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Prenatal Choline Supplementation and MK-801 Toxicity: Protecting Memory and Preventing Neurodegeneration

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Abstract

Choline is essential to the development and function of the central nervous system. Supplemental choline is neuroprotective against a variety of insults, including neurotoxins like dizocilpine (MK-801). MK-801 is an NMDA receptor antagonist that is frequently used in rodent models of psychological disorders. At low doses, it causes cognitive impairments, and at higher doses it induces motor deficits, anhedonia, and neuronal degeneration. The primary goals of the present study were to investigate whether prenatal choline supplementation protects against the cognitive impairments, motor deficits, and neuropathologies that are precipitated by MK-801 administration in adulthood. Adult male Sprague Dawley rats were fed either a standard or supplemented choline diet prenatally. Using the novelty preference test of object recognition, we examined three aspects of memory in relation to choline and MK-801: encoding, consolidation, and retrieval. Our first main finding was that choline protected memory consolidation; presently, we are not able to draw clear conclusions regarding memory encoding and retrieval, as control rats did not exhibit the expected patterns. We also observed that choline alleviated the motor response to MK-801, particularly ataxia. Using doublecortin to mark neurogenesis in the hippocampus and Fluoro-Jade B to mark degenerating axons in the anterior commissure, we found that this prenatal choline supplementation, in the face of MK-801 toxicity, protects against reduced hippocampal neurogenesis and may prevent neurodegeneration. Taken together, the current findings suggest that prenatal choline supplementation protects against a variety of behavioral and neural pathologies induced by the neurotoxin MK-801. This research contributes to the growing body of evidence supporting the robust neuroprotective capacity of choline.

Introduction

Choline is an essential nutrient crucial to the development and function of the central nervous system due to its roles in cell signaling, cell membrane structure, neurotransmission, and gene expression. The availability of choline modulates behavior and the brain, particularly in development. During development in rats, prenatal choline supplementation enhances cognitive functioning throughout adulthood via the adjustment of neuronal networks in cholinergic-rich brain areas (Meck and Williams, 2003). Supplemental choline in development, in addition to enhancing normal cognitive functioning, also protects against a diverse range of cognition-impairing neural insults. The neuroprotective capacity of choline is not well understood but may stem from its numerous functions crucial to cellular function, especially its roles in gene expression and neurotransmission. Within a larger ongoing investigation of the neuroprotective mechanisms and qualities of choline, the present study aims to elucidate the role of prenatal choline supplementation in preventing the cognitive impairments, motor deficits, and neuropathologies induced following neural insult by the neurotoxin MK-801.

Since choline is crucial to proper biological functioning, choline deficiency creates several problems that may provide insight as to how supplemental choline can exert protection. Choline is vital to cell structure because it is a component of the phosopholipid cell membrane, comprising both phosphatidylcholine and sphingomyelin. A lack of phosphatidylcholine, resulting from choline deficiency, contributes to fatty liver and general liver damage (Yao & Vance, 1988; Zeisel et al., 1991). Choline deficiency also causes muscle damage (da Costa et al., 2004) likely because of the weakened cell membrane (Zeisel, 2006). In addition to its role in cell membrane structure, choline is necessary for neurotransmission since it is the precursor to the neurotransmitter acetylcholine. Choline availability directly determines the rate of acetylcholine

synthesis and release (Blusztajn & Wurtman, 1983). Acetylcholine release in the hippocampus of 3-4 week-old rats is greater in prenatally choline-supplemented rats compared to prenatally choline-deficient rats; prenatal choline supplementation enhances cholinergic neurotransmission via increased levels of acetylcholine release (Blusztajn et al., 1998). Choline is also involved in gene expression through its metabolite *S*-adenosylmethionine. *S*-adenosylmethionine acts as a methyl group donor to epigenetically alter gene expression. This methylation of promoter regions of DNA is associated with decreased gene expression, or gene silencing. Dietary choline deficiency leads to reduced *S*-adenosylmethionine, which results in a reduction of DNA methylation and increased gene expression (reviewed in Zeisel, 2007). This function of choline is of particular interest for the current research regarding choline's protective capacity because choline's broad neuroprotective effects are likely mediated by its fundamental role in the expression of various genes. All of these processes in which choline is involved are often at risk of malfunctioning, as many people do not acquire a sufficient amount of choline.

Choline is synthesized in the body, but the amount produced is inadequate for proper biological functioning. Therefore, individuals must obtain this vitamin from foods such as eggs, beef, chicken, fish, broccoli, cauliflower, and milk (Zeisel et al., 2003). Typically, men and women require an intake of 550 and 425 mg of choline per day, respectively (Zeisel & Caudill, 2010); however, less than 10% of people consistently meet this standard (Jensen et al., 2007). Adequate choline intake is crucial throughout the lifespan, but particularly in development as this is an especially sensitive period for the brain (Zeisel, 2006). For example, pregnant women with low choline intake have a higher risk of having a child with a neural tube defect or cleft palate (Shaw et al., 2004; Shaw et al., 2006). While choline deficiency is detrimental, choline supplementation is protective in a variety of ways.

Choline supplementation is protective throughout the lifespan (Zeisel, 2006), but a greater protective capacity is exhibited when the supplementation occurs in development as opposed to in adolescence or in adulthood (Meck et al., 2008; Wong-Goodrich et al., 2008b; Glenn et al., 2012). At varying stages of the lifespan, choline has the ability to protect against several different neural insults. In several models, choline exerts neuroprotection against epilepsy (Wong-Goodrich et al., 2008a), Down syndrome (Moon et al., 2010), Rett syndrome (Nag & Berger-Sweeney et al., 2007), and fetal alcohol syndrome (Thomas et al., 2003), among other insults. One of these additional insults includes the neurotoxin MK-801 (Guo-Ross et al., 2002; 2003).

MK-801 is a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist (Wong et al., 1986) that blocks the activity of the excitatory neurotransmitter glutamate, which also binds to and activates the NMDA receptor. Because glutamate is the primary excitatory neurotransmitter in the nervous system, the disruption of its activity by MK-801 causes a variety of problems. In a rodent model, chronic or large acute doses of MK-801 induce motor deficits (Andiné et al., 1999), anhedonia (Vardigan et al., 2010), and neuronal degeneration (Guo-Ross et al., 2002; 2003), while low acute doses induce cognitive impairment in both spatial memory (Åhlander et al., 1999) and object recognition memory (de Lima et al., 2005). These are all symptoms observed in several psychological disorders for which pathology is associated with reduced excitatory neurotransmission. Consequently, MK-801 is frequently used as a tool to examine one or more of these symptoms in isolation.

MK-801 induces neurodegeneration, which is prevented by prenatal choline supplementation: Rats fed supplemental choline prenatally are protected from neurotoxicity induced by an acute dose of MK-801 in adolescence (Guo-Ross et al., 2002; 2003). However,

this finding has yet to be directly linked to a behavioral outcome. One goal of the present investigation was to discern whether prenatal choline supplementation protects against MK-801-induced neurodegeneration. To relate this finding to behavior, the present study also explored choline's protective capacity against MK-801-induced motor deficits and cognitive impairment.

Cognition is the primary behavior of interest in the current investigation. By disrupting the activity of glutamate, MK-801 is believed to disturb learning and memory functioning through the blockage of long-term potentiation (LTP; Coan et al., 1987). LTP, the persistence of synaptic strengthening between two neurons, is a widely accepted mechanism of synaptic plasticity: the changing connections between neurons thought to underlie learning and memory processes (reviewed in Lynch, 2004). The form of LTP described by Bliss and Lomo (1973) and predicted by Donald Hebb boasts the motto 'fire together, wire together,' meaning simultaneous electrical activity of both pre- and post-synaptic neurons is required for the two neurons to develop a lasting connection. In accordance with another popular phrase 'use it or lose it,' the connection degrades without this coordinated electrical activity. By preventing the activity of glutamate at the NMDA receptor, MK-801 disrupts the electrical activity mediated by the receptor, ultimately inhibiting LTP. Another NMDA receptor antagonist, AP5, inhibits LTP and also impairs spatial memory (Morris et al., 1986). This was one of the first pieces of evidence suggesting LTP is the molecular mechanism behind learning and memory. This finding also emphasizes the importance of the NMDA receptor for LTP as well as learning and memory processes. However, other NMDA antagonists block LTP without impairing spatial memory (Saucier & Cain, 1995). Nonetheless, NMDA-mediated LTP remains a widely believed mechanism underlying learning and memory. In the present study, a low acute dose of the

NMDA receptor antagonist MK-801 was used to induce learning and memory deficits possibly via the disruption of LTP or LTP-like processes.

Cognitive impairment is the primary focus of the current study for a couple of reasons. For one, adolescent choline supplementation prevented anhedonia induced by chronic exposure to MK-801, but it did not protect against the resulting cognitive deficits (Glenn et al., unpublished data). However, adolescent choline supplementation did prevent cognitive impairment in a two-hit model of schizophrenia in which rats were prenatally stressed and then administered an acute dose of MK-801 as adults (Corriveau & Glenn, 2012). It remains unclear why there was a discrepancy between the cognitive outcomes in these two studies. The length of MK-801 exposure may contribute to the difference since choline was protective against cognitive deficits induced by the acute but not the chronic dose. The chronic dose may have been too damaging for adolescent choline supplementation to adequately protect, especially since supplemental choline generally exerts greater neuroprotection when provided in development rather than in adolescence (Meck et al., 2008; Wong-Goodrich et al., 2008b; Glenn et al., 2012). In the present investigation, to eliminate the late timing of the choline diet as a possible reason for lack of cognitive protection, choline supplementation is provided earlier in development (prenatally rather than in adolescence) when it exerts greater neuroprotection (Glenn et al., 2012).

In addition to the discrepancy between the cognitive outcomes of the two studies (Glenn et al., unpublished data; Corriveau & Glenn, 2012), conclusions cannot be drawn regarding whether or not choline was protective against the stress- or the MK-801-induced component of memory impairment in the model of schizophrenia because memory tests were conducted after both the prenatal stress and MK-801 toxicity had occurred; neither component's effects were

isolated. In the present study, the factor of stress is removed to isolate the effects of MK-801, and MK-801 exposure is acute since the chronic dose appears to induce deficits beyond the scope of choline's neuroprotective capacity. More specifically, the present research investigates whether or not this supplemental choline regimen protects against cognitive impairments in memory encoding, consolidation, and/or retrieval induced by MK-801. Researchers have primarily focused on the ability of MK-801 to disrupt memory consolidation: the process of forming new memories (de Lima et al., 2005; van der Staay et al., 2011). The current study explores the impact of MK-801 on specific memory processes by investigating the encoding and retrieval (recall) of previously acquired information in addition to the memory consolidation process. These three aspects of memory are assessed using three trials of the novel object recognition test, in which only the timing of the MK-801 administration is altered among trials. A small acute dose of MK-801 is used to isolate the cognitive response and prevent induction of motor deficits (de Lima et al., 2005).

The present investigation also examined changes in motor behaviors across diet conditions following a large acute dose of MK-801. In the two-hit model of schizophrenia in which rats were prenatally stressed and treated with MK-801 in adulthood, motor responses to MK-801 were mildly attenuated by adolescent choline supplementation (Corriveau & Glenn, 2012). Now, though, there was no prenatal stress component, so the effects of MK-801 and choline diet on motor deficits were investigated in isolation. In the present study, the rats were assessed on the same three scales of motor behavior: locomotion, stereotypy, and ataxia. However, the time frame of behavioral observation was made longer because the schizophrenia model revealed slight neuroprotection by choline against MK-801-induced motor deficits within

a 30-minute period; the current observation period was expanded to 3 hours to more comprehensively characterize the progression of motor effects.

