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NMDA Receptor Involvement in Spatial Delayed Alternation in Developing Rats

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Abstract

Two experiments examined the effect of the non-competitive NMDA receptor antagonist, dizocilpine maleate (MK-801), on spatial working memory during development. Rats were trained on spatial delayed alternation (SDA) in a T-maze following i.p. administration of 0.06 mg/kg MK-801, 0.1 mg/kg MK-801, or saline on postnatal days (P) P23 and P33 (Experiment 1), or following bilateral intrahippocampal administration of 2.5 or 5.0 micro-g per side MK-801 or saline on P26 (Experiment 2). In Experiment 1, MK-801 dose-dependently impaired SDA learning at both ages. Because the same doses of systemic MK-801 have no effect on T-maze position discrimination learning, impairment of SDA by MK-801 likely reflects disruption of spatial working memory. Both doses of MK-801 abolished acquisition of SDA performance in Experiment 2. Disruption of hippocampal plasticity may account for the effects produced by systemic MK-801 administration. These results confirm and extend earlier lesion studies by implicating plasticity of hippocampal neurons in the ontogeny of spatial delayed alternation.

Keywords

Development; Working Memory; MK-801; Rat; Spatial Learning

There are several known subtypes of glutamate receptors; however, the N-methyl-D-Aspartate (NMDA) subtype of glutamate receptors, in particular, plays a significant role in the molecular/cellular basis of short- and long-term memory formation, as well as induction and maintenance of long-term potentiation (LTP), a long-lasting form of synaptic plasticity. LTP and behavioral learning and memory, depend on the NMDA receptor, especially in the hippocampus, is well established (Morris, 2006; Morris, Anderson, Lynch, & Baudry, 1986; Shapiro, 2001; Wang, Hu, & Tsien, 2006).

Studies using radio-labeled receptor antagonists to ascertain receptor binding densities in different rat brain regions have revealed that the highest densities of NMDA receptors are found in the hippocampus, cerebral cortex, and striatum and the lowest receptor densities

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were found in the brainstem and cerebellum (Boje & Skolnick, 1992; Sircar & Li, 1994; Valles, Felipe, Montoliu, & Guerri, 1995; Wong et al., 1986; Wong, Knight, & Woodruff, 1988). This regional distribution is also consistent with a role of NMDA receptors in spatial learning and memory, contextual learning, as well as the formation of long-term memories, at least in adulthood. A limited number of developmental lesion studies suggest that these same brain regions may also be engaged during spatial working memory tasks in developing animals (Altemus & Almli, 1997; Freeman & Stanton, 1991; Green & Stanton, 1989; Ivkovich & Stanton, 2001; Kolb, Buhrmann, McDonald, & Sutherland, 1994). For example, the ontogenetic emergence of spatial delayed alternation (SDA) between P15 and P21 parallels the cellular and neurochemical maturation of the hippocampus (Green & Stanton, 1989). Fimbria-fornix transection or hippocampal aspiration lesions on P10 severely disrupt T-maze SDA in weanling rats (Freeman & Stanton, 1991; Ivkovich & Stanton, 2001). It is not known, however, whether these effects reflect damage to fibers of passage from extra-hippocampal neurons or whether NMDA-receptor-dependent plasticity in hippocampal neurons is necessary for spatial working memory in weanling rats, as it appears to be in adult rats (Yoshihara & Ichitani, 2004).

In order to examine the role of NMDA receptor development in the ontogeny of learning and memory, it is necessary to determine the behavioral effects of drugs that are active at this receptor. A limited number of studies have investigated the role of NMDA receptors in learning and memory using pharmacological manipulations in developing animals (Chadman, Watson, & Stanton, 2006; Griesbach, Hu, & Amsel, 1998; Highfield, Nixon, & Amsel, 1996). These studies confirm NMDA involvement in spatial discrimination reversal learning and non-spatial working memory during the periweanling period in rats. However, little is known concerning the role of NMDA receptors in specific brain regions on memory development and nothing is known concerning its role in spatial working memory in developing rats. SDA engages different memory processing mechanisms relative to those engaged during acquisition or reversal of a simple spatial discrimination (Freeman & Stanton, 1992; Freeman & Stanton, 1991; Green & Stanton, 1989) and these additional processes may alter the dose-response properties of MK-801 on spatial working memory in developing rats.

To address these issues, two experiments examined the effects of NMDA receptor antagonism on spatial working memory in weanling rats. Experiment 1 examined different doses of systemically administered MK-801 to determine for the first time whether this drug impairs SDA in two weanling age groups, P23 and P33. Finally, Experiment 2 tested the effect of intrahippocampal administration of MK-801 on SDA in P26 rats. We predicted that sensitivity to MK-801 would be greater in younger animals because an adult-like NMDA receptor profile is just emerging at this age but has reached maturity by P33 (see Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994). Hence, P23 animals would show poorer performance relative to P33 animals (Experiment 1). We also predicted that the source of the MK-801 impairment was blockade of hippocampal NMDA receptors (Experiment 2).

