HIPPOCAMPAL PLASTICITY AND GENE EXPRESSION UNDERLIE OBJECT LOCATION MEMORY IN ADOLESCENT AND JUVENILLE RATS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Neuroscience

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ABSTRACT

Novel object recognition (NOR) is a form of incidental learning that relies on a rat's innate tendency to explore novel stimuli (Dix & Aggleton 1999). Object recognition (OR) is a non-spatial NOR variant that requires rats to learn the identity of objects, resulting in the preferential exploration of a novel object opposed to a familiar object. Object location (OL) evaluates an animal's ability to learn the spatial location of each object as opposed to its identity by assessing exploration of a moved object. During ontogeny, OR is present as early as Postnatal Day (PD) 17 whereas OL first develops later on PD21 (Westbrook et al., 2014), corresponding with hippocampal maturation. While a number of lesion studies have shown that OL is hippocampus-dependent in adult rats, few have looked at the underlying neuroplastic changes thought to mediate the OL task in developing animals.

The current set of experiments examined the role NMDA receptors (NMDAR) and the upregulation of immediate early genes in developing rats. Experiment 1 found that bilateral microinfusions of MK-801, a non-competitive NMDAR antagonist, into the dorsal hippocampus of adolescent (PD33) rats impairs spatial learning in the OL task, but does not impair learning in the non-spatial OR task. Experiment 2 confirmed in juvenile rats (PD26) that bilateral microinfusions of MK-801 into the dorsal hippocampus also impairs test performance in the OL task. Lastly, Experiment 3 used RT-PCR to examine relative expression of immediate early genes in the dorsal hippocampus and the perirhinal cortex of PD26 rats immediately, 15min, 30min, and 90min post OL testing. We found that hippocampal c-Fos expression was upregulated

immediately, 15min, and 90min, while BDNF was upregulated 15min and 90min after testing in comparison to homecage control rats, suggesting a possible modulatory role for these neuroplasticity molecules in spatial learning as it first emerges during ontogeny. In the perirhinal cortex, c-Fos was upregulated immediately, 15min, and 30min after OL testing, while BDNF was only upregulated 15min after OL testing. Taken together, these studies suggest that neuroplastic mechanisms of OL performance in adult rats are evident as early as PD26 in the developing rat.

Chapter 1

GENERAL INTRODUCTION

The hippocampus is one of the most extensively studied brain region in respect to learning and memory. While a number of behavioral tasks have been used to assess hippocampal involvement in memory, novel object recognition paradigms have become an increasingly popular alternative to study memory. Novel object recognition (NOR) is an incidental learning task that allows investigators to evaluate various memory systems through a rapid one-trial session free of reinforcing or aversive stimuli (Ennaceur & Delacour, 1988). While a large number of NOR variants exist, two of the most commonly used are the object recognition task (OR) and object location task (OL). In these tasks rodents are presented with two identical objects during a sample phase followed by a delay. In OR animals are then presented with a familiar object and a novel object. If rats have learned the identity of these objects they will preferentially explore the novel object over the familiar. OL on the other hand assesses an animal's ability to tell if a familiar object has moved to a new location. Here, a change in location rather than object identity elicits object exploration.

While both these tasks employ novelty to induce preferential exploration, the OR and OL tasks recruit differential neuronal circuits. Traditionally OR is thought to rely on the perirhinal cortex but not hippocampus. Conversely, the OL task depends on the hippocampus and task performance is impaired following hippocampal lesions (Mumby et al., 1994; Mumby et al., 2002; Brown, Warburton, & Aggleton, 2010; Albasser et al., 2011, Jablonski et al., 2013). Furthermore, previous work has shown

that the hippocampus continues to develop postnatally and its spatial memory functions emerge around post natal (PD) 21 (Rudy & Morledge, 1994, Jablonski et al., 2013; Schiffino et al., 2011). Recently our lab has paralleled these findings by showing differential ontogenetic profiles of the OR and OL tasks (Westbrook et al., 2014). OR (hippocampal independent) emerges at PD17, while OL (hippocampal dependent) emerges on PD21 (Westbrook et al., 2014). Moreover, while it has been shown that hippocampal development correlates with the ontogeny of OL task performance, the role of the hippocampus in this task during development is largely unknown.

NMDARs (N-methyl-D-aspartate receptors), have been found to play a critical role in the induction of long-term memory (LTM). In response to a learning event glutamate binds to NMDARs, which subsequently results in an influx of calcium. There is considerable evidence that these receptors are activity-dependent and results in synaptic remodeling that strengthen synapses after a learning event (Xia et al., 1996; Madison et al., 1991; Bliss & Collingridge, 1993; Kessels & Malinow, 2009). More importantly, the synaptic modifications driven by NMDARs are necessary for hippocampal-dependent behavioral learning (Schiffino et al., 2011; Heroux et al., 2016; Morris et al., 1986; Heale & Harley, 1989; Jablonski et al., 2013).

Similarly to NMDARs, immediately early genes (IEGs) are also necessary for proper encoding and consolidation of a memory. IEGs are a set of genes that are rapidly transcribed following synaptic activation. Like NMDARs, IEGs play an important role in synaptic alterations and strengthening (Okuno 2011, Guzowski et al, 1999; Vann et al., 2000). Since their discovery IEGs have become a hallmark for quantifying neuronal activation following a learning event. These changes in gene expression have been commonly observed during a number of behavioral tasks and inhibition of IEG

transcription impairs learning of these tasks (Guzowski et al., 1999; Vann et al., 2000; Schreiber et al., 2014; Seoane et al., 2012; Guzowski et al., 2000).

While NMDAR and IEG inhibition have been seen to disrupt performance of hippocampal tasks, very few studies have investigated the role of NMDAR and IEG expression in NOR paradigms. More significantly, while many of these plasticity markers have been quantified in adults, few studies investigated these effects in developing animals. Knowing that hippocampal tasks emerge later in development, it begs the question whether or not adolescent and juvenile rats rely on NMDARs during learning as well as if they show similar IEG patterns as adults. In an attempt to fill a gap in the literature the current study aims to investigate the necessary role of NMDAR activation and changes in IEGs expression in developing animals following the OL task. Experiment 1 aimed to confirm that hippocampal NMDARs are necessary for the OL task, but not the OR task in adolescent rats (PD33) by bilaterally infusing an NMDAR antagonist (MK-801) prior to the OL and OR task. Experiment 2 aimed to extend these findings to juvenile animals (PD26) by bilaterally infusing a NMDA antagonist prior to the OL task. Finally, Experiment 3 investigated if IEGs are upregulated at various time points following the OL task in juvenile (PD26) animals.

Chapter 2

EXPERIMENT 1: THE ROLE OF NMDA RECEPTORS IN OBJECT RECOGNITION AND OBJECT LOCATION MEMORY IN ADOLESCENT RATS

Introduction

Our lab has previously shown that systemic injections of MK-801, an NMDAR antagonist, impairs OL performance, but not OR performance (Jablonski et al., 2013). While previous lesion literature provides a strong inference that the impaired performance is due to the blockage of hippocampal NMDARs, there have been no subsequent studies to confirm this hypothesis. Experiment 1 aims to extend our previous results and confirm that dorsal hippocampal NMDARs are necessary for the OL, but not the OR task in PD33 rats by bilaterally infusing MK-801 or PBS into the dorsal hippocampus prior to training and testing.

Materials and Methods

Subjects

Animal colony maintenance has been previously detailed in Jablonski et al., 2013 and Westbrook et al., 2014. The subjects used in all experiments were Long-Evans rats bred and housed in accordance to NIH guidelines at the University of Delaware, Office of Laboratory Animal Medicine (OLAM). Females were time bred and housed in white polypropylene cages 45cm x 24cm x 21cm with standard bedding and were allowed *ad libitum* to food and water. During the light cycle (12:12) cages were

checked for births. The day a newborn litter was found was designated as postnatal (PD) 0. On PD2 litters were moved from the breeding facility to the laboratory colony room. On PD3 the litters were culled to eight pups (typically four females and four males) and paw marked for identification with a subcutaneous injection of nontoxic black ink. On PD21 subject were weaned and housed with same-sex littermates in white polypropylene cages (45cm x 24cm x 21cm) with *ad libitum* food and water.

A total of 61 (32F and 29M) PD33 Long-Evans rats from 16 litters were used as the subjects of Experiment 1. Subjects were assigned to a primary task (OL or OR) and drug condition (phosphate buffer saline (PBS) or MK-801 (dizocilpine maleate)), which was counterbalanced across all littermates to ensure no more than one same-sex littermate per group.

