Sex and strategy use matters for pattern separation, adult neurogenesis and immediate early gene expression in the hippocampus

by

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ABSTRACT

Adult neurogenesis in the dentate gyrus (DG) plays a crucial role for pattern separation and there are sex differences in the regulation of neurogenesis. Although sex differences, favoring males, in spatial navigation have been reported, it is not known whether there are sex differences in pattern separation. The current study was designed to determine whether there are sex differences in the ability for separating similar or distinct patterns, learning strategy choice, adult neurogenesis and immediate early gene (IEG) expression in the DG in response to pattern separation training. Male and female Sprague-Dawley rats received a single injection of the DNA synthesis marker, bromodeoxyuridine (BrdU) and were tested for spatial pattern separation in a delayed nonmatching to place task using the 8-arm radial arm maze. Twenty eight days following BrdU injection, rats received a probe trial to determine whether they were idiothetic or spatial strategy users. We found that male spatial strategy users outperformed female spatial strategy users only when separating similar, but not distinct, patterns. Furthermore male spatial strategy users had greater neurogenesis in response to pattern separation training than all other groups. Interestingly neurogenesis was positively correlated with performance on similar pattern trials during pattern separation in female spatial strategy users but negatively correlated with performance in male idiothetic strategy users. These results suggest that the survival of new neurons may play an important positive role for pattern separation of similar patterns in females.

Furthermore, we found sex and strategy differences in IEG expression in the CA1 and CA3 regions in response to pattern separation. These findings emphasize the importance of studying biological sex on hippocampal function and neural plasticity.

PREFACE

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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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ANOVA	Analysis of variance
BrdU	Bromodeoxyuridine
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

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CHAPTER 1: Introduction

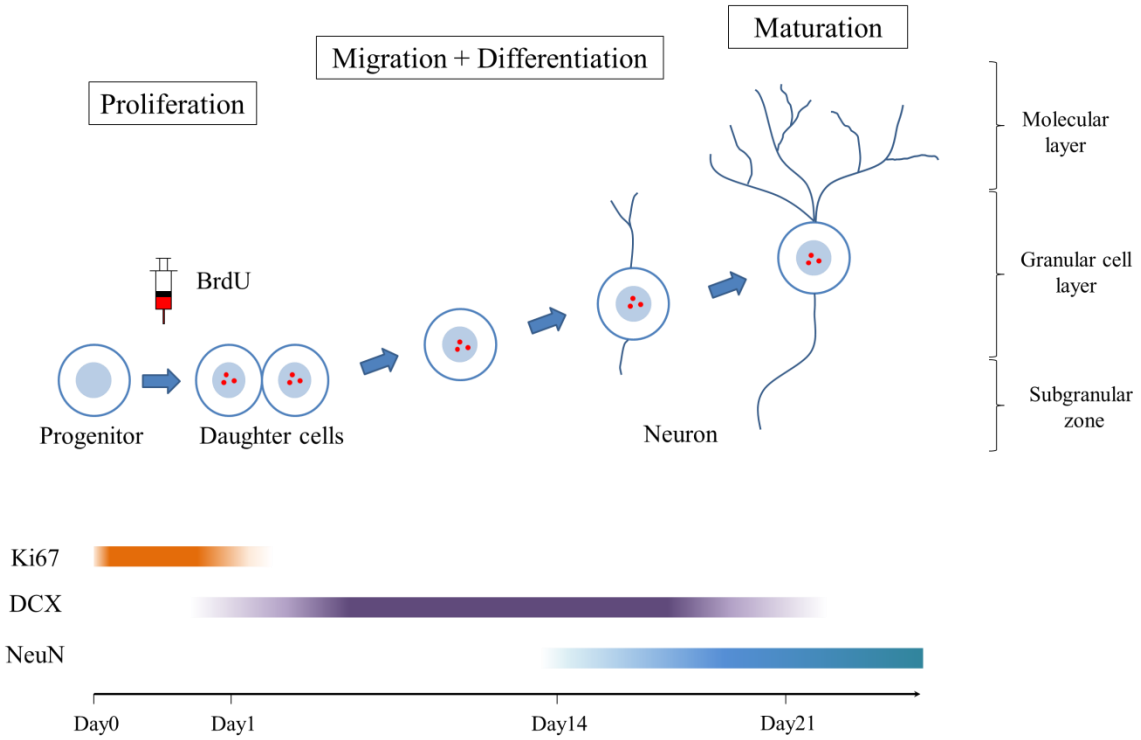
1. 1. Hippocampal adult neurogenesis

Adult neurogenesis refers to the ability of the adult mammalian brain to produce new neurons throughout adulthood. It is well-supported that there are two main regions in the brain in which neurogenesis occurs, the subgranular zone of dentate gyrus and the subventricular zone of olfactory bulb (Christie and Cameron, 2006; Eriksson et al., 1998). Adult neurogenesis in the dentate gyrus consists of at least four processes: cell proliferation (production of new cells), migration (migration of new cells to the appropriate place), differentiation (into a neuron or glia cell or the phenotype of new cells) and cell survival (cells surviving to maturity; see Figure 1). The amount of neurogenesis is determined by changes in any one of these components independently or in concert. For instance, chronic antidepressants increase neurogenesis via increases in cell proliferation independent of any changes in cell survival (Malberg et al., 2000), while testosterone increases neurogenesis via increases in cell survival independent of any changes in cell proliferation (Hamson et al., 2013; Spritzer and Galea., 2007). On the other hand prenatal alcohol exposure decreases neurogenesis in female rats due to reduction in the percentage of new cells differentiating into neurons independent of changes to cell proliferation or survival (Uban et al., 2010). Theoretically modification during any of the four stages may lead to changes in the function of the dentate gyrus. The integrity of the hippocampus is important for

successful spatial learning and memory, however, the role that adult hippocampal neurogenesis plays in spatial learning and memory is complicated. For example, studies show that ablation of neurogenesis impairs long-term spatial memory (Snyder et al., 2005) and pattern separation (Clelland et al., 2009) but does not affect acquisition or short-term spatial memory (Shors et al., 2002).

Stages of neurogenesis can be studied along with stage specific endogenous cell markers such as Ki67 (proliferating cells) or doublecortin (immature neurons), or exogenous markers such as bromodeoxyuridine (BrdU). BrdU incorporates into the DNA of mitotic cells during S-phase of the cell cycle, over a period of 2 hours (Nowakowski et al., 1989). Thus, BrdU is often used for birthdating and monitoring the fate of divided cells [for discussion on markers see (Taupin, 2007)]. Because BrdU does not exclusively label neurons but also other cell types such as glial cells (Taupin, 2007), it is necessary to co-label BrdU with endogenous markers, such as doublecortin (immature neurons expressed 1-21 days after production) or NeuN (expressed in mature neurons beginning approximately 14 days after production in a rat), depending on your timeline, to determine neuronal phenotype (Brown et al., 2003; Snyder et al., 2009).

Figure 1. Timeline of adult neurogenesis in the rat hippocampus. Dividing cells will form two daughter cells and express Ki67. Doublecortin (DCX) is expressed in new immature neurons from approximately day 1 until day 21 (Brown et al., 2003). BrdU incorporates into the DNA of mitotic cells during S-phase of the cell cycle, over a period of 2 hours. BrdU-labeled cells also co-express markers for mature neuronal proteins (such as NeuN) as early as 2 weeks after production.



1. 2. Pattern separation and pattern completion

The hippocampus plays critical roles for learning and recall of memory through two processes called pattern separation and pattern completion (Marr, 1971). Pattern separation is defined as the process that renders the pattern of information to be stored as distinct from each other during memory encoding and storage, while pattern completion is the process to recover the stored pattern from a degraded or partial retrieval cue during the time of recall and retrieval of memory (Marr, 1971). The computational model of pattern separation emphasizes the neural anatomy of hippocampus, in which there are divergent synaptic projections from ~200,000 cells in the entorhinal cortex to ~1,000,000 dentate granule neurons that project to the ~160,000 CA3 pyramidal neurons through mossy fiber pathway (Amaral and Witter, 1989). This feature allows the hippocampus to separate overlapping inputs from entorhinal cortex by increasing neural sparseness at the dentate gyrus before the information is transferred to the CA3, and subsequently to the CA1 hippocampal subregions. In contrast, the computational model of pattern completion emphasizes auto-associative networks in the hippocampus such as the recurrent collaterals circuitry in the CA3 region (Marr, 1971). Auto-associative networks were suggested to be able to store different patterns that are separated by dentate granule neurons (Levy, 1996; Levy et al., 2005; Rodriguez and Levy, 2004).