Experiment 1

Spatial working memory emerges between P15 and 21, when assessed with a discrete-trials SDA task (Green & Stanton, 1989). Neonatal damage to hippocampus and related structures selectively interferes with the ontogeny of SDA (Freeman & Stanton, 1991; Ivkovich & Stanton, 2001) but the role of NMDA receptor function in this ontogeny is unknown. The present study tested the hypothesis that SDA in weanling rats would be disrupted by systemic administration of the non-competitive NMDA receptor antagonist, MK-801. Doses of 0.06 and 0.10 mg/kg of MK-801 were used because they impair reversal but not acquisition of position discrimination in weanling rats (Chadman et al., 2006). Impairment of reversal shows that these doses of MK-801 are effective behaviorally in weanling rats; whereas, the failure to impair acquisition indicates that these doses do not disrupt reference memory or basic sensory, motor, or motivational processes involved in T-maze performance. The ages of test were selected to use the youngest age where we reliably expected to see learning (i.e. P23); as well as, the oldest age we can test using our apparatus (i.e. P33).

Six separate groups of P23 and P33 rats received SDA training in a T-maze following i.p. administration of one of 3 doses of MK-801, 0 (saline vehicle), 0.06, or 0.10 mg/kg of MK-801 at each age group. On the basis of previous reports (Chadman et al., 2006; Griesbach et al., 1998), we predicted that MK-801 would impair SDA learning. If sensitivity to MK-801 increases with age, we predicted that the dose-response effect would be shifted down in the older group. Whereas if sensitivity decreases with age, we predicted that the younger group would show poorer performance relative to the older treatment groups, particularly at the highest dose of MK-801.

Method

Subjects—Seventy-three (39 female, 34 male) Long-Evans rat pups derived from 25 litters served as subjects, with 12 litters common to both age groups. Thirty-six rats (20 female, 16 male) were run on P23 and 37 rats (19 female, 18 male) were run on P33. Breeding females and males were obtained from Harlan Laboratory breeders (Frederick, Maryland) and bred at the animal facility at the University of Delaware. Females remained undisturbed during gestation and lactation except for weekly cage changes.

After birth, the pups were housed with their mothers until P21 in transparent polypropylene cages measuring 8" high × 18" long × 9" wide. The housing facility was illuminated on a 12:12 hr light-dark cycle; with light onset occurring at 0700 hr. Age of birth for each litter was determined by confirming the presence of newborn pups during the light phase and designating that day as P0. The litters were culled to 8 pups (as close to 4 females and 4 males as possible) on P3. Excluding routine cage changes, the litters were left undisturbed from P4 to P21.

All pups from each litter were weaned on P21 and housed with same-sex littermates with *ad libitum* access to food and water until the deprivation procedure began (see *Procedure*, below). Subjects were housed individually without food or water for the remainder of the experiment. The average weight at deprivation for subjects run on P23 was 50.0 ± 1.203 g

(range: 30.0–62.0 g); and for subjects run on P33, it was 100.7 ± 2.14 g (range: 69.0–121.0 g). A 2 (age) $\times$ 3 (treatment) ANOVA was performed on the deprivation weight data that revealed a main effect of age ($F(1, 67) = 397.17, p < .0001$), such that the P33 animals were significantly heavier than the P23 animals. No more than one same-sex littermate was assigned to a given experimental group.

Drugs—The experiment involved administration of the non-competitive NMDA-receptor antagonist, dizocilpine (the compound is also referred to as MK-801). MK-801 was purchased commercially from Tocris (Ellisville, MO). It was dissolved in sterile saline. (Treatment doses are described under training procedure below.)

Apparatus—As described by Freeman and Stanton (1991), subjects were trained in four Plexiglas T-mazes scaled to the size of weanling rats. Briefly, the T-mazes consisted of three equal length arms: a left and a right choice arm that were perpendicular to the start arm. The start arm was separated from a central choice point and the choice point was separated from the choice arms by pneumatically-operated doors.

Computer-controlled syringe pumps that dispensed light cream reward (commercially available half and half) were connected to a small metal cup that was located at the ends of both the left and right choice arms. When subjects broke a photoelectric beam placed in front of the feeding cup, the computer recorded the latency to make the response, lowered the maze doors, and when appropriate, activated the syringe pump (and delivered .07 ml light cream). Intertrial interval (ITI) boxes of clear Plexiglas were used to house subjects between trials.

Design—Discrete-trials SDA training consisted of a total of 60 trials over two sessions (5 12-trial blocks) divided into 2 blocks and 3 blocks, respectively (Freeman & Stanton, 1991). Drug doses were administered 30 min prior to the start of each session. The same drug treatment administered in the morning session was also given in the afternoon. This yielded a 3 (treatment) $\times$ 5 (trial blocks) mixed factorial design. The P23 animals were divided equally across the treatment groups ($n = 12$ for each dose). For P33 animals, both the saline and 0.1 mg/kg MK-801 contained the same numbers of animals ($n = 12$) and 13 animals were run in the 0.06 mg/kg MK-801 dose group.

Procedure—All subjects underwent the following procedure of deprivation, maze acclimation, and training. Table 1 illustrates the timeline of the procedures used for Experiment 1.

Deprivation and maze acclimation: Subjects were deprived of food, water, and maternal/sibling contact approximately 16 hr prior to initial T-maze exposure (usually between 1600 and 1700 hr). The animals run on P23 were weaned and deprived on P21 and acclimated on P22. The subjects tested on P33 were weaned on P21, group reared undisturbed for 9 days, deprived on P31 and acclimated on P32.