Apparatus

The arena used in all experiments was a white circular chamber made of white polyester resin panels 78.7cm in diameter, 48.9 cm walls, and raised 26.7 cm off the floor. The arena contained two local spatial cues, a black "X" and a striped circle that were placed on the north and west walls of the arenas respectively out of reach of the rat. All sessions were recorded with a digital video camera mounted on a tripod placed behind the south wall of the arena.

Stereotaxic Surgery

Surgical implantation of intrahippocampal cannula in juvenile rats has been detailed previously (Schiffino et al., 2011; Watson & Stanton. 2009; and Robinson-Drummer et al., 2016). Rats were taken from post-weaning group housing on PD30± 1 and were anesthetized with ketamine/xylazine mixture of .1mg/kg prior to surgery with

an injection volume of .85ml/kg. Guide cannula (Plastics One, Roanoke, VA) were bilaterally implanted into the dorsal hippocampus at the following coordinates anteroposterior(AP), +3.3 mm and mediolateral (ML), ±2.3 mm relative to interaural midline coordinates, and dorsoventral (DV), -2.1 mm relative to the top of the skull. Cannulas were then fixed in position with dental acrylic and 'skull hooks'. Following surgery, dummy cannulas attached to dust caps were inserted to reduce obstruction. Post operatively rats were allowed to recover in clear individual housing cages (20.3cm x 33.02cm x 17.78cm) on an electric heating pad placed under half the cage. The following day all the animals underwent cannula clearing were they received .25µl of phosphate buffer saline (PBS) to each hemisphere to adapt them to the infusion procedure and reduce occlusion on the experimental day.

Drug Infusion

On PD33 rats received bilateral microinfusions of either MK-801 (Tocris; Ellisville, Missouri) or PBS (Fisher Scientific, Waltham, MA.) approximately 15 minutes before the OL/OR testing session. During the administration the animals were held while either MK-801 (10µg/µL) or PBS was infused into the dHPC at a rate of .25µL/min for a one minute administering a total of 2.5µg of MK-801 to each hemisphere. Following drug administration, dummy cannulas were replaced and animals were taken back to the colony room were they were remained undisturbed until the start of the testing session.

Procedure

Habituation

Rats were habituated to the arena by undergoing 3 habituation sessions during the light cycle (Westbrook et al., 2014; Ramsaran et al., 2016). The first two habituation sessions occurred one day prior to the testing session on PD32±1. The first habituation session took place between 0700am-1200pm and the second session occurred 5(±1) hrs later. The third habituation session took place the following morning 5(±1) hrs before the testing session. Prior to each habituation session, rats were handled for 3 minutes to familiarize rats with the experimenter and decrease anxiety. At the start of each habituation session animals were weighed and carted to the behavioral testing room in their homecage. Prior to each session the arena was cleaned with a 70% ethanol solution. For all sessions rats were placed in the center of the arena facing the north wall and were allowed to explore the arena freely for 10min.

Object Recognition (OR)

The object recognition task (see Fig. 1A) occurred on the afternoon of PD33±1. Each session consisted of one training and one testing session. During the training session rats were placed in the chamber facing the north wall. Rats were then allowed to freely explore a set of identical objects (fake apples or glass jar; handle always pointing to the east wall; see Jablonski et al., 2013) for 5min. Subjects were then removed for a 5min delay where the arena and object were cleaned with a 70% ethanol solution. Following the delay, rats were placed back into the arena with one previously experienced object and a novel object, not present during the training session, for a 3min test. Additionally, objects and object configuration were counterbalanced across sex and drug condition (Westbrook et al., 2014).

Object Location (OL)

The protocol for the OL (see Fig. 1B) task is similar to the OR task with the exception that one object is moved to a new spatial position during testing. Like the OR task, rats are exposed to two identical objects (flat bottomed hooks see Jablonski et al., 2013) for a 5min training session. Following training subjects are subsequently removed for a 5min delay. For the testing session rats are placed back into the chamber where one object has moved to a new spatial location for a 3min test. Object configuration and spatial movement was counterbalanced across sex and drug condition.

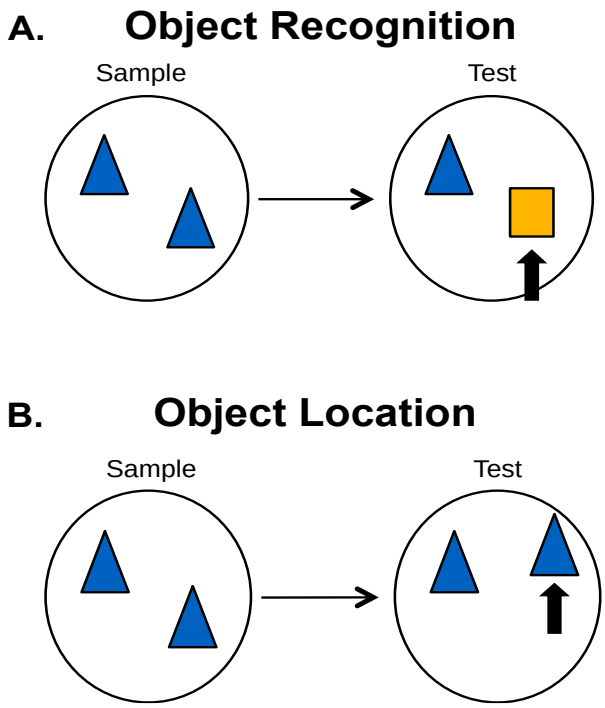

Figure 1 A schematic representation of the Panel A shows the OR task where animals were train on two identical objects during that sample phase and were then presented with a familiar and novel object during the test phase. Panel B show the OL task where animals learn the location of the objects during the sample then during test one object moved to a new location (novel) while the other object does not (familiar). Novel object are indicated in each panel by the black arrow

Data Collection and Analysis

Exploration Ratios

Exploration during all sessions (habituation and testing) was recorded using a video camcorder (Pansonic USA, Model #SDR-85P). During the testing and training sessions, exploration was scored as described by (Jablonski et al., 2013; Westbrook et al., 2014). Digital recordings of all sessions were scored by a blind experimenter using a dual-button timer (Arun Asok, University of Delaware) allowing investigators to score each object independently. Exploration was defined as active sniffing, whisking, or pawing directed toward the object, if animals were sitting on top of an object this was not considered exploration and was excluded. Another observer subsequently analyzed a subset of data in order to calculate inter-observer reliability. Analyses revealed a high agreement between observers (mean r= .816, SEM= .029 p=0.001).

Histology

Within 24-48hrs after behavioral testing rats were sacrificed by rapid decapitation. Brains were removed and frozen in -45° C isopentane and kept in a -81° C freezer until sectioned. Using a microtome brains were sectioned at 40μm slices and mounted on positively charged slides. Following mounting, slides were counter stained with Neutral Red (1%) and photo-captured. Images were then examined to confirm the placement of the guide cannula. This was accomplished by verifying that the tip of the guide cannula terminated in the dorsal hippocampus, defined as Plates 54-72 of the *Rat Brain in Stereotaxic Coordinates 6th Edition* by Paxinos and Watson. Cannula placements of all animals analyzed in Experiment 1 can be seen in Figure 2.

Statistical Analysis

STATISTIC 12 software was used for statistical analyses. Sample phase exploration times were analyzed by sex and by experimental group (experiment dependent) by using a factorial ANOVA. Preference for the novel object during the test phase was obtained by converting exploration times into an exploration ratio defined as: $[t_{novel} / t_{novel +} t_{familiar}]$ (Mumby et al., 2002). Exploration ratios of each group were compared to chance performance (.5), by using a one-sample *t*-test as opposed to an ANOVA. The used of a *t*-test is commonly and consistently used in the novel object recognition literature and can be further explained in Dix and Aggleton (1999) and our previous reports (Jablonski et al., 2013; Westbrook et al., 2014; Ramsaran et al., 2015, 2016). Exploration ratios did not differ across sex (all *p*s<.29.) and therefore this factor was collapsed across groups in all experiments described.

Results

Subjects

Ten of the 61 subjects were excluded from analysis. Of these, 2 animals were dropped due to technical errors (PD33, F, MK-801, OL, n=1; PD33, M, MK-801, OL, n=1). Additionally, 4 animals were lost to post operative surgical complications (PD33, F, PBS, OL, n=1; PD33, F, MK-801, OR, n=1; OR, M, PBS, n=1; M, MK-801, OL, n=1). Another 4 animals were excluded from analysis since they fell outside the accepted dHPC plate ranges (PD33, M, PBS, OL, n=1; PD33, M, MK-801, OL, n=1; PD33, M, PBS, OR n=1; PD33, M, MK-801, OR, n=1). Finally, 4 animals were excluded by meeting outlier criterion defined as any exploration ratio the exceeds of ±1.96 standard deviations away from the group mean (PD33, F, OR, MK-801, n=1; PD33, F, MK-801, OL, n=1; PD33, F, PBS, OL, n=1; PD33, M, OR, MK-, n=1). The

remaining 47 animals were then analyzed (OR-PBS, n=12 (5F; 7M); OR-MK-801, n=12 (6F; 6M); OL-PBS, n=12 (6F; 6M); OL-MK-801, n=11 (5F; 6M)).