Both human and rodent studies support the finding that pattern separation occurs at the level of dentate gyrus (Gilbert et al., 2001; Yassa et al., 2011). Human studies with fMRI demonstrated that activity in dentate gyrus/CA3 was more active when detecting small differences and CA1/Subiculum was more active during generalization of information (Yassa et al., 2011). In particular adult neurogenesis in the dentate gyrus plays a critical role in pattern separation, as disruption of hippocampal neurogenesis interrupted the ability of mice to distinguish between similar patterns in multiple tests of pattern separation in female mice (Clelland et al., 2009). Furthermore increased neurogenesis was commensurate with greater ability of pattern separation in male mice (Chen et al 2012). These studies collectively suggest that neurogenesis in the dentate gyrus is important for the separation of similar patterns.

1. 3. Sex differences in hippocampal learning and memory

There are numerous examples of sex differences in hippocampal learning and memory, favoring males. Meta-analyses indicate that in both humans and rodents, males outperformed females in spatial tasks (Voyer et al., 1995; Jonasson, 2005). It is important to note that sex differences favoring men generally appear more prominently when there is greater cognitive demand, and perhaps when similar patterns are used and thus more attention is needed to process extramaze cues (Chamizo et al., 2011; Gibbs and Johnson, 2008). Furthermore there are sex

differences in hippocampal plasticity (Miranda et al., 1999; Dalla et al., 2009) including adult neurogenesis, for example cell proliferation is higher in proestrous females compared to males (Tanapat et al., 1999; reviewed in Galea et al., 2013). However, to date, there are no studies examining sex differences in pattern separation ability and the relationship to neurogenesis in the dentate gyrus. Studying sex differences in hippocampal function and plasticity are important as they may lead us to better understand why women are more vulnerable to neuropsychiatric and neurodegenerative disorders that negatively impact cognition and target the hippocampus such as depression (Gutiérrez-Lobos et al., 2002) and Alzheimer's disease (Beinhoff et al., 2008). Furthermore in both these disorders women suffer from cognitive impairment to a greater extent than men (Henderson and Buckwalter., 1994; Barnett and Gotlib, 1990), indicating that innate differences in the female processing of hippocampal function may lead us to discover the particular vulnerability of women to these diseases.

1. 4. Multiple memory systems and sex differences in learning strategy

The multiple memory system suggests that subjects learn the location of a goal by adopting two main cognitive strategies, hippocampus dependent spatial cue strategy and striatum dependent response cue strategies (McDonald and White, 1993). Hippocampus dependent spatial strategy users rely on knowledge of one's position and a goal that obtained from extra-maze

spatial cues (Morris et al., 1982), whereas striatum-dependent idiothetic strategy users rely on proprioceptive cues (Cook and Kesner, 1988).

Males and females can use different strategies to solve spatial navigation tasks, which is seen in both humans (Dabbs et al., 1998; Lawton, 1994; Silverman and Choi, 2006) and rodents (Korol, 2004; Hawley et al., 2012; Grissom et al, 2013). Females preferentially use striatum dependent strategies, although this is dependent on ovarian hormone status, while males preferentially use hippocampus dependent strategies to solve the same tasks (Williams et al., 1990; Galea and Kimura, 1993; Cherney et al., 2008; Grissom et al., 2013). This sex difference in strategy choice may be attributed to different usage of hippocampal neurons, because strategy preference can be shifted by inactivation of striatal or hippocampal neurons (Packard & McGaugh, 1996). In order to measure the amount of neuronal activation in a specified brain area, immediate early gene (IEG) expression, such as c-Fos and zif268, is often examined.

1. 5. Immediate early gene (IEG) expression in the hippocampus: relation to memory

IEGs are transcribed rapidly after neuronal stimulation and encode transcription factors that modify gene expression in response to the neuronal stimulation (Sheng and Greenberg, 1990), and play an important role in neural plasticity and memory consolidation (Guzowski et al., 2001; Jones et al., 2001). The IEG *c-fos* encodes a transcription factor, c-Fos, and is induced by learning

related stimulation in spatial working memory (Vann et al., 2000). Although long-term potentiation (LTP) is not always required for c-fos induction (Douglas et al., 1988; Wisden et al.,1990), ablation of *c-fos* gene impaired LTP and memory consolidation in spatial water maze training (Fleischmann et al., 2003; Guzowski et al., 2001). IEG zif268 encodes zinc finger transcription factors zif268/Egr1. Egr1 plays a critical role in the maintenance of LTP in the hippocampus through regulating gene expression of synapsin, and plays a crucial role in the consolidation of long-term memory (Jones et al., 2001; Penke et al., 2014; Petersohn et al., 1995). One of important differences of zif268 from c-fos is that zif268 is rapidly induced in association with LTP (Jones et al., 2001).

Other IEGs such as Arc (activity-regulated cytoskeleton-associated protein) have received considerable attention because it is suggested tight association with the induction of synaptic plasticity. Transcription of Arc gene is induced in response to synaptic activity or learning experience (Lyford et al., 1995; Guzowski et al., 1999). Specifically, activity of NMDA receptor is required for Arc induction (Czerniawski et al., 2011). mRNA of Arc is rapidly transported and accumulates at recently activated dendrites, and Arc protein is locally translated in the dendrites (Steward and Woorley, 2001; Farris et al., 2014). Because disruption of Arc synthesis impaired late-LTP and consolidation of memory, Arc is implicated the association with synaptic activity coupling to transcription and translation dependent synaptic plasticity

(Guzowski et al., 2000; Messaoudi et al., 2007). Although it is suggested that Arc is more tightly associated with synaptic plasticity, few studies have been conducted using Arc and will not be used in the present thesis but would be an important IEG to use in future.

1. 6. Sex differences in expression patterns of immediate early gene (IEG)

Male and female rats show different patterns of c-Fos expression in the hippocampus during spatial learning (Méndez-López et al., 2009). Méndez-López et al.(2009) showed that c-Fos expression was greater in the CA1 and CA3 regions in females but not in males after spatial reference memory retrieval in the Morris water maze task. Furthermore activation of adult-born granule neurons was associated with better memory retrieval in the Morris water maze in female, but not in male, rats (Chow et al., 2013). Thus collectively these studies suggest that there may be sex differences in activation of new neurons with pattern separation.

Therefore, the aim of this study was to explore sex differences in pattern separation ability, strategy choice, neurogenesis and immediate early gene expression in the hippocampus in response to a pattern separation task. To examine this, male and female rats were tested on their ability to separate similar and distinct patterns with a delayed non-match to place (DNMP) task in the radial arm maze (RAM). We also examined the neurogenesis levels in the hippocampus in response to pattern separation training 27 d after an injection of the DNA synthesis marker,

bromodeoxyuridine (BrdU) and after a probe trial to measure cell survival and IEG expression in the hippocampus. We hypothesized that males would outperform females only when separating similar patterns. We also predicted that better performance during pattern separation would be associated with greater cell survival and greater activation of new neurons that would be accompanied by sex differences possibly related to strategy use.

CHAPTER 2: Methods

2. 1. Subjects

Thirty-six 8 week old Sprague Dawley rats (males: n = 19; females: n = 17), were purchased from Charles River Canada (St-Constant, Quebec, Canada) for this study. Rats were housed in same sex pairs for 2 weeks after arrival and single-housed throughout the entire experiment. Males and females were housed in separate rooms to avoid odor cues of opposite-sex conspecifics interfering with performance. Rats were housed in opaque polyurethane bins (48 × 27 × 20 cm) with paper towels, polyvinylchloride tube, cedar bedding, and free access to food and water, and maintained under a 12 : 12 hour light/ dark cycle (lights on at 07:00 h). All animals were handled every day for 2 minutes beginning one week after arrival. All experiments were carried out in accordance with Canadian Council for Animal Care guidelines and were approved by the animal care committee at the University of British Columbia. All efforts were made to reduce the number of animals used and their suffering during all procedures.

2. 2. Apparatus

The radial arm maze had 8 arms (53 cm long × 10 cm wide) and an octagonal center platform (36 cm in diameter) and was set 80 cm above the floor in the center of a dimly lit room. Metal gates were used to block entries to arms. Large extramaze cues were placed on all four

walls of the room and were not moved throughout the study. At the end of each arm was a small cup securely glued to hold a sugar reward.

2. 3. Procedure

2. 3. 1 Experimental timeline

One intraperitoneal injection of bromodeoxyuridine (BrdU; 200mg/kg; Sigma-Aldrich, Oakville, ON, Canada) was administered to all animals at 11 weeks of age (experimental Day1). Four days after BrdU injection, all animals were food restricted and maintained at 87-92% of their original weight throughout the entire experiment (Day4-27). One week after BrdU injection, all animals were habituated to the radial arm maze for 2 days (Day 8, 9). Following habituation, rats were shaped for 3 consecutive days for 5 minutes each day (Day10-12). Following shaping, all rats were tested in the delayed nonmatching to place radial arm maze task for 14 days (Day 13-26), followed by a day of probe trial (Day 27) (Figure 2A).

