased across conditions between these two time points. Interestingly, mean locomotion score was not significantly different at the two time points in rats that received MK-801 but did decrease between the two time points in those females that received saline. Ataxia was only demonstrated to decrease in the MK-801 treated females, as those that received saline scored only 0’s across the two assessments. This behavioral profile over time demonstrates that

hyperlocomotion was conserved in response to acute and sub-chronic dosing regimens of MK-801 in female rats. This is potentially explained by the study design, wherein the third dose received prior motor observation was preceded by a 5-day washout period. However, this is the only result that confirmed our hypothesis that motor responses following MK-801 injection would remain stable throughout the injection period. Hyperlocomotion in MK females was not habituated in response to repeated administration, as was observed in MK-801-treated males. These sex differences again have profound implications for pharmaceutical models of schizophrenia that utilize male and female subjects.

4.3 Social behavior

In males, across diet conditions it was demonstrated that MK-801 administration facilitated social interaction with their cage-mate, an effect that could potentially be explained by the effect of NMDA hypofunction on amygdala activity, the same mechanism through which social withdrawal is proposed to be decreased in MK-801-treated rats (Matsuoka et al., 2008). In this case, however, social interaction between cage-mates was measured, in contrast to the social interaction task. So, this dysregulated amygdala response may promote sleeping together as a compensatory behavior. In DEF-female rats, a similar pattern was observed wherein the MK-801-treated rats were more likely to sleep together and less likely to be awake apart than saline-treated females. However, DEF-MK females were less likely to be awake together than were DEF-SAL females. Again this result points to aberrant GABA-mediated neurotransmission in the amygdala (Wilson & Koenig, 2011), but it is inconsistent and requires further investigation. As we assessed social interaction between cage-mates, the results are less transparent compared to those found through the social interaction task as it has been utilized with greater frequency. This

effect could also reflect a limitation of acute MK-801 administration in inducing negative symptoms, as we only assessed social interaction following one MK-801 injection and it was not directly following injection, during the period during which the drug is still active in the animal. Some limitations of this study is that we did not expand our analyses to other social videos throughout the injection period, we did not assess social interaction between rats that were unfamiliar with one another, as well as the reduced statistical power in our analyses as we could only analyze behavior at the level of the dyad but not at the level of the individual.

5. Conclusion

The findings of the current investigation reveal that the effect of prenatal choline deficiency on behavioral changes induced following adult MK-801 toxicity. Prenatal choline deficiency enhanced MK-801-induced anhedonia in males but not females. Beyond this finding, the results of this study did not definitively reveal any effect of prenatal choline deficiency on the expression of anhedonia, social withdrawal or hyperlocomotion in response to acute or sub-chronic MK-801 treatment. Sex differences in response to MK-801 treatment were apparent in motor assessments immediately following treatment, wherein MK females had greater motor impairment overall and these changes persisted throughout the injection period. Although choline's organizational impact on the developing brain likely intersects with the glutamate hypothesis of schizophrenia, the effect of prenatal choline deficiency on later expression of negative symptoms following neurotoxic treatment is unclear.

