ADULT HIPPOCAMPAL NEUROGENESIS AND CELL ACTIVATION ARE REGULATED BY SEX DIFFERENCES IN SPATIAL LEARNING

by

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ABSTRACT

Adult hippocampal neurogenesis is associated with hippocampus-dependent learning and memory. Throughout the course of a new neuron's development, it is differentially sensitive to factors that can influence its survival and subsequent functionality. Previous research shows that in male rats, spatial training that occurred 6 to 10 days after an injection of the DNA synthesis marker, bromodeoxyuridine (BrdU), increased cell survival, but no change was observed in animals trained on days 1 to 5 or 11 to 15 and perfused 16 days after BrdU injection (Epp et al., 2007). Because sex differences favouring males in spatial cognition and in hippocampal neurogenesis have been reported, it is unclear whether spatial learning would influence hippocampal neurogenesis in the same way in males and females. Therefore, this study aimed to compare sex differences in hippocampal neurogenesis relative to training in a spatial task. Male and female rats were exposed to training in the spatial or cued version of the Morris Water Maze 6 to 10 days after one injection of BrdU (200mg/kg). Twenty days following BrdU injection, all animals were given a 30-second probe trial and perfused. Males showed better performance in the spatial task, but not cue task, than females. Spatial learning increased the density of BrdU-labeled cells relative to cue training only in males, but both males and females showed greater cell activation (BrdU co-labeled with immediate early gene product zif268) after spatial training compared to cue training. Furthermore, performance during spatial training and testing were positively correlated with cell activation in females but not males. This study shows that while spatial learning differentially regulates hippocampal neurogenesis in males and females, the activity of new neurons in response to spatial memory is similar. These findings highlight the importance of sex on neural plasticity and cognition.

PREFACE

An abbreviated version of this thesis has been submitted on June 1, 2012.

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Ethics approval for this experiment was obtained from the animal care committee at the University of British Columbia. Certificate #: A10 0080.

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ABBREVIATIONS

ANOVA	Analysis of variance
BrdU	Bromodeoxyuridine
CORT	Corticosterone
CREB	Cyclic adenosine monophosphate response binding protein
DAB	Diaminobenzidine
DCX	Doublecortin
GCL	Granule cell layer
IEG	Immediate early gene
I.p.	Intraperitoneal
LTP	Long-term potentiation
NDS	Normal donkey serum
NeuN	Neuronal nuclei
PBS	Phosphate buffered saline
PSA-NCAM	Polysialylated neural cell adhesion molecule
PVA-DABCO	Polyvinyl alcohol - 1,4-Diazabicyclo[2.2.2]octane
TBS	Tris buffered saline
Zif268	Zinc finger protein

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CHAPTER 1: INTRODUCTION

Adult hippocampal neurogenesis

The hippocampus is a highly plastic structure that has an important role in spatial learning and memory (Morris et al, 1990) and is one of two main areas that harbours continual neurogenesis in adulthood (Altman & Das, 1965). Adult hippocampal neurogenesis exists in most mammalian species including humans (Eriksson et al, 1998) and can be subdivided into cell proliferation, migration, differentiation, and maturation.

Cell proliferation occurs in the subgranular zone of the dentate gyrus and refers to the production of new cells through cell division. Daughter cells either continue to proliferate or migrate to the innermost granule cell layer (GCL) where they will differentiate into either a neuron or glial cell (Cameron et al, 1993). At 4 to 10 days of age, cells begin to extend their axons towards CA3 and continue to mature and integrate into the hippocampal network (Hastings & Gould, 1999); however, these timelines may be different for mice (Snyder et al, 2009). Altering the progression of development at any stage of neurogenesis can lead to changes in the levels of neurogenesis.

To track how a given manipulation affects a group of cells during certain stages of development, an exogenous marker can be used. Bromodeoxyuridine (BrdU) is a thymidine analog that is incorporated into dividing cells within a 2-hour period (Nowakowski et al, 1989). Examination of cells up to 24 hours after injection, approximately the length of one cell cycle (Cameron & McKay, 2001), provides a measure of cell proliferation, while examining the tissue any time after 24 hours gives a measure of cell survival.

BrdU coupled with other labels, such as the endogenous markers doublecortin (DCX) or NeuN, can be used as an indicator for neurogenesis (Cameron & McKay, 2001). DCX is a

microtubule-associated protein expressed in immature neurons while the neuronal marker NeuN is expressed in more mature cells (Brown et al, 2003; Mullen et al, 1992). This method makes it possible to know the age of labeled cells and to track the fate of a group of cells that are at similar stages of developmental upon examination.

Adult hippocampal neurogenesis and learning

The function of new neurons in the hippocampus has been linked to hippocampus-dependent learning and memory (for review see Winocur et al., 2006). Hippocampus-dependent learning tasks such as the spatial Morris Water Maze task can increase hippocampal neurogenesis (Gould et al, 1999). Increasing hippocampal neurogenesis through exercise or environmental enrichment facilitated spatial learning (van Praag et al, 1999; Nilsson et al, 1999), while manipulations that decrease hippocampal neurogenesis, such as irradiation, impaired contextual fear conditioning and long-term spatial memory (Saxe et al, 2006; Snyder et al, 2005). Recent studies have found that a partial reduction of hippocampal neurogenesis via genetic knockdown was sufficient to interfere with spatial learning and spatial discrimination (Zhang et al, 2008; Clelland et al, 2009), both of which require the dentate gyrus. Furthermore, Jessberger and colleagues (2009) showed that greater reductions in hippocampal neurogenesis impaired spatial memory and novel object recognition memory, whereas animals with lower reductions in hippocampal neurogenesis performed similarly to controls, suggesting that there is an optimal level of neurogenesis required for the regulation of learning and memory.

Exposure to hippocampus-dependent learning also regulates hippocampal neurogenesis and can exert different effects on cell survival depending on the type of task (Leuner et al, 2006), quality of learning (Sisti et al, 2007; Epp et al., 2007), task difficulty (Epp et al, 2010), and/or the age of cells at the time of exposure and perfusion (Epp et al, 2007; Epp et al, 2011). We have

previously found that the effects of spatial learning to promote hippocampal neurogenesis depend on when during development immature neurons were exposed to spatial learning (Epp et al., 2007). Spatial training 6 to 10 days after BrdU injection increased cell survival; however, spatial training on days 1 to 5 or 11 to 15 after BrdU injection did not increase cell survival (Epp et al, 2007). These data suggest that there is a critical period in new neuron development during which the survival of immature neurons are more prone to influence from spatial learning.

Hippocampus-dependent learning and cell activation

Studies have also investigated the effects of hippocampus-dependent tasks to activate new neurons under different conditions. Cell activation can be quantified using immediate early genes (IEG) such as c-Fos, *arc*, and *zif268* (Guzowski et al, 2001). These genes are transiently expressed in response to neuronal activation and have a role in neural plasticity and memory consolidation (Jones et al, 2001; Fleischmann et al, 2003). IEG expression in adult-born neurons is increased in response to exploration of a new environment (Ramirez-Amaya et al, 2006), re-exposure to a familiar environment (Tashiro et al, 2007), spatial learning (Jessberger & Kempermann, 2003) and memory retrieval (Kee et al, 2007; Epp et al, 20011).

The age of cells during which an event occurs can also affect the activation of new neurons as assessed by immediate early genes. In male rats, induction of long-term potentiation (LTP) two weeks, but not one week, after BrdU injection increased expression of the IEG product zif268 in new neurons (Bruel-Jungerman et al, 2006). Additionally, 11-15 day old neurons stimulated by spatial training showed increased cell activation when examined 20 days following BrdU injection (Epp et al, 2011). Animals trained on days 6-10 and given a probe trial on day 15 resulted in significantly less cell activation, indicating that at this age fewer cells are

physiologically active. Taken together, the survival and function of adult-born hippocampal neurons appears to be mediated in a timing and experience-specific manner.

Sex differences in neurogenesis and learning

Thus far the majority of research in this area has been conducted in male animals. However there are significant sex differences in neurogenesis in the dentate gyrus. For example, there are greater levels of both cell proliferation and survival in male, compared to female, rodents (Galea and McEwen, 1999; Tanapat et al, 1999) and several studies have demonstrated sexually dimorphic regulation of cell proliferation and cell survival by factors such as stress and gonadal hormones (Falconer & Galea, 2003; Westenbroek et al, 2004; Galea & McEwen, 1999; Barker and Galea, 2008). Furthermore, there are sex differences in spatial performance across a wide variety of species, with males typically outperforming females (Galea et al, 1996 review; Astur et al, 1998; Gaulin & Fitzgerald, 1986).