2. 3. 2. Habituation and shaping

During habituation, rats were placed on the center platform and allowed to explore all arms freely for 10 minutes. During the first day of shaping, 3 quartered pieces of a Froot Loop (Kellog's) were placed along the length of each arm at equidistant intervals and a quarter was placed in a cup (3 cm in diameter) at the end of each arm. During the second and the third day of

shaping, each arm was baited with a quarter of Froot Loops placed in each cup.

2. 3. 3. Behavioral testing for spatial pattern separation

All testing began at approximately the same time every day at 08:00 h. Rats received 4 trials a day for 14 consecutive days (56 trials total with 28 trials of each separation – adjacent or separate). There was approximately 45 minutes interval between trials for each rat. The orders of testing for each rat were randomized every day. One trial consisted of a sample phase and a choice phase (40 seconds interval between the two phases – see Figure 2B). Rats were tested in their ability to discriminate the newly-opened arm during choice phases. During the sample phase, only a start arm and a sample arm were open and all other arms were closed. A rat was placed on the start arm and the rat was allowed to visit the sample arm and retrieve a quarter of Froot Loop (reward). Rats were retrieved from the maze after eating the reward (max 10 seconds). During the choice phase, all arms were blocked off except the start, sample and an additional arm (correct arm). The additional/correct arm, but not the sample arm, was baited during the choice phase. Rats that made incorrect choices (sample arm or start arm) were permitted to self-correct and retrieve the reward from the additional arm. A choice was defined as being made when the entire body, excluding the tail, had entered the arm. Rats were retrieved from the maze after they ate the reward or after 60 seconds had passed and returned to the colony room. During the interval between a sample phase and a choice phase, the maze was rotated to minimize the ability of rats

to utilize intramaze cues such as odor. After the rotation, the location of the start and sample arms relative to extramaze cues, but not the arms themselves, were held constant during each trial. The radial arm maze was wiped with distilled water after each rat.

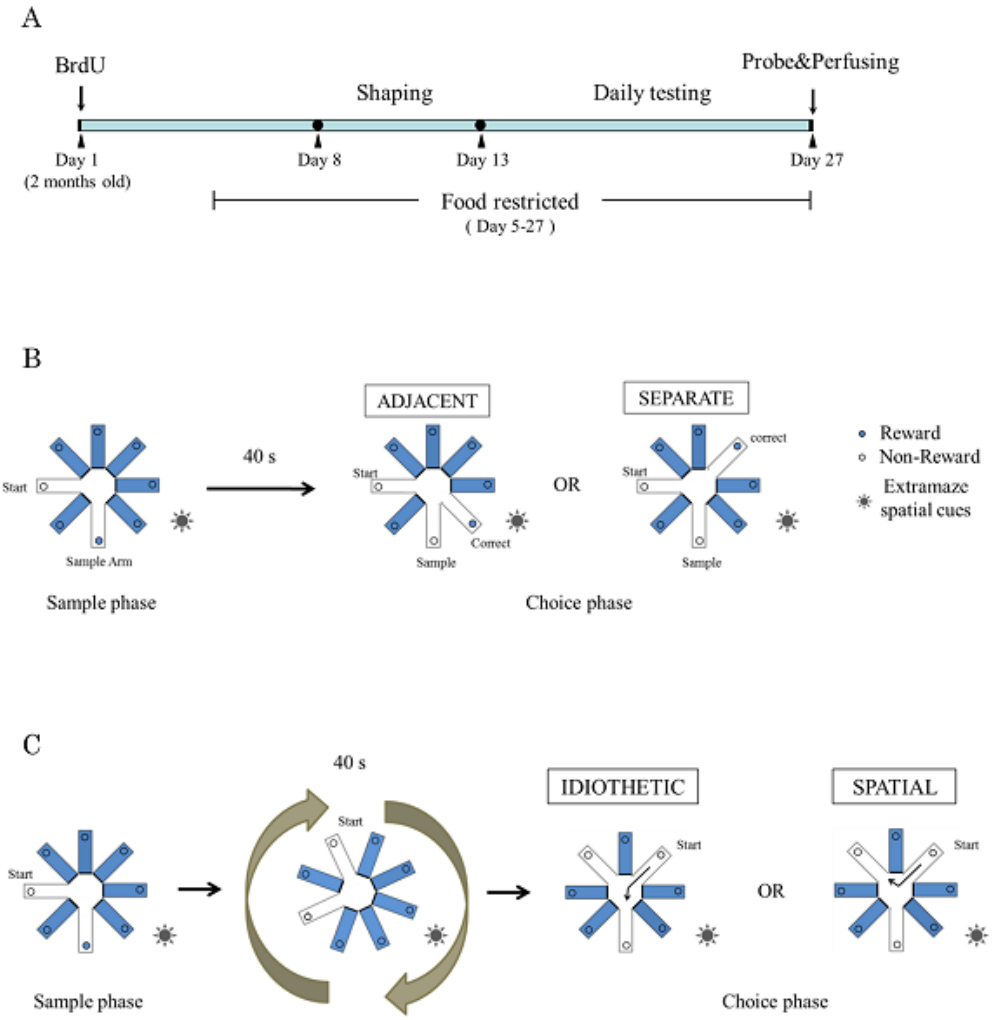
Two patterns of sample-correct arm pairs were used in this study, ADJACENT and SEPARATE. Correct arms during ADJACENT trials were 45° away from the sample arm and correct arms during SEPARATE trials were 135° away from the sample arm. Start arms were located perpendicular to either the correct or sample arms (Figure 2B). Sample-correct-start arm combinations were pseudo-randomly chosen for each day from the pool of possible combinations so that overlaps in the presentation of arms were minimized both within each day and across the entire experiment.

2. 3. 4. Probe trial

On the last day, Day 27, rats received one testing trial in the morning between 08:00 and 10:00 h. Eighty minutes after the testing trial, rats received a probe trial to determine whether they relied on idiothetic cues or spatial (extramaze) cues to solve the delayed non-match to place task. A probe trial consisted of a sample phase and a choice phase with a 40 second interval between the two phases. During a sample phase of the probe trial, the same rules as a sample phase of testing trials were applied (Figure 2C). The start arm during sample phase was perpendicular to the sample arm that was located to the right from the start arm. After the sample

phase, all arms were blocked off and the maze was rotated. A new start arm was moved to a new location (unlike in the testing trials) and two choice arms were opened after the rotation. The sample arm during choice phase was held the same position as the sample phase, relative to the extramaze cues, and the correct arm was located 135° away from the sample arm. The location of new start arm was perpendicular to the correct arm that was located to the right from the new start arm. In short, the orientation of start-sample arm pair during the sample phase was the same as that of start-correct arm pair. If a rat chose the correct arm, they were categorized as a spatial strategy user but if a rat chose the sample arm they were categorized as an idiothetic strategy user (Figure 2C). Immediately after the probe trial, rats were perfused (about 90 minutes after the testing trial).

Figure 2. Experimental design to examine spatial pattern separation for similar (ADJACENT) and distinct (SEPARATE) patterns. (A) The experimental timeline. (B) Pattern separation was tested with delayed non-match to place RAM task with two separation patterns, ADJACENT and SEPARATE. (C) Probe trial to determine whether a spatial or idiothetic strategy was used.



2. 4. Tissue processing

Rats were administered an overdose of sodium pentobarbitol and perfused transcardially with 60 ml of 0.9% saline followed by 120 ml of 4% paraformaldehyde (Sigma-Aldrich). Brains were extracted and post-fixed in 4% paraformaldehyde overnight, then transferred to 30% sucrose (Fisher Scientific) solution for cryoprotection and remained in the 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 anti-freeze solution consisting of ethylene glycol, glycerol and 0.1M PBS at -20°C.