In addition to exploring the behavioral effects of adult MK-801 administration and prenatal choline diet, neural changes were also investigated. One effect of MK-801 on the brain is neurodegeneration (e.g. Olney et al., 1991), and prenatal choline supplementation protects against this neurotoxicity induced by MK-801 in adolescent rats (Guo-Ross et al., 2002; 2003). Neurodegeneration occurs in many psychological disorders involving memory deficits including Alzheimer's disease and dementia. The present study seeks evidence that the behavioral effects of MK-801 are accompanied by MK-801-induced neurodegeneration that is attenuated by choline. In addition to confirming past findings concerning neurodegeneration, the current research seeks to extend the investigation to include a marker of learning and memory: neurogenesis.

Another effect of MK-801 on the brain is altered neurogenesis. Neurogenesis, the birth of new neurons (e.g. Shors et al., 2001; reviewed in Koehl & Abrous, 2011), occurs in both the dentate gyrus of the hippocampus and the olfactory bulb of the mammalian brain (Gage, 2000). Increased neurogenesis, like LTP, is an indicator of enhanced neural plasticity: the ability of the brain to reorganize and form new connections. With enhanced neural plasticity, the brain has a greater capacity for learning and memory since this plasticity is thought to underlie these cognitive processes. Choline increases hippocampal neurogenesis (Glenn et al., 2007), but the role of MK-801 exhibits mixed results in that MK-801 both increases neurogenesis (Nacher et al., 2003) and decreases neurogenesis (Arvidsson et al., 2001). Therefore, the present research seeks to clarify this inconsistency as well as determine the interaction between MK-801 and prenatal choline diet.

The current study proposes the overarching question: Does *prenatal* choline supplementation protect against cognitive and motor deficits induced by *acute* MK-801 administration? In addition to the behavioral investigation, the present study also proposes the following questions regarding changes in the brain: Can choline prevent MK-801-induced neuronal degeneration? Is choline able to combat MK-801-induced deficits in neural plasticity, as indicated by reduced hippocampal neurogenesis?

To answer these questions, a 2x2 design was utilized in which there were two diet groups and two drug groups. Half of the rats were on a standard choline diet prenatally, and the remaining rats were on a supplemented choline diet during development. Within each diet group, half the rats received saline injections in adulthood, while the other half were treated with MK-801 in adulthood. When the rats reached adulthood, three trials of the object recognition test were conducted to investigate memory encoding, consolidation, and retrieval. The motor response to the large acute dose of MK-801 was assessed following cognitive testing, and histological analyses followed to assess neurodegeneration in the anterior commissure and neurogenesis in the hippocampus.

Prenatal choline supplementation enhances neurotransmission mediated by NMDA receptors (Montoya & Swartzwelder, 2000) as well as LTP (Pyapali et al., 1998), suggesting choline may protect against the cognitive deficits induced by the NMDA receptor antagonist MK-801. Therefore, prenatal choline supplementation is hypothesized to protect against MK-801-induced impairment in memory encoding, consolidation, and retrieval. Adolescent choline supplementation prevented ataxia, the loss of body control, in rats administered MK-801 and lacking prenatal stress in the two-hit model of schizophrenia (Corriveau & Glenn, 2012). In the current model, prenatal choline supplementation is hypothesized to prevent deficits in general

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locomotion, stereotypy, and ataxia over the 3-hour time span. Concerning the neural measures, it

is also expected that the prenatal choline supplementation will prevent increased

neurodegeneration and decreased hippocampal neurogenesis. This work will help elucidate the

discrepancies regarding cognitive and neural outcomes following choline supplementation and

MK-801 administration; furthermore, this research will contribute to the rapidly expanding

collection of evidence revealing the robust neuroprotective capacity of choline.

Materials and Methods

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., Hazleton, PA). Pregnant dams and dams

with litters were housed individually; after weaning, pups were housed in same-sex cage pairs.

All cages contained a thin layer of corncob bedding. The colony room was maintained at 20-

24°C with 10-55% humidity and was kept on a 12-hour light/12-hour dark cycle, with the lights

turned on at 08:00 daily; every procedure was conducted in the light phase of the cycle. All rats

had *ad lib* access to food and water throughout the experiment. Food and water intake were

recorded every other day throughout the experiment, and body weights were noted once a week.

All testing procedures were approved by the Colby College Institutional Animal Care and Use

Committee and performed in accordance with federal standards.

Prenatal Choline Manipulation, Cross-Fostering, and Experimental Groups

Twelve timed-pregnant female Sprague Dawley rats arrived at the lab from Charles River

Laboratories (Wilmington, MA) 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 choline diet prepared based on formulations by the American Institute of Nutrition (AIN76A; Dyets Inc., Bethlehem, PA). Half of the pregnant dams (n = 6) 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 = 6) were placed on a choline-supplemented diet (SUP; AIN76A with 5 g/kg choline chloride).

When all mothers gave birth on GD 22, pups were marked by toe-clipping to indicate the prenatal diet condition. On postnatal day (PD) 2, male and female pups from standard-fed and choline-supplemented mothers were gathered, sorted, and cross-fostered among all dams so that each litter contained 11-13 pups with a mix of males and females, both standard-fed and choline-supplemented. Also on PD 2, all dams were switched to the standard choline diet (1.1 g/kg). On PD 23, rat pups were weaned into same-sex cage litters and switched to the regular, commercially available rat chow, as described above. Rats were separated into same-sex cage pairs on PD 30.

The male offspring were the subjects of the current study (n = 40): 20 were on the standard choline diet prenatally (STD), while 20 were fed supplemental choline prenatally (SUP). Within each diet condition, half (n = 10) were randomly selected to receive MK-801 injections (MK), and the remaining half (n = 10) was selected to receive 0.9% saline (SAL). Therefore, there were four experimental groups: SUP-MK (n = 10), SUP-SAL (n = 10), STD-MK (n = 10), and STD-SAL (n = 9; one rat in the STD-SAL condition died of unknown causes prior to behavioral testing on PD 65).

Drug Administration

MK-801 (5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate) exists in two enantiomers. (-)-MK-801, as opposed to (+)-MK-801, is the less active enantiomer, binding to NMDA receptors with a slightly decreased affinity (Wong et al., 1986). The present study utilizes (-)-MK-801 because the primary goal is to investigate cognitive deficits in relation to choline. A lower dose (de Lima et al., 2005; van der Staay et al., 2011) of the less active form of MK-801 may aid in generating cognitive impairment without causing the other deficits induced by larger doses of MK-801 or by the (+)-MK-801 enantiomer with higher activity. This allows for the isolation of the cognitive deficits in the current rat model.

The (-) enantiomer of MK-801 hydrogen maleate (MK-801) was used in all behavioral tests (Sigma-Aldrich, St. Louis, MO). Testing began when rats reached adulthood (PD 82) and continued until sacrifice (PD 127 and PD 128). As described in the following sections, MK-801 was administered before the study phase of the object recognition test to assess memory encoding, after the study phase to assess memory consolidation, and before the test phase to assess memory retrieval. In each of the three object recognition test trials, the MK-801/saline dosage was 0.2 mg/kg i.p. in a volume of 1 mL/kg. This dosage was chosen because it was large enough to induce cognitive deficits in the rats without causing changes in motor behaviors (de Lima et al., 2005; van der Staay et al., 2011). A larger dose of MK-801, 3 mg/kg i.p. in a volume of 1 mL/kg, was used for the motor response test that followed cognitive assessments and just preceded sacrifice. This dose was chosen because it was large enough to induce motor deficits and neuronal degeneration in the rats, the two additional focuses of the current study (Andiné et al., 1999; Guo-Ross et al., 2002; 2003).

Novelty Preference Test of Object Recognition

The novelty preference test of object recognition was used to assess cognition in response to low-dose MK-801 administration and prenatal choline diet. This test operates on the premise that rats have a tendency to explore novel objects in a familiar environment: When presented with a novel object and a previously studied object, a typical rat will spend more time investigating the novel object relative to the studied object (e.g. Dix & Aggleton, 1999). Rats with intact memory spend more time investigating the novel object compared to the total time spent exploring both the studied and novel objects. This task was performed three times, assessing three aspects of memory: memory encoding, memory consolidation, and memory retrieval.

Apparatus and Materials

The testing apparatus was a 70 x 70 x 60 cm painted wooden box lined with corncob bedding. The arena was placed in a room that could be closed off from the larger lab space so that distracting noises were minimized. In each corner of the box, under the bedding, a jar lid was screwed to the wood, upside down, so that objects could be glued to glass jars and firmly secured to the apparatus. This allowed for rats to explore the objects without knocking any over; a suddenly fallen object is an inherently more interesting object and skews the outcome of the test. All objects used were ceramic figurines of different colors and shapes, and all were approximately 3-6 inches tall. The objects were cleaned with OdoBan® thoroughly between each trial. A video camera (Logitech HD Pro Webcam C920, Fremont, CA), placed directly above the arena, was used to record each study and test phase. Each rat's behavior was tracked with ANY-maze software (Stoelting Co., Wood Dale, IL).

Procedure

For the study phase of object recognition, each rat spent 5 minutes alone in the arena investigating two identical objects placed in opposite corners of the apparatus. There was a 3-hour retention delay between the study phase and test phase. This time frame was chosen when designing the initial trial in which MK-801 or saline were administered directly after the study phase; this allowed the test phase to occur at a time when the drug had taken effect but had since subsided (de Lima et al., 2005). This 3-hour retention delay was maintained for the following trials. After the retention delay, each rat was tested by spending 3 minutes in the arena with two objects: One from the study phase and one novel object. For each trial of the object recognition test, there were two sets of objects for a total of six objects. Objects and object positions were counterbalanced within and between conditions. The memory consolidation trial was first conducted, followed by the memory encoding and memory retrieval trials.

Habituation. Before the object recognition tests, the rats were habituated to the testing apparatus for 3 consecutive days. The apparatus contained no objects, only the jar lids and corncob bedding as described above. On the first day of habituation, rats spent 10 minutes in the arena with their cage mate. On days 2 and 3 of habituation, each rat spent 5 minutes in the arena alone.

Memory consolidation. The first object recognition test conducted was designed to explore the effects of MK-801 and choline on memory consolidation. Immediately following the study phase, rats were injected with either MK-801 or saline as described above (de Lima et al., 2005; van der Staay et al., 2011). As the rats had already studied the objects, but had yet to process them, MK-801 influenced consolidation of memories for the objects just investigated while leaving encoding and retrieval processes intact.

Memory encoding. To investigate the influence of MK-801 and choline on memory encoding, MK-801 and saline injections were performed 20 minutes prior to the study phase of the object recognition test (de Lima et al., 2005; van der Staay et al., 2011). This injection time point allowed for MK-801 to disrupt memory encoding specifically, since the rats were studying objects 20 minutes after drug treatment.

Memory retrieval. The third trial of the object recognition test was conducted to examine the influence of MK-801 and choline on memory retrieval. MK-801 and saline were administered 20 minutes before the test phase (de Lima et al., 2005; van der Staay et al., 2011), which disturbed exclusively memory retrieval since the rats had already studied and processed object memories for 2.5 hours; the rats only remaining task post-injection was to recall the studied object.