To our knowledge only one study has examined the role of sex in the effects of a hippocampus-dependent task on hippocampal neurogenesis (Dalla et al., 2009). Dalla and her colleagues (2009) showed that faster acquisition of a trace eyeblink conditioning task in females was correlated with a greater percent increase in cell survival compared to males. Intriguingly, sex differences in performance of the trace eyeblink conditioning task favours females, unlike performance in the Morris Water Maze, which typically favours males (e.g. Galea et al, 1996). To our knowledge there have been no studies examining the effect of spatial learning on hippocampal neurogenesis or activation of new neurons in males and females.

Thus the current study aims to determine whether there are sex differences in the survival of new neurons after exposure to Morris Water Maze training and whether there is differential activation in response to spatial memory retrieval. Adult male and female rats were trained in the

Morris Water Maze 6-10 days after BrdU injection and given a probe trial on day 20 to examine new cell activation via the immediate early gene product zif268. We hypothesized that males would outperform females in the acquisition of the Morris Water Maze and would have higher levels of hippocampal neurogenesis in response to learning and show greater activation of new neurons in response to memory retrieval compared to females.

CHAPTER 2: METHODS

Subjects

Sixty-three Sprague Dawley rats (males: $n = 29$; females: $n = 34$) between 58-62 days old that were bred and raised in the Department of Psychology at the University of British Columbia were used in this study. All animals were pair-housed in standard cages with a polyvinylchloride tube, paper towels, cedar bedding, and free access to food and water. Animals were given one week to acclimatize to their environment and the 12/12h light-dark cycle. Five days prior to the start of the experiment, rats were handled five minutes per day. All testing was carried out in accordance with the Canadian Council for Animal Care guidelines and was approved by the animal care committee at the University of British Columbia. All efforts were made to reduce the number of animals used and to minimize their suffering.

Apparatus

The Morris Water Maze was a white circular pool that was 180cm in diameter and filled with water mixed with white tempura (non-toxic) paint to render it opaque. Large distal cues were placed on all four walls of the room surrounding the pool and remained constant throughout the study. A camera installed above the center of the pool was connected to a computer running ANY-maze (Stoelting, Wood Dale, IL, USA) in order to record measures of performance such as latency, swim distance and percentage of time spent in the quadrant with the platform.

Procedure

One intraperitoneal injection of bromodeoxyuridine (BrdU; 200mg/kg; Sigma-Aldrich, Oakville, ON, Canada) was administered to all animals at the start of the experiment (day 0). Six days later, female and male rats were either exposed to four trials of training per day for five

consecutive days in the spatial (n = 25; 11 males and 14 females) or cued (n = 24; 11 males and 13 females) version of the Morris Water Maze (see Figure 1A) or served as cage controls and were left undisturbed in their home cage except for weekly cage changing (n = 14; 7 males and 7 females).

In the spatial task, the platform was submerged roughly 2cm beneath the pool surface and remained in the northeast quadrant throughout training. In the cue task, the platform was raised roughly 2cm above the water and the location of this platform changed after every trial to ensure that animals relied on the visible platform as a cue rather than extramaze spatial information. Training began at approximately the same time each day and occurred over five consecutive days, with four trials per day. Each trial ended when the animal reached the platform or when 60s had elapsed. Animals that were unable to locate the hidden or visible platform within the allotted time were guided to the platform and left there for 10s before removing from the pool. Inter-trial interval was approximately 5 minutes. For each day, performance on the four trials was averaged to obtain a measure of performance per day on the water task.

In females, estradiol levels fluctuate over the estrous cycle, which has been shown to influence both hippocampal plasticity (Tanapat et al, 1999; Rummel et al, 2009) and spatial learning (Warren & Juraska, 1997; Frick & Berger-Sweeney, 2001), therefore the estrous cycles of female rats were monitored in our study. Animals were lavaged on the day of BrdU injection, every day after water maze training, and the probe trial. Vaginal cells were collected by lavage. The lavage sample was transferred onto microscope slides and stained with Cresyl Violet (Sigma) before leaving to dry. Slides were analyzed using a 20x objective (200x magnification). A rat was determined to be in the proestrous stage if at least 70% of cells were nucleated epithelial cells.

After training, animals were returned to the colony rooms and remained undisturbed until ten days later (20 days after BrdU injection) when animals received a 30-second probe trial, during which the platform was removed from the pool. Percentage of time spent in the target quadrant (that previously contained the hidden platform) was recorded on the probe trial. Ninety minutes after probe trial, animals were administered an overdose of sodium pentobarbitol and perfused transcardially with 0.9% saline followed by 4% formaldehyde (Sigma-Aldrich). Brains were extracted and post-fixed in 4% formaldehyde overnight, then transferred to 30% sucrose (Fisher Scientific) solution 24h later and remained in solution until sectioning. Brains were sliced into 40 μm coronal sections using a Leica SM2000R microtome (Richmond Hill, Ontario, Canada). Sections were collected in series of ten throughout the entire rostral-caudal extent of the hippocampus and stored in ethylene glycol, glycerol and 0.1M PBS at -20°C.

BrdU immunohistochemistry

Brains were sliced into 40 μm thick coronal sections using a Leica SM2000R freezing sliding microtome (Richmond Hill, Ontario, Canada). Sections were collected in series of ten throughout the entire rostral-caudal extent of the hippocampus. Tissue slices were rinsed with 0.1M TBS three times, incubated in H_2O_2 for 30 minutes, then rinsed with TBS again. Tissue were then transferred to 2N HCl and incubated in a water bath for 30 minutes at 37°C to denature the DNA. Tissue were then rinsed with 0.1M borate buffer for 10 minutes then rinsed with TBS to remove any background staining. After blocking the tissue with TBS+ solution, consisting of 0.3% Triton-X (Sigma), and 3% normal horse serum (Vector Laboratories; Burlingame, CA, USA) in 0.1M TBS, slices were incubated in a primary antibody solution, which contained 1:200 mouse anti-BrdU (Roche; Mississauga, ON, Canada) and TBS, for 48 hours at 4°C then rinsed with TBS. Then tissue was incubated in biotinylated secondary antibody solution

containing 1:500 horse anti-mouse IgG Biotynlated (Vector Laboratories) in TBS+ for 4 hours at room temperature. Excess antibodies were then washed off with TBS and then an ABC kit (Vector Laboratories) was used and prepared according to instructions on the kit. Brain sections were then transferred to diaminobenzidine (DAB; Sigma) solution and incubated for 5 minutes in a dark room, then rinsed with TBS. The tissue was mounted onto glass microscope slides, counterstained with cresyl violet, and cover-slipped with Permount (Fisher Scientific; Ottawa, ON, Canada).

BrdU/NeuN double labeling

Brain sections were rinsed three times with 0.1M PBS and left to sit overnight at 4°C. The tissue was transferred to a NeuN primary antibody solution containing 1: 250 mouse anti-NeuN (Millipore; MA, USA), 3% normal donkey serum (NDS; Vector Laboratories), and 0.3% Triton-X in 0.1M PBS then left to sit for 24hrs at 4°C. Tissue were washed again with PBS then incubated in a secondary antibody solution, which contained 1: 200 donkey anti-mouse ALEXA 488 (Jackson ImmunoResearch; PA, USA) in 0.1M PBS, for 18 hours at 4°C. Following three rinses with PBS, sections were washed once with 4% paraformaldehyde (PFA) then twice in 0.9% NaCl. A 37°C water bath was prepared and used to incubate the tissue in 2N HCl for 30 minutes before tissue slices were incubated in a BrdU primary antibody solution consisting of 1: 500 rat anti-BrdU (AbD Serotec; Raleigh, NC, USA), 3% NDS, and 0.3% Triton-X in 0.1M PBS for 24 hours at 4°C. Tissue were rinsed with PBS again and followed by incubation in a secondary antibody solution containing 1:500 donkey anti-rat Cy3 (Jackson ImmunoResearch; PA, USA) in 0.1M PBS for 24 hours at 4°C. After rinsing with PBS, tissue were mounted onto microscope slides and cover-slipped with PVA DABCO.