2. 5. Immunohistochemistry

2. 5. 1. BrdU

Brain tissue slices were washed with 0.1 M TBS (pH 7.4) three times between each of the following steps. Tissue was pretreated in 0.6% H_2O_2 for 30 minutes, transferred to 2N HCl and incubated at 37 °C for 30 minutes. Then the tissue was rinsed with 0.1 M borate buffer (pH 8.5) for 10 minutes. After blocking the tissue with TBS+ solution, consisting of 0.3% Triton-X 100 (Sigma), and 3% normal horse serum (Vector Laboratories; Burlingame, CA, USA) in 0.1 M TBS, slices were incubated in a primary antibody solution 1:200 mouse anti-BrdU (Roche

Diagnostics, Laval, QC, Canada) and TBS, for 24 hours at 4 °C. This was followed by incubation with the secondary antibody solution containing 1:200 horse anti-mouse biotinylated IgG (Vector Laboratories, Burlington, ON, Canada) in TBS+ for 4 hours at room temperature. Tissue was incubated for 1.5 hour in ABC solution (Vector Laboratories). Tissue slices were then visualized with diaminobenzidine (DAB; Vector Laboratories) solution. The tissue was mounted onto microscope slides, followed by counterstaining with cresyl violet, dehydrated, cleared with xylene and cover-slipped with Permount (Fisher Scientific; Ottawa, ON, Canada).

2. 5. 2. Zif268 / c-Fos

Brain tissue was rinsed overnight with 0.1 M PBS at 4 °C. The tissue was incubated in 0.6% H_2O_2 for 30 minutes and then incubated in primary antibody solution containing 1:1000 Rabbit anti-Erg-1 (Santa Cruz Biotechnologies; CA, USA) or 1:1000 anti-c-Fos (Santa Cruz Biotechnologies), 0.04% Triton-X, and 3% normal goat serum (NGS; Vector Laboratories) in 0.1 M PBS for 24 hours at 4 °C. Following rinsing the tissue four times, the tissue was incubated in secondary antibody solution consisting of 1:1000 goat anti-rabbit biotinylated IgG (Vector Laboratories, Burlington, ON, Canada) in 0.1 M PBS for 24 hours at 4 °C. The tissue was then incubated in ABC solution (Vector Laboratories) for 1 hour at room temperature. Tissue slices were then visualized with diaminobenzidine (DAB; Vector Laboratories) solution and mounted onto microscope slides, followed by dehydrated, cleared with xylene and cover-slipped with

Permount (Fisher Scientific; Ottawa, ON, Canada).

2. 5. 3. BrdU/NeuN double labelling

Brain tissue was prewashed three times with 0.1 M PBS and left overnight at 4 °C. The tissue was incubated in a primary antibody solution containing 1:250 mouse anti-NeuN (Millipore; MA, USA), 0.3% Triton-X, and 3% normal donkey serum (NDS; Vector Laboratories) in 0.1 M PBS for 24 hours at 4 °C. Tissue was incubated in a secondary antibody solution containing 1:200 donkey anti-mouse ALEXA 488 (Invitrogen, Burlington, ON, Canada) in 0.1 M PBS, for 18 hours at 4 °C. After rinsing three times with PBS, tissue was washed with 4% paraformaldehyde, and rinsed twice in 0.9% NaCl, followed by incubation in 2N HCl for 30 minutes at 37 °C. Tissue was then 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 0.1 M PBS for 24 hours at 4 °C. Tissue was then incubated in a secondary antibody solution containing 1:500 donkey anti-rat Cy3 (Jackson ImmunoResearch; PA, USA) in 0.1 M PBS for 24 hours at 4 °C. Following three rinses with PBS, tissue was mounted onto microscope slides and cover-slipped with PVA DABCO.

2. 5. 4. BrdU/zif268 double labeling

The tissue was prewashed with 0.1 M TBS and left to sit overnight at 4 °C. The next day, tissue was incubated in zif268 primary antibody solution made with 1:1000 Rabbit anti- Egr-1

(Santa Cruz Biotechnologies; CA, USA), 3% NDS, and 0.3% Triton-X in 0.1 M TBS for 24 h ours at 4 °C. Then the sections were incubated in secondary antibody solution, consisting of 1:500 Donkey anti-Rabbit ALEXA 488 (Invitrogen, Burlington, ON, Canada) in 0.1 M TBS, for 18 hours at 4 °C. The tissue was then rinsed with 4% paraformaldehyde and washed twice in 0.9% NaCl. After incubation in 2N HCl for 30 minutes at 37 °C, slices were incubated with BrdU primary antibody solution consisting of 1:500 mouse anti-BrdU (Roche), 3% NDS, and 0.3% Triton-X in 0.1 M TBS for 24 hours at 4 °C. Then the tissue was incubated with secondary antibody solution consisting of 1:250 Donkey anti-Mouse Cy3 (Jackson ImmunoResearch; PA, USA) in 0.1 M TBS for 16 hours at 4 °C. After three rinses with TBS, slices were mounted onto slides and cover-slipped with PVA DABCO.

2. 6. Cell counting

All counting was conducted by an experimenter blind to the group assignment of each animal using a Nikon E600 microscope. Immunoreactive cells were determined to be in the dorsal or ventral DG using the criterion defined by Banasr et al. (2006), with sections 6.20-4.00mm from the interaural line defined as dorsal and sections 4.00-2.28mm from the interaural line as ventral. Cells were counted separately in each region because the dorsal hippocampus is associated with spatial learning and memory, while the ventral hippocampus is

associated with stress and anxiety-like responses (Moser et al., 1993; Kjelstrup et al., 2002).

2. 6. 1. BrdU immune-reactive(ir) cells

BrdU-ir cells were counted under a 100x oil immersion objective lens (Figure 3B) using light microscopy. For BrdU-ir cells, every 10th section of the granule cell layer (GCL) that includes the subgranular zone were counted. Total immunoreactive cells per region were estimated by multiplying the aggregate number of cells per region by 10 (Epp et al., 2007). Density of BrdU-ir cells was calculated by dividing the total immunoreactive cells in the GCL by volume of the corresponding region. Volume estimates of the dentate gyrus were calculated by multiplying the summed areas of the dentate gyrus by distance between sections (400μm; using Cavalieri's principle; Gundersen and Jensen, 1987). Area measurements for the dentate gyrus were obtained using digitized images on the software ImageJ (NIH).

2. 6. 2. BrdU/NeuN, BrdU/zif268 double labeled cells

The percentages of BrdU/NeuN and BrdU/zif268 double-labeled cells were obtained by randomly selecting 50 BrdU-ir cells and calculating the percentage of cells that coexpressed NeuN or zif268 under 400x magnification using a Nikon E600 epifluorescent microscope (Figure 3D-F).

2. 6. 3. zif268 and c-Fos expression

Optical density of zif268 and c-Fos expression in the dentate gyrus, CA1 and CA3 were analyzed as an estimate of the proportion of immunoreactive cells in the subregions. Images of the hippocampus were acquired at 40× magnification from three sections from the dorsal hippocampus and three sections from the ventral hippocampus on a Nikon E600 light microscope (see Figure for zif268: 4C-E; c-Fos: 4H-J). The proportion of area that exhibited above-threshold zif268 and c-Fos immunoreactive intensity in the corresponding subregions was obtained using ImageJ with digitized images. The threshold was set to 2.5 times above the background gray levels (Hartig, 2013). The background gray levels were the mean gray values that were obtained from three randomly selected areas without immunoreactivity. The total value of optical density for each brain was calculated by dividing the total immunoreactive areas by the total area of the corresponding subregions on the three sections.

2. 7. Data analyses

All analyses were conducted using Statistica (Statsoft Tulsa, OK) and significance level was set at $\alpha = 0.05$. The percentage of correct choices during ADJACENT and SEPARATE trials in the radial arm maze were each analyzed using analysis of variance (ANOVA), with strategy choice (spatial, idiothetic) and sex (male, female) as between-subject variables. Chi-square

analysis was used for strategy choice across sex and estrous cycle phase. Repeated-measures ANOVAs were used to separately analyze the density of BrdU-ir cells, optical density of zif268 and c-Fos expression, and volume of dentate gyrus with strategy choice and sex as between subject factors and hippocampal subregion (dorsal, ventral) as within-subject factors. For percentage of cells co-expressing BrdU/NeuN or BrdU/zif268, repeated-measures ANOVAs were performed with sex and strategy choice as between-subject variables. Pearson product-moment correlations were calculated to examine the relationship between spatial pattern separation performance and density of BrdU/NeuN cells, zif268 expression, c-Fos expression or cells co-expressing BrdU/zif268. Post-hoc tests utilized the Neuman-Keuls procedure. A priori comparisons were subjected to Bonferroni corrections.

CHAPTER 3: Results

3. 1. Behavioral testing

3. 1. 1. Male spatial strategy users made more correct choices than females in ADJACENT trials, but not in SEPARATE trials

Males had significantly greater percentage of correct arm choices than females for ADJACENT trials [main effect of sex: $F(1, 32) = 4.39$, $p = 0.044$, all other main and interaction effects: $p > 0.15$; see Figure 2A]. However a priori we expected there to be a strategy difference and when broken down by strategy group, a priori tests revealed that only male spatial strategy users chose significantly greater percentage of correct choices than female spatial strategy users in ADJACENT trials ($p = 0.011$; see Figure 3B), but not in idiothetic strategy users ($p = 0.69$) (Figure 3C).