Motor Responses Following MK-801 Administration

Ten days after all object recognition tests were completed, the motor reactions of rats following a high dose of MK-801 were observed. Rats were given a single injection of either MK-801 (3 mg/kg i.p. in a volume of 1 mL/kg) or saline. As mentioned earlier, this dose was chosen because it induces motor deficits in the rats and causes neuronal degeneration (Andiné et al., 1999; Guo-Ross et al., 2002; 2003). Directly following MK-801 or saline administration, rats were placed in a clear polycarbonate cage lined with corncob bedding and covered with a metal cage top in a room closed off from the larger lab area. Their behavior was recorded via a camera connected to a computer in an adjacent room for a total of 3 hours. Following the three-hour observation period, rats were moved back to their home cages and returned to the colony rooms. The video footage was analyzed in 5-minute increments (including $t = 0$ and $t = 180$ minutes) for

a total of 37 assessments per rat. Each rat's behavior was coded for locomotion, stereotypy, and ataxia at each time point based on published scales specifically designed to assess motor effects of drugs like MK-801 (Andiné et al., 1999; Manahan-Vaughan et al., 2008; Corriveau & Glenn, 2012).

Locomotion. The rating scale of locomotion ranged from 0-5: 0 = stationary, 1 = movements within a localized area forelimbs only, 2 = intermittent movements within half of the area of the cage, 3 = continuous movement within half of the cage, 4 = intermittent movements within the whole area of the cage, and 5 = continuous movement within the whole area of the cage).

Stereotypy. The rating scale of stereotypy ranged from 0-2: 0 = no sniffing, 1 = discontinuous sniffing ('normal' sniffing), and 2 = continuous sniffing.

Ataxia. The ataxia rating scale ranged from 0-3: 0 = normal body control, 1 = falling tendency upon movement, 2 = falling upon movement, and 3 = almost unable to move.

Histology: Neurogenesis and Neurodegeneration

Rats were sacrificed three days after the MK-801 administration and motor assessments on PD 127 and PD 128. This three-day delay between MK-801 administration and sacrifice was chosen to allow MK-801 sufficient time to induce degeneration in the axons of the rat brains (Guo-Ross et al., 2003). Each rat was deeply anesthetized with isoflurane in 1.5% O_2 prior to decapitation. The brains were extracted and post-fixed in 4% paraformaldehyde in 1M phosphate buffer (PB) at 4°C for 4-8 weeks before vibratome sectioning. One hemisphere from each rat brain was sectioned through the prefrontal cortex and hippocampus. During sectioning, the vibratome well was filled with 0.2 M PB. Coronal sections of the hippocampus were taken at a

thickness of 60 μm, while frontal cortex coronal sections were sliced at a thickness of 30 μm. Both hippocampal and frontal cortical sections were stored in 0.1% sodium azide at 4°C until staining was performed, as described below.

 Neurogenesis: Doublecortin (DCX) Immunohistochemistry. Protocols for DCX immunohistochemistry were based on methods as described previously (Rao & Shetty, 2004; Glenn et al., 2007). The hippocampal tissue sections, free-floating, were rinsed in tris-buffered saline (TBS), incubated in 0.6% hydrogen peroxide for 30 minutes at room temperature, rinsed again in TBS, incubated in a 3% normal horse serum (NHS; Vector Laboratories) and 0.1% triton-X-100 (TTX) solution for 30 minutes at room temperature, and then incubated with the primary DCX antibody (1:200; Santa Cruz Biotechnology, Inc.). The sections remained in this primary antibody solution overnight on a shaker kept at 4°C. The following day, the tissue was rinsed with TBS and then incubated with the secondary antibody (biotinylated horse anti-goat immunoglobulin G; 1:200; Vector Laboratories) for one hour at room temperature. After the incubation, the sections were rinsed in TBS and transferred to an avidin-biotin complex (ABC; Vector Laboratories) for one hour at room temperature. The tissue was rinsed in TBS and vector grey was used to visualize the stained neurons (Vector Laboratories). Following a final rinse in TBS, tissue was mounted on 1% gelatin-coated slides and counterstained using methyl green. For this counterstain, the tissue was submerged sequentially into the following solutions: dH_2O (1 minute), methyl green (5 minutes), dH_2O (30 seconds), 95% ethanol (EtOH; 10 dips), 100% EtOH (10 dips), an additional solution of 100% EtOH (10 dips), and xylene (30 seconds). The slides were then left submerged in a second xylene dish while cover slips were applied to the slides using permount.

Neurodegeneration: Fluoro-Jade B Stain. The frontal cortex sections were first rinsed in 0.2 M phosphate buffer and then mounted onto 2% gelatin-coated slides. Directly after the tissue was set on the slides, the slides were placed on a slide warmer at 50°C to dry for at least 30 minutes. Slides were then immersed in the following solutions, in sequence: 1% NaOH in 80% EtOH (2 minutes), 70% EtOH (2 minutes), dH_2O (2 minutes), 0.06% $KMnO_4$ (10 minutes with agitation), dH_2O (2 minutes), 0.0001% Fluoro-Jade B in 0.1% acetic acid (20 minutes with agitation), dH_2O (1 minute), dH_2O (1 minute), and dH_2O (1 minute). After staining, the slides were again placed on the slide warmer at 50°C for 5-10 minutes or until they were completely dried. All slides were then immersed in xylene for one minute before being moved to another dish of xylene. Slides remained submerged in xylene until coverslipped with DPX (a mixture of distyrene, a plasticizer, and xylene).

Quantification of Neurogenesis and Neurodegeneration

Neurogenesis: DCX-labeled cells using unbiased stereology. DCX-labeled neuronal cell bodies in the dentate gyrus of the hippocampus were systematically counted using the StereoInvestigator computer program (Microbrightfield Inc., Williston, VT) and optical fractionator method (West, 1993, 1999). Hippocampal sections chosen for DCX quantification had the highest consistency of staining, were the most intact sections post-staining, and represented the correct structural location of the hippocampus: the dorsal and ventral blades of the dentate gyrus. The program selected approximately 15-30 sites within a 100x100μm frame of the dentate gyrus to count DCX-labeled cells for a total of 75-150 sites. These methods eliminated bias when counting labeled cells.

Neurodegeneration: Fluoro-Jade B quantification using optical density. For each brain, three coronal sections of the anterior commissure were quantified. Images of the three anterior commissure sections per brain were captured with the 20X objective using DVCView 3.5. The images were then analyzed using the ImageJ computer program to quantify optical density of the Fluoro-Jade B fluorescence. Each image was adjusted to 8-bit, and the threshold was adjusted so that the highlighted regions represented the fluorescence in the image. A 670 x 470 pixel oval was created around a representative area of the image. Areas with background interference and air bubbles were avoided when possible. ImageJ then analyzed the particles, and the optical densities were recorded.

Statistical Analyses

Means and standard errors of the means, displayed in the figures, were calculated for all behavioral and histological results including the object recognition tests, the motor response test, neurogenesis, and neurodegeneration. For all three object recognition tests, the time spent investigating the novel object was divided by the total time spent exploring both the novel and studied objects in the test phase to calculate the exploration ratio of novelty preference. Values close to 0.5 indicated that the rats spent equal time investigating both objects, an indication of chance exploration. Values closer to 1.0 demonstrated that rats spent more time investigating the novel object than the studied object, inferring memory for the studied object.

Two-way analyses of variance (ANOVAs) comparing between subject factors of Diet (STD and SUP) and Drug (SAL and MK) were conducted for all three object recognition tests to compare the exploration ratios of the four groups. One-sample t-tests were conducted comparing the mean exploration ratio of each group to the no preference value of 0.5 to validate the object

recognition task as a test of memory. Values significantly higher than the no preference value of 0.5 were taken to indicate memory for the studied object. Rats that did not spend any time investigating either object were removed from the analysis.

For both locomotion and stereotypy behaviors of the motor response test, a three-way ANOVA was conducted comparing the effects of Diet, Drug, and Time on the motor behavior score. For ataxia, a two-way ANOVA was conducted comparing the effects of Diet and Time on average ataxia score; the drug component was eliminated from statistical analysis because only rats given MK-801 exhibited ataxia. For ataxia and stereotypy, average scores were collapsed over two or three time points, respectively, to elucidate patterns over time. Additional two- and three-way ANOVAs, respectively, were conducted on the time-collapsed ataxia and stereotypy scores.

For histology data of neurodegeneration and neurogenesis, two-way ANOVAs of between subject factors Diet and Drug were conducted to compare both average optical density of fluorescence and average number of DCX-labeled cells among the four groups. For all analyses, planned or post hoc t-tests were conducted as appropriate. The significance level was 0.05 for all tests unless otherwise stated.

<div style="text-align:center">

Results

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Novelty Preference Test of Object Recognition

Memory consolidation. Figure 1 shows the mean novelty preference exploration ratios calculated over the full three-minute test phase and evaluated across prenatal choline diet and adult drug conditions. A 2x2 ANOVA revealed a main effect of prenatal choline diet on the exploration ratios ($F_{(1, 34)} = 5.789$, $p = 0.022$): Overall, SUP rats had higher exploration ratios

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than STD rats. However, there was not a significant main effect of adult drug administration (F (1, 34) = 2.879, $p > 0.05$) nor was there a significant interaction between Diet and Drug ($F < 1$).

To confirm that STD-SAL rats exhibited novelty preference and to pursue the hypothesis that prenatal choline supplementation protects memory consolidation against adult MK-801 toxicity, one-sample t-tests of each of the four groups were performed comparing the mean novelty preference ratios to 0.5. STD-SAL rats displayed a significant preference for the novel object (t (8) = 4.350, $p = 0.002$), and STD-MK rats did not (t (9) = 0.988, $p > 0.05$). The SUP-SAL rats also exhibited a significant preference for the novel object (t (8) = 4.062, $p = 0.004$); unlike the STD-MK rats, the SUP-MK rats displayed significant novelty preference (t (9) = 4.041, $p = 0.003$).

Memory encoding. Figure 2 shows the mean novelty preference exploration ratios calculated over the full three-minute test phase and evaluated across prenatal choline diet and adult drug conditions. A 2x2 ANOVA revealed no significant main effects of either Drug or Diet, nor was there a significant interaction between Drug and Diet ($F < 1$). To assess each group's memory, one-sample t-tests were performed to compare the mean exploration ratios to the 0.5 no preference line. None of the four groups were significantly different than chance exploration (p's > 0.05).

Figure 3 shows the mean novelty preference exploration ratios calculated over the final third minute of the test phase. Because the SAL rats did not exhibit novelty preference over the full three-minute test phase, an examination of the final third minute in isolation was conducted. The final minute of the test phase was analyzed because there may have been decreased exploration in the first two minutes of the test phase; returning to the apparatus for the test phase possibly reminded the rats of the injection they had received 20 minutes prior to the study phase,

so they may have been slow to adjust to the arena and thus may have explored less overall during the first two minutes. A 2x2 ANOVA on the ratios for the final minute of the test phase revealed no significant main effect of either Diet or Drug, nor was there a significant interaction between Diet and Drug ($F < 1$). Using a one-sample t-test comparing the ratios to 0.5 again revealed that none of the four groups were significantly different from chance exploration ($p > 0.05$).

Memory retrieval. Figure 4 shows the mean novelty preference exploration ratios calculated over the full three-minute test phase and evaluated across prenatal choline diet and adult drug conditions. A 2x2 ANOVA revealed no significant main effects of either Diet or Drug, nor was there a significant interaction between Diet and Drug ($F < 1$).