The pups were weighed to the nearest gram, tail-marked for identification, and individually housed in white polycarbonate “mouse” cages with a metal spoon secured to one side of the

cage. About 0.1 ml of the light cream reward was infused directly into each animal's mouth and an additional 1 ml of cream was placed in the spoon. This pre-exposure to light cream minimized taste-related neophobia when subjects were presented with the reward later during acclimation and experimentation.

Acclimation sessions began the following morning and occurred at 4 hr intervals. Pups learned to consume the light cream from the reward cups at the end of each choice arm and then to run in the maze. Two goal-box training sessions occurred in the morning (0800 – 0900 hr) and early afternoon (1200 – 1300 hr). Subjects were placed into either the right or left choice arm facing the center choice point. Each arm was closed off from the rest of the maze by a guillotine door. The subject was given up to 3 min to locate and consume 0.07 ml of half and half that was placed in the reward cup before the introduction of each pup. Subjects were removed from the maze after consumption of all of the light cream. A goal box session consisted of 6 placements in one maze arm or the other, counterbalanced across the successive sessions. A forced-run session concluded acclimation training (1600 – 1700 hr). One choice arm in the T-maze was opened on each trial. Animals were placed facing forward in the start box and ran to the open arm to trigger the photoelectric beam, causing the door to the open arm to lower and light cream to be infused into the reward cup. The forced run session consisted of a total of 12 runs with an equal number to each choice arm (left = 6, right = 6; presented in pseudorandom order). To maintain body weight at 80–85% of that recorded at deprivation (i.e. target body weight), a light cream post-acclimation supplement was administered. Amount of the supplement was calculated as follows: 6 ml – (body weight at start of forced run session – target body weight).

Training: The design involved the between-subjects variables of treatment: 0.06 mg/kg MK-801, 0.1 mg/kg MK-801, or saline vehicle. Doses were administered 30 min prior to the start of each session. The sessions were spaced 6 hr apart to maintain adequate motivation (Freeman & Stanton, 1991). This also took into account the 2–3 hr half-life of MK-801 in the rat brain (confirmed behaviorally in our previous studies, Chadman et al., 2006). The rewarded goal arm, maze, and sex were counterbalanced across treatment groups. Littermates were assigned to ages and groups such that a maximum of 1 male and 1 female per litter contributed to a given group. Subjects were trained in squads of eight animals, with four pups assigned to each maze. A total of 4 mazes were used.

Trials consisted of a pair of runs separated by a delay of approximately 2–5 sec. The first run of each trial was a forced run (an information run) where only one goal arm was accessible and subjects were always rewarded for reaching the end of that arm. The direction of forced runs was balanced across trials in a pseudorandom sequence (Freeman & Stanton, 1991). For the second run, the choice run, both arms were open. Reward was contingent upon the subject choosing the arm opposite to that entered during the forced run. For each trial, a pup was removed from the ITI box and placed into the start arm facing the central choice point. The experimenter controlled the start of the trial by pressing a button. The computer-controlled apparatus caused the door to the choice point to be raised. The door to one arm was raised following a 2-s delay. After the photobeam in the open arm was crossed by the subject, the door to that arm automatically lowered, and the animal was allowed 20 sec for reward consumption. Once this period elapsed, the subject was placed immediately

in the start box (2–5 sec delay) and the choice run was initiated by the experimenter. The start box door raised and 2 sec later the doors to both arms lifted. All doors lowered once the animal broke the photoelectric beam at the end of either choice arm. A correct choice of the opposite goal arm resulted in the infusion of the cream reward into the cup (.07 ml). Including infusion time, the subject remained in the arm for 20 s. If the incorrect arm was selected, subjects remained in the goal arm for a 20 sec without reward. After both runs were completed, the subject was returned to its ITI box. The four pups in a squad were run in rotation such that the ITI for a given pup was the sum of the trial times for the other three pups in the squad (~ 3 min). After completion of the first session, pups received a supplementary cream reward to maintain 80–85% of deprivation weight and compensate for incorrect, non-rewarded trials.

Data Analysis—Body weight, forced run, and choice run latencies and the total percent of correct trials per block were collected for each subject during testing sessions. The percent correct data were then subjected to analysis of variance (ANOVA) as well as post-hoc paired comparisons (Newman-Keuls). The between-groups variables used in the analysis were age (P23 or 33), sex (male or female), maze (1–4), and treatment (MK-801 dose or saline). The within-group variable was blocks (5 blocks of 12 trials).

Results and Discussion

In Experiment 1, a 2 (age) $\times$ 3 (treatment) factorial ANOVA was performed on the weights at Session 1 (sex and maze had no effect in initial analyses, thus data were combined across these factors). A significant main effect of age was found ($F(1, 67) = 375.18, p < .0001$) such that the P33 animals were heavier than the P23 animals. The average weight at Session 1 was 41.36 ± 1.02 g and 83.35 ± 1.83 g, for P23 and 33 animals respectively. Body weight did not differ across treatment groups ($F < .12$).