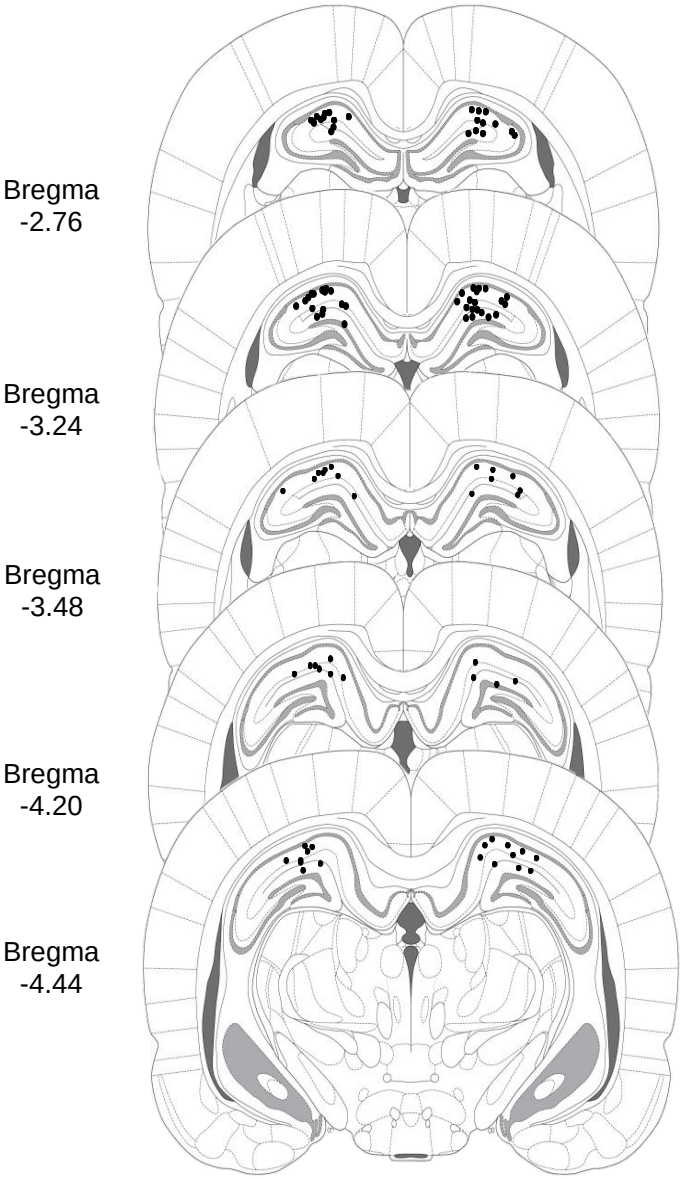
Bregma
-2.76
Bregma
-3.24
Bregma
-3.48
Bregma
-4.20
Bregma
-4.44

Figure 2 Schematic representation of injection cannula tips placements in the dHPC. Animals included in final analyses are represented by black dots, animals excluded from analysis by falling outside of the accepted plate ranges are not shown. Placements ranged from -2.52 mm to -4.68 mm (Plates 54-72) from Bregma. Images are adapted with permission from The Rat Brain in Stereotaxic Coordinates (6th ed.), Paxinos & Watson, 2007, New York, NY: Academic Press. Copyright, 2005 by Elsevier Academic Press.

Sample Phase

Sample exploration was analyzed by a 2 (Sex) x 2 (Drug) x 2 (Task) factorial ANOVA. A main effect of sex was observed [$F(1, 39) = 6.796$, $p=.013$], where females explored significantly less than males. A Drug x Sex interaction was also observed ($F(1, 39) = 16.146$, $p=.00024$). A post-hoc Newman-Keuls tests revealed that this interaction was driven by MK-801 groups where MK-801 (OR & OL) males explored significantly higher that MK-801 (OR & OL) females (PD33, MK-801, OL, F= 46.482± 4.590; PD33, MK-801, OR, F= 44.455± 7.71; PD33, MK-801, OL, M= 77.725± 6.978; PD33, MK-801, OR, M= 77.328± 11.529; all p's<.04; see Fig. 3). These sex differences in sample exploration time did not cause novelty preference ratios to differ across experimental groups (all p's>.311), therefore animals were collapsed across sex in further analyzes. No other main effects or interactions were seen in the exploration time measure (all F's< .239).

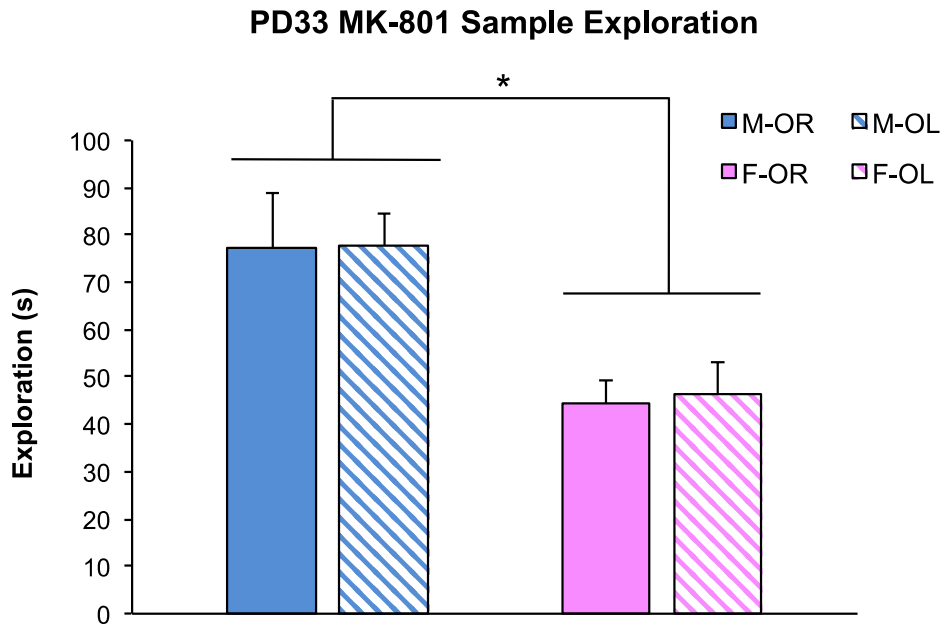

Figure 3 Sample exploration times for the MK-801 group. MK-801 OR and OL males explored significantly more the MK-801 OR and OL females *p<.05.

Test Phase

Figure 4 presents the results obtained from Experiment 1. Figure 4 shows the novelty ratios of P33 rats who received microinfusions of either MK-801 or PBS into the dHPC prior to the OL or OR task. Each group was compared to chance performance of .5. It was found that OR-PBS, OR-MK-801 and OL-PBS groups significantly preferred the novel object in comparison to the familiar object (all ps<.0002). However, the OL-MK-801 group performed at chance performance and did not show a preference for the novel object (p=.16).

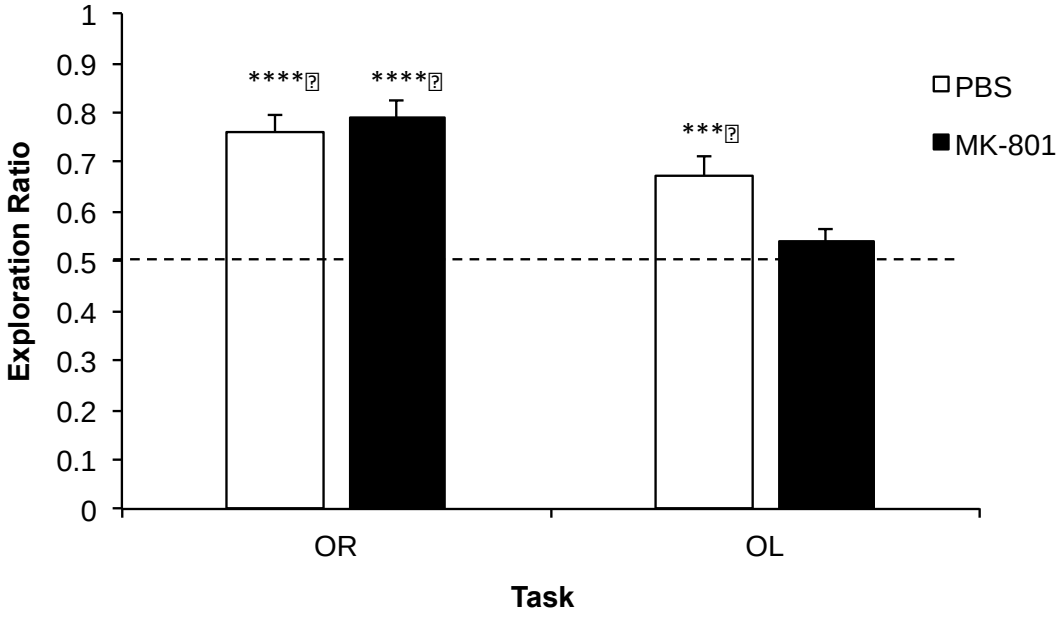

Figure 4 Mean exploration ratios (±SEM) during the OR and OL task by drug condition. The dashed line indicates a chance performance of .5. The OR-PBS group, OR-MK-801, and OL-PBS group preferential explored the novel object (****p<.00001; ***p<.001). The OL-MK-801 group however, failed to exhibit a novelty preference (p=.16).