One-sample t-tests revealed that while none of the four groups were significantly different from chance exploration ($p > 0.05$), the average mean for each group followed an encouraging trend: STD-SAL rats had a higher mean novelty preference ratio compared to STD-MK rats, whereas SUP-SAL and SUP-MK rats exhibited mean ratios closer to the STD-SAL controls.

Figure 5 shows the mean novelty preference exploration ratios calculated over the final third minute of the test phase. As in the test of memory encoding, the SAL rats did not exhibit significant novelty preference over the full three-minute test phase, so the final third minute of the test phase was isolated for analysis. The final minute was chosen because the injection administered 20 minutes before the test phase was likely disrupting overall object exploration in the first two minutes of the test. A 2x2 ANOVA revealed no main effect of either Diet or Drug, nor was there a significant interaction between Diet and Drug ($F < 1$). A one-sample t-test comparing the mean ratios to 0.5 once again showed that none of the four groups had mean ratios significantly different from chance exploration ($p > 0.05$). While there was no significant novelty

preference in any of the four groups, average novelty preference ratios gave an indication of an interesting trend in the third minute of the test phase. As can be seen in Figure 5, SAL rats had higher mean ratios compared to MK rats: The ratios of both STD-SAL and SUP-SAL rats were higher than both STD-MK and SUP-MK rats.

Motor Responses Following MK-801 Administration

 Locomotion. Figure 6 shows mean locomotion scores over three hours following injection of either saline or MK-801. A 2x2x37 ANOVA revealed main effects of both Time (F (36, 1260) = 9.258, $p < 0.001$) and Drug (F (1, 35) = 19.797, $p < 0.001$) on locomotion score, but no main effect of Diet (F (1, 35) = 2.239, $p > 0.05$). There was neither a significant interaction between Time and Diet ($F < 1$) nor a three-way interaction among Time, Diet, and Drug ($F < 1$); however, there was a significant interaction between Time and Drug (F (36, 1260) = 1.920, $p = 0.001$) as well as between Diet and Drug (F (1, 35) = 5.255, $p = 0.028$).

 To pursue the significant Diet by Drug interaction, independent sample t-tests were conducted first comparing SAL and MK rats within the STD group, and then comparing SAL and MK rats within the SUP group. STD-MK rats had a significantly higher mean locomotion score compared with STD-SAL rats (t (17) = 4.086, $p = 0.001$). SUP-MK and SUP-SAL rats did not have significantly different locomotion scores (t (18) = 1.858, $p > 0.05$).

 Stereotypy. Figure 7 shows mean stereotypy scores over three hours following injection of either saline or MK-801. A 2x2x37 ANOVA revealed that while there was no main effect of Diet on mean stereotypy score ($F < 1$), there were both main effects of Drug (F (1, 35) = 29.898, $p < 0.001$) and Time (F (36, 1260) = 16.132, $p < 0.001$). There was no three-way interaction among Time, Diet, and Drug (F (36, 1260) = 1.281, $p > 0.05$). There was, however, a significant

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interaction between Time and Drug ($F_{(36, 1260)} = 2.524$, $p < 0.001$), and both the interactions between Diet and Drug ($F_{(1, 35)} = 3.894$, $p = 0.056$) and Time and Diet ($F_{(36, 1260)} = 1.331$, $p = 0.092$) approached significance.

Figure 8 shows mean stereotypy scores over the three-hour period, where the original 37 time points were collapsed intro three: the first, second, and final hours of the three-hour time period. A 2x2x3 ANOVA revealed the same main effects as previously: There was no main effect of Diet ($F < 1$), but there were both main effects of Drug ($F_{(1, 35)} = 30.104$, $p < 0.001$) and Time ($F_{(2, 70)} = 78.280$, $p < 0.001$). Again, there was no three-way interaction among Time, Diet, and Drug ($F < 1$). However, compared to the 2x2x37 ANOVA analysis, there was no Time by Drug interaction ($F_{(2, 70)} = 2.123$, $p > 0.05$), there was a significant Time by Diet interaction ($F_{(2, 70)} = 3.170$, $p = 0.048$), and the interaction between Diet and Drug again approached significance ($F_{(1, 35)} = 3.936$, $p = 0.055$).

Ataxia. Figure 9 shows the mean ataxia scores over three hours following injection of either saline or MK-801. To assess ataxia in response to the acute, high dose of MK-801, a 2x37 ANOVA was conducted comparing Diet and Time; the drug condition was not analyzed because none of the saline rats exhibited ataxia during the three hours following injection. There were both main effects of Time ($F_{(36, 648)} = 23.125$, $p < 0.001$) and Diet ($F_{(1, 18)} = 4.998$, $p = 0.038$), as well as a significant interaction between Time and Diet ($F_{(36, 648)} = 1.824$, $p = 0.003$).

Figure 10 shows mean ataxia scores collapsed over the first and second halves of the three-hour observation period to elucidate the Time by Diet interaction. When split in this fashion, the significant effects of both Time ($F_{(1, 18)} = 36.803$, $p < 0.001$) and Diet ($F_{(1, 18)} = $

5.011, $p = 0.038$) remained, but there was no longer a significant interaction between Time and Diet ($F (1, 18) = 2.305, p > 0.05$).

Quantification of Neurogenesis and Neurodegeneration

     Neurogenesis: DCX-labeled cells using unbiased stereology. Figure 11 shows the mean number of DCX-labeled neurons per 10^4 μm^2 area of the dentate gyrus calculated to quantify neurogenesis in the dentate gyrus of the hippocampus. A 2x2 ANOVA revealed no significant main effects of either Diet or Drug, nor was there a significant interaction between Diet and Drug ($F < 1$). However, a planned comparison utilizing an independent sample one-tailed t-test revealed that STD-MK rats had a significantly lower number of DCX-positive neurons compared with SUP-MK rats ($t (12) = -2.033, p = 0.033$).

     Neurodegeneration: Fluoro-Jade B quantification using optical density. Figure 12 shows the mean fluorescent optical densities, marked by Fluoro-Jade B staining, calculated to quantify neurodegeneration in the anterior commissure. A 2x2 ANOVA revealed no significant main effects of either Diet or Drug, nor was there a significant interaction between Diet and Drug ($F < 1$). However, although not statistically significant, an encouraging trend was evident in the mean optical densities. As seen in Figure 12, the highest optical density was exhibited in the STD-MK rats, while lower densities of similar values were observed in STD-SAL, SUP-SAL, and SUP-MK rats.

Discussion

     The present research sought to investigate the neuroprotective capacity of developmental choline supplementation against adult MK-801 toxicity. Prenatal choline supplementation was

hypothesized to protect against cognitive impairments, motor deficits, neurodegeneration, and reduced hippocampal neurogenesis in the rat model. Behaviorally, this developmental supplementation of choline clearly protected the memory consolidation process against an acute low dose of MK-801 as measured by the novelty preference object recognition test. In addition, choline prevented locomotor deficits, most evidently in ataxia and general locomotion, induced by an acute high dose of MK-801. The influence of diet and drug on memory encoding and consolidation, however, was not obvious as the control rats (saline-treated) did not exhibit memory in the novelty preference task. Histologically, as reflected in the significant difference in the number of DCX-positive neurons between MK-801-treated diet groups, prenatal choline supplementation significantly prevented reductions in hippocampal neurogenesis caused by MK-801 administration. In the anterior commissure, there was a trend that reflects choline's neuroprotection against MK-801-induced neurodegeneration, although differences in optical density of fluorescently-labeled neurodegeneration among experimental groups were not significant. These findings indicate that developmental choline supplementation is neuroprotective against adult MK-801 toxicity both behaviorally and within the brain. This research helps elucidate discrepancies regarding cognitive and neural outcomes in past studies and also contributes to the vast body of evidence supporting the neuroprotective capacity of choline.

Memory Consolidation

Because MK-801 disrupts memory consolidation in rats (de Lima et al., 2005; van der Staay et al., 2011), it was hypothesized that MK-801 would also impair memory consolidation in the current rat model. To combat this impairment, prenatal choline supplementation was

expected to protect memory consolidation since cognition was protected in a rat model of schizophrenia in which rats were prenatally stressed, supplemented with choline in adolescence, and given the MK-801 insult as adults (Corriveau & Glenn, 2012). In this model of schizophrenia, no conclusion can be drawn regarding whether the choline protected memory deficits induced by prenatal stress, the MK-801 insult, or a combination of the two.

In the current study, prenatal choline supplementation clearly protected memory consolidation in response to MK-801 toxicity, indicated by significant preference for a novel object in all groups except the STD-MK rats. This supports the protection gained from adolescent choline supplementation observed in the model of schizophrenia (Corriveau & Glenn, 2012). However, this current finding conflicts with another previous study in which adolescent choline supplementation protected against MK-801-induced anhedonia, but not cognitive deficits (Glenn et al., unpublished data). This discrepancy likely arises from the difference in timing of the choline supplementation. The present study utilizes prenatal, rather than adolescent, choline supplementation which generally exerts greater neuroprotection (Meck et al., 2008; Wong-Goodrich et al., 2008b; Glenn et al., 2012). The enhanced neuroprotection from the developmental choline supplementation may have been responsible for preventing the deficits in memory consolidation. This result from the present study also contradicts findings in mice, where MK-801 was shown to facilitate, rather than impair, memory consolidation (Nilsson et al., 2007). Although both are rodents, mice and rats are two unique animal models that do not always demonstrate identical outcomes. This discrepancy in memory consolidation is likely due to the species difference; as rats are more commonly used to model cognitive behaviors and are thought to be better models of humans in this respect, the current hypotheses and discussion regarding cognition are based on findings in rat models.

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Motor Responses

Chronic or large acute doses of MK-801 cause motor deficits in rats (Andiné et al., 1999). This was undoubtedly supported in the current results: MK-801-treated rats exhibited increased ataxia, locomotion, and stereotypy compared with saline-treated rats.

Ataxia. In the rat model of schizophrenia in which rats were prenatally stressed, supplemented with choline in adolescence, and given MK-801 in adulthood, choline-supplemented rats were most protected from the MK-801-induced ataxia observed in the standard-fed rats (Corriveau & Glenn, 2012). Choline was expected to prevent MK-801-induced ataxia in the current model as well, especially since the supplementation occurred in development when choline exerts greater neuroprotection (Meck et al., 2008; Wong-Goodrich et al., 2008b; Glenn et al., 2012). Over the three-hour observation period in the current study, there was a significant interaction between Time and Diet. When the five-minute time points over the three-hour span were collapsed into just two time points (the first and second halves of the three-hour observation period), the interaction disappeared but main effects of both Time and Diet remained. Therefore, when analyzed in this manner, the level of ataxia clearly declined over time, as expected knowing that the motor effects of MK-801 fade with time. In addition to this effect, the SUP rats exhibited reduced ataxia compared to the STD rats, in accordance with the initial hypothesis that prenatal choline supplementation would prevent MK-801-induced ataxia.

Locomotion. Since there were hints that adolescent choline supplementation was neuroprotective against general locomotion impairments in the rat model of schizophrenia (Corriveau & Glenn, 2012), prenatal choline supplementation was hypothesized to prevent these locomotion deficits caused by MK-801. In the current study, the MK-801 treatment in both diet groups caused increased locomotion compared to the saline-treated groups, and activity levels

decreased over time in all groups. In agreement with the hypothesis, STD-MK rats exhibited significantly higher locomotion compared to STD-SAL rats, while both SUP-SAL and SUP-MK animals demonstrated levels of locomotion that were not significantly different. Therefore, the prenatal choline supplementation prevented MK-801-induced increases in locomotion as expected.