A 2 (age) $\times$ 3 (treatment) $\times$ 5 (trial blocks) repeated measures ANOVA was performed on the forced run latency data (sex and maze had no effect in initial analyses). A significant main effect of blocks was found ($F(4, 268) = 5.75, p < .0002$). No other effects or interactions were significant (all F 's < 1.88). The forced run latencies were 4.028 ± 0.145 s for saline-treated animals, 4.134 ± 0.326 s for 0.06 mg/kg MK-treated animals, and 3.893 ± 0.105 s for 0.10 mg/kg MK-treated animals ($F < .35$).

A 2 (age) $\times$ 3 (treatment) $\times$ 5 (trial blocks) repeated measures ANOVA was performed on the choice run latency data (sex and maze had no effect in initial analyses). Small, but significant, main effects of treatment ($F(2, 67) = 11.90, p < .0001$) and blocks ($F(4, 268) = 14.52, p < .0001$) were found. In general, the MK-treated animals had slightly faster choice run latencies relative to saline-treated animals. The average choice run latencies were 3.787 ± 0.119 s for saline-treated animals, 3.308 ± 0.087 s for 0.06 mg/kg MK-treated animals, and 3.138 ± 0.085 s for 0.10 mg/kg MK-treated animals. The MK-801 effect on choice run latency was likely secondary to the cognitive deficit on percent correct data (see below). There was no evidence that the drug decreased motivation to solve the task as evidenced by the lack of a treatment effect in the forced run latency data.

The percent correct data are shown as a function of drug treatment, age and trial-blocks in Figure 1 (left panel: P23, right panel: P33). Although an increase in percent correct choice throughout acquisition occurred for all treatment groups, the MK-801 treated groups learned more slowly and made, on average, fewer correct choices than the control groups. In general, the P23 drug treatment groups began training in Block 1 with lower percent correct choices relative to the P33 groups and on average, performance of all groups increased across age. At the highest MK-801 dose (0.10 mg/kg), the P23 animals did not acquire the SDA task. All P33 treatment groups demonstrated learning by Block 5 of acquisition.

A 2 (age) $\times$ 2 (sex) $\times$ 3 (treatment) $\times$ 5 (trial blocks) ANOVA was performed on the percent correct data (data were combined across the maze factor, which had no effect in initial analyses). The ANOVA yielded significant main effects for age ($F(1, 61) = 44.27, p < .0001$), sex ($F(1, 61) = 6.26, p < .015$), treatment ($F(2, 61) = 19.37, p < .0001$), and blocks ($F(4, 244) = 24.62, p < .0001$). The sex effect did not further interact with MK-801 treatment ($p > .10$). In general, the females had worse performance relative to the males, the P23 rats had worse performance than the P33 rats, and the dose response effect reflected differences between each of the doses. The absence of interactions of Age $\times$ Treatment or Age $\times$ Treatment $\times$ Blocks suggests that both age groups showed similar sensitivity to MK-801.

Experiment 2

Experiment 1 demonstrated that MK-801 impairs SDA learning in P23-P33 rats. These findings are consistent with the role of the hippocampal system in the ontogeny of SDA (Freeman & Stanton, 1991; Ivkovich & Stanton, 2001) and the role of NMDA receptors in hippocampal plasticity during development and in adulthood (Morris, 2006; Morris et al., 2003; Shapiro, 2001; Wang et al., 2006). In adult rats, the role of hippocampal NMDA receptors in memory has been more directly tested by examining the effects of local infusions of NMDA antagonists into the hippocampus on spatial working memory (Kawabe, Ichitani, & Iwasaki, 1998; Steele & Morris, 1999; Yoshihara & Ichitani, 2004). However, such effects in developing rats have not yet been examined. The present study examined the effects of intra-hippocampal administration of different doses of MK-801 on SDA performance in P26 rats.

Three treatment groups received 5 blocks of SDA training as previously described. All groups received bilateral infusions in dorsal hippocampus (0.5 μ l per side). Separate groups were administered a high dose of MK-801 (5.0 μ g per side); an intermediate dose (2.5 μ g per side); or vehicle (sterile saline). If hippocampal receptors are involved in the effects of systemic MK-801 observed in Experiment 1, then the present experiment should reveal dose-related impairment of SDA learning.

Method

The methods, apparatus, etc. in Experiment 2 were the same as those detailed for Experiment 1 except where noted below.

Subjects—Thirty-five (17 female, 18 male) Long-Evans rat pups derived from 28 litters served as subjects. All pups from each litter were weaned on P21 and housed with same-sex littermates until P23. Weaned pups had uninterrupted access to food and water until the onset of behavioral procedures. The surgical procedure began on approximately P23 (range: 22–24). Subjects were housed individually in cages following surgery for the duration of the experiment. Pups were allowed to recover from surgery for 1 day before behavioral procedures were initiated with the onset of deprivation (see below). The average weight at deprivation for subjects was 59.2 ± 1.31 g (range: 43.0 – 75.0 g). A 3 (treatment) ANOVA was performed on the deprivation weight data that did not reveal differences among subjects in different treatment groups ($F < 1.98$). No more than one same-sex littermate was assigned to a given experimental group.