BrdU/zif268 double labeling

The tissue was washed with 0.1M PBS and left to sit overnight at 4°C. The next day, slices were transferred to zif268 primary antibody solution made with 1: 1000 Rabbit anti-Egr-1 (Santa Cruz; CA, USA), 3% NDS, and 0.3% Triton-X in 0.1M PBS to be incubated for 24hours at 4°C. After three rinses with PBS, the tissue was incubated in zif268 secondary antibody solution, which consisted of 1: 500 Donkey anti-Rabbit ALEXA 488 (Molecular Probes, CA, USA) in 0.1M PBS, for 18 hours at 4C. The tissue was washed with PBS three times then washed once with 4% paraformaldehyde and twice in 0.9% NaCl. Following incubation in 2N HCl for 30 minutes at 37°C, slices were washed with PBS. The BrdU primary antibody solution was prepared with 1: 500 mouse anti-BrdU (Roche), 3% NDS, and 0.3% Triton-X in 0.1M PBS and tissue sections were incubated in this solution 24 hours at 4°C. After rinsing with PBS, a secondary antibody solution containing 1:250 Donkey anti-Mouse Cy3 (Jackson ImmunoResearch; PA, USA) in 0.1M PBS was used to incubate tissue slices for 16 hours at 4°C. After rinsing with PBS, tissue slices were mounted onto glass slides and cover-slipped with PVA DABCO.

Cell counting

All microscope slides were coded to ensure that counting was done by an experimenter blind to the group assignment of each animal. BrdU-labeled cells were counted using a Nikon E600 light microscope under a 100x oil immersion objective lens (1000x magnification; see Figure 1B) while BrdU/NeuN and BrdU/zif268 positive cells were counted at 400x magnification (See Figure 1E and 1H) using a Nikon E600 epifluorescent microscope. For BrdU-labeled cells, every 10th section of the granule cell layer (GCL; including the subgranular zone) and hilus was counted separately and an estimate of total immunoreactive cells per region was

obtained by multiplying the aggregate number of cells per region by 10 (Epp et al, 2007; Epp et al, 2011). Cells in the hilus were counted for several reasons: 1) cells in the hilus give rise to a different population of cells, 2) mature granule cells in the hilus are generally considered ectopic and 3) to determine whether cells in both the GCL and hilus were affected similarly. Area measurements for the GCL and hilus were obtained with digitized images and the software ImageJ (NIH). Volume estimates of the dentate gyrus were calculated using Cavalieri's principle (Gundersen & Jensen, 1987) by multiplying the summed areas of each region by distance between sections (400μm). Density of BrdU-labeled cells in the GCL and hilus were calculated by dividing the sum of BrdU-labeled cells in the GCL or hilus by volume of the corresponding region.

The percentages of BrdU/NeuN and BrdU/zif268 double-labeled cells were obtained by randomly selecting, respectively, 50 or 100 BrdU-labeled cells and determining the percentage of cells that coexpressed NeuN or zif268. We also noted whether labeled cells were located in the dorsal or ventral GCL using the criterion defined by Banasr and others (2006), with sections 6.20-3.70mm from the interaural line defined as dorsal and sections 3.70-2.28mm from the interaural line as ventral.

Data analyses

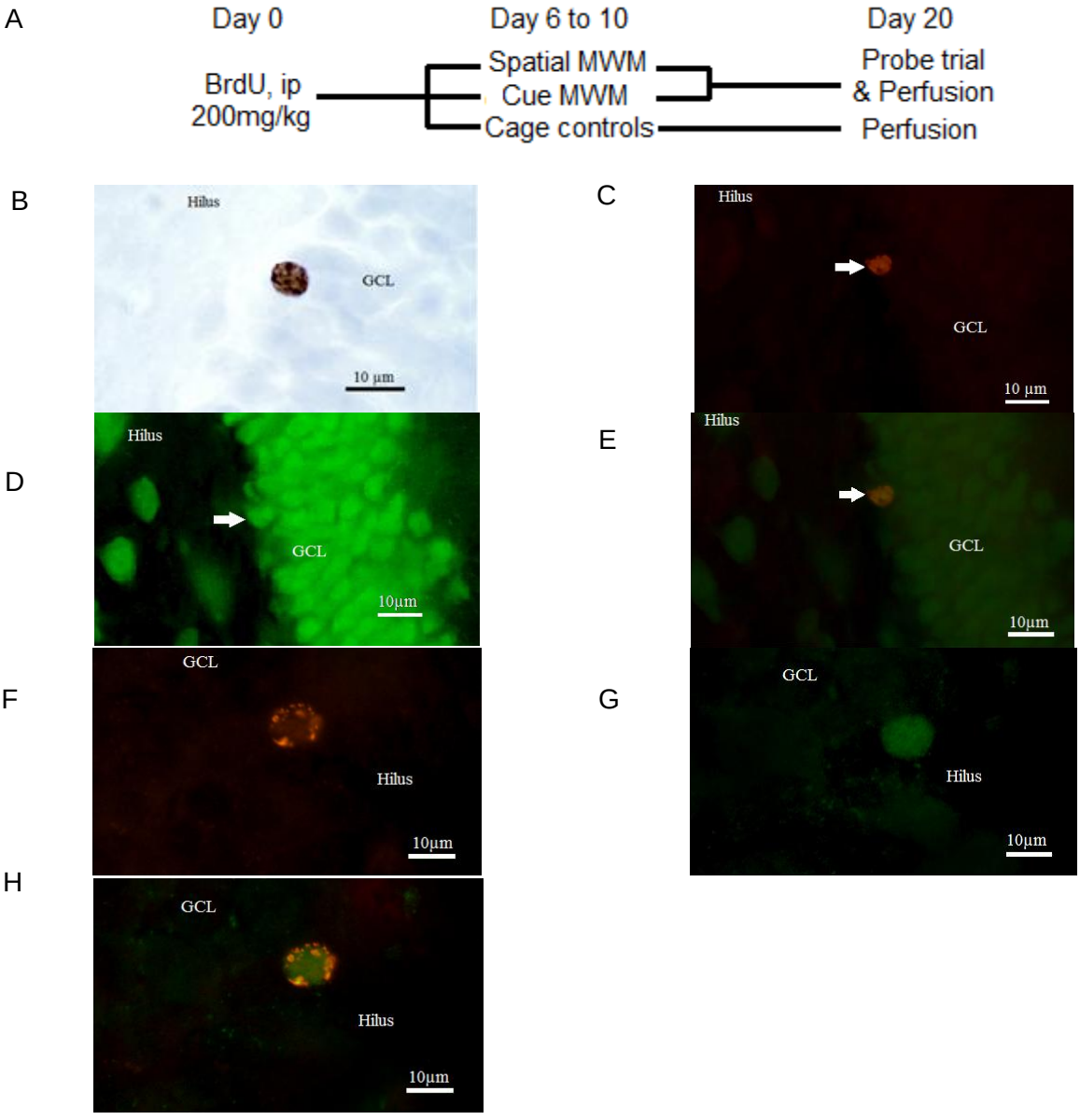
All analyses were conducted using Statistica (Statsoft Tulsa, OK). Swim distance and latency to reach the platform were each analyzed using repeated measures analysis of variance (ANOVA), with training group (spatial, cue) and sex (male, female) as between-subject variables and training day (1 to 5) as the within-subject variable. Repeated-measures ANOVAs were used to analyze total number of BrdU-labeled cells, volume of GCL and hilus, and cell density with

sex and training group (spatial, cue, cage controls) as between subject factors and region (GCL, hilus) as within-subject factors.

For percentage of cells co-expressing BrdU/NeuN or BrdU/zif268, repeated-measures ANOVAs were performed with dentate gyrus subregion (dorsal, ventral) as the within-subject variable and with sex and training group as between-subject variables. Pearson product-moment correlations were calculated to examine the relationship between spatial performance and density of BrdU-labeled cells or cells co-expressing BrdU/zif268.

To examine spatial training performance across the estrous cycle in female rats, an analysis of covariance (ANCOVA) was used, with estrous state (proestrus, non-proestrus) as the covariate, group (spatial, cue) as the between-subject variable, and training day (1 to 5) as the within-subject variable. For probe trial performance across the estrous cycle, an ANOVA was used with estrous state and training group as between-subject variables. Post-hoc tests were performed with the Neuman-Keuls procedure. A priori comparisons were subjected to Bonferroni corrections.