   Stereotypy. Again, because there were hints that adolescent choline supplementation protected against stereotypy in the rat model of schizophrenia (Corriveau & Glenn, 2012), prenatal choline supplementation was expected to prevent stereotypy following the high dose of MK-801. When stereotypy scores were analyzed at every five-minute time point over the three-hour observation period, there was a significant interaction between Time and Drug. To clarify this interaction, the data were collapsed over three time points: hours one, two, and three. This analysis revealed a different interaction than previously discussed, an interaction between Time and Diet. While there were signs of neuroprotection against MK-801-induced stereotypy at certain time points within the three hours, the trend across the full three hours remains unclear. In the future, a modification of the stereotypy rating scale may clarify these results: While 0 indicated no sniffing and 2 indicated pathological stereotypy, a 1 indicated normal sniffing behavior. Noting normal sniffing behavior appeared a distracting measure from the true MK-801-induced behavior of interest. In the future, this scale could be changed to mark either presence or absence of head-weaving (stereotypy) behavior. This may eliminate noise in the data to uncover a clearer trend over time.

Memory Encoding and Retrieval

Based on findings showing MK-801-induced disruption of memory encoding and consolidation (de Lima et al., 2005), MK-801 was hypothesized to impair all three memory phases investigated in this study: encoding, consolidation, and retrieval. Although, in mice, MK-801 impaired only memory encoding and actually enhanced memory retrieval, this same model also demonstrated enhanced memory consolidation following MK-801 treatment (Nilsson et al., 2007); this finding regarding memory consolidation is inconsistent with studies conducted with rats (de Lima et al., 2005) as well as with the results of the current investigation. The current hypotheses are therefore based on rat models, once again because of the discrepancies between rats and mice. In the rat model of schizophrenia, because adolescent choline supplementation had protected against deficits in cognition (Corriveau & Glenn, 2012), prenatal choline supplementation was expected to prevent MK-801-induced deficits in memory encoding and retrieval. In addition to this behavioral reasoning, prenatal choline supplementation augments NMDA receptor-mediated neurotransmission (Montoya & Swartzwelder, 2000) as well as long-term potentiation (Pyapali et al., 1998); this suggests that choline may protect against a wide range of cognitive impairments via its actions at the cellular level.

Unfortunately, control rats in the present investigation (saline-treated rats) did not exhibit novelty preference in the object recognition task over the full three-minute test phase in either the memory encoding or retrieval trials. In both the encoding and retrieval trials, the injection of either MK-801 or saline was 20 minutes prior to either the study phase (encoding) or test phase (retrieval). The proximity of the injection to the task likely hindered the overall level of exploration in both of these trials. In the case of encoding, the rats were distracted from the initial studying of the objects, which may have rendered the test phase irrelevant. In the retrieval

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trial of object recognition, the injection disrupted rats from performing the test phase. Since the injection likely interfered with the initial minutes of the test phase in the retrieval trial, the final third minute of the test phase was isolated to calculate novelty preference ratios; this was used to determine if the control rats exhibited novelty preference by the end of the test when the animals had presumably recovered from the injection. This analysis was also conducted for the memory encoding trial in the case that the rats were slow to adapt to the arena during the test phase, although the last minute of the test phase was unlikely to reveal novelty preference in the control rats since the study phase itself was disturbed by the injection.

When the analysis of the final third minute of the test phase in the object recognition task for memory retrieval was conducted, the saline-treated controls rats exhibited a trend more closely resembling novelty preference compared to the full three-minute analysis; however, the novelty preference ratios for control animals were still not significantly above the no preference value of 0.5. While this analysis revealed only a trend, it is also interesting to note that MK-801-treated rats had an overall lower novelty preference ratio, within the no preference range, compared with saline-treated rats across both diet conditions. Future investigations with altered injection timings or procedures to habituate the rats to the injection may reveal that choline is able to protect memory consolidation, but not memory retrieval.

The same analysis of the final third minute of the test phase for the memory encoding trial did not uncover either a significant novelty preference or even a trend toward this preference in control rats. This is consistent with the theory that the injection disrupted the study phase in the encoding trial. Isolating the last minute of the test phase for analysis was not effective in revealing novelty preference in the controls likely because the study phase itself was disturbed, so the entire test phase was also disrupted because of the lack of investigation in the study phase.

Neurogenesis

MK-801 has been shown to both increase (Nacher et al., 2003) and decrease neurogenesis (Arvidsson et al., 2001). Since hippocampal neurogenesis is a marker of neural plasticity, a process thought to underlie learning and memory processes, MK-801 was hypothesized to reduce neurogenesis in the hippocampus to match the hypothesized MK-801-induced memory deficits. In the standard-fed animals, there was no significant difference in the number of new neurons between the saline- and MK-801-treated rats. Although the difference was not significant, the average number of new neurons for the standard-fed group was lower in MK-801-treated rats compared to the saline-treated rats. This suggests that MK-801 acts to reduce hippocampal neurogenesis, but the current data cannot conclusively clarify the discrepancy between claims of both increased and decreased neurogenesis following MK-801 administration.

Choline increases hippocampal neurogenesis (Glenn et al., 2007), so the prenatal choline supplementation of the current study was expected to enhance neurogenesis to combat reductions caused by MK-801. In accordance with this hypothesis, the current investigation revealed that SUP-MK rats had a significantly higher number of new neurons compared to the STD-MK animals. This difference in hippocampal neurogenesis between the two diet groups may be responsible for differences in cognitive behaviors, namely the protected memory consolidation observed in the SUP-MK rats.

Neurodegeneration

Prenatal choline supplementation in the present study was expected to prevent MK-801-induced neurodegeneration in the prefrontal cortex of adult rats as was demonstrated in past research with adolescent rats (Guo-Ross et al., 2002; 2003). While examining neurodegeneration

in the prefrontal cortex during the present investigation, substantial fluorescence was observed in the anterior commissure region of MK-801 treated animals. This observation was consistent with findings in mice in which increased degeneration was observed in the anterior commissure of both aged mice and the genetic mouse model of Alzheimer's disease (Chen et al., 2011). Therefore, as this region appears to be implicated in conditions of impaired memory, neurodegeneration in the anterior commissure, rather than in the prefrontal cortex, was analyzed in the current study.

While there is a neuroprotective trend of prenatal choline supplementation against neurodegeneration caused by MK-801 in adulthood, the differences in fluorescence among the groups were not significant. However, these data were gathered using the computer program ImageJ, which quantified fluorescence by optical density. This was a very rough initial measurement to gauge general patterns of neurodegeneration among experimental groups. A significant effect of prenatal choline supplementation against the MK-801-induced neurodegeneration may be revealed in the future by using more exact methods of quantification such as the unbiased stereology technique used to quantify neurogenesis.

Conclusion

The findings of the current investigation reveal that prenatal choline supplementation is protective against a variety of behavioral and neural changes induced following adult MK-801 toxicity. Regarding behavior, this choline supplementation protected memory consolidation as well as prevented motor deficits especially in locomotion and ataxia. Within the brain, developmental choline supplementation protected against reductions in hippocampal

neurogenesis and may have prevented neurodegeneration in the anterior commissure as well, although this compelling trend was not significant.

Future investigations should include another examination of memory encoding and retrieval, to fit these pieces with the current result concerning memory consolidation. The injections for these tests must occur close enough to the study or test phases so that they disrupt either encoding or retrieval, respectively, but they also need to occur longer than 20 minutes prior to the test so that the injections themselves do not disturb object exploration. Alternatively, an additional habituation period within the object recognition test could be implemented so as to familiarize the rats to injections and eliminate the problem with timing. As mentioned above, alterations of the stereotypy rating scale may clarify the effects of diet and drug over time and generate a better understanding of overall motor behaviors following MK-801 toxicity. In addition, a more accurate method of quantification should be applied to the fluorescent-tagged neurodegeneration in the anterior commissure; perhaps this would reveal a significant effect of diet on neurodegeneration. As well as modifying the methods of the current investigation, future research could add the measure of anhedonia to compare with the adolescent choline supplementation investigation in which choline prevented anhedonia, but not cognitive deficits, followed by MK-801 administration (Glenn et al., unpublished data). In addition, while this project isolated the adult MK-801 toxicity component of the schizophrenia model (Corriveau & Glenn, 2012), a next step could be to explore these same measures of behavior and the brain following prenatal stress.

Taken together, these results contribute to the growing evidence in support of the broad neuroprotective capacity of choline. These findings also help elucidate discrepancies regarding cognitive and neural outcomes from previous work. This investigation and other research

exploring the protective ability of choline demonstrate that a mere modification of diet may

prove a viable, safer alternative to drug treatment in the face of behavioral impairments and