Discussion

As an extension of our previous report, Experiment 1 provides confirmation that hippocampal NMDARs are necessary for spatial learning OL, but not non-spatial OR learning. This is seen in the results of Experiment 1 where animals in the MK-801 OR group preferentially preferred the novel object as opposed to the MK-801 OL group, which failed to show preference for the novel object.

Chapter 3

EXPERIMENT 2: THE ROLE OF NMDA RECEPTORS IN OBJECT LOCATION MEMORY IN JUVENILE RATS

Introduction

Experiment 1 provided confirmation that hippocampal NMDARs are necessary for the OL task, but not the OR task in adolescent animals. In an extension of Experiment 1, Experiment 2 aims to confirm the role of NMDARs in juvenile (PD26) animals, closer to the ontongenic emergence of the OL task.

Materials and Methods

Subjects

The subjects used were 37 (19F; 18M) PD26 Long-Evans rats from 10 liters. Animal colony maintenance was the same as described in Experiment 1. Animals were assigned to two drug conditions (MK-801 or PBS), and received microinfusions of either PBS or MK-801 bilaterally into the dorsal hippocampus, all conditions were counter balanced to avoid over sampling of littermates.

Stereotaxic Surgery

The stereotaxic surgery protocol used was identical to the one used in Experiment 1 with minor changes. Since the subjects of Experiment 2 were juveniles (PD26), rats underwent surgery on PD23±1. The stereotaxic coordinates were also adapted to accommodate the juvenile animals. The coordinates used were as follow:

anteroposterior (AP), +2.6 mm, mediolateral (ML), ± 2.3 mm, relative to interaural measurements and -2.0mm dorsoventral (DV) from the top of the skull. Placement of cannula hits can be seen in Figure 5.

Drug Infusion

The drug infusion procedure was the same as Experiment 1, with the exception that rats received microinfusion of MK-801 or PBS on PD26 before training and testing as opposed to PD33.

Apparatus

The apparatus used was the same chamber described previously in Experiment 1.

Procedure

Habituation

Rats received 3 10min habituation sessions identical to the protocol outlined in previous experiment.

Object Location (OL)

The OL task remained the same as Experiment 1, but adjustments were made to the training, delay and testing times used. The PD26 group underwent a 4 min training session followed by a 50 min delay interval, and a 4 min test, as opposed to the 5min training, 5min delay, and 3min testing used in Experiment 1. This change in protocol was used to match the procedures in Experiment 3 that examined task-related gene expression (see below).

Data Collection and Analysis

Exploration Ratios

Data was collected and analyzed in the same fashion as Experiment 1. Similarly, a blind experimenter analyzed a subset of the data to calculate inter-observer reliability. Analyses reveled a high agreement between observers (mean *r*=.85, SEM=. 04 p=.03)

Histology

All histological procedures were the same as Experiment 1.

Statistical Analysis

Sample exploration data was analyzed as previously described in Experiment 1 by using factorial ANOVAs.

Results

Subjects

Seventeen of the 37 subjects were excluded from analysis. Of these, 7 animals were dropped due to technical errors (PD26, F, PBS, n=1; PD26, M, PBS, n=2; PD26, F, MK-801, n=3; PD26, M, MK-801, n=1). Additionally, one was lost to post operative surgical complications (PD26, F, PBS, n=1). Another 9 animals were excluded from analysis by falling outside the accepted dHPC plate ranges (PD26, F, PBS, n=2; PD26, M, PBS, n=2; PD26, F, MK-801, n=2; PD26, M, MK-801, n=3). Lastly 2 animals were excluded by meeting outlier criterion of ±1.96 standard deviations away from the group

mean (PD26, F, PBS, n=1; PD26, M, MK-801, n=1). The remaining 18 animals were then analyzed (PBS=9(5F; 4M); MK-801=9(5F; 4M)).

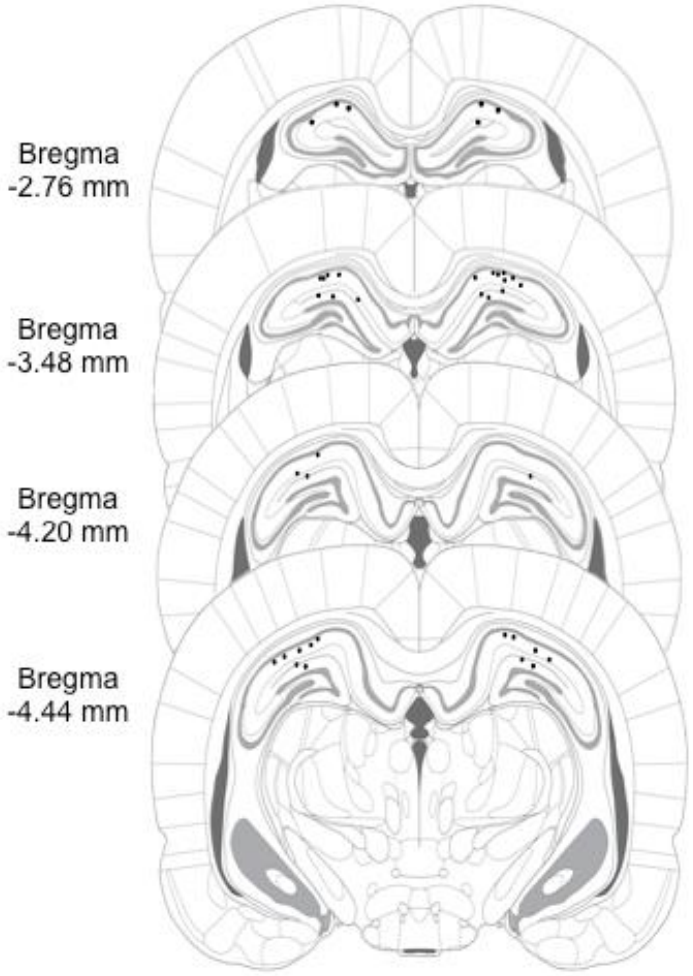

Figure 5 Schematic representation of injection cannula tips placements in the dHPC. Animals included in final analyses are represented by filled black dots, animals excluded from analysis are not shown. Placements ranged from -2.52 mm to -4.68 mm from Bregma. From The Rat Brain in Stereotaxic Coordinates (6th ed.), Paxinos & Watson, 2007, New York, NY: Academic Press. Copyright, 2005 by Elsevier Academic Press. Adapted with permission.

Sample Phase

Sample exploration was analyzed by to observe any difference between drug condition and sex by using a 2 (Sex) x 2 (Drug) factorial ANOVA. No main effects or interactions were observed (all F's≤.441). Mean exploration time was 52.62 ±4.975.

Test Phase

Figure 6 displays the results obtained from Experiment 2. Figure 6 shows the mean exploration ratios of PD26 rats who received microinfusion of MK-801 or PBS into the dHPC prior to OL training and testing. Each group was compared to chance performance of .5. The OL-PBS group significantly preferred the novel object ($p<.0001$). However the, OL-MK-801 group performed at chance and did not show a preference for the novel object ($p=.79$).