Figure 1: A) Experimental outline. MWM = Morris Water Maze. (B) BrdU-labeled cells in the GCL viewed at 1000x magnification. Cells in the GCL labeled with BrdU (C) and the neuronal marker NeuN (D). (E) Merged image with the arrow pointing to cells co-labeled with BrdU (red) and NeuN (green). Images in figures C to E were captured at 400x magnification. (F) BrdU-labeled cells in the GCL. (G) Cells expressing the IEG product zif268 in the GCL. (H) Merged image showing co-expression of BrdU (red) and zif268 (green). Images in figures F to H were captured at 600x magnification. Scale bar = 10µm. GCL = granule cell layer.



CHAPTER 3: RESULTS

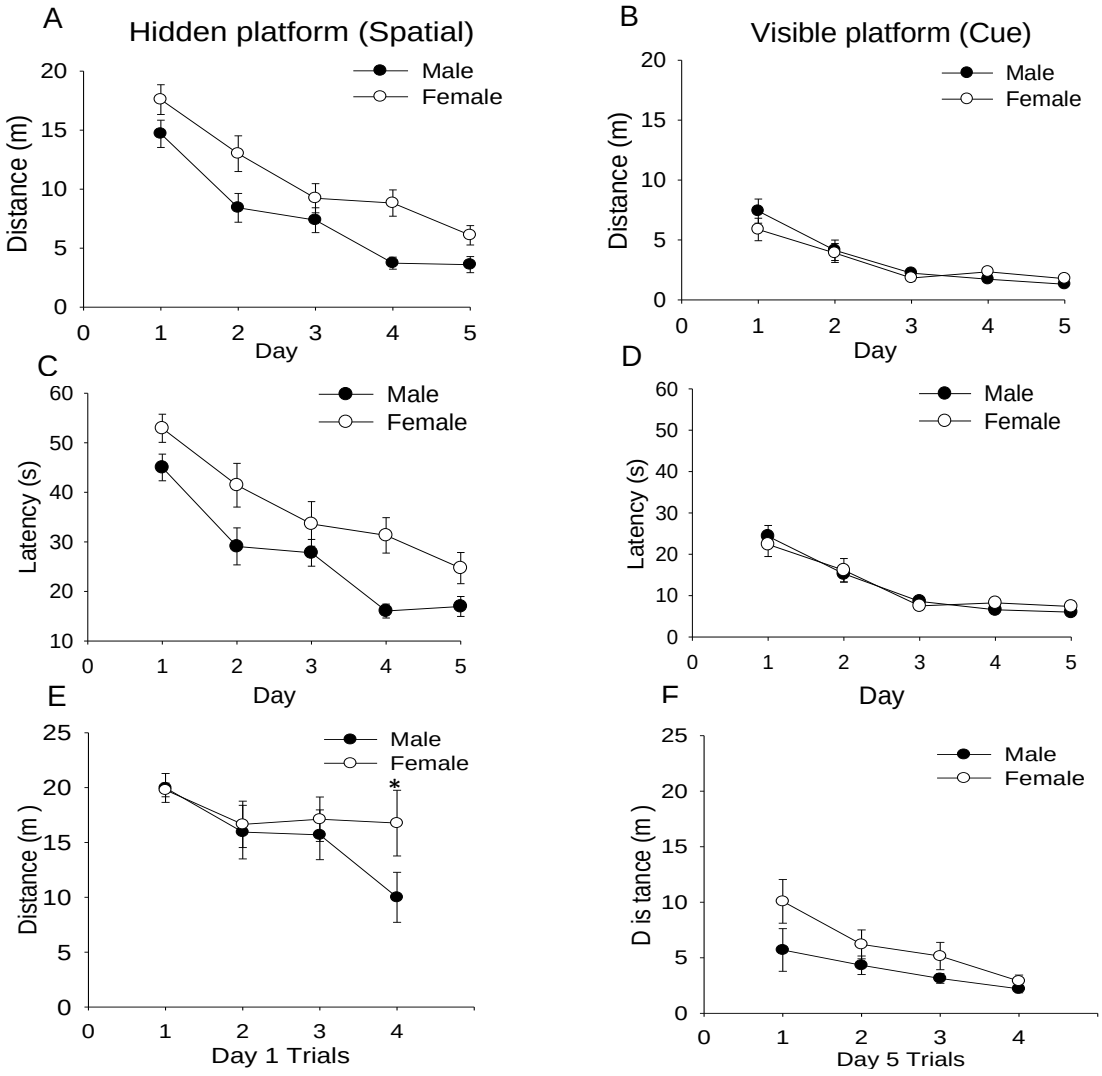
Females swam greater distances and required more time than males to locate the hidden, but not visible, platform

There was a significant sex by group interaction for swim distance ($F(1,45) = 7.76$, $p < .008$) and latency ($F(1,45) = 7.45$, $p < .01$) to reach the platform across training days (Figure 2A and B). Post-hoc analyses revealed that for the spatial training group, females swam longer distances ($p < .001$) and required more time ($p < .001$) than males to locate the hidden platform. There was also a significant interaction of day by group on distance travelled ($F(4,180) = 11.22$, $p < .0001$) and on latency to reach the platform ($F(4,180) = 4.42$, $p < .002$). Post-hoc tests revealed that for the spatial group, distance traveled during training decreased significantly between days 1 to 4 (p's $> .008$), whereas for the cue group, swim distance decreased significantly only between days 1 to 3 (p's $> .02$). For latency to reach the platform, post-hoc tests showed that in the spatial group, latency decreased significantly between days 1 to 4 (p's $> .02$), and for the cue group, latency was significantly decreased only between days 1 to 3 (p's $> .002$). There were also main effects of day, sex, and group but no other significant effects for either distance or latency to reach the platform were found (p's > 0.19).

Training performance (distance travelled) in females was analyzed using estrous state as a covariate across training days with training group as the between-subject factor. There was a significant day by group interaction ($F(4,80) = 4.92$, $p < .001$) and a main effect of group and day (p's < 0.026), but no significant effect of the estrous cycle covariate (p's > 0.21). Post-hoc tests showed that distance to reach the platform was greater in the spatial compared to cue group on each training day ($p > .001$).

We also analyzed distance to reach the platform across each trial (trial 1 to 4) on the first and final day (day 1 and 5) in order to determine whether there were preexisting sex differences on the first day of training and whether performance was equivalent on the first and last trial of training in the spatial group. There was a significant interaction of sex by day by trial ($p < .013$) and main effects of sex, day, and trial (p's $< .03$). Post-hoc comparisons revealed that males outperformed females for distance travelled only on day 1 trial 4 ($p = 0.006$) but not on day 1 trial 1 ($p = 0.93$) or on day 5 (trial 1: $p = 0.08$ and trial 4: $p = 0.73$; Figures 2E and 2F). This indicates that there were no preexisting (or lasting) sex differences in performance on the first or last trial of training in the spatial group.

Figure 2: Mean distance to the hidden (A) and visible (B) platform in males and females. Mean latency to locate the hidden (C) and visible (D) platform in males and females. Females swam significantly greater distances and longer times before reaching the hidden platform compared to males (p's < .001). There were no significant differences in the cue group. Mean distance to the hidden platform on the first and final trials of training days 1 (E) and 5 (F). Females swam significantly greater distances than males on the final trial of day 1 (p < .01) and showed a trend toward significance on first trial of day 5 (p = .09) but there were no significant sex differences on the first trial of day 1 or the last trial of day 5. Error bars represent ± standard error of the mean (± SEM).