neural pathologies observed in numerous psychological disorders.
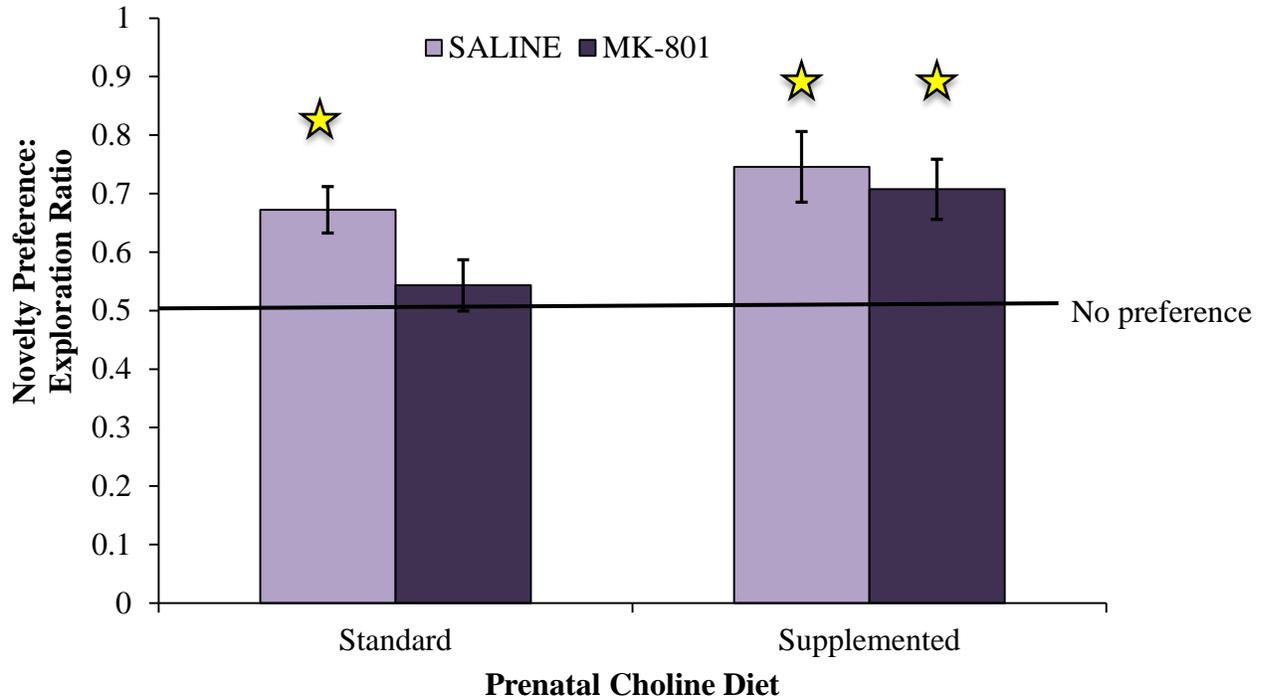
Figure 1. Mean novelty preference exploration ratios as a function of prenatal diet condition and adult drug administration as a test of memory consolidation. The exploration ratio is defined as the time spent investigating the novel object divided by the total time spent investigating both the novel and studied objects throughout the 3-minute test phase. Stars indicate significance ($p < 0.05$, one-sample t-test) from the no preference line (exploration ratio of 0.5; no preference for the novel nor studied object). Error bars represent ±1SE.
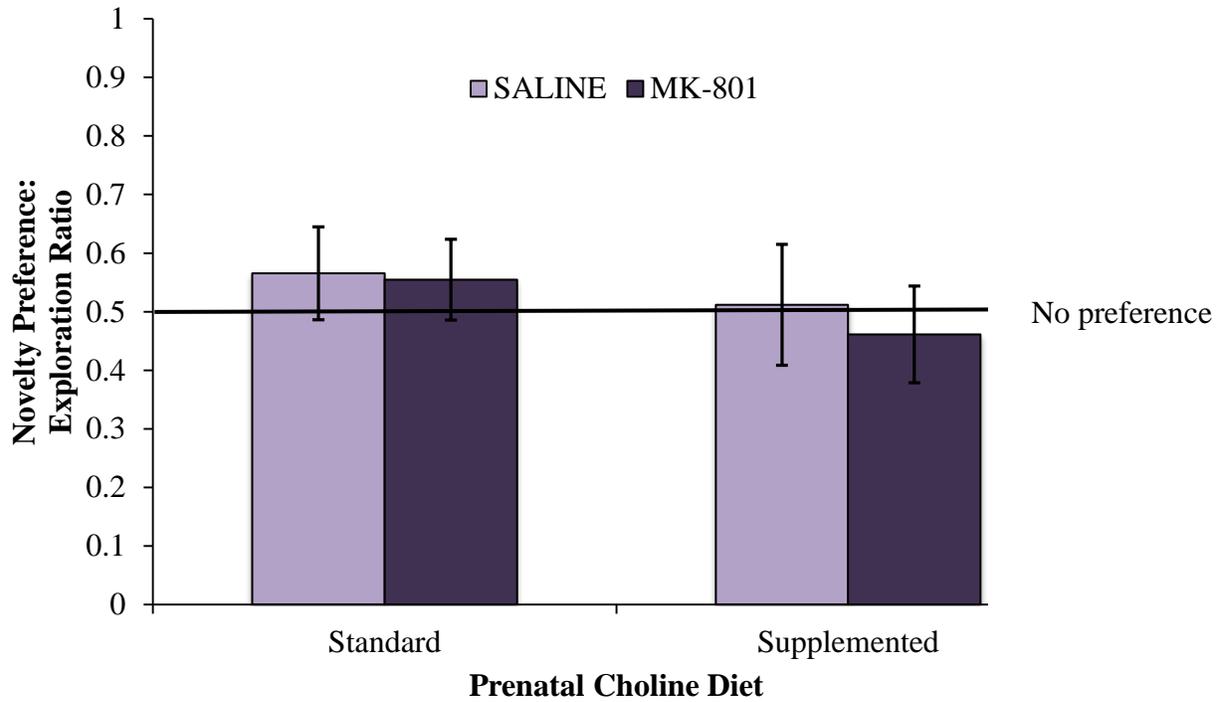
Figure 2. Mean novelty preference exploration ratios as a function of prenatal diet condition and adult drug administration as a test of memory encoding. The exploration ratio is defined as the time spent investigating the novel object divided by the total time spent investigating both the novel and studied objects throughout the 3-minute test phase. The no preference line indicates an exploration ratio of 0.5, meaning no preference for the novel or studied object. Error bars represent ±1SE.
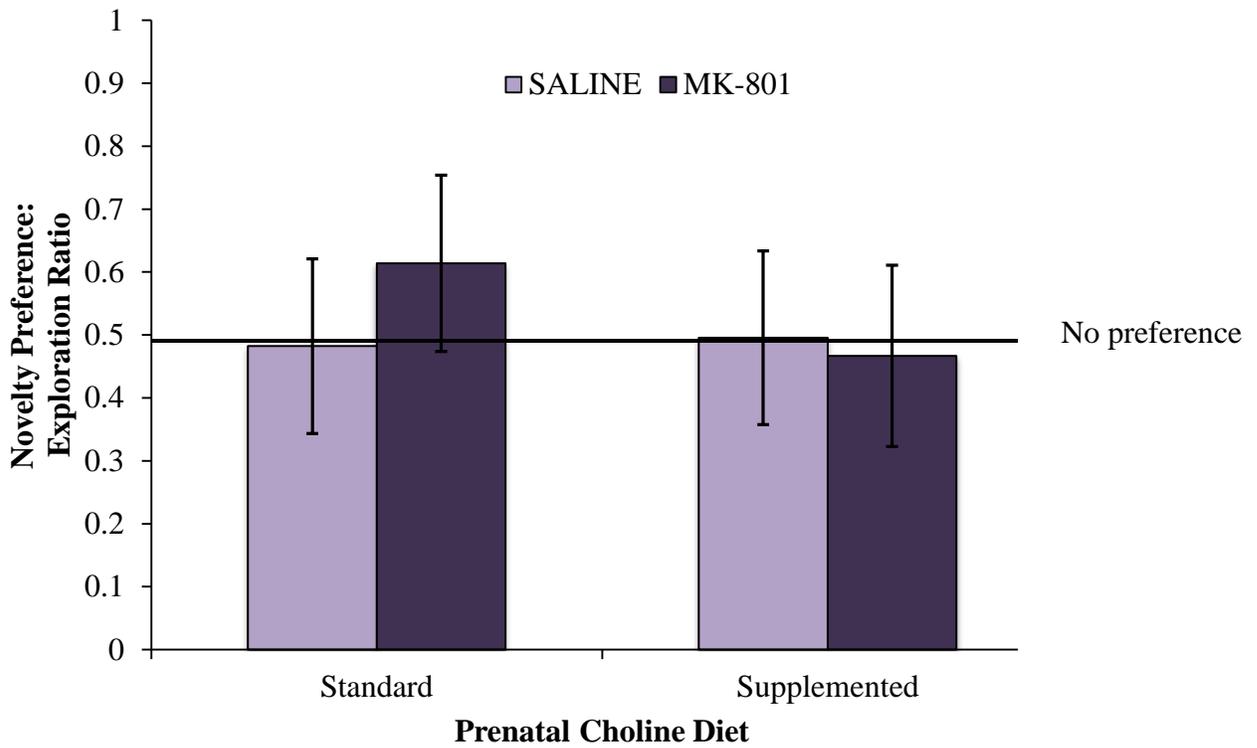
Figure 3. Mean novelty preference exploration ratios for the final third minute of the test phase

as a function of prenatal diet condition and adult drug administration as a test of memory

encoding. The exploration ratio is defined as the time spent investigating the novel object

divided by the total time spent investigating both the novel and studied objects during the final

third minute of the test phase. The no preference line indicates an exploration ratio of 0.5,