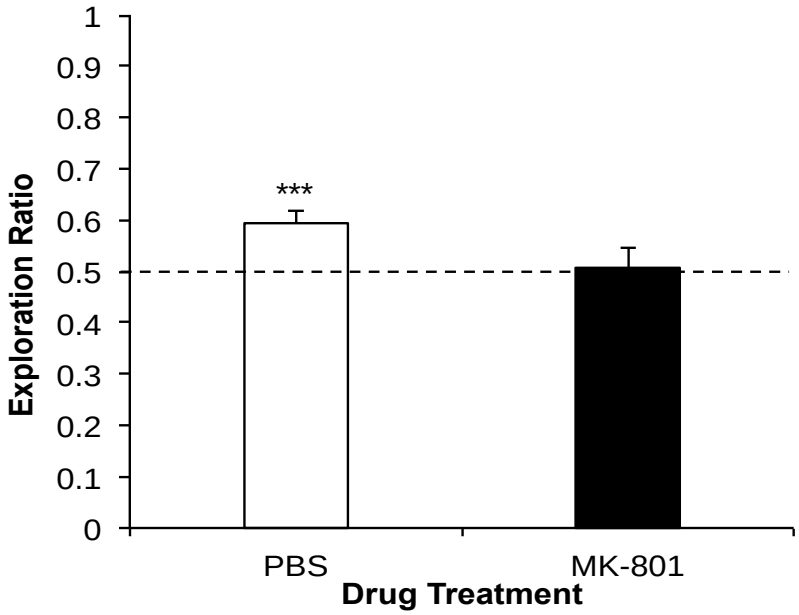

Figure 6 Mean exploration ratios (±SEM) during the OL task by drug condition. The dashed line indicates a chance performance of .5. The OL-PBS group preferential explored the displaced object (***p<.001). The OL-MK-801 group however, failed to exhibit a novelty preference (p=.79).

Discussion

Similarly to Experiment 1, bilateral infusions of MK-801, an NMDAR antagonist, impaired OL performance in juvenile rats. Collectively these results suggest the NMDARs in the hippocampus are necessary for OL task performance.

Chapter 4

EXPERIMENT 3: CHANGES IN IMMEDIATE EARLY GENE EXPRESSION AFTER OBJECT LOCATION LEARNING IN JUVENILE RATS

Introduction

Experiments 1 and 2 investigated the role of hippocampal NMDAR antagonism on the OL task. Experiment 3 aimed to observe if immediate early genes are upregulated after the OL task. In the scope of Experiment 3, we investigated changes in two of the most commonly studied IEGs: c-Fos and BDNF. Both of these genes have been implemented in synaptic remodeling and long-term potentiation. Furthermore c-Fos and BDNF have are upregulated after hippocampal dependent learning tasks, and therefore an upregulation should be expected to be seen following the OL tasks in juvenile animals (Guzowski et al, 2002; Mendez et al., 2016; Alonso et al., 2002).

Materials and Methods

Subjects

The subjects used were 60 (30F; 30M) PD26 Long-Evans rats from 14 liters. Animal colony maintenance was the same as described in Experiment 1. Animals were assigned to either the OL task or homecage (HC) control groups at random. OL animals were then assigned to one of four sacrificing time delays (Immediately (IMM), 15min, 30min, or 90min after OL testing). All conditions were counter balanced across cohorts and littermates to ensure no oversampling occurred.

Apparatus

The apparatus used in Experiment 3, was the same apparatus used in Experiment 1 and 2.

Procedure

Habituation:

All subjects that underwent the OL task under went three habituation sessions, outlined in Experiment 1.

Object Location (OL)

The protocol for the OL task is the same as Experiment 2. Animals receive a 4min training session followed by a 50min delay. Following the delay animals underwent a 4 min test. This protocol was adopted from Mendez et al., 2015 which looked at IEG expression in adults following the OL task. Following OL testing, animals were placed back in the colony room were they were kept until they were sacrificed by rapid decapitation immediately, 15min, 30min, or 90min after testing.

Realtime-PCR

The dorsal hippocampus (Bregma −3.00 mm to approximately −4.68 mm), and the perirhinal cortex (dissected from the same sections as the hippocampus), were collected from one hemisphere from each animal (hemispheres taken from was counter balanced across all groups of animals) for gene expression analysis. mRNA was extracted from the hippocampus and perirhinal cortex by using RiboZol RNA Extraction Reagent (Cat. No. 97064-950, VWR, Radnor, PA). Extracted RNA then underwent DNase treatment and cDNA synthesis using the protocol and QuantiTect Reverse Transcription Kit from QIAGEN (Cat. No. 205314). Relative gene expression

was measured using Quanta-Bio SYBR Green Supermix (Cat. No 101414-210) in 10-µl reactions on a CFX96 Touch™ real-time PCR machine. All primers were ordered (Integrated DNA Technologies) and diluted to a concentration of 0.65 µM used in the real-time PCR reaction. The primer sequences used are: 18s: Forward: ATGGTAGTCGCCGTGCCTA; Reverse: CTGCTGCCTTCCTTGGATG; c-fos: Forward: CAGCCTTTCCTACTACCATTCC; Reverse: ACAGATCTGCGCAAAAGTCC; BDNF: Forward: ATCCCATGGGTTACACGAAGGAAG; Reverse: GTAAGGGCCCGAACATACGATTG.

Data Collection and Analysis

Object Location Analysis

Data was collected and analyzed in the same fashion as Experiment 1 and 2. Similarly, a blind experimenter analyzed a subset of the data to calculate inter-observer reliability. Analyses reveled a high agreement between observers (mean r=.821, SEM=.045, p=.024).

RT-PCR Analysis

For each sample the quantitative threshold amplification cycle number (C_q) was obtained, and the $2^{-\Delta\Delta Cq}$ method was used using 18s as the house keeping reference gene to calculate the relative gene expression (Posillico et al., 2015). House keeping gene expression did not differ across groups when a one-way ANOVA was used to analyze gene expression by time sacrificed for both the pRh cortex and the dHPC (all F's <.79; Mean expression= 12.71± .11).

Statistical Analysis (Extended)

Sample exploration data was analyzed in a similar way as previously described in Experiment 1 and 2. Factorial ANOVAs were used on both the sample exploration data and relative gene expression data in regards to sex and groups (Time Sacrificed). Gene expression data was then further analyzed by using one-way ANOVAs and planned comparisons to compare gene expression to a homecage average value (explained further in results).

Results

Subjects (Behavior)

Four of the 60 subjects were excluded from analysis. Of these, 1 animal was dropped due to technical errors (PD26, M, OL-15min; n=1). Additionally, three animals were dropped from the analysis, as they did not reach the minimum criteria for exploration (≥1s of exploration); PD26, M, OL-IMM; n=1; PD26, F, OL-IMM; n=1; PD26, F, OL-90min; n=1). Additionally, 5 animals were excluded from behavior analysis by meeting outlier criterion of ±1.96 standard deviations away from the group mean (PD26, F, OL-IMM; n=2; PD26, F, OL-15 min; n=1; PD26, M, OL-30 min; n=1; PD26, F, OL-90 min; n=1). The remaining 51 animals were then analyzed (OL-IMM n=9 (6M; 3F); OL-15 min n=11 (5M; 6F); OL-30 min n= (5M; 6F); OL-90 min (6M; 5F).

Sample Phase

Sample exploration was analyzed by a 2 (Sex) x 5 (Time Sacrificed: *HC, IMM, 15min, 30min, & 90min*) factorial ANOVA. No main effects or interactions were observed (all *F*'s≤.1.40). Mean exploration time was 40.86± 2.206.

Test Phase

Behavioral results from Experiment 3 are depicted in Figure 7. The mean exploration ratios of PD26 rats indicate rats who underwent the OL task preferred the novel object above chance (all *p*s<.01). Additionally, the mean exploration rations between the OL groups did not differ (all *p*s>.51)

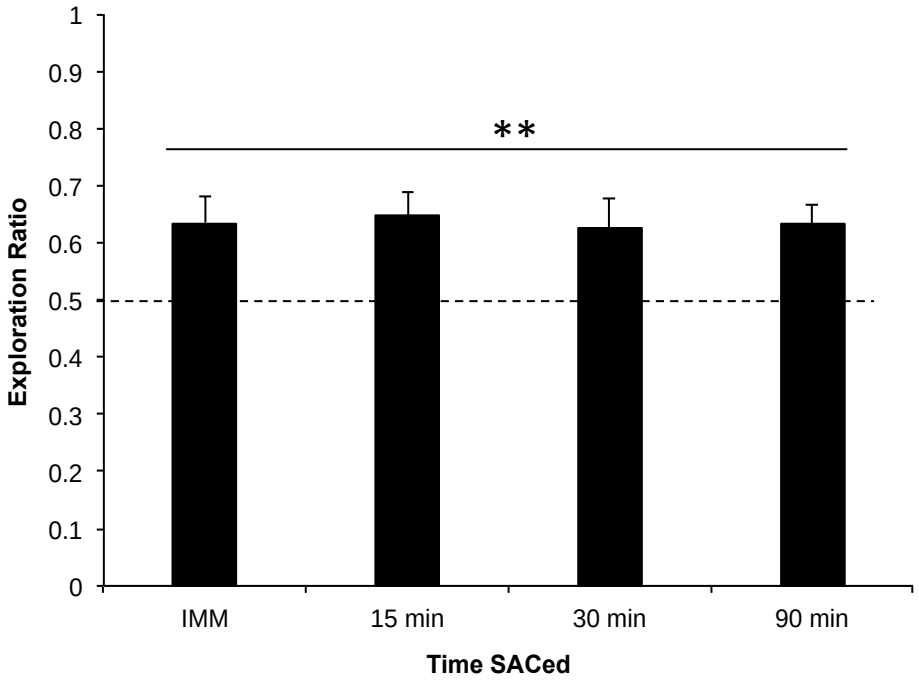

Figure 7 Mean exploration ratios (±SEM) for the OL task. The dashed line indicates a chance performance of .5. All OL groups preferential explored the displaced object over the familiar object (**p<.01).