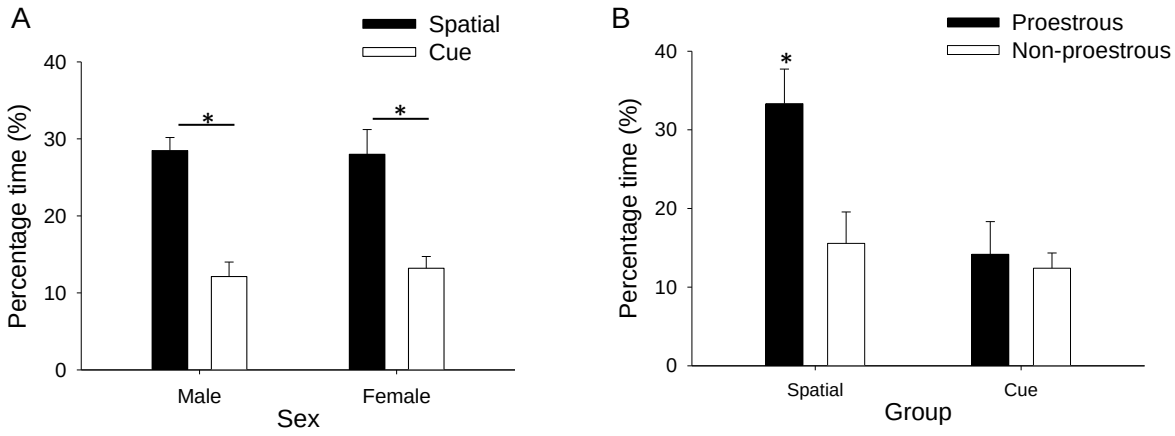
Spatial-trained animals spent a greater percentage of time in target quadrant than cue-trained animals during the probe trial

There was a significant effect of group on time spent in the target quadrant during the probe trial ($F(1,36) = 46.55$, $p < .0001$) with spatial-trained rats spending more time in target quadrant than cue-trained rats (Figure 3A). No other significant main or interaction effects were found (p's > 0.70).

Proestrous females in the spatial group spent a significantly greater percentage of time than non-proestrous females in the platform quadrant during the probe trial.

For the probe trial, there was a main effect for estrous cycle status on percentage time spent in the target quadrant ($F(1, 15) = 6.17$, $p = .025$) and a main effect of group ($F(1, 15) = 8.08$, $p = .012$); see Figures 3B), with proestrous females in the spatial group spending a significantly greater percentage of time in the target quadrant relative to non-proestrous females in the spatial ($p = .005$), but no differences were found in the cue ($p's < .52$), groups.

Figure 3: (A) Mean percentage of time spent in the target quadrant in spatial and cue-trained groups. Spatial-trained groups spent more time in the target quadrant than cue-trained groups (* indicates $p < .001$). (B) Mean percentage of time spent in the target quadrant for proestrous versus non-proestrous females. For proestous females in the cue group, $n = 3$, and $n = 5$-6 proestrous females in the spatial group and non-proestrous females. Females in proestrous spent significantly more time in the target quadrant than non-proestrous females (* indicates $p < .005$). Error bars represent $\pm$ SEM.



Males had larger dentate gyrus volumes than females

As expected, the dentate gyrus volume in males was significantly larger than females (main effect of sex: $F(1,48) = 12.55$, $p < .0001$; region by sex interaction: $F(1,48) = 4.26$, $p < .044$; see Table 1). Despite the interaction, post-hoc tests revealed that there were sex differences favoring males for both the GCL and hilus (all p's < 0.04). No other significant main or interaction effects in dentate gyrus volume (all p's $> .40$) were found. Because there were sex differences in dentate gyrus volume BrdU-labeled cell density were used instead of total cell counts.

Table 1: Mean (± SEM) volume of the GCL and hilus in male and female rats

	Volume (mm^3)	
	GCL	Hilus
Male – Spatial	1.02 ± .07 mm^3	2.16 ± .05 mm^3
Male – Cue	1.05 ± .09 mm^3	2.34 ± .08 mm^3
Male - Control	1.03 ± .06 mm^3	2.18 ± .09 mm^3
Female – Spatial	.90 ± .08 mm^3	1.96 ± .14 mm^3
Female - Cue	.88 ± .04 mm^3	1.94 ± .11 mm^3
Female - Control	.86 ± .05 mm^3	1.82 ± .16 mm^3

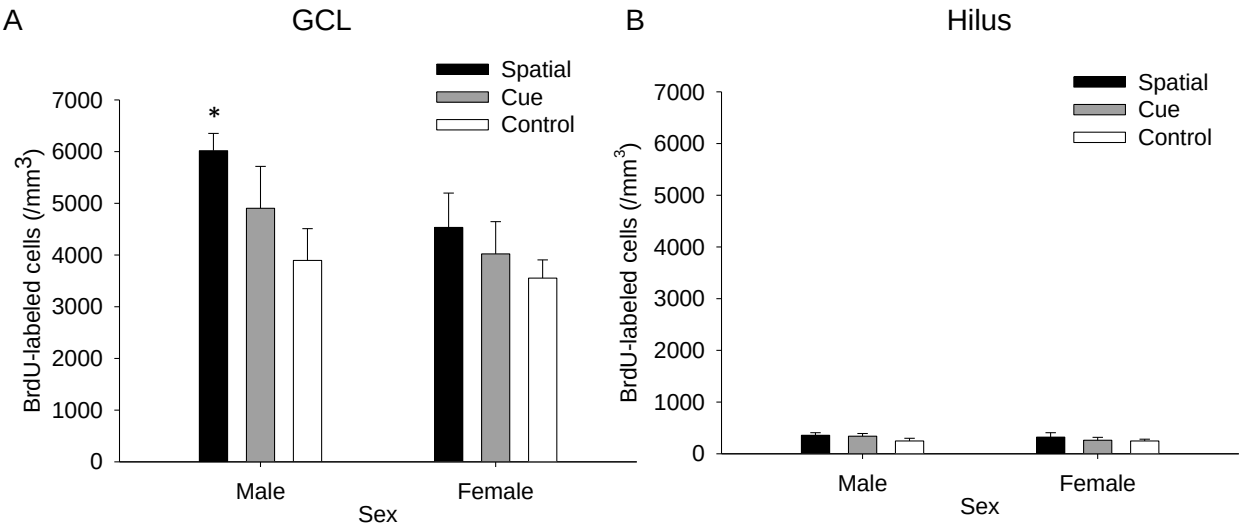
Males, regardless of group, had significantly greater GCL and hilus volume than females, regardless of group (p's < .04).

Males trained in the spatial task but not the cue task showed significantly greater cell survival compared to females

Spatial-trained rats had a greater density of BrdU-labeled cells in the GCL, but not hilus, than cued-trained or cage-control rats (Group by region: $F(2,45)= 3.22$, $p < .049$). Post-hoc tests revealed that spatial-trained rats had higher density of BrdU-labeled cells than both cue-trained and control rats (all *p's* <.002). A priori we wanted to determine whether there were sex differences in BrdU-labeling after spatial learning, and we found that spatial-trained males had significantly greater BrdU-labeled cell density than cue-trained males and cage-control males (all p's <.007) but that there were no significant differences between any of the female groups (all p's > 0.36). Furthermore spatial-trained males had higher levels of BrdU-labeled cell density than spatial-trained females ($p < .010$) but there were no sex differences in BrdU-labeled cell

density for cue-trained ($p < .4$) or control animals ($p < .6$; see Figure 4A). As expected, there was also a main effect of region ($p < .001$) and group ($p < .05$) but no other significant effects were found.

Figure 4: Mean (+SEM) density of BrdU-labeled cells in the GCL (A) and hilus (B) in males and females. Males in the spatial group showed greater BrdU cell density in the GCL than males in the cue and control groups, as well as females. No significant differences were found in the hilus. Error bars represent ± SEM. * indicates $p < .05$ compared to all other groups.



The majority of BrdU-labeled cells co-expressed NeuN

The majority of BrdU-labeled cells were colabeled with NeuN. There was a significantly greater proportion of new neurons in the dorsal compared to ventral hippocampus (main effect of region (dorsal, ventral): $F(1,36) = 74.32$, $p < .001$; see Table 2). However there were no significant sex or group main or interaction effects in proportion of BrdU cells co-expressing NeuN (all p's $> .2$).

Table 2: Mean (±SEM) percentage of cells co-expressing BrdU and NeuN in the GCL in male and female rats.