Analyzing the percent correct responses in SEPARATE trials revealed a trend for spatial strategy users to have more correct choices than idiothetic strategy users (main effect of strategy, $p<0.06$) but no other significant effects (all p's >0.57).

3. 1. 2. There were no significant sex or estrous cycle differences in the number of strategy users

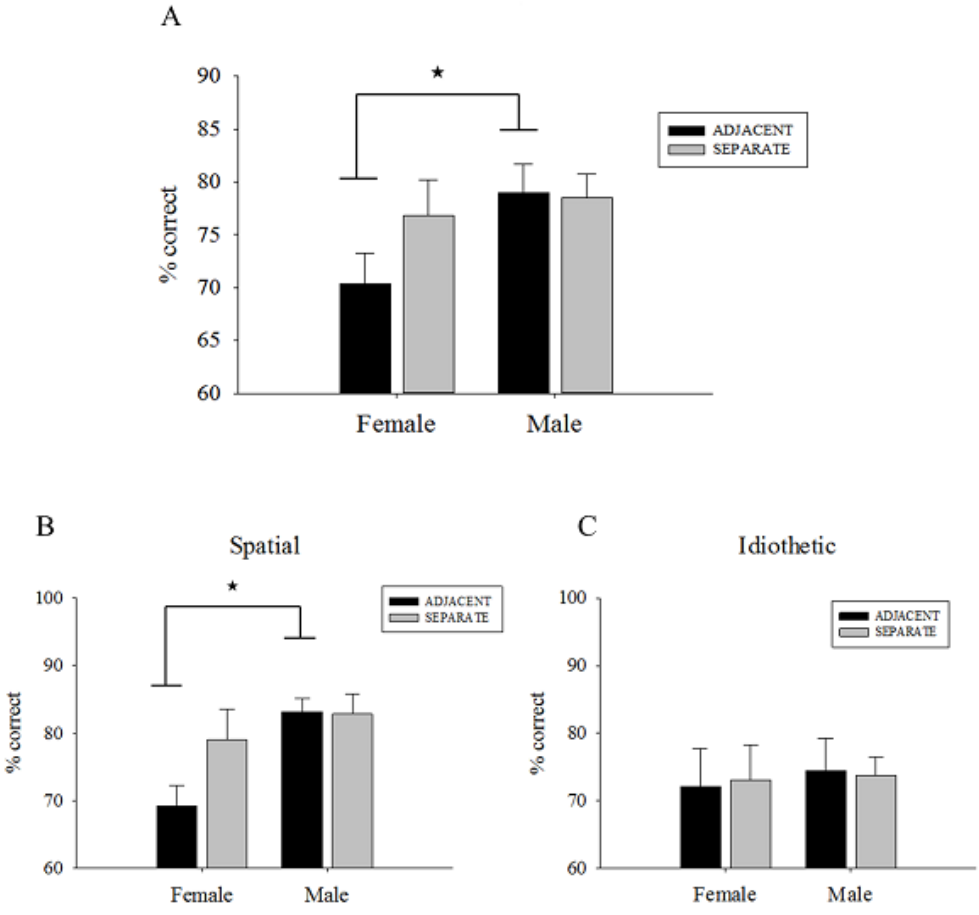
There was no significant difference in the distribution of strategy choices between males and females ($\chi2 = 0.139$, $p = 0.709$), nor between proestrous and non-proestrous females ($\chi2 = 0.131$, $p = 0.252$; see Table 1).

Table 1 The number of subjects within each strategy

		Strategy	
	Estrus state	Spatial	Idiothetic
Male	-	10	9
Female	Proestrus	4	1
	Non-proestrus	6	6

There were no significant differences in strategy choice.

Figure 3. (A) Mean (+SEM) percentage of correct choices during ADJACENT and SEPARATE trials in females versus males with data from the spatial and idiothetic strategy users combined, and (B and C) shown separately with strategy users; (B) with spatial strategy users and (C) with idiothetic strategy users. Males had significantly greater percentage of correct choices than females during ADJACENT trials but not during SEPARATE trials. Male spatial strategy users had significantly greater percentage of correct choices than female spatial strategy users during ADJACENT trials. * indicates $p < 0.05$.



3. 2. Males had a significantly larger dentate gyrus than females

Males had a larger dentate gyrus volume than females in both dorsal and ventral regions despite the significant interaction [region by sex interaction: $F(1,34) = 11.72$, $p = .0016$, main effect of sex: $F(1,34) = 51.41$, $p < .0001$; main effect of region: $F(1.34) = 74.38$, $p < .0001$; see Table 2]. Because there were sex differences in the volume of dentate gyrus, cell density was used in order to directly compare the sexes without volume being a confounding variable.

Table 2 Mean (+SEM) volume of the dorsal and ventral GCL

			Volume (mm^3)	
		N	Dorsal	Ventral
Female	Spatial	10	1.56 ± .088	1.98 ± .105
Female	Idiothetic	9	1.43 ± .149	1.88 ± .082
Male	Spatial	10	1.90 ± .096	2.90 ± .180
Male	Idiothetic	7	1.87 ± .138	2.87 ± .130

Males had significantly greater dentate gyrus volume than females (p's < 0.004)

3. 3. Male spatial strategy users had greater neurogenesis in the dorsal dentate gyrus than male idiothetic strategy users and females

Male spatial strategy users had greater density of BrdU-ir cells in the dorsal DG compared to all other groups [all p's <0.004; sex by strategy interaction: p = 0.031; see Figure 4A also a main effect of region: F(1,26) =7.63, p =0 .011;]. A 'neurogenesis index' was also calculated by multiplying the density of BrdU-ir cells by percentage of BrdU-ir cells that also co-expressed NeuN (Snyder et al., 2009). Male spatial strategy users had significantly greater BrdU/NeuN cell density than all other groups in the dorsal dentate gyrus [all p's <0.005; region by sex by strategy interaction: F(1,26) = 4.09, p = 0.05; main effect of region: F(1,26) =9.98, p < 0.004; sex by strategy interaction p < 0.07; see Figure 5A].

There was greater percentage of BrdU-ir cells co-expressing NeuN in the dorsal compared to the ventral dentate gyrus [main effect of region: F(1,27) = 13.175, p = 0.001] and a trend for males to have a greater percentage of BrdU/NeuN co-labelled cells than females (p = 0.061; see Table 3).

There were very few cells co-expressing BrdU/zif268 (see Table 4). There was no significant difference between groups for the percentage of cells co-expressing BrdU/zif268 (all p's > 0.09).

Table 3 Mean (+SEM) percentage of cells co-expressing BrdU and NeuN in the GCL in female and male rats

			BrdU/NeuN co-expressing cells (%)	
		N	Dorsal	Ventral
Female	Spatial	8	82.75 ± 3.34	74.00 ± 2.70
Female	Idiothetic	5	84.40 ± 4.12	74.00 ± 4.34
Male	Spatial	9	86.67 ± 1.05	81.56 ± 2.10
Male	Idiothetic	9	82.67 ± 2.05	80.00 ± 2.67

There was greater percentage of BrdU-ir cells co-expressing NeuN in the dorsal compared to the ventral dentate gyrus but no significant differences between groups.

Table 4 Mean (+SEM) percentage of cells co-expressing BrdU and zif268 in the GCL in female versus male rats.

			BrdU/zif268 co-expressing cells (%)	
		N	Dorsal	Ventral
Female	Spatial	8	0.00 ± 0.00	0.00 ± 0.00
Female	Idiothetic	5	0.80 ± 0.80	0.00 ± 0.00
Male	Spatial	9	0.22 ± 0.22	0.44 ± 0.44
Male	Idiothetic	9	0.44 ± 0.44	1.56 ± 0.80

No significant differences between groups in percentage of BrdU/zif268 co-expressing cells were found.

Figure 4. (A) Mean (+SEM) density of BrdU-ir cells in the dentate gyrus and (B) photomicrograph of BrdU-ir cell stained with DAB in the granule cell layer (GCL). Male spatial strategy users had significantly greater density of BrdU-ir cells in the dorsal dentate gyrus than the other groups. Images were captured at 1000× magnification. White arrows indicate immune-reactive cells. * indicates $p < 0.05$.