Dorsal Hippocampus

Subjects (General):

Of the 56 animals ran through the OL task, outliers (±1.96 standard deviations away from the group mean) were determined separately for each gene and for behavior, unless a significant correlation was found between novelty scores and relative gene expression. In such a case, the both behavioral and gene-expression outliers were removed from that group's data set.

c-Fos dHPC Subjects

For the c-Fos relative gene expression data, 1 sample was dropped due to aberrant mRNA concentration after mRNA extractions (PD26, OL-90min, M, n=1). An additional 3 data points were also removed by meeting outlier criterion of ±1.96 standard deviations away from the group mean (PD26, OL-15min, M, n=1; PD26, OL-30min, M, n=1; PD26, OL-90min, M, n=1). Additionally, only the OL-IMM group showed a correlation between novelty scores and relative gene expression (r=-.723), therefore both behavioral outliers were removed from the data set (PD26, OL-IMM, F, n=2). The remaining 51 data points were then analyzed (OL-IMM n= 9 (3F; 6M); OL-15min (n=10 (5F; 5M) OL-30min n=11 (6F; 5M); OL-90min n=11 (6F; 5M); Homecage n=10 (4M; 6F).

BDNF dHPC Subjects

For the relative gene expression, 2 data points were removed for meeting outlier criterion of ±1.96 standard deviations away from the group mean (PD26, OL-30min, M, n=1; PD26, HC, M, n=1). The remaining 53 gene expression data points were then

analyzed (OL-IMM n= 11 (F5; M6); OL-15min (n=10 (5F; 5M) OL-30min n=11 (6F; 5M); OL-90min n=12 (6F; 6M); Homecage n=9 (3M; 6F).

Changes in Gene Expression after OL Testing

All relative gene expression results are graphically represented in the Figure 8 and 9 and specific results per region and gene is outlined below.

c-Fos dHPC Gene Expression Results

Relative gene expression was first analyzed by a factorial ANOVA 2 (Sex) x 5 (Time Sacrificed: *HC, IMM, 15min, 30min, & 90min*). No main effects or interactions involving sex were observed (all *F*'s≤.323). Sex was then dropped as a variable to which a one-way ANOVA (Time Sacrificed: *HC, IMM, 15min, 30min, 90min*) was used. No main effect was seen (*F*≤.2.407). A planned comparison against HC reveled a significant difference between the HC group and the OL-IMM, OL-15min and OL-90min groups (all *p*'s <.033). Furthermore, no differences were seen between the HC group and the OL-30min group (*p*=.37).

BDNF dHPC Gene Expression Results

Relative gene expression was first analyzed by a factorial ANOVA 2 (Sex) x 5 (Time Sacrificed: *HC, IMM, 15min, 30min, & 90min*). No main effect or interactions were observed (all *F*'s≤.323). Sex was then dropped as a variable to which a one-way ANOVA (Time Sacrificed: *HC, IMM, 15min, 30min, 90min*) was used. A main effect of Time sacrificed was seen [$F(4, 47) = 4.992$, $p=.002$]. Planned comparisons against HC and time sacrificed reveled a significant differences between the HC group and the OL-15min and OL-90min groups (all *p*'s <.033). However, no differences were seen between the HC group and the OL-IMM and OL-30min group (all *p*'s>.42).

Perirhinal Cortex

Subjects (General)

Similarly to the dHPC gene analysis, outliers determined for each gene were determined separately from behavioral outliers, unless a significant correlation was found between novelty scores and relative gene expression.

c-Fos pRh Subjects

For the c-Fos relative gene expression data, 3 samples were dropped due to aberrant mRNA concentration after mRNA extractions (PD26, OL-30min, F, n=1; PD26, OL-30min, M, n=1; PD26, OL-IMM, M, n=1;). An additional 4 data points were also removed by meeting outlier criterion of ±1.96 standard deviations away from the group mean (PD26, OL-IMM, M, n=2; PD26, OL-30min, M, n=1; PD26, OL-90min, F, n=1). The remaining 49 gene expression data points were then analyzed (OL-IMM n= 8 (5F; 3M); OL-15min n=11 (6F; 5M) OL-30min n=9 (5F; 4M); OL-90min n=11 (5F; 6M); Homecage n=10 (4M; 6F).

BDNF pRh Subjects

For the relative gene expression, 5 data points were removed as outliers (PD26, OL-IMM, F, n=1; PD26, OL-15min, F, n=1; PD26, OL-15min, M, n=1; PD26, OL-30min, M, n=1; PD26, OL-90min, M, n=1). The remaining 48 gene expression data points were then analyzed (OL-IMM n= 9 (F4; M5); OL-15min (n=10 (5F; 5M) OL-30min n=9 (5F; 4M); OL-90min n=11 (6F; 5M); Homecage n=9 (4M; 5F).

Results

c-Fos pRh Gene Expression Results

Relative gene expression was first analyzed by a factorial ANOVA 2 (Sex) x 5 (Time Sacrificed: *HC, IMM, 15min, 30min, & 90min*). No main effects or interactions were observed (all *F*'s≤.9825). Sex was then dropped as a variable to which a one-way ANOVA (Time Sacrificed: *HC, IMM, 15min, 30min, 90min*) was used. A main effect was seen ([$F(4, 44) = 6.796$, p=.013]). A planned comparison against HC reveled a significant difference between the HC group and the OL-IMM, OL-15min and OL-30min groups (all *p*'s <.03). No differences were seen between the HC group and the OL-30min group (p=.33).

BDNF pRh Gene Expression Results

Relative gene expression was first analyzed by a factorial ANOVA 2 (Sex) x 5 (Time Sacrificed: *HC, IMM, 15min, 30min, & 90min*). No main effects or interactions were observed (all *F*'s≤1.98). Sex was then dropped as a variable in a one-way ANOVA (Time Sacrificed: *HC, IMM, 15min, 30min,& 90min*). Again no main effect was seen (F=1.6256). A planned comparison against HC revealed a significant difference between the HC group and the OL-15min group (all p<.03). No differences were seen between the HC group and the OL-IMM, OL-15min, OL-30min, and OL-90min groups (p<.08).