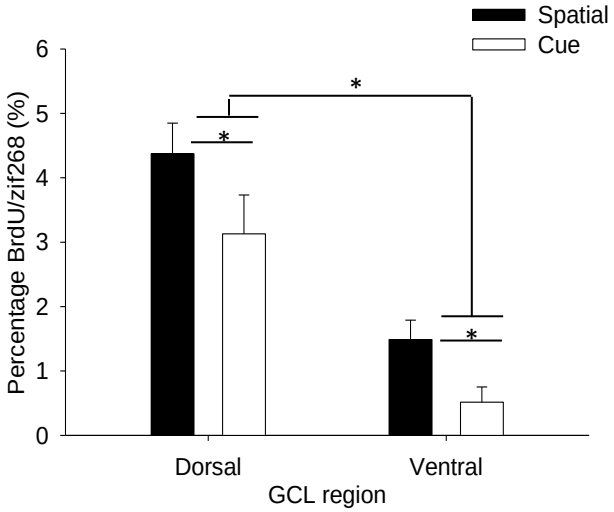
	BrdU/NeuN labeled cells (%)		
	Dorsal	Ventral	Dorsal and ventral
Male - Spatial	88.07 ± 1.78	76.52 ± 1.44	82.47 ± 1.254
Female - Spatial	87.27 ± 1.62	78.83 ± 2.78	82.68 ± 1.926
Male – Cue	86.40 ± 1.66	77.67 ± 2.63	81.71 ± 1.903
Female - Cue	82.15 ± 3.41	73.88 ± 2.49	77.76 ± 2.651

No significant differences between groups in percentage of BrdU/NeuN co-labeled cells were found.

Spatial-trained rats had significantly greater percentage of BrdU/zif268 co-expression than cue-trained rats and greater activation was found in the dorsal compared to ventral GCL

Spatial-trained rats had greater activation of BrdU-labeled cells in response to spatial memory retrieval compared to cue-trained rats ($F(1,28) = 20.52$, $p < .023$; Figure 5). Furthermore there were significantly greater numbers of BrdU-labeled cells in the dorsal GCL that co-expressed the immediate early gene product zif268 compared to the ventral GCL ($F(1,28) = 39.89$, $p < .001$). No other significant main or interaction effects were found (all p's > .28) and there were no significant main or interaction effects of estrous cycle on activation of BrdU-labeled cells (all p's > .06), which may have been due to low sample size especially from proestrous females in the cue group ($n = 1$), as some animals were excluded due to lack of BrdU labeling in the GCL.

Figure 5: Mean (±SEM) percentage of cells co-expressing BrdU and the immediate early gene product zif268 in the dorsal and ventral GCL for animals in the spatial versus cue group. The dorsal GCL had significantly greater levels of BrdU/zif268 co-labeling compared to the ventral region ($p < .001$) and spatial trained animals had significantly increased level of cell activation compared to the cue group ($p < .022$). * indicates $p < .05$



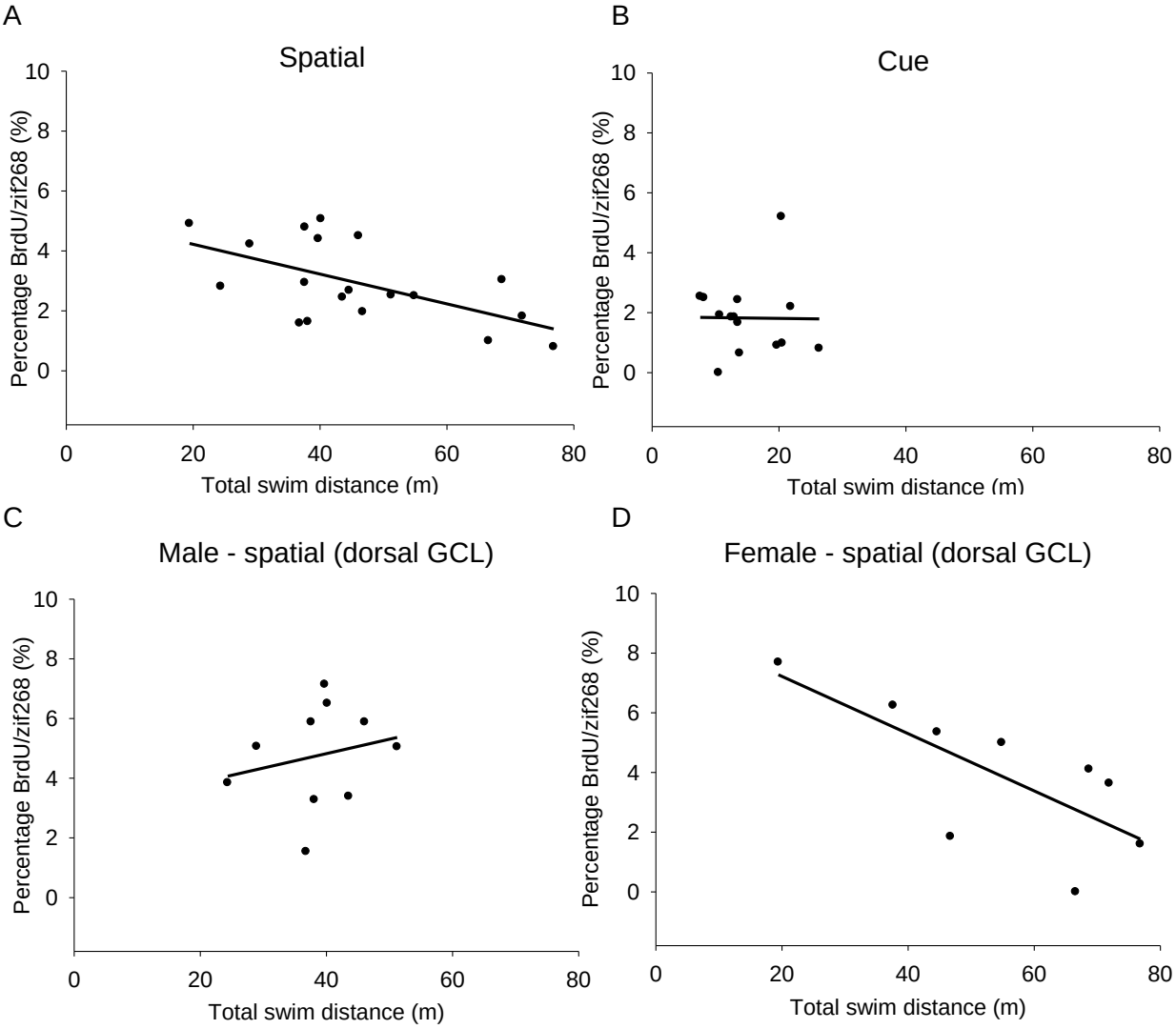
In females, better spatial learning performance was correlated with more cell activation in the dorsal GCL

Total swim distance was negatively correlated with the percentage of BrdU/zif268 co-labeled cells in the GCL (dorsal and ventral) for animals in the spatial ($r(19) = -.49$, $p = .027$) but not cue group ($p = .63$; see Figures 6A and 6B). This shows that more activation in new neurons was associated with better overall performance during acquisition in the water task, a relationship that was stronger in spatial-trained females ($r(8) = -.82$, $p = .007$) than males ($r(10) = .14$, $p = .68$). Additionally, this significant negative correlation was driven by the relationship in the dorsal GCL in spatial-trained females ($r(8) = -.73$, $p = .02$; see Figure 6D) rather than spatial-trained males ($r(10) = .12$, $p = .73$; see Figure 6C). No significant correlations were found

in the ventral GCL for either males ($r(10) = .10$, $p = .77$) or females ($r(8) = -.47$, $p = .20$) in the spatial group.

We also correlated the change in swim distance between training days 1 and 5 with percentage of BrdU/zif268 co-labeled cells in the dorsal GCL and found that there was a trend toward a positive correlation in males ($r(9) = .57$, $p = .088$) but not females ($r(8) = -.49$, $p = .18$). No significant correlations were found in the ventral GCL for spatial-trained males ($p = .67$) or females ($p = .45$), and no significant correlations were found for animals in the cue group ($p = .85$).

Figure 6: (A, B) Correlations between total swim distance during spatial (A) and cue (B) training and percentage of cells co-expressing BrdU and zif268 in the dorsal GCL for both males and females. There was a significant negative correlation between total swim distance and BrdU/zif268 co-labeling for the spatial group. (C,D) Correlation between total swim distance during spatial training and percentage of cells co-expressing BrdU and zif268 in the dorsal GCL for males (C) and females (D).