One pup was discarded from analysis for failure to select a maze arm within 3 minutes from the start of the trial for three or more consecutive trials during the forced run acclimation session. Of the remaining 34 pups, 8 pups were excluded from further analysis following histological analysis of cannula placement. These pups were excluded due to incorrect cannula placements in the corpus callosum or overlying cortex rather than the dorsal hippocampus. The incorrect placements were typically unilateral in nature and disrupted SDA to a much lesser extent, indicating that bilateral cannula placement in the dorsal hippocampus was necessary for the MK-801 impairment on percent correct choice data (see *Results* below). Data from the remaining 26 pups are reported below.

Surgery—The surgical procedure was based on one we routinely use in rats this age to implant recording and stimulating electrodes in eyeblink conditioning studies (Stanton & Freeman, 1994). Commercially-obtained cannulas (Plastics One, Roanoke, VA) were implanted bilaterally under stereotaxic guidance in the brains of weanling rats under ketamine/xylazine anesthesia (52.2–60.9 mg/kg ketamine/7.8–9.1 mg/kg xylazine in a 0.7 – 0.8 ml/kg injection volume). Buprenorphine (0.03 mg/kg in a volume of 0.05 ml/100 gm) was also administered s.c. to alleviate pain during and following the surgical procedure. The hair overlying the dorsal skull surface was shaved and washed with Betadine and rinsed with isopropyl alcohol and then a scalpel blade was used to make a midline incision in the scalp. The skin and fascia were then retracted and small holes were drilled in the skull with a 1/16" drill bit for the cannulas. The location of the drill holes and the stereotaxic coordinates were estimated from a stereotaxic atlas of the developing rat brain (Sherwood & Timiras, 1970) and adjusted empirically by trial and error in previous pilot experiments. Implantation of cannulas was done under stereotaxic guidance to ensure consistent placement; and histological analysis to confirm location of cannula placement occurred following training (see *Histological Analysis* below). Guide cannulas were bilaterally implanted in the dHPC (AP +2.6 mm, ML ± 2.3 mm, DV -1.3 mm). All AP and ML coordinates were based on interaural coordinates as measured from the horizontal zero plane, such that the ear bars and incisor bar were set to zero, as this was the method that was used to create the developing rat stereotaxic atlas (Sherwood & Timiras, 1970). Dummy cannulas were inserted into the guide cannula to prevent obstruction until infusions were made (see below). For weanling rats, two “skull hooks,” fashioned from stiff wire, were inserted superficially under the skull surface and were used to secure the cannula assembly

to the skull with dental acrylic at the end of surgery (Gilbert & Cain, 1980; Stanton & Freeman, 1994). The dental acrylic completely filled the opening in the scalp, thereby serving to “close” the incision. Rats were then returned to their home cages with food and water. Rats were monitored and kept warm until they recovered from anesthesia. Rats received 1 day of recovery prior to the deprivation procedure that started the T-maze protocol. (Thus, surgery preceded acclimation training by 2 days and SDA training by 3 days.) This amount of recovery time has been found sufficient for weanling/juvenile rats having undergone stereotaxic surgery (Freeman, Rabinak, & Campolattaro, 2005).

Drug infusion procedure—Five minutes prior to the start of each SDA training session, the rats were infused with MK-801 or vehicle. More specifically, the dummy cannulas were removed and an injection cannula was lowered through each guide cannula extending 1 mm below the guide cannula. An injection cannula was connected to polyethylene tubing attached to a 10 μ L Hamilton syringe mounted on a microinfusion pump. Following previous reports in the literature (Palencia & Ragozzino, 2004), MK-801 was dissolved in sterile saline at a concentration of either 5 or 10 μ g per μ L. MK-801 was delivered at a rate of 0.5 μ L per minute for 1 minute, for a total volume of 0.5 μ L per side. This volume delivered either 2.5 or 5.0 μ g of MK-801 per side, for the two concentrations of MK-801, respectively. These infusion doses and volumes are within the range of doses that have been used in a variety of learning studies in adult rats (Bast, Zhang, & Feldon, 2003; Levin, Sledge, Baruah, & Addy, 2003; May-Simera & Levin, 2003; Zhang, Bast, & Feldon, 2000; Zhang, Bast, & Feldon, 2001). The same volume of saline was used for the control infusions. One minute after infusion, the injection cannula were removed and replaced with the dummy cannulas.

Design—The experimental design was a 3 (treatment) $\times$ 5 (trial blocks) mixed factorial design. (Sex and maze were additional factors, but since they failed to reveal effects in any analysis, 3 $\times$ 5 ANOVAs were performed.)

Procedure—No intrahippocampal infusions occurred during maze acclimation. Infusions occurred before each SDA training session as described above. The sessions were separated by approximately 5hr. The subjects were weaned on P21, received cannula implantation on P23, were deprived on P24, acclimated on P25, and trained on P26.

Histological Analysis—Within 24–72 hrs after completion of behavioral testing, pups were deeply anesthetized with an injection (IP) of a ketamine/xylazine cocktail following a 0.5 μ L injection of 2% pontamine skyblue dye solution through each guide cannula. The stain showed the position of then internal cannula tip. Animals were perfused intracardially with 0.9% saline followed by 10% neutral buffered formalin. The brains were removed and post-fixed via placement into 10% neutral buffered formalin. The following day, brains were placed in 30% sucrose in 10% buffered formalin. After the brains sank, coronal sections (40 μ m) were taken using a cryostat (Leica CM3050 S). Sections were mounted on slides, and then counterstained with Neutral Red (1%). Slides were examined under a microscope for cannula tip placement.