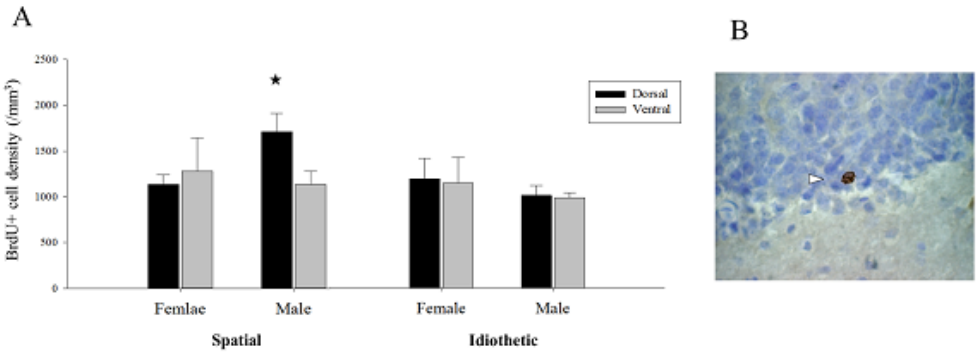
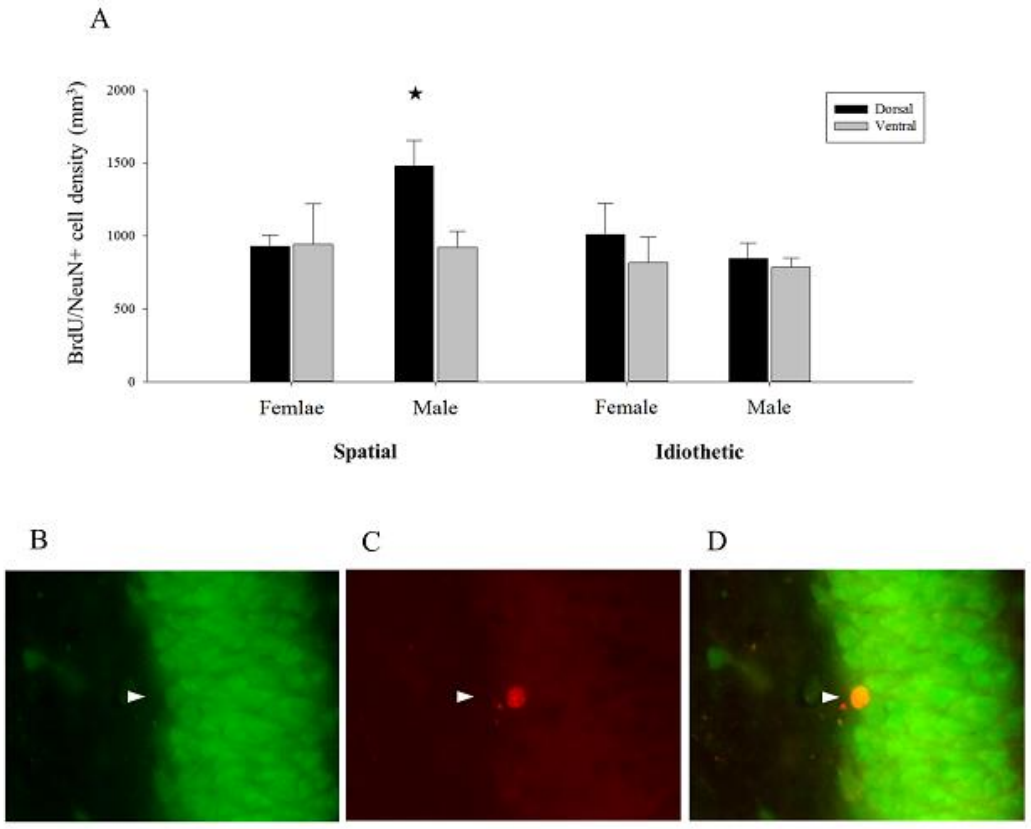


Figure 5. (A) Mean (+SEM) density of BrdU/NeuN coexpressing cells in the dentate gyrus. Male spatial strategy users had significantly greater density of BrdU/NeuN coexpressing cells in the dorsal dentate gyrus than the other groups. (B-D) photomicrographs of cells labeled with the fluorescent neuronal marker NeuN (green) (B), BrdU (red) (C), and merged image indicating a double-labelled cell (D). 400× magnification. White arrows indicate immune-reactive cells. * indicates $p < 0.05$.



3. 4. zif268 expression in the dorsal CA3 was greater in females than males

Females had significantly greater density of zif268 expression in the dorsal CA3, but not in the ventral CA3, than males [sex by region: $F(1, 31) = 12.79$, $p = 0.001$; see Figure 6A]. Zif268 expression in the dorsal CA1 was greater than in the ventral CA1 [region: $F(1,30) = 105.2$, $p < 0.001$; see Figure 6B]. There were no other significant main or interaction effects (p's > .20). There were no significant main or interaction effects in expression of zif268 in the dentate gyrus (p's > 0.22).

3. 5. c-Fos expression in the CA1 region was greater in idiothetic strategy users than spatial strategy users, while in the CA3 region males had greater expression in the dorsal CA3 than females, and there was greater expression in the dorsal dentate gyrus compared to ventral dentate gyrus

In the dentate gyrus, there was greater c-Fos expressing cell density in the dorsal dentate gyrus than ventral dentate gyrus [main effect of region: $F(1, 32) = 14.7$, $p < 0 .001$]. For the CA3 region, males had greater expression of c-Fos in the dorsal CA3 than all other groups but there were no other significant effects [sex by region: $F(1, 32) = 8.05$, $p < 0.001$; main effects of sex and region: both p's <0.001; see Figure 7A]. In the CA1 region, idiothetic strategy users had greater c-Fos expression than spatial strategy users [main effect of strategy: $F(1,32) = 7.20$, $p <$

0.011; see Figure 7B] and greater c-Fos expression in ventral compared to dorsal CA1 [$F(1,32) = 56.3$, $p < 0.0001$] but no other significant effects (p's > 0.28).

Figure 6. (A) Mean (+SEM) density of zif268 expressing cells in the CA3 in all females versus all males. Females had significantly increased zif268 expression in the dorsal CA3 than males. (B) Mean (+SEM) density of zif268 expressing cells in the CA1. Dorsal CA1 had significantly greater zif268 expression compared to the ventral CA1 in all groups. (C-E) Photomicrographs of zif268 immunoreactive cells in the CA1 (C), in the CA3 (D) and in the dentate gyrus (DG) (E). Images were captured at 200× magnification.* indicates $p < 0.05$.

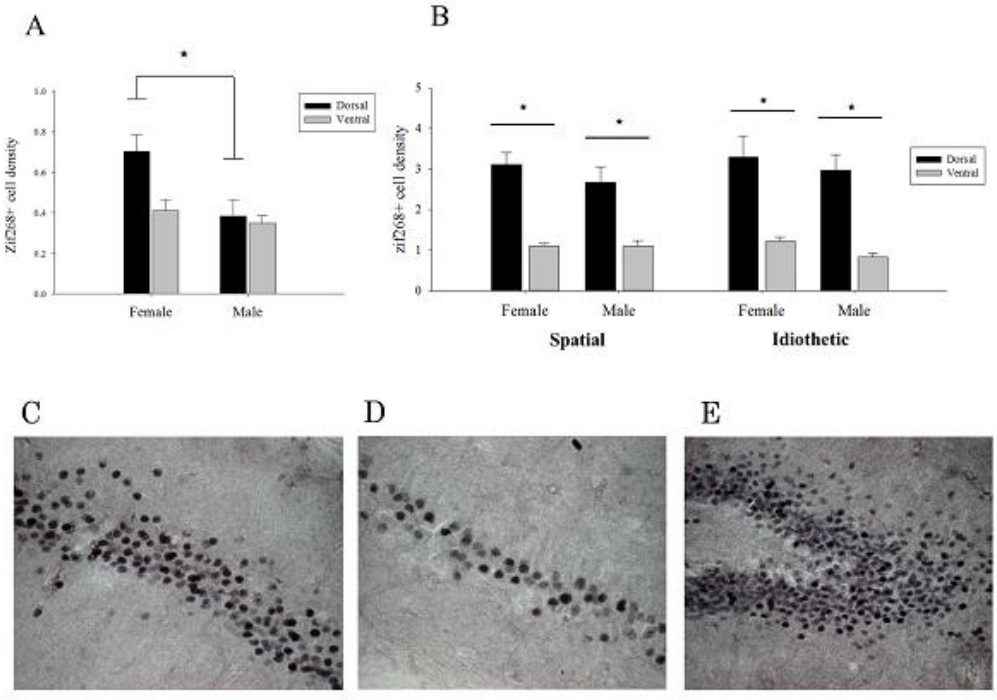
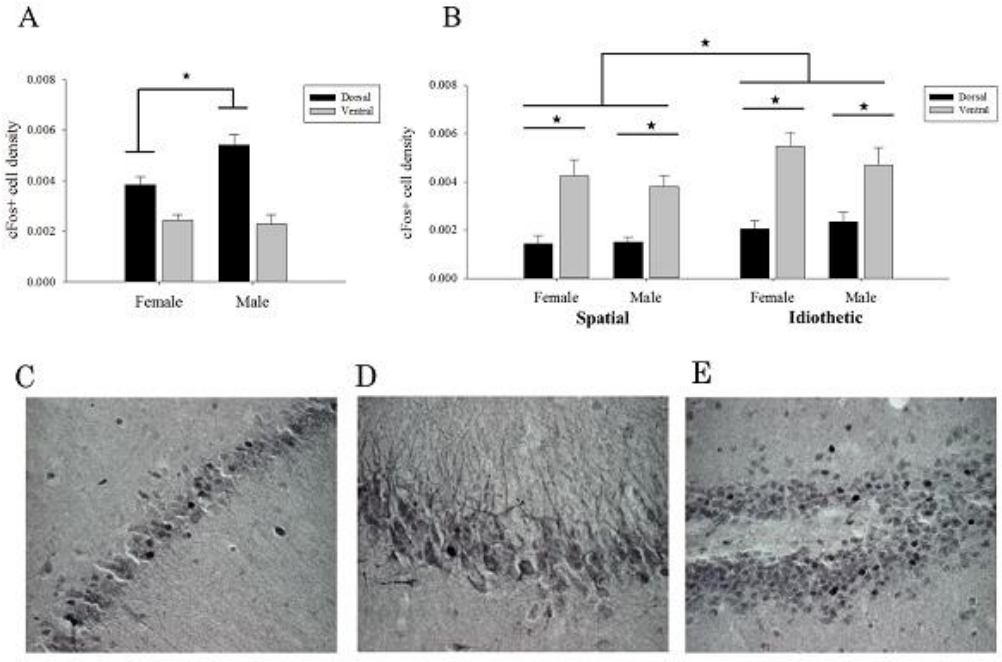