CHAPTER 4: DISCUSSION

There were significant sex differences favouring males in acquisition of the spatial Morris water task; however, no sex differences were found in memory retention during the probe trial, which is consistent with previous findings (Galea et al., 1996). Females in the proestrous stage showed significantly better memory for platform location during the probe trial than non-proestrous females, which is supported by studies demonstrating the importance of estradiol in spatial memory retrieval (Packard & Teather, 1997; Chen et al, 2002). However, estrous cycle did not affect spatial or cue learning in the water maze. In the present study, consistent with past literature, males trained on the spatial task 6-10 days after BrdU injection showed significantly greater cell survival in the dentate gyrus than males trained on a cued task or cage controls (Epp et al, 2007; Epp et al., 2011; Gould et al., 1999). Furthermore we found that spatial-trained females did not show the same change in density of BrdU-labeled cells compared to cued-trained females, suggesting that the enhancement in cell survival is related to spatial learning rather than memory retention. However we saw that spatial-trained rats, regardless of sex, had greater activation of new neurons in response to spatial retrieval memory compared to cue-trained rats. In addition, we found that activation of new neurons was negatively correlated with total swim distance during spatial training and that this correlation was stronger in the dorsal GCL in female rats. This demonstration of the relationship between sex differences in spatial learning, cell survival (neurogenesis), and cell activation suggests that spatial training differentially affects hippocampal plasticity and function in males and females.

Sex differences in spatial learning may be linked to differences in cell survival

In our study, males swam shorter distances to reach the hidden platform than females, but did not show significant differences in performance on the final day of training or subsequent

spatial memory retention compared to females, which indicates sex differences in learning strategy rather than learning ability, and is consistent with previous studies (Beiko et al, 2004; Williams et al, 1990; Galea et al, 1996; Galea & Kimura, 1993; Grön et al, 2000).

Furthermore, we found that spatial, but not cue, learning increased cell survival only in males. Results from previous literature show that increases in hippocampal neurogenesis after spatial learning (Gould et al, 1999) is dependent on a number of factors such as task difficulty (Epp et al, 2010) and quality of learning (Epp et al, 2007; Sisti et al, 2007). Therefore enhancement of cell survival in males, but not females, suggests that sex differences in learning strategy, which affects task difficulty and learning quality, may have differentially altered hippocampal involvement during spatial training. Support for this supposition extends from the spatial strategy literature. Sex differences in spatial learning strategies have been well documented in both humans and rodents. In general, males focus more on spatial and geometric cues in the environment, which is a hippocampus-dependent strategy, while females rely more on landmark cues, which activates other systems such as the striatum (Willams et al., 1990; McDonald & White, 1994; Miranda et al, 2006). Therefore, although sex difference in strategy choice did not affect mastery of the spatial task, use of less hippocampus-dependent strategies may have contributed to less neurogenesis in females (Rummel et al, 2009).

As mentioned above, it is possible that choice of learning strategy may influence difficulty of the task in some way, as a more efficient strategy would obviously make learning less difficult, and vice versa. Because our study and previous research have demonstrated that males perform better than females during spatial training, one may infer that males employ a more efficient strategy than females. Epp and his colleagues (2010) have shown that increasing difficulty of the spatial water maze task reduced cell survival in male rats. Perhaps this can also

be generalized to females such that favoring a less efficient and less hippocampus-dependent strategy rendered the spatial task more difficult, and thus down-regulated the survival of new neurons. All together, our study and the current literature suggest that learning strategy choice influences hippocampal activation and task difficulty, which in turn affects the regulation of cell survival.

Dalla and colleagues (2009) were the first to directly show that sex differences in hippocampus-dependent learning produce sex differences in neurogenesis. Females acquired the trace eyeblink conditioning task faster than males and subsequently showed greater increases in cell survival. Because all animals performed similarly by the end of training, the initial acquisition phase of the learning process appears to have a greater impact on cell survival. We showed a similar pattern of results using a hippocampus-dependent task that favours learning in males and showed that this resulted in increased neurogenesis in males but not females. However, in our study, spatial performance was not significantly correlated with cell survival, which is consistent with work by Epp and colleagues (2011), and may have been due to the fact that we used a different learning task than Dalla and colleagues (2009). Ablation studies suggest that new hippocampal neurons have different roles in spatial learning versus trace conditioning (Snyder et al, 2005; Shors et al, 2001); therefore it is possible that quality of spatial learning has a smaller influence on cell survival than other factors such as learning efficiency. Taken together, both studies support the idea that acquisition rate, perhaps as a function of learning strategy choice, can influence cell survival depending on the degree to which the strategy engages the hippocampus. Intriguingly, the use of hippocampus-dependent learning strategies appears to produce sexually-dimorphic effects on cell proliferation. Males that favoured a spatial strategy when navigating the Morris water task showed lower levels of cell proliferation compared to

males that chose a cue strategy, whereas the opposite was true in females (Epp & Galea, 2009; Rummel et al, 2009).

Differences in water maze training procedures may differentially alter learning and neurogenesis in males and females

Exposure to novel environments such as the water maze can be a source of stress in rodents (Hennessy, 1991). Stress can impair learning (Bodnoff et al, 1995) as well as neurogenesis in male rodents (Westenbroek et al, 2004; Brummelte & Galea, 2010). Additionally, Beiko and colleagues (2004) showed that females that were naive to the water maze had higher serum corticosterone (CORT) levels and performed more poorly than males. If animals received pre-training, however, sex differences were eliminated and CORT levels were reduced relative to naive animals, although female CORT levels were still elevated compared to males. It is possible that in our study, elevated stress levels contributed to a slower rate of spatial learning in females and subsequently resulted in lower rates of cell survival relative to males.

Other aspects of water maze training may also have influenced learning and indirectly affected cell survival. Epp and colleagues (2010) observed that increasing task difficulty by reducing the number of extramaze cues increased latency for platform location and decreased cell survival in males. Additionally, Roof and Stein (1999) found that manifestation of sex differences in spatial learning varied with slight alterations in task parameters. Releasing animals at different points of the maze between trials impaired learning in females. When the release points were constant between trials, no sex differences were observed. Furthermore, when release points were different between trials but the experimenter remained in the same location when animals were released, females learned as quickly as males. In our study, both the release points and experimenter location varied across trials, which may have prevented females from

using their preferred strategy, as altering the position of landmark cues impairs spatial learning in females but not males (Suzuki et al, 1980; Williams & Meck, 1991). Therefore, sex differences in sensitivity to water maze task parameters may have influenced learning and indirectly affected cell survival.

Spatial training increased activation of 20-day-old new neurons in both males and females

No significant sex differences in new neuron activation, as assessed by expression of the IEG product zif268 in response to spatial memory retrieval, were found, which is consistent with the lack of sex differences observed in probe trial performance.

Water maze training in general increased activation of 20 day old neurons in the dorsal relative to the ventral GCL, consistent with previous studies showing that the cells in the dorsal dentate gyrus are generally more active compared to the ventral region (Snyder et al, 2009b; Snyder et al, 2009c). Additionally, in both the dorsal and ventral GCL, spatial-trained animals had significantly greater levels of cell activation compared to cue-trained animals, which is consistent with past findings (Epp et al., 2011; Kee et al, 2007).

To our knowledge, only one other study has directly examined the activation of immature neurons across the dorsal-ventral axis of the dentate gyrus in response to spatial learning. Snyder and colleagues (2009b) has found that, in male rats, younger neurons in the ventral dentate gyrus were more likely to be activated by spatial training than the dorsal region. However, this is in contrast to our finding that spatial training increased cell activation compared to cue training in both the dorsal and ventral GCL. This may have been due to the use of different markers to identify immature neurons or in the timing between training and tissue examination. As in the study by Snyder and his colleagues (2009), activation of immature neurons was quantified by co-labeling cells for PSA-NCAM and the IEG c-fos. PSA-NCAM is an endogenous protein

expressed in developing neurons for 2 to 4 weeks (Seki, 2002). In our study, the exogenous marker BrdU, which is incorporated into dividing cells within a 2-hour time window (Nowakowski et al, 1989), was used; therefore our sample of activated new neurons would have been more specifically aged at 3 weeks and thus showed a slightly different pattern of activation. It is possible that activated neurons in the study by Snyder and others (2009b) predominantly contained neurons younger than 3 weeks of age. Taken together, results from both studies suggest that the shift in expression patterns of new neurons across the dorsal-ventral axis of the dentate gyrus may occur between 2 to 4 weeks after neuronal birth.