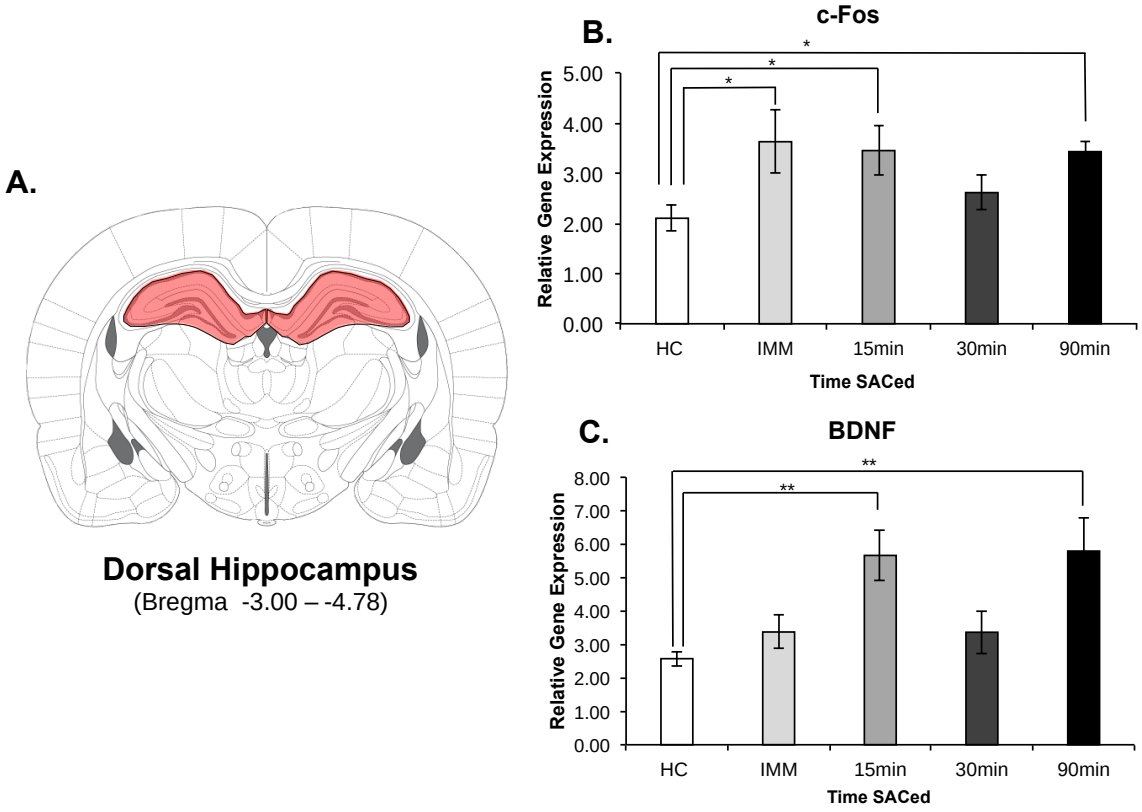

Figure 8 Relative gene expression (±SEM) in the dorsal hippocampus of PD26 rats after OL testing. Figure 6A show a schematic of the dHPC region (highlighted in red) taken for RNA extraction and cDNA synthesis used for real-time PCR. Figure 6B represents the relative gene expression (c-Fos) of OL animals sacrificed at difference time intervals (IMM, 15min 30min, 90min) in comparison to homecage controls. The IMM, 15min and 90min groups showed a significant increase in c-Fos expression in comparison to HC animal (*p<.05). Figure 6C depicts the relative gene expression (BDNF) of OL animals. Only the OL-15min and OL-90min groups showed a significant increase in gene expression in comparison to HC controls (**p<.01).

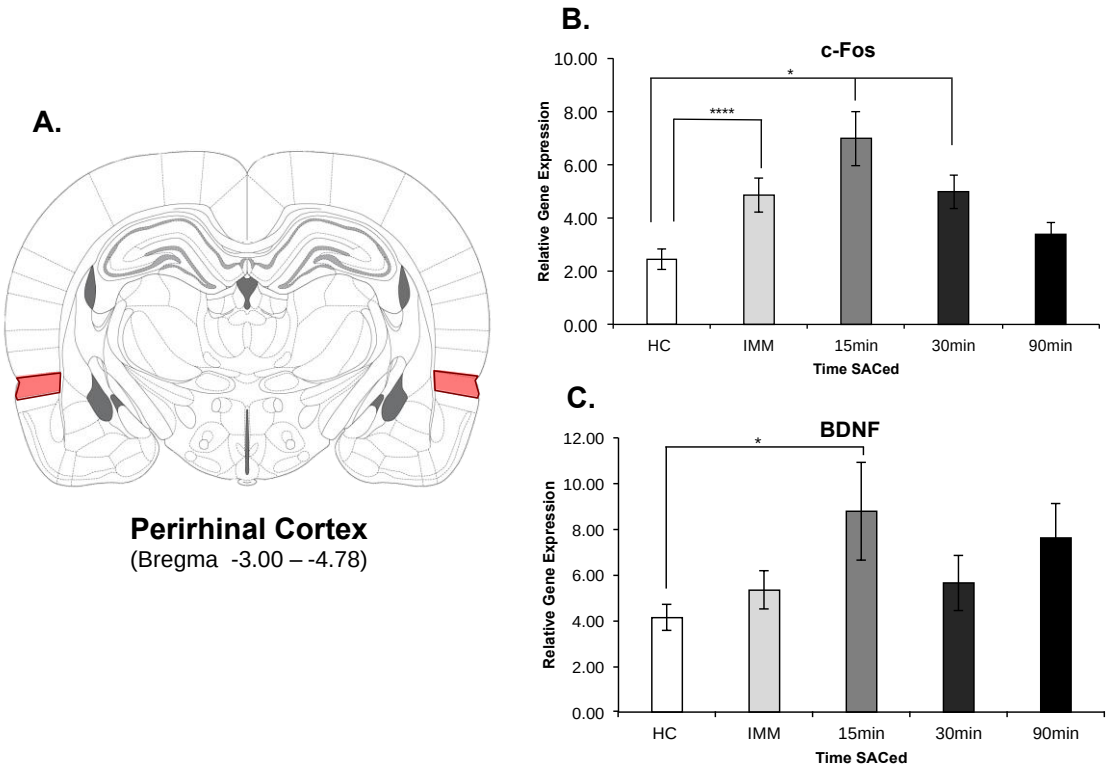

Figure 9 Relative gene expression (±SEM) in the perirhinal cortex of PD26 rats after OL testing. Figure 6A show a schematic of the pRh region (highlighted in red) taken for RNA extraction and cDNA synthesis used for real-time PCR. Figure 6B represents the relative gene expression (c-Fos) of OL animals sacrificed at difference time intervals (IMM, 15min 30min, 90min) in comparison to homecage controls. The IMM, 15min and 30min groups all showed a significant increase in c-Fos expression in comparison to HC controls(*p<.05; ****p<.0001). Figure 6C depicts the relative gene expression (BDNF) of OL animals. Only the OL-15min group showed a significant increase in gene expression in comparison to HC controls (*p<.05).

Discussion

The results of Experiment 3 provide evidence that IEGs are differentially upregulated in regards to time sacrificed and brain region of interest. In the dorsal hippocampus c-Fos and BDNF were seen to be upregulated in a biphasic pattern.

However, in the perirhinal cortex we do not see a biphasic wave of expression in c-Fos or BDNF. Instead we see bell shape curve for pRh c-Fox expression and a single rise in pRh BDNF. These results provide evidence that IEGs are upregulated in the juvenile animals after undergoing a hippocampal learning event (see General for in depth analysis).

Chapter 5

GENERAL DISCUSSION

The results of the present study begin to elucidate the underlying neuronal mechanisms necessary for object location learning in developing animals. Experiment 1 extended our previous report by providing evidence that NMDARs in the dorsal hippocampus (dHPC) are necessary for the object location (OL) task, but not the object recognition (OR) task in adolescent rats (PD33). Experiment 2 extends these results by confirming that NMDAR antagonism impairs OL performance in juvenile (PD26) rats. Lastly, Experiment 3 sought to observe changes in immediate early gene (IEG) expression at various time points following OL testing. Results from Experiment 3 presents evidence that IEG upregulation is dependent on the time sacrificed, brain region, and gene of interest. Within the hippocampus c-Fos expression peaked immediately, 15min, and 90min after OL testing when compared to homecage controls. Hippocampal BDNF expression however, peaked only 15min and 90min following OL testing. In regards to regional differences, peak expression times in the perirhinal cortex (pRh) differed from peak expression times seen in the hippocampus. Within the pRh c-Fos expression rose immediately, 15min, and 30min after OL testing, while BDNF expression only reached a peak level 15min following testing.

Experiment 1 and 2 extended our previous findings that NMDARs in the hippocampus are required for OL performance in adolescent animals. The critical role of the NMDARs in learning and memory has been extensively studied since their discovery in 1981 (Watkin, 1981). Since their discovery, many studies have

demonstrated the necessity of NMDARs in hippocampal dependent learning task in adult and developing rats (Schiffino et al., 2011; Heroux et al., 2016; Morris et al, 1986; Heale & Harley, 1989; Jablonski et al., 2013). In regards to novel object recognition, more specifically object location, only one other study to our knowledge has investigated the role of hippocampal NMDARs in the OL task. Similarly to Experiments 1 and 2, intracranial administration of MK-801, an NMDAR antagonist, into the hippocampus disrupts OL performance in adult rats (Luiz et al, 2009). Our findings extend this study by demonstrating that NMDARs are necessary for hippocampal learning closer to the time when OL performance emerges ontogenetically (i.e., PD19-23, Rudy & Morledge, 1994, Westbrook et al., 2014; Jablonski et al., 2013; Schiffino et al., 2011).