Figure 7. (A) Mean (±SEM) density of c-Fos expressing cells in the CA3 in all females versus all males. Males had significantly increased c-Fos expression in the dorsal CA3 than females. (B) Mean (+SEM) density of c-Fos expressing cells in the CA1. Idiothetic strategy users had significantly greater expression of c-Fos in the dorsal CA1 than spatial strategy users, and greater c-Fos expression in the ventral than dorsal CA1 in all groups. (C)-(E) Photomicrographs of c-Fos immunoreactive cells in the CA1 (C), in the CA3 (D) and in the DG (E). Images were captured at 200× magnification.* indicates $p < 0.05$.



3. 6. Correlations:

3. 6. 1. Greater BrdU/NeuN cell density was associated with better performance during a pattern separation task in spatial strategy users but not in idiothetic strategy users. These correlations were stronger in females than in males.

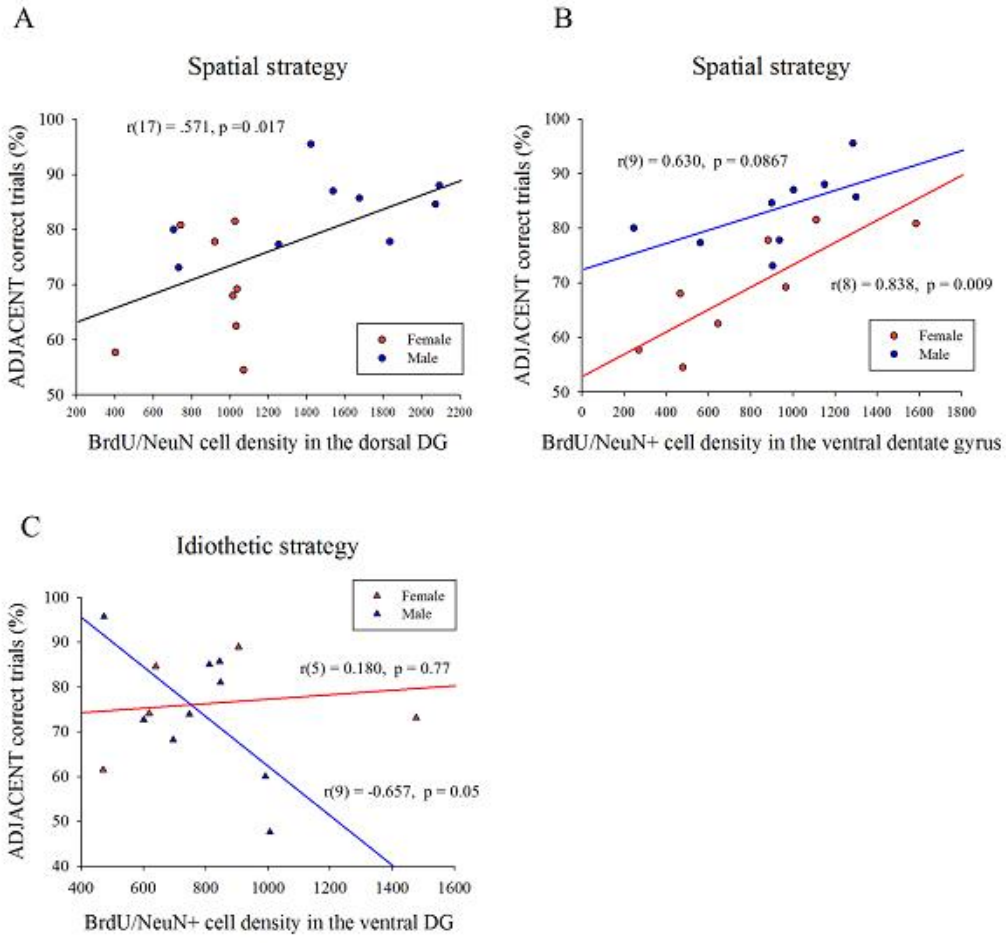
Proportion of correct choices during ADJACENT trials was positively correlated with BrdU/NeuN co-expressing cell density in the dorsal dentate gyrus [r(17) = .571, p =0 .017] and in the ventral dentate gyrus [r(17) = .663, p = 0.004] in spatial strategy users, but not in idiothetic strategy users (p's > .44; see Figure 8). Proportion of correct choices in SEPARATE trials and BrdU/NeuN co-expressing cell density in the ventral dentate gyrus was positively correlated in spatial strategy users [r(17) = .600, p = 0.011] and negatively correlated in idiothetic strategy users [r(14) = -.626, p =0 .017].

When broken down by sex, in female rats, proportion of correct choices during ADJACENT trials was positively correlated with BrdU/NeuN co-expressing cell density in the ventral dentate gyrus [r(13) = .574, p = .040], but not in male rats [r(18) = .627, p < 0.81]. When broken down by sex and strategy users, proportion of correct choices during ADJACENT trials was positively correlated with BrdU/NeuN co-expressing cell density in the ventral dentate gyrus in female spatial strategy users [r(8) =0 .838, p =0.009; see Figure 8B], negatively correlated in male idiothetic strategy users [r(9) = -0.657, p = 0.05; see Figure 8C] and a trend for a positive

correlation in male spatial strategy users [r(9) = 0.603, p = 0.086; see Figure 8B] but no other significant correlation in other groups (p > .10).

There were also positive correlations with total dentate gyrus volume and proportion of correct choices during ADJACENT trials in spatial strategy users (r(19) =0.5197, p =0.019) driven by ventral dentate gyrus volume (r(19) =0.4855, p =0.03). There were negative correlations with total dentate gyrus volume and proportion of correct choices during SEPARATE trials in idiothetic male stategy users (r(8) =-0 .7075, p =0.033) driven again by ventral dentate gyrus volume(r(8) =-0 .7104, p =0.032).

Figure 8. Correlations between performance during a spatial pattern separation task and neurogenesis. (A and B) Correlation in spatial strategy users with data from both males (blue) and females (red) between proportion of correct choices during ADJACENT trials and density of BrdU/NeuN coexpressing cells in the dorsal (A) and ventral dentate gyrus (B). (C) Correlation between proportion of correct choices during ADJACENT trials and density of BrdU/NeuN coexpressing cells in the ventral dentate gyrus in idiothetic strategy users with data from both males (blue) and females (red).



3. 6. 2. Zif268 expression in the ventral CA3 and CA1 was negatively correlated to performance of pattern separation in male spatial strategy and idiothetic strategy users but not in females.