Data Analysis—Body weight, forced run and choice run latencies, and the total percent of correct trials per block were collected for each subject during testing sessions. These measures were subjected to analysis of variance (ANOVA) as well as post-hoc paired comparisons (Newman-Keuls). The between-groups variables used in the analysis were sex (male or female) and treatment (2.5 µg MK-801, 5.0 µg MK-801, or saline). The within-group variable was blocks (5 blocks of 12 trials).

Results and Discussion

Histology – Cannula Placement—Rats were included in the analysis if the cannulas were located within or around the borders of the dorsal aspect of the hippocampus (8 excluded, 26 included; see cannula placements in Figure 2). Thus, both the saline and 5.0 µg MK-801 groups contained the same numbers of animals ($n = 10$). Six animals were used from the 2.5 µg MK-801 dose.

Behavioral Effect of Intrahippocampal administration of MK-801—In Experiment 2, a 3 (treatment) ANOVA was performed on body weight at Session 1, that were 51.4 ± 1.81 g, 45.0 ± 1.15 g, and 52.0 ± 2.57 g for the saline, 2.5 µg, and 5.0 µg MK-treated animals, respectively. Treatment had no effect on body weight ($F < 2.58$). The average forced run latency for each treatment group across training blocks is shown in Table 2. A 3 (treatment) $\times$ 5 (trial blocks) repeated measures ANOVA revealed a significant Blocks $\times$ Treatment interaction ($F(8, 92) = 2.68, p < .011$). Post-hoc analysis revealed that the 5.0 µg MK-treated animals had a slower forced run latency on Block 3 ($p < .04$) relative to the 2.5 µg MK- and saline-treated animals, which did not differ ($p > .90$). This may be a spurious effect considering the lack of group differences in latencies on the other 4 blocks.

A 3 (treatment) $\times$ 5 (trial blocks) repeated measures ANOVA performed on the choice run latency data (see Table 2) revealed small, but significant, main effects of treatment ($F(2, 23) = 5.52, p < .011$), blocks ($F(4, 92) = 14.98, p < .0001$), and Blocks $\times$ Treatment ($F(8, 92) = 6.26, p < .0001$). In general, the MK-treated animals had slightly faster choice run latencies relative to saline-treated animals. Post-hoc analysis of the Blocks $\times$ Treatment interaction revealed that the saline-treated animals had slightly slower choice run latencies ($p < .0002$) on Blocks 1–2 relative to the 5.0 µg MK-treated animals and the 2.5 µg MK- and saline-treated animals, which did not differ ($p > .69$). There was no evidence that the drug decreased motivation to solve the task as the latency effects do not correlate with the effects on percent correct choice (see below).

The percentage of correct responses for each treatment group over the course of training (Figure 3) clearly shows greatly impaired performance in the MK-801 treatment groups relative to the vehicle treated animals. The saline-administered subjects readily acquired the DA task. In contrast, performance of neither MK-801 group exceeded chance levels by the end of training.

A 3 (treatment) $\times$ 5 (trial blocks) mixed factorial ANOVA yielded main effects for treatment, $F(2, 23) = 43.20, p < .0001$; and blocks, $F(4, 92) = 3.84, p < .006$; but no interaction of Treatment $\times$ Blocks ($F < 1.2$). Both MK-801 dose groups significantly differed

from the vehicle group (p 's < .01), but *not* each other (p > .20): saline (79.62 %), 2.5 µg MK-801 (53.90 %), and 5.0 µg MK-801 (58.00 %).

The absence of a Treatment x Blocks interaction likely reflected the treatment effect that was present on Block 1 of training. This effect was likely produced by spontaneous alternation in the saline group (Green & Stanton, 1989) that was not evident in the MK-801 groups because hippocampal disruption impairs spontaneous alternation in this task (Freeman & Stanton, 1991). Alternatively, the saline group may have benefited from some learning during the first 12 trials. To examine these alternatives, performance of the three groups was examined on Trials 1–4 from Block 1 only. Again, each MK-801 dose group was significantly different from vehicle treatment, but *not* each other (p > .94): saline (70.0 %), 2.5 µg MK-801 (41.67 %), and 5.0 µg MK-801 (42.5 %). ANOVA revealed a significant main effect of treatment ($F(2, 23) = 4.17, p < .029$). Thus, MK-801 has a behavioral effect on performance as early as the first 4 trials of the session that impaired spontaneous alternation behavior. The saline group demonstrated further improvement over the five blocks of trials. Both drug-treated groups had flat acquisition curves and did not significantly improve across training.

General Discussion

Two experiments in developing rats examined the effects of NMDA receptor antagonism on a discrete-trials SDA task. Experiment 1 determined which doses of systemically administered MK-801 disrupted SDA acquisition at two developmental ages, P23 and P33. MK-801 dose-dependently impaired performance at both ages, with 0.06 mg/kg modestly impairing and 0.10 mg/kg severely disrupting learning. Experiment 2 showed that hippocampal administration of MK-801 disrupted SDA acquisition in P26 rats. Doses of 2.5 µg and 5.0 µg per side were found to equally impair SDA acquisition. This impairment resembled that produced by the systemic 0.10 mg/kg dose in Experiment 1. In both experiments, MK-801 treatment had no effect on body weight, negligible effects on forced run latencies, and caused slightly faster choice run latencies. The selective effect of MK-801 on choice run latency suggests this was secondary to the cognitive deficit. There was no evidence that the drug decreased motivation to solve the task as the latency effects do not correlate with the effects on percent correct choice.