Immediate early genes (IEGs) are a set of genes that are rapidly transcribed in neuronal assembles in association with learning and memory. More specifically, IEG expression is upregulated in response to hippocampal dependent-learning paradigms (Guzowski et al, 1999; Guzowski et al, 2002 Farina & Commins, 2016; He et al., 2002). Knowing IEG expression is a hallmark of neuronal activation, Experiment 3 sought to observe changes in key IEGs after completing object location testing. Similarly to other behavioral tasks we saw an increase in c-Fos and BDNF mRNA expression in the hippocampus following OL testing. Additionally, our results provided a novel insight that peak IEG expression differed temporally across brain regions (dHPC/pRh) and the genes of interest (c-Fos/BDNF). We found hippocampal c-Fos exhibits a biphasic pattern of expression with the first wave of upregualtion occurring immediately and 15min after testing, followed by a second wave of upregulation occurring 90min after testing. These results converge on recent findings that c-Fos shows early and late

biphasic activity. This biphasic upregulation is commonly seen in response to hippocampal learning, providing support for the validity of the results found in the current study (Katche et al., 2009, Ivashkina et al., 2016).

Our results regarding hippocampal BDNF yield a similar biphasic pattern of expression where the first wave of upregulation is seen at 15min followed a successive wave at 90min. These results are in agreement with studies that have shown that activity dependent BDNF expression increases 60min-120min in the hippocampus (Igaz et al., 2002; Alonso et al., 2002, Pollak et al., 2005). Surprisingly, our results also indicate an early onset peak at 15min, which has not to our knowledge been previously reported in the literature and is discussed in further detail below.

Experiment 3 also looked at the time course of both c-Fos and BDNF expression in the perirhinal cortex, which have been seen as critical neural substrates of OR (Brown & Aggleton, 2001). While many studies have looked at c-Fos upregulation at single time points ranging from 30-120min, very few have looked at a time course of expression across behavioral measures (Barbosa et al., 2013; Mendez et al., 2015; Beck & Fibiger, 1995). The results acquired from Experiment 3, provides evidence that pRh c-Fos shows a gradual rise and decline in gene expression, as opposed to the biphasic upregulation seen in the dHPC. Unlike c-Fos, BDNF expression following learning events has been reported to peak at 120min, and has not been demonstrated a short-term upregulation (Klaus et al., 2013). Our data from Experiment 3 however, shows an early increase in pRh BDNF at 15min following OL learning opposing to what has been reported previously.

While this experiment is the first to exhibit regional gene specific IEG time courses after the OL task, limited studies have been conducted investigating the role of

gene expression in the OR and OL tasks. The most comprehensive study looking at OL performance saw an upregulation in c-Fos in the hippocampus, specifically the dentate gyrus, CA1 and CA3, 90min after OL testing in adult rats (Mendez et al., 2015). While our study replicates this subregion specific trend regionally in our 90min hippocampal c-Fos group, the current study extends these findings by discovering an initial wave of hippocampal c-Fos expression immediately and 15min after OL learning. Furthermore, Mendez et al. failed to see a rise in pRh c-Fos expression at 90min. While we replicated their pRh c-Fos results at 90min, we also found an upregulation immediately, 15min, and 30min after OL learning not previously reported. In regards to BDNF expression to our knowledge this is the first evidence presented that pRh BDNF expression is upregulated following the object location task in juvenile animals. Altogether, the current experiment provides novel evidence that juvenile animals may exhibit similar gene profiles as their adult counter parts. This similarity in expression profiles between adults and juveniles may suggest that the hippocampus is showing adult like functioning at PD26, which may not be present at younger ages where the hippocampus fails to show adult like functioning.

In interpreting the collective data set, a broad set of conclusions can be drawn in regards to the role of NMDARs and the cyclic nature of IEG upregulation expression. The literature indicates that c-Fos and BDNF expression is necessary for long-term memory (LTM). Additionally, the inhibition of c-fos and BDNF transcription impairs hippocampal learning (i.e contextual fear conditioning and spatial learning) (Paylor et al., 1994; Alonso et al., 2002; Bramham Guzowski & McCaugh 1997; Mizuno et al., 2000). Furthermore, in recent years evidence has suggested that IEG upregulation is not necessary for the acquisition or expression of short-term memory (STM), but IEG

upregulation is needed for the maintenance and potentiation of memories instead (for review see Guzowski, 2002). Knowing that IEG upregulation is necessary for LTM, but not STM we suggest that the differential waves of IEG expression, are representative of different phases of the learning event. Notably it is possible that the early wave of hippocampal c-Fos expression is representative of the encoding of the new spatial location of the moved object, while the subsequent wave represents a long-term consolidation of the displaced object in an effort to update the spatial map constructed during training.

In addition to the biphasic pattern seen in hippocampal c-Fos upregulation, we see a similar biphasic pattern in hippocampal BDNF as well. Here we speculate the uprgulation of BDNF may act upon c-Fos expressing neurons promoting subsequent wave of c-Fos expression promoting long term potentiation of the moved object. While this claim is highly speculative, there is evidence that c-Fos and BDNF expression is colocalized, and that c-Fos and BDNF are both able to induce transcription of each-other (Dong et al. 2006; Cohen et al., 2011; Kellner et al., 2014). In regards to the current study, we speculate that co-activation and biphasic patterns of hippocampal IEGs upregulation may be necessary to remodel synaptic connections and thereby update the spatial map that has changed since training.

Another interesting finding of this report is the rise and fall of c-Fos expression in the perirhinal cortex. While the pRh provides input to the hippocampus, this parahippocampal region has been more commonly implicated in the OR task as opposed to the OL task (Buckley, 2011; Ennaceur & Aggleton, 1997; Mumby et al., 1997; Seoane et al., 2012). Since pRh detects differences in object identity, we suggest that the early rise of c-Fos and BDNF are involved with recognition of the object identity

(Seoane et al., 2012). In contrast to the hippocampal results, no second wave is needed in the OL task due to a lack of novel object identity. Unlike OR, the object identity in the OL task does not change between the training and testing phases, therefore there is no need to update the object identity representation formed during the training session and subsequently no need for a second consolidating wave a gene upregulation in the OL task.

In connection to Experiment 1 and 2, there is also the strong possibility that NMDARs are necessary for the induction of these IEG waves to occur. Previous studies have presented evidence that NMDAR driven depolarization induces IEG expression (Cole et al., 1989; Bading et al., 1995; Beilharz et al., 1993). Knowing that NMDARs depolarization is capable of inducing IEG expression, it is probable that the changes in IEG expression needed for LTM rely on NMDAR depolarization. While this claim lacks confirmation in the literature, this theoretical framework allows us to consider an interesting synergetic relationship between NMDAR dependence (Exp. 2) and IEG expression (Exp. 3) in the OL task.

While the current study presents novel findings regarding the role of NMDARs and IEGs in learning during ontogeny, there are a number of caveats present in the current data that would need to be addressed when moving forward. While our time intervals for Experiment 3 were derived from a previous study (Martinez et al. 2016), it is possible that the first wave of gene expression is an artifact of the training session experienced 50min prior to the testing session. To confirm our proposed two wave theory, we propose to complete the same study but with a 24hr retention interval, to temporally separate IEG upregulation during training from testing. Additionally, in order to control for the effect of object exposure alone, a no-novelty control group

should be added to all future studies to confirm that all IEG changes observed are the results of a learning event as opposed to object exposure per se. Finally while we speculate about the role of NMDAR activation in regards to regulating IEG expression, it is necessary to perform a study looking at IEG expression following bilateral infusions of an NMDAR antagonist to confirm our hypothesis.

In addition to addressing certain speculative questions to further support the current findings, these findings open an interesting set of hypotheses regarding the ontogenetic emergence of the OL task. As briefly described earlier, performance on hippocampal dependent tasks emerges between PD19 and PD21. While the time course for IEG expression for the OL task was conducted on PD26 rats, the results obtained begs the question of whether or not IEG upregualtion is seen in younger ages. Further studies are needed to determine whether the developmental emergence of hippocampal dependent learning correlates with IEG upregulation.

In summary the current report suggests there are a number of activity dependent plastic changes necessary and transcribed during the OL task. This report starts to shed light on the fact that OL learning in developing animals is dependent on hippocampal NMDARs, while OR is not. Additionally, the current study also provides novel evidence that c-Fos and BNDF exhibit temporally distinct upregulation patterns, which differ per brain region in the developing animal.

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Appendix

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APROVAL FORM

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

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Title of Protocol: Rodent Models of Cognitive Development	
AUP Number: 1104-2016-2	← **(4 digits only)**
Principal Investigator: Mark E. Stanton, Ph.D.	
Common Name: Rat **Genus Species:** Rattus novegicus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ **B**	Breeding or holding where NO research is conducted
☐ **C**	Procedure involving momentary or no pain or distress
☐ **D**	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☒ **E**	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: Glen Talbot, DVM

Date of Approval: 6/1/16