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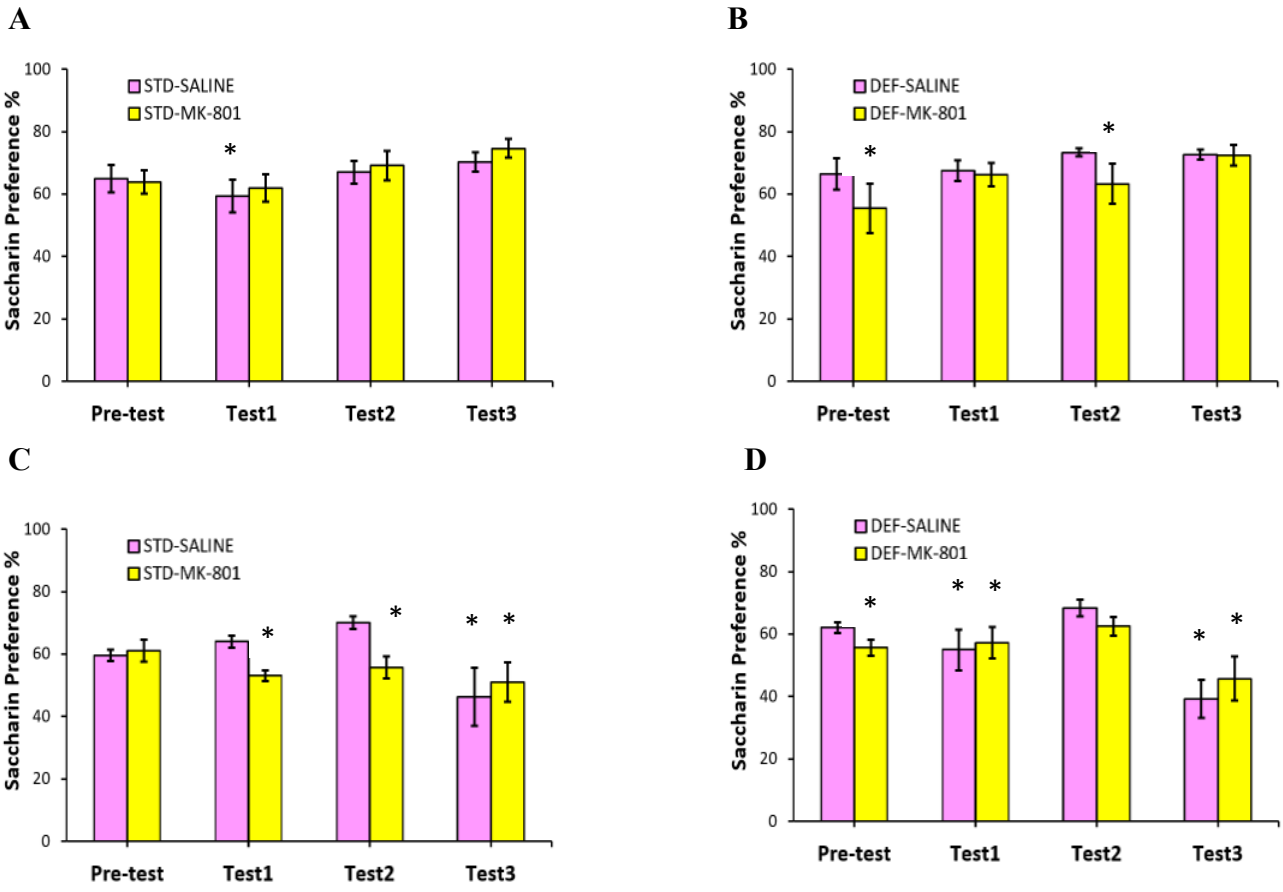
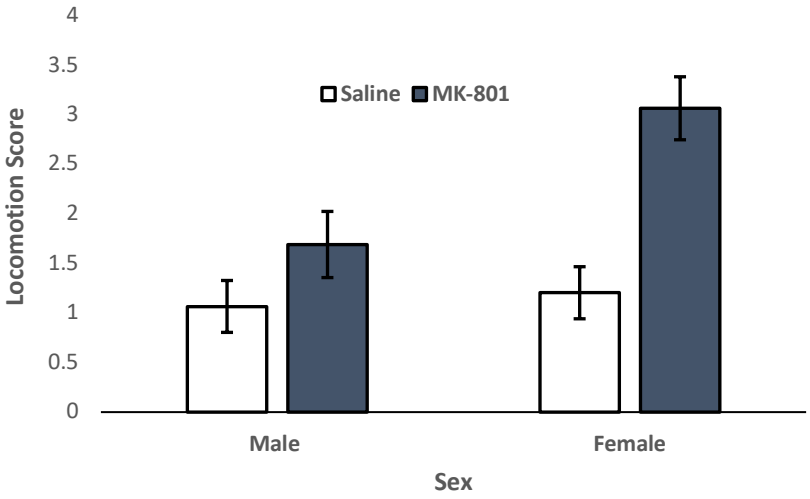


Figure 1. Saccharin preference at four time points as a function of prenatal dietary condition and low dose sub-chronic MK-801 or saline administration. Scores of standard fed males as a function of drug condition are shown in 1A while scores of deficient fed males as a function of drug condition are shown in 1B. Scores of standard fed females are shown in 1C and deficient fed females are shown in 1D. Saccharin preference scores that do not differ significantly from 50 are marked (* > 0.05).