In the rat, acquisition of SDA emerges by the end of the third postnatal week (Green & Stanton, 1989). Fimbria-fornix transections or hippocampal aspiration lesions performed on P10 severely impair SDA performance of weanling rats at a range of ages tested (approximately P19–32) (Freeman & Stanton, 1991; Ivkovich & Stanton, 2001). Early damage to the medial prefrontal cortex on P10 impaired SDA acquisition on P19–23, but this performance deficit recovered between P27–33 (Carter, Freeman, & Stanton, 1995; Freeman & Stanton, 1992). Early exposure to different neurotoxic agents that target hippocampus and related structures severely impairs SDA performance in weanling rats (Freeman, Barone, & Stanton, 1994; Stanton, Jensen, & Pickens, 1991; Stanton, Mundy, Ward, Dulchinos, & Barry, 1994). Disruption of SDA by early hippocampal injury could not be attributed to general “performance effects” (sensory, motor, motivational, or reference memory processes) because acquisition of a control task—position discrimination—was generally

not (or only marginally) impaired by this damage. A task distinction between spatial working memory and reference memory has been demonstrated in normally developing rats (Freeman & Stanton, 1991; Green & Stanton, 1989). Rats as young as P15 acquired the position discrimination (reference-memory) task; but SDA (working memory) did not emerge until later in ontogeny, between P19-21. The present study did not include this control task because we have previously shown that neither percent correct choice nor choice run latency is impaired by the doses of systemic MK-801 used in Experiment 1 during acquisition of position discrimination (Chadman et al., 2006). We have also completed experiments on position discrimination showing the same lack of impairment by the doses of intra-hippocampal MK-801 used in Experiment 2 (Watson, Burr, Cohen, & Stanton, 2007). The results of Experiment 2 suggest that antagonism of NMDA receptors specifically in dorsal hippocampus is sufficient to account for the effects of systemic drug administration demonstrated in Experiment 1. The current findings importantly extend the previous developmental studies by showing, for the first time, that plasticity of hippocampal neurons are specifically involved and that fibers of passage or extra-hippocampal structures do not play a necessary role in SDA in weanling rats.

Systemically administered MK-801 disrupts patterned single alternation in a runway (Griesbach & Amsel, 1998; Highfield et al., 1996), olfactory discrimination and reversal in a Y-maze (Griesbach et al., 1998), and spatial reversal in a T-maze (Chadman et al., 2006). Amsel and colleagues evaluated the ability of MK-801 (0.5 mg/kg i.p.) to disrupt patterned single-alternation (PSA), a nonspatial task dependent on working memory, as well as the hippocampus at long inter-trial intervals (ITIs) in preweanling rat pups (P16-17) (Highfield et al., 1996). In another task, position discrimination reversal in a T-maze, a high dose of MK-801 (0.1 mg/kg) administered prior to both acquisition and reversal training impaired spatial reversal in P26 rat pups without affecting acquisition performance and these effects were solely due to the effect of the drug on reversal learning, not acquisition (Chadman et al., 2006). Although the hippocampus has been implicated in many of these behavioral pharmacology studies in peri-weanling animals, none have directly implicated the hippocampus by investigating the effects of intrahippocampal infusions of MK-801 on learning and memory.

Thus far, there are no studies of the effects of infusion of NMDA antagonists into specific regions on SDA learning in adult rats. However, effects of systemic injections have been examined. MK-801 impairs adult SDA performance in a T-maze or water T-maze (Hauber, 1993; Locchi, Dall'olio, Gandolfi, & Rimondini, 2007; Verma & Moghaddam, 1996). These studies, together with studies of the effects of adult lesions on SDA (reviewed below), studies of intra-hippocampal NMDA antagonists and other hippocampal-dependent memory tasks, and the present findings, suggest that intra-hippocampal infusions of MK-801 in the adult rat would also impair SDA.

The sites with the greatest receptor binding density of NMDA receptors, as shown by [3H] MK-801 labeling, are the hippocampal formation, cerebral cortex, olfactory bulb, striatum, thalamus, medulla-pons; with the lowest NMDA receptor densities were found in brainstem and cerebellum (Reynolds & Palmer, 1991; Sakurai, Cha, Penney, & Young, 1991; Wong et al., 1986; Wong et al., 1988). The intrahippocampal dye infusion in Experiment 2 was

localized to the dorsal hippocampus, which suggests that MK-801 did not spread to extra-hippocampal regions. This further suggests that SDA is disrupted by an action of the drug bilaterally on dorsal hippocampus rather than in other areas.