meaning no preference for the novel or studied object. Error bars represent ±1SE.
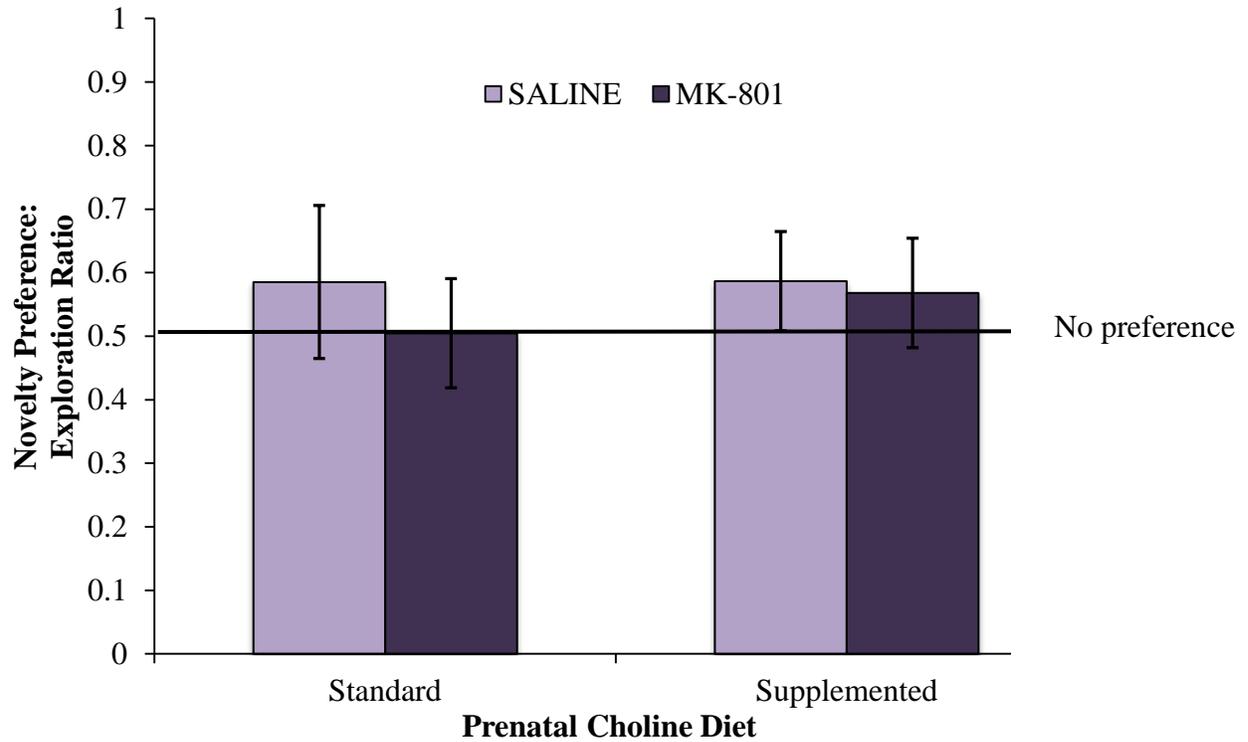
Figure 4. Mean novelty preference exploration ratios as a function of prenatal diet condition and adult drug administration as a test of memory retrieval. The exploration ratio is defined as the time spent investigating the novel object divided by the total time spent investigating both the novel and studied objects throughout the 3-minute test phase. The no preference line indicates an exploration ratio of 0.5, meaning no preference for the novel or studied object. Error bars represent ±1SE.
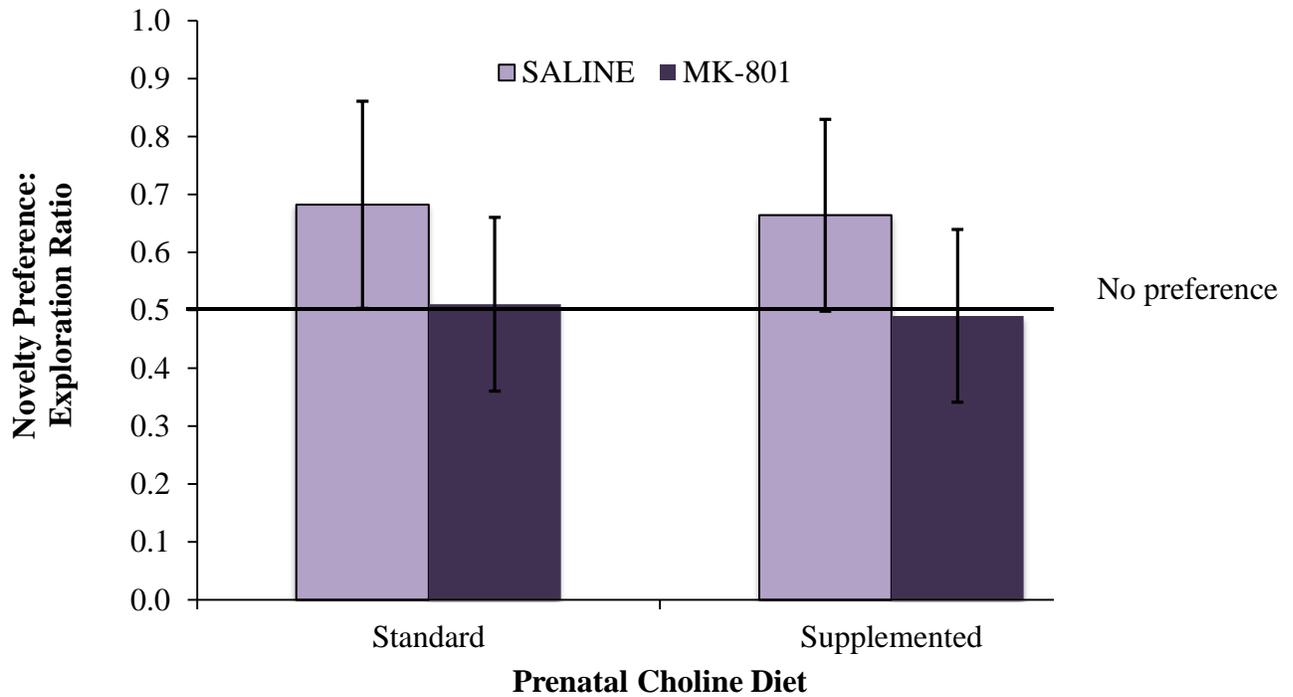
Figure 5. Mean novelty preference exploration ratios for the final third minute of the test phase as a function of prenatal diet condition and adult drug administration as a test of memory retrieval. The exploration ratio is defined as the time spent investigating the novel object divided by the total time spent investigating both the novel and studied objects during the final third minute of the test phase. The no preference line indicates an exploration ratio of 0.5, meaning no preference for the novel or studied object. Error bars represent ±1SE.
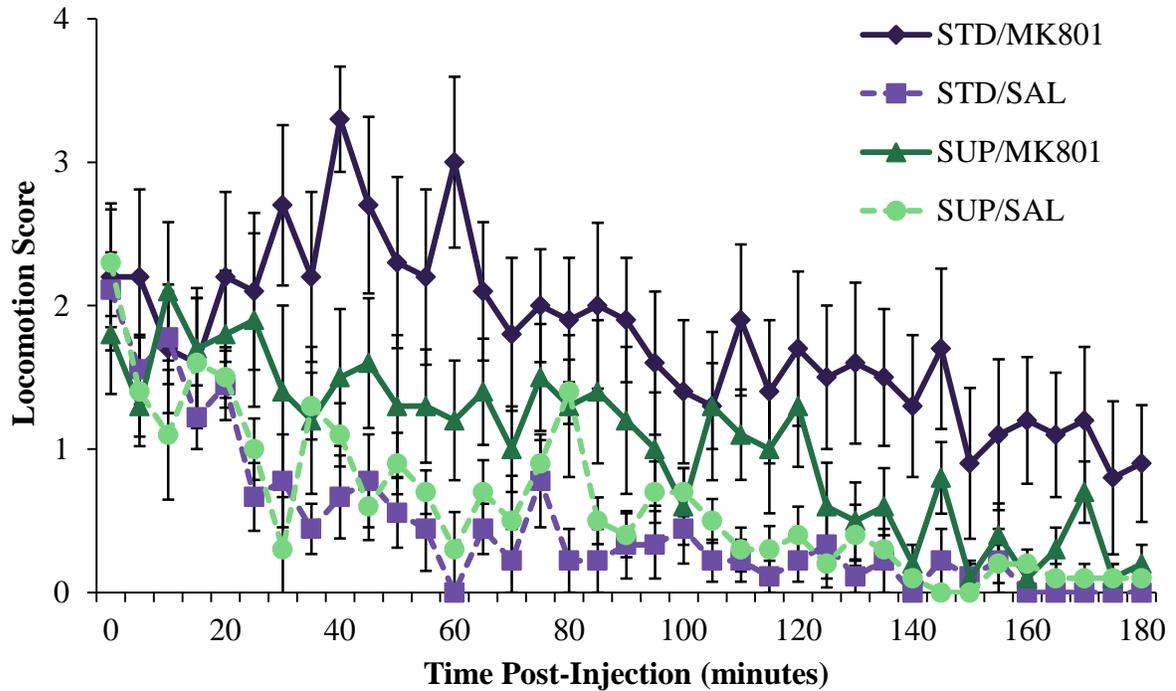
Figure 6. Mean locomotion scores over three hours following MK-801 or saline injection for both standard-fed and choline-supplemented rats. Scores range from 0-5: 0 = stationary, 1 = movements within a localized area forelimbs only, 2 = intermittent movements within half of the area of the cage, 3 = continuous movement within half of the cage, 4 = intermittent movements within the whole area of the cage, and 5 = continuous movement within the whole area of the cage. Error bars represent ±1SE.
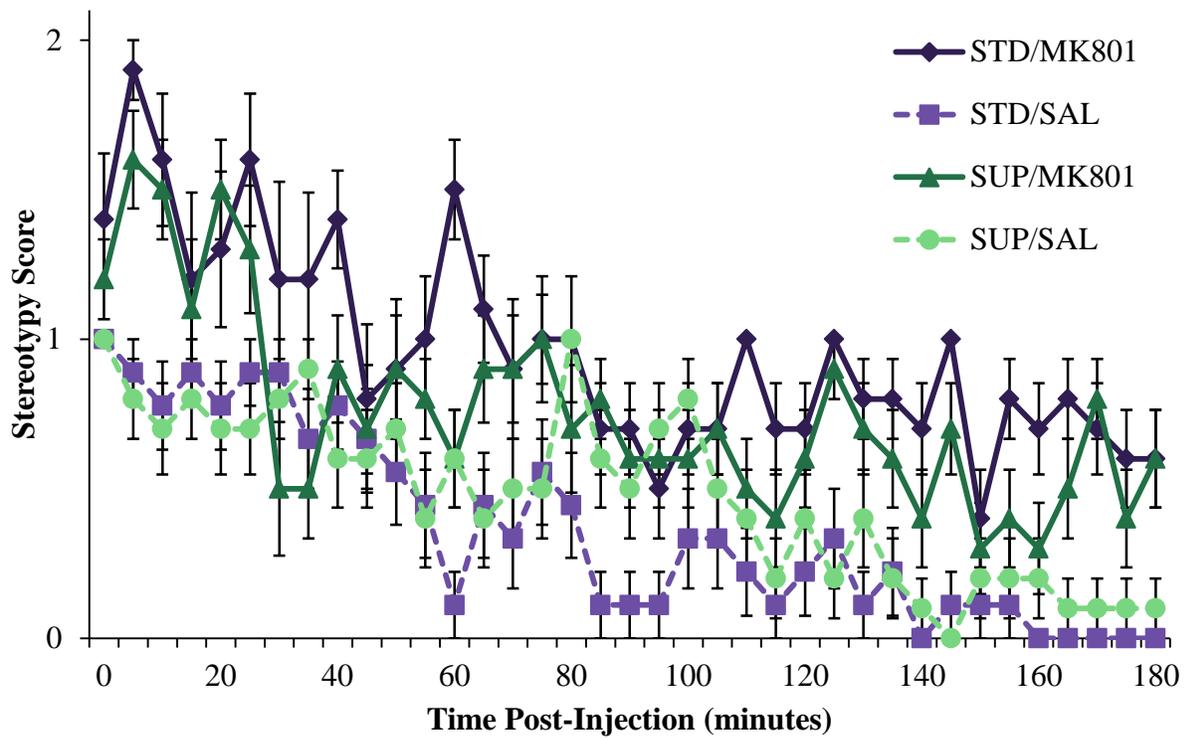
Figure 7. Mean stereotypy scores over three hours following MK-801 or saline injection for both standard-fed and choline-supplemented rats. Scores range from 0-2: 0 = no sniffing, 1 = discontinuous sniffing ('normal' sniffing), and 2 = continuous sniffing. Error bars represent ±1SE.