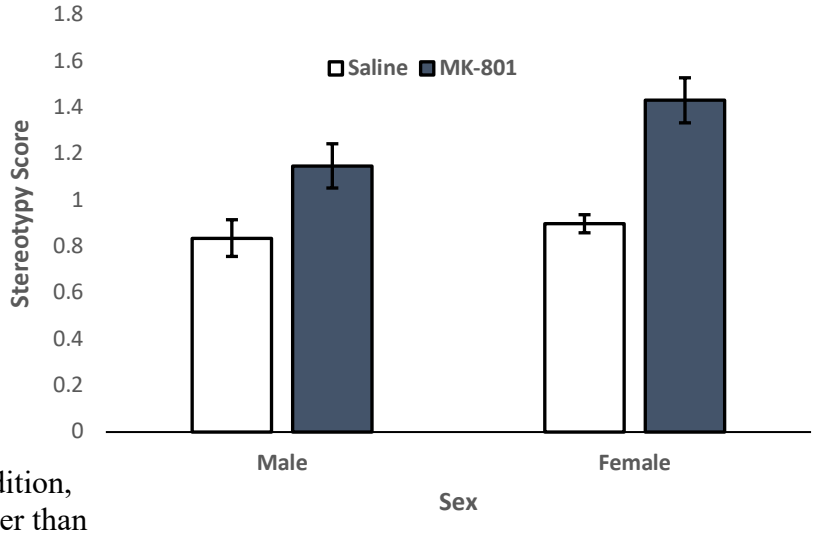
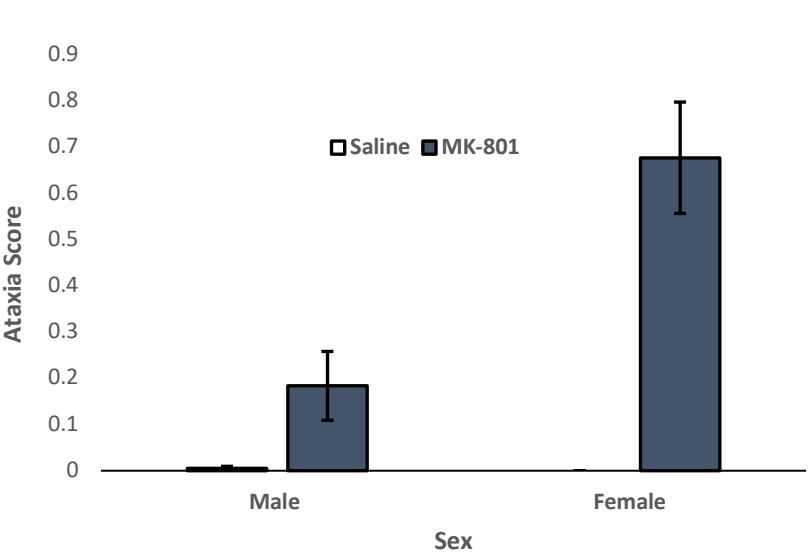
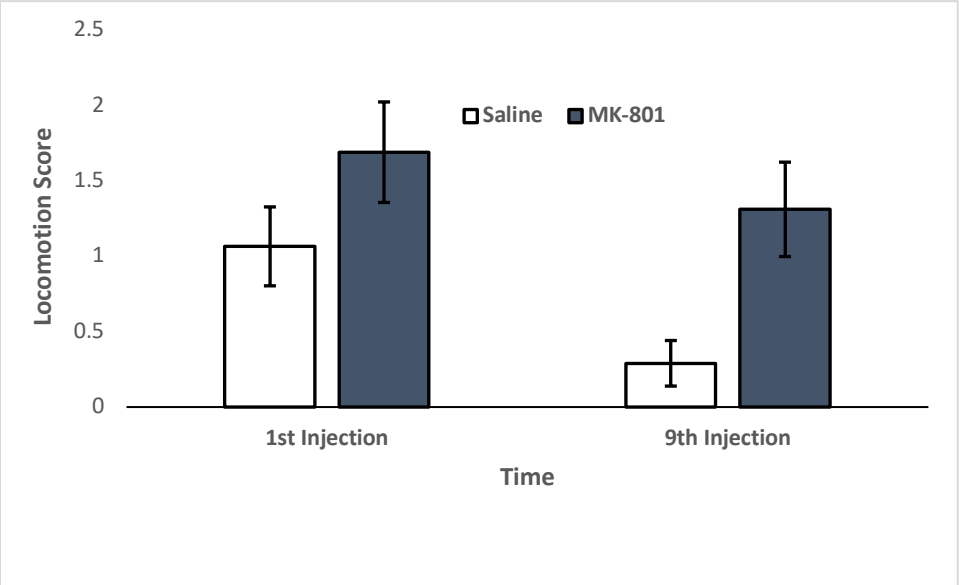