The hippocampus and related structures (i.e. fornix and cingulate bundle); medial prefrontal cortex (mPFC); anterior thalamus; and mammillary bodies are necessary for SDA performance in adult rats. The impairment following hippocampal administration of MK-801 seen in our data is consistent with data showing impairment of SDA learning in a T-maze following lesion of the hippocampus (Ainge, van der Meer, Langston, & Wood, 2007; Bardgett, Baum, O'Connell, Lee, & Hon, 2006a; Bardgett et al., 2006b; Dudchenko, Wood, & Eichenbaum, 2000), the fornix (Aggleton, Neave, Nagle, & Hunt, 1995; Bussey, Duck, Muir, & Aggleton, 2000; Mogensen, Hjortkjaer, Ibervang, Stedal, & Mala, 2007; Shaw & Aggleton, 1993), and the cingulate bundle (Aggleton et al., 1995b; Neave, Nagle, Sahgal, & Aggleton, 1996). Our results in weanling rats are consistent with SDA findings in adult rats, such that hippocampal lesions, fornix lesions, and mPFC lesions impair SDA performance; which suggests that adult-like spatial working memory mechanisms seem to be operating in weanling rats. This general principle is supported also by data from adult studies of other spatial memory tasks. Systemic administration of MK-801, hippocampal infusion of NMDA receptor antagonists, and hippocampal lesions all impair spatial memory (Aggleton, Hunt, Nagle, & Neave, 1996; Caramanos & Shapiro, 1994; Kawabe et al., 1998; Shapiro & Caramanos, 1990; Shapiro & O'Connor, 1992; Steele & Morris, 1999; Woodside, Borroni, Hammonds, & Teyler, 2004; Yoshihara & Ichitani, 2004).

Deficits in working memory, cognitive flexibility, and other learning problems are common in neurodevelopmental disorders, such as schizophrenia and autism (Coldren & Halloran, 2003; Steele, Minshew, Luna, & Sweeney, 2007). NMDA hypofunction in prefrontal cortex has been suggested to occur in human schizophrenia and autism (Carlsson, 1998; Harris, Sharp, Gartlon, Jones, & Harrison, 2003; Laruelle, Kegeles, & Abi-Dargham, 2003; Ossowska et al., 2000) and this phenomenon has been modeled in rodents using MK-801 administration (Braun et al., 2007; Csernansky et al., 2005; Harris et al., 2003; Rujescu et al., 2006). Further investigation into the exact role of NMDA receptors in spatial memory tasks across ontogeny could advance the development of animal models of both autism and schizophrenia. The results of the present study show that such models can be implemented in developing rats. This creates an opportunity to incorporate the developmental profile of NMDA-receptor function in animal models of these developmental disorders.

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Figure 1.

Mean ($\pm$ SE) percentage of correct responses for the three treatment groups beginning on P23 (left panel) or P33 (right panel) in Experiment 1 as a function of blocks and dose. The treatment groups were vehicle (SALINE: closed circles), or one of two drug doses (0.06 mg/kg MK-801: open circles; 0.1 mg/kg MK-801: open triangle). The same treatment was administered prior to each session. Dashed line at 50 percent indicates chance performance.

Figure 2. Schematic representation of cannula placements targeted to the dHPC. Coordinates represent distances posterior to Bregma (Paxinos & Watson, 2005).

Figure 3.

Mean ($\pm$ SE) percentage of correct responses for the three treatment groups beginning on P26 in Experiment 2 as a function of blocks and dose. The intrahippocampal treatment groups were vehicle (SALINE: closed circles), or one of two drug doses (2.5 micro-g MK-801: open circles; 5.0 micro-g MK-801: open triangle). The same treatment was administered prior to each session.

Table 1

Timeline of behavioral procedures for Experiments 1 and 2. For groups trained at P23 in Experiment 1, day 0 was P21 and for groups trained at P33, day 0 was P31. For groups trained at P26 in Experiment 2, day 0 was P24. Cannula implantation occurred on P23 in Experiment 2.

<u>Day</u>	<u>Time</u>	<u>Procedure</u>
0	1600 hr	Deprivation
1	800 hr	GBT 1
1	1200 hr	GBT 2
1	1600 hr	F Run
2	800 hr	DA - 1
2	1300 – 1400 hr	DA - 2

Table 2

Mean ($\pm$ SE) of forced run (upper panel) and choice run (lower panel) latencies for the three treatment groups beginning on P26 in Experiment 2 as a function of blocks and dose. The same treatment was administered prior to each session.

Forced Run Latency										
	Block 1	Block 1 - SEM	Block 2	Block 2 - SEM	Block 3	Block 3 - SEM	Block 4	Block 4 - SEM	Block 5	Block 5 - SEM
SAL	5.941	0.923	4.650	0.555	3.658	0.186	3.907	0.147	3.765	0.190
MK (2.5 μ g)	3.710	0.233	3.513	0.129	3.337	0.106	3.319	0.337	3.665	0.277
MK (5.0 μ g)	4.531	0.536	4.151	0.293	6.222	1.331	4.815	0.378	4.344	0.305
Choice Run Latency										
	Block 1	Block 1 - SEM	Block 2	Block 2 - SEM	Block 3	Block 3 - SEM	Block 4	Block 4 - SEM	Block 5	Block 5 - SEM
SAL	4.311	0.286	4.485	0.454	3.294	0.251	2.997	0.174	3.019	0.088
MK (2.5 μ g)	3.115	0.115	3.003	0.108	2.680	0.036	2.737	0.067	2.628	0.070
MK (5.0 μ g)	3.354	0.119	3.030	0.083	3.169	0.222	3.100	0.151	2.871	0.077