Alternatively, differences in the time point at which activation was examined may also have contributed to differences between the two studies. Snyder and colleagues (2009b) examined cell activation in response to spatial learning, while our study examined cell activation in response to retrieval of long-term spatial memory. Gusev and colleagues (2005) found that expression of the IEG *arc* in the dorsal hippocampus remained stable regardless of the interval between spatial learning and memory retrieval, whereas activation in the ventral hippocampus decreased with longer retention periods. Therefore, the dorsal hippocampus appears to have a greater role in long-term spatial memory retrieval. Further research examining the region-specific activation of young neurons over different memory retention periods may resolve the differences between these studies.

Sex differences in learning strategies may influence activation of new neurons in response to spatial memory

In the present study, better spatial learning and memory retention was associated with greater activation of 20-day-old neurons only in females. The greater activation by spatial memory retrieval in females suggests that either these new neurons are more excitable in females

than in males or that new neurons mature faster in females. Both explanations are plausible, as estradiol influences both neuronal activity and phosphorylation of cyclic AMP response element binding (CREB) protein, which regulates cell development (Terasawa & Timiras, 1968; Lee et al, 2004; Fujioka et al, 2004). However, given that there were no sex differences in proportion of cells expressing a mature phenotype, new neurons reached similar levels of maturity by day 20 in both males and females. Thus it is more likely that 20-day old neurons were more excitable in response to spatial memory retrieval in females than in males.

While there is evidence that female rodents (and humans) use less hippocampus-dependent strategies (Galea et al, 1996; Miranda et al, 2006; Galea and Kimura, 1993; Grön et al, 2000), there may be more overall activation in the hippocampus during spatial learning compared to males (Méndez-López et al, 2009). Previous studies have found that females attend to both geometric and landmark cues during spatial learning (Kant et al, 2000; Tropp & Markus, 2001), thus increased processing of information during navigation may enhance hippocampal activation in females despite using less-hippocampus dependent strategies during spatial learning. There is also evidence that estradiol increases neuronal excitability and potentiates LTP in rodents (Terasawa & Timiras, 1968; Smith & McMahon, 2006) and intriguingly, higher ovarian hormones increase overall brain activity in response to spatial tasks in women (Dietrich et al, 2001).

As mentioned earlier, females may experience greater difficulty in spatial learning due to their choice of learning strategy. Coupled with the fact that task difficulty increases hippocampal involvement (Beylin et al, 2001) while also down-regulating neurogenesis (Epp et al, 2010) it is possible that, in females, immature neurons receive more activation during acquisition and were more responsive to excitatory input. Further research is needed to determine if maturation and

activation of adult-born neurons follow a similar time course in males and females as well as how task difficulty and/or how estradiol alters activation of new neurons.

Limitations and future directions

There were several factors in the present study that may influence the interpretation of the results reported above and are useful to keep in mind when designing future experiments.

The first limitation is that spatial-trained animals spent less time in the target quadrant during the probe trial compared to previous studies (e.g. Epp et al, 2011), and some animals spent little to no time in the target quadrant, suggesting either a failure or difficulty in retrieving previously learned information. As all animals showed significant improvement across each day of water maze training, and the spatial group spent significantly more time in the target quadrant than the cue group, it is unlikely that the lower level of performance is due to a lack of leaning. Rather, it is more likely that more training trials were needed to facilitate memory retention over the relatively long time interval between spatial training and the probe trial (10 days), as Snyder and colleagues (2005) have shown that intact male rats were able to retain spatial memories for up to 4 weeks after a more rigorous training schedule.

It is unlikely that this lower level of probe trial performance had significant effects on cell survival, as previous studies have shown that neurogenesis is more tightly linked to the learning process (Kempermann & Gage, 2002), and indeed, we showed that there were sex differences in cell survival despite a lack of sex differences in probe trial performance. However, it is possible that the level of activated new neurons were underestimated in this study, and perhaps we might have more robust effects of spatial learning on new neuron activation. But as discussed above, spatial-trained animals showed a higher percentage of BrdU/zif268 co-labeled

cells compared to the cue group, suggesting that these animals still retained more information about their environment than the cue group.

Sex differences in stress was a potential confound that may have mediated the effects of sex on spatial learning, neurogenesis, and new neuron activation in our study. As Beiko and colleagues (2004) have shown, females exhibit an enhanced stress response to the novelty of the Morris Water Maze compared to males, an effect paralleled by reduced spatial performance in the initial training trials. Because sex differences in performance during the initial phases of learning have produced differences in neurogenesis previously (Dalla et al, 2009), our findings may have been indirectly impacted by the effects of stress. Therefore, in order to parse out the effects of sex differences in spatial learning on neurogenesis and more definitively confirm that the differences in neurogenesis and new neuron activation are due mainly to sex differences in spatial learning strategies, future studies should control for the effects of stress either by pre-exposing animals to the water maze prior to training, as Beiko and colleagues (2004) had demonstrated, or increase the number of training trials to allow females more time to habituate to novelty-related stress.

Though sex differences in stress could certainly have influenced our findings for cell survival, our results for new neuron activation cannot be explained by stress. Stress has been shown to influence immediate early gene expression in the brain (Cullinan et al, 1999), and the hippocampus both responds to and is affected by stress (McEwen, 1999), thus it is plausible that sex differences in stress response to the novelty of the Morris Water Maze could have accounted for the correlation between spatial performance and BrdU/zif268 co-expression in females. However, we observed only a significant relationship between spatial performance and new neuron activation in the dorsal GCL. The dorsal hippocampus has a more important role in

spatial learning and memory, whereas the ventral hippocampus regulates stress and emotional responses (Moser et al, 1993; Kjelstrup et al, 2002). If new neuron activation in females was a result of stress, we should have seen a negative correlation between BrdU/zif268 expression in the ventral GCL and spatial performance. Furthermore, spatial-trained females did not show significantly more in BrdU/zif268 expression in the ventral GCL compared to males, suggesting again that activation of new neurons during the probe trial was due more to retrieval of spatial memories rather than stress.

An interesting finding in the present study was that there were sex differences in the regulation of new neuron activation, as defined by expression of the immediate early gene product zif268 in BrdU-labeled cells, such that spatial learning performance was positively correlated with more new neuron activation in the dorsal GCL in females but not males. This suggests that estradiol may influence the excitability of new neurons and/or synaptic integration of new neurons (Terasawa & Timiras, 1968; Smith & McMahon, 2006). Examining baseline sex differences in IEG expression in comparison to IEG expression following spatial training and correlating serum estradiol levels with BrdU/zif268 co-expression may provide a more exact explanation.

Currently, the only study to examine the development of adult-born neurons in rats was done in males (Snyder et al, 2009a). It would be interesting to conduct a similar time course study to pinpoint any sex differences in the characteristics of immature neurons (e.g. development of dendritic spines, expression of different estrogen receptor subtypes) and potentially provide more insight into other sex differences in regulation of adult neurogenesis. Furthermore, because we found a different in the pattern of IEG expression along the dorsal-

ventral axis of the GCL compared to the study by Snyder and colleagues (2009b), a time course study may also be able to characterize the shift in IEG expression as new neurons develop.

Finally, in our study, we examined the effects of exposing 6 to 10 day old neurons to spatial or cue learning on cell survival in the present study. Previous work by Epp and colleagues (2007; 2011) have identified days 6 to 10 as the critical period for spatial learning to enhance cell survival in male rats. Due to the possible confound of sex differences in stress in the present study, and the fact that stress differentially affects neurogenesis in males and females (Falconer & Galea, 1993; Westenbroek et al, 2004), we cannot say for certain whether or not females have a similar critical period, given that such a critical period exists. Therefore, a follow-up study could be conducted with a greater number of training trials per day to allow more time for stress habituation in females, and also with the addition of a group trained 11 to 15 days after BrdU injection (Epp et al, 2011) as another potential critical time point.

Conclusion

In the present study males performed better during spatial learning and subsequently had enhanced levels of cell survival compared to females. However, males and females showed similar levels of spatial memory retention, indicating equivalent mastery of the spatial task. Although no sex differences in new neuron activation were found, activation of new neurons seems to be regulated differently between the sexes. Better performance during spatial learning was correlated with greater cell activation only in females, which may be due to estradiol's facilitatory effects on neuronal excitability (Smith & McMahon, 2006) and/or increased task difficulty, which also increases hippocampal involvement (Beylin et al, 2001), due to use of less efficient learning strategies. Overall, our results show that sex differences in spatial learning

regulated cell survival, but not the subsequent ability of these new neurons to become functionally integrated into the hippocampal circuitry.

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