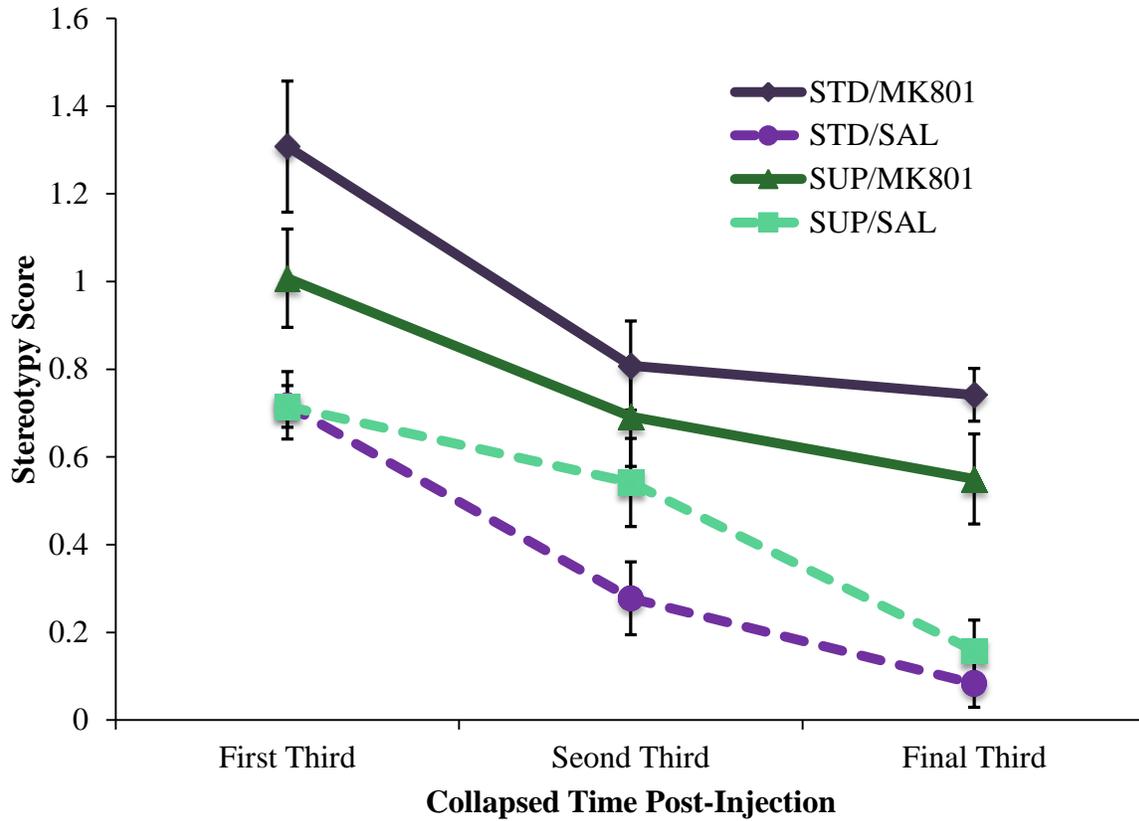
Figure 8. Mean stereotypy scores for standard-fed and choline-supplemented rats given MK-801 or saline collapsed over the first, second, and final thirds of the three-hour observation period of motor behaviors. Scores range from 0-2: 0 = no sniffing, 1 = discontinuous sniffing ('normal' sniffing), and 2 = continuous sniffing. Error bars represent ±1SE.
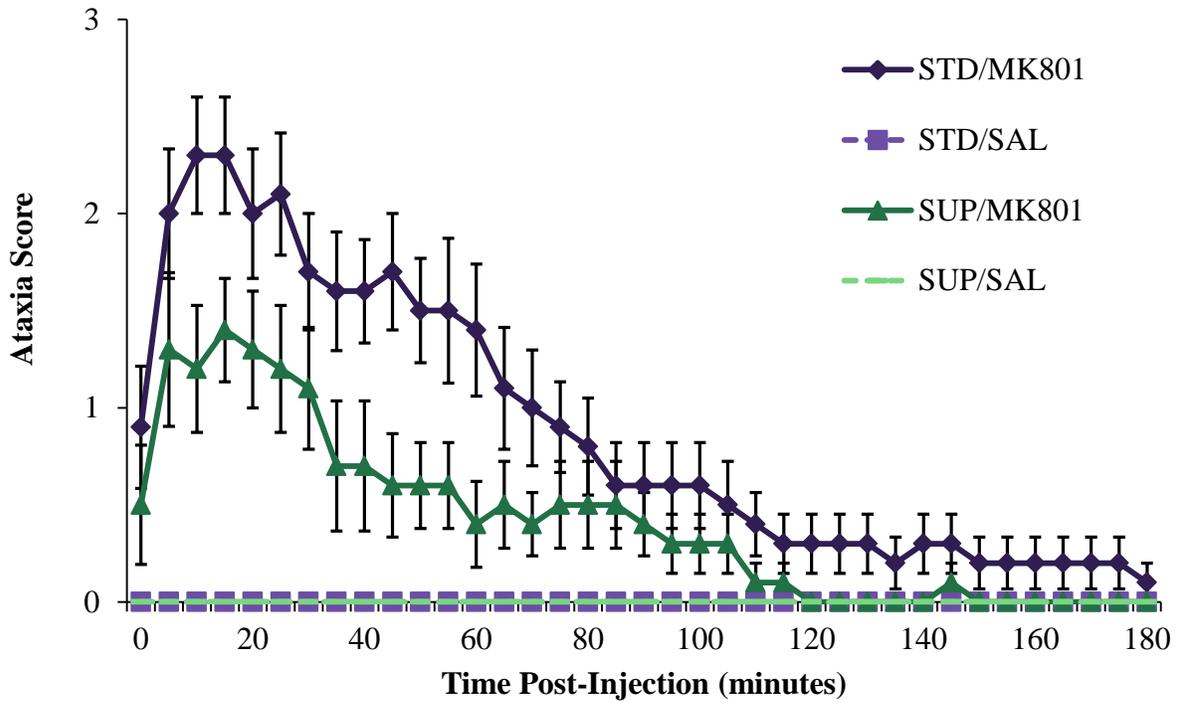
Figure 9. Mean ataxia scores over three hours following MK-801 or saline injection for both standard-fed and choline-supplemented rats. Scores range from 0-3: 0 = normal body control, 1 = falling tendency upon movement, 2 = falling upon movement, and 3 = almost unable to move. Error bars represent ±1SE.
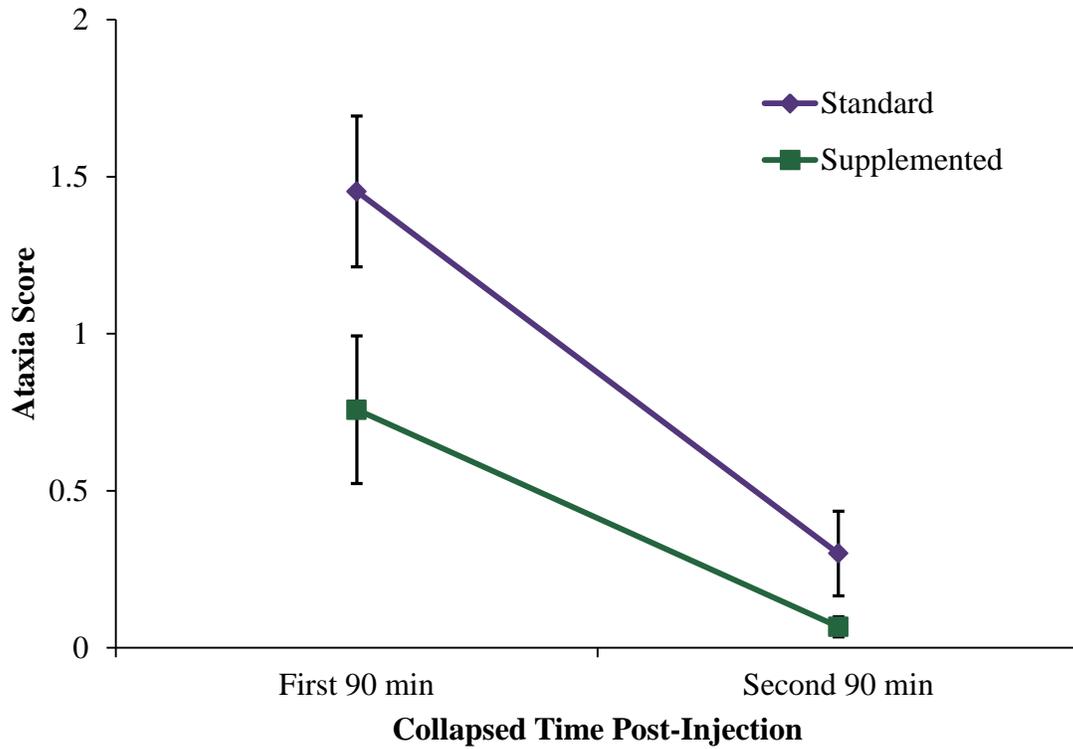
Figure 10. Mean ataxia scores for standard-fed and choline-supplemented rats given MK-801 collapsed over the first and second halves of the three-hour observation period of motor behaviors. Scores range from 0-3: 0 = normal body control, 1 = falling tendency upon movement, 2 = falling upon movement, and 3 = almost unable to move. Error bars represent ±1SE.
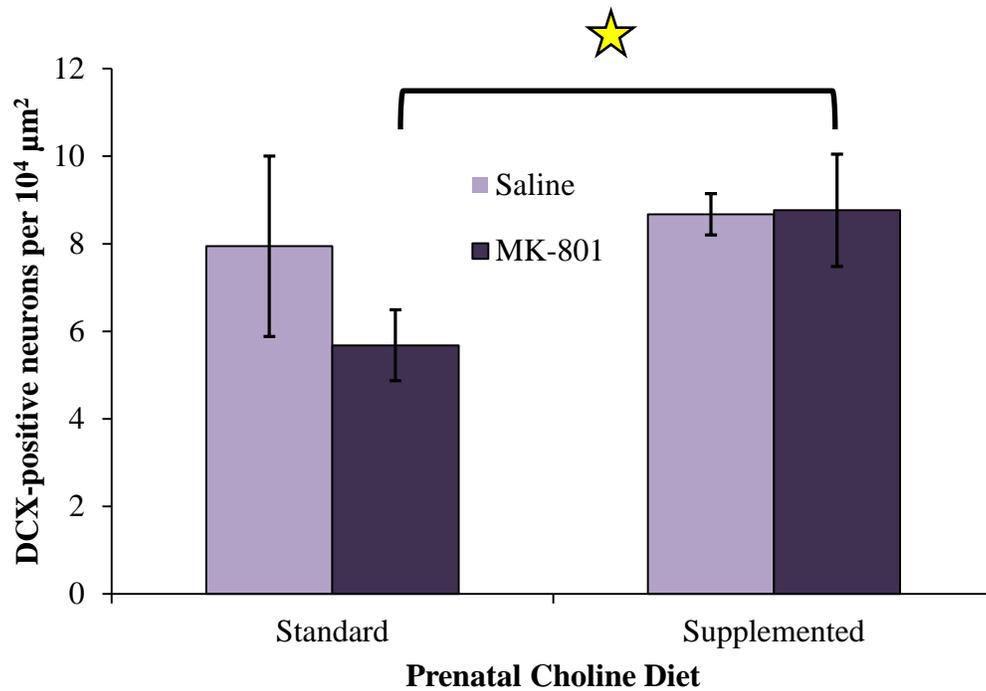
Figure 11. Neurogenesis, indicated by the average number of DCX-labeled cells per 10^4 μm^2, in the dentate gyrus of the hippocampus as a function of prenatal choline diet and adult drug administration. The star indicates a significant difference ($p < 0.05$, one-tailed t-test) between the STD-MK and SUP-MK groups. Error bars represent ±1SE.
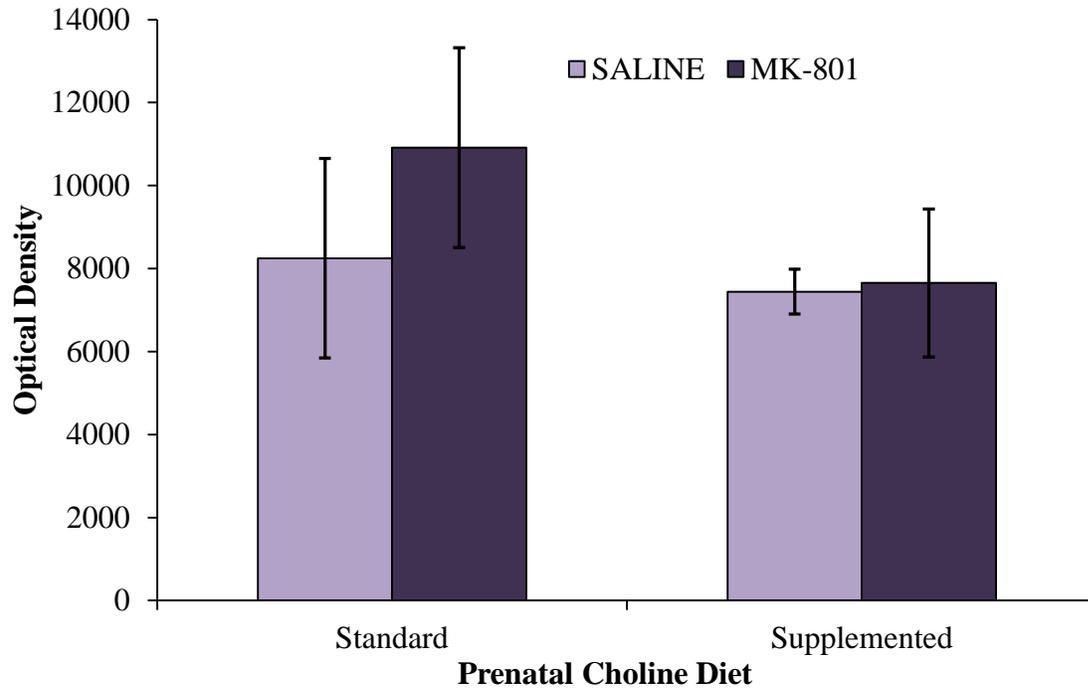
Figure 12. Neurodegeneration in the anterior commissure as a function of prenatal choline diet and adult drug administration. Neurodegeneration was marked by Fluoro-Jade B staining and was quantified by the optical density of the fluorescence. Error bars represent ±1SE.

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