Figure 2. Motor responses following an acute dose of MK-801. Locomotor scores for males and females at each level of the drug condition are shown in 2A. Stereotypy scores for males and females at each level of the drug condition are shown in 2B. Ataxia scores for males and females at each level of the drug condition are shown in 2C. Overall, males and females administered MK-801 demonstrated higher scores on each motor scale. In addition, MK female locomotion scores were higher than MK males.



A



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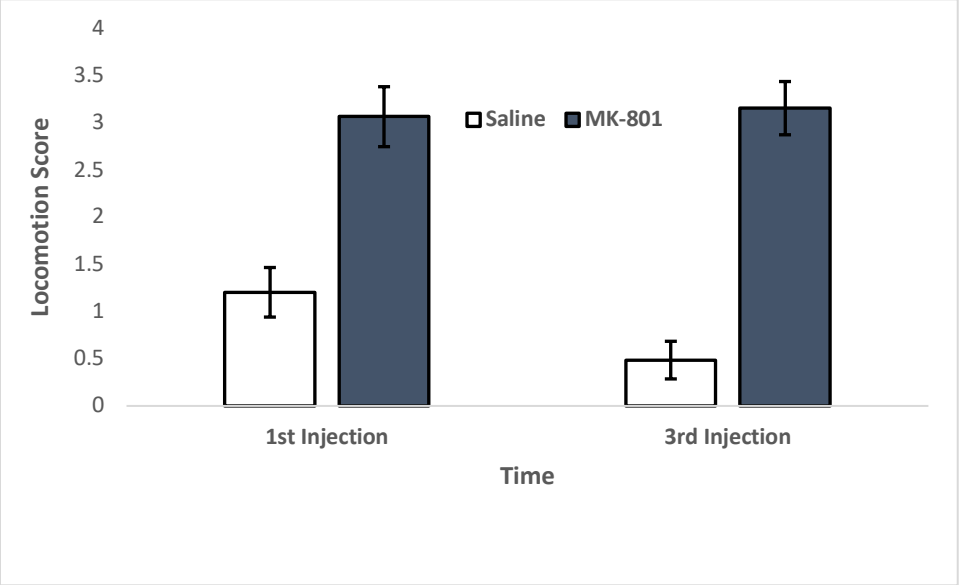

Figure 3. Mean locomotion scores compared across two time points, specifically the first and last motor observations. Mean locomotion scores following the first and ninth injection in the males at each level of the drug condition are shown in 3A. Mean locomotion scores following the first and third injection in the females at each level of the drug condition are shown in 3B. At each time point in males and females, MK-801-treated rats demonstrated higher mean locomotion scores than saline-treated rats, however, over time the females showed no reduction in this elevated average. MK males had a reduced mean locomotion score over the injection period.