Proportion of correct choices during ADJACENT trials was negatively associated with zif268 expressing cell density in the ventral CA3 in spatial strategy users [all spatial strategy users: $r(20) = -.440$, $p = 0.05$; see Figure 9A], and in both males and females [all males: $r(19) = -.446$, $p = 0.056$; all females: $r(17) = -.465$, $p = 0.06$]. However, when broken down by sex and strategy, there were no significant correlations (p's > 0.12).

Zif268 expression in the ventral dentate gyrus was negatively correlated with proportion of correct choices during ADJACENT trials in male idiothetic strategy users [$r(9) = -.725$, $p = 0.027$, See Figure 9B] and during SEPARATE trials in male spatial strategy users [$r(10) = -.648$, $p = 0.043$] but no significant correlations were seen in females (p's > 0.30).

Zif268 expression in the ventral CA1 was negatively correlated to proportion of correct choices during ADJACENT trials in male idiothetic strategy users [$r(9) = -.745$, $p = .021$; see Figure 9C]. There was no other significant correlations between zif268 expression in the CA1, DG or CA3 and performance in ADJACENT or SEPARATE trials (p's $>.10$).

3. 6. 3. Performance in SEPARATE trials was positively correlated with dorsal CA3 c-Fos expression in male spatial strategy users, but negatively correlated in dorsal CA1 c-Fos expression in male idiothetic and female spatial strategy users. Performance during ADJACENT trials was positively correlated with dorsal CA1 c-Fos expression in female spatial strategy users.

Greater c-Fos expression in the dorsal CA3 was associated with better performance in SEPARATE trials in males [$r(19) = 0.534$, $p = 0.019$], in spatial strategy users [$r(20) = 0.533$, $p = 0.016$], and specifically in male spatial strategy users [$r(10) = 0.806$, $p = 0.005$]. There was no other significant correlations in any other groups in the CA3 (p's $> .26$) or in the dentate gyrus (all p's > 0.05).

In males, greater c-Fos expression in the dorsal CA1 was negatively correlated with proportion of correct choices during SEPARATE trials in males [$r(19) = -0.542$, $p = 0.017$] and females [$r(19) = -.577$, $p = 0.010$]. When broken down by strategy users, greater c-Fos expression in the dorsal CA1 was negatively correlated with proportion of correct choices during SEPARATE trials in male idiothetic strategy users [$r(9) = -.706$, $p = 0.034$] and in female spatial strategy users [$r(9) = -.810$, $p = 0.008$]. In ADJACENT trials, c-Fos expression in the dorsal CA1 was negatively correlated with proportion of correct trials [$r(9) = -.775$, $p = 0.014$; see Figure 10],

in female spatial strategy users only. However, there were no significant correlations between c-Fos expression in the dentate gyrus and performance (all p's > 0.06).

Figure 9. Correlations between performance during a spatial pattern separation task and immediate early gene expression. (A) Correlation in spatial strategy users with data from both males (blue) and females (red) between proportion of correct choices during ADJACENT trials and density of zif268 expressing cells in the ventral CA3. (B and C) Correlation between proportion of correct choices during ADJACENT trials and zif268 expressing cell density in the ventral dentate gyrus (B), and in the ventral CA1(C) in male idiothetic strategy users.

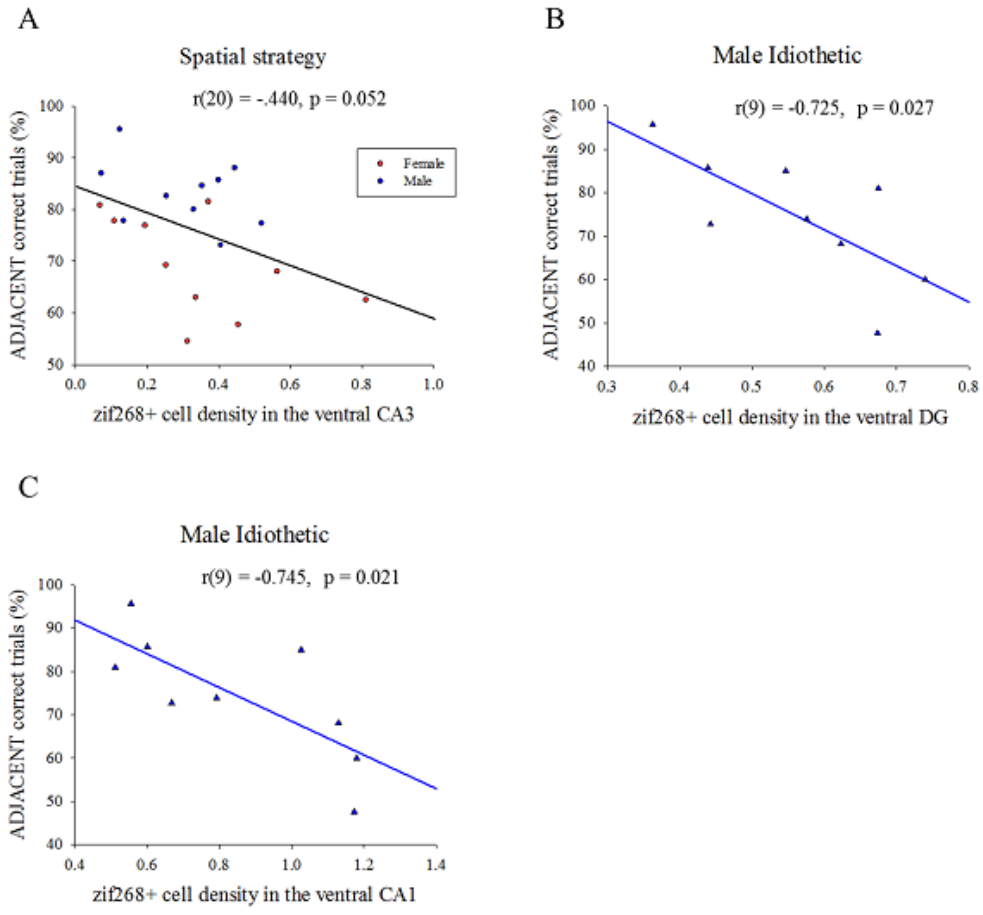
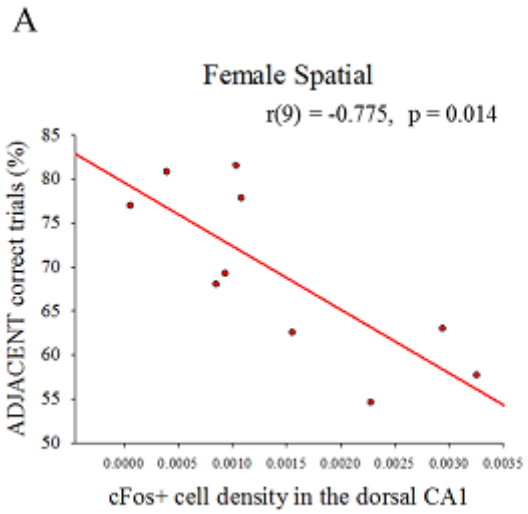


Figure 10. Correlation between proportion of correct choices during ADJACENT trials and c-Fos expressing cell density in the dorsal CA1 in female spatial strategy users.



CHAPTER 4: Discussion

In the present study there were sex differences in pattern separation performance, neurogenesis in response to pattern separation training and in associations of neurogenesis and activation with performance. While males outperformed females on similar patterns (during ADJACENT trials), there was no sex difference in performance of distinct patterns (during SEPARATE trials) in the delayed non-match to place radial arm maze task. This effect was stratified by strategy users as the sex difference in performance in similar pattern (ADJACENT) trials emerged only in the spatial strategy users, but not in idiothetic strategy users. Furthermore, male spatial strategy users had greater neurogenesis in the dorsal dentate gyrus than all other groups. In addition in the dorsal CA3 there were sex differences in zif268 and c-Fos expression patterns with females exhibiting greater expression of zif268 and males exhibiting greater expression of c-Fos. Furthermore, c-Fos expression in the CA1 region was greater in idiothetic strategy users than spatial strategy users. There were also regional differences with greater dorsal expression in IEG expression in the CA1 and DG regions. Finally neurogenesis and IEG expression were significantly correlated with performance stratified by sex and strategy use. Greater levels of neurogenesis was associated with better performance during ADJACENT trials in the female spatial strategy users, but worse performance during ADJACENT trials in male idiothetic strategy users. Zif268 expression in the ventral dentate gyrus and CA1 was negatively

correlated to performance of pattern separation in male idiothetic strategy users. c-Fos expression in the dorsal CA1 was negatively correlated with pattern separation (SERPATE and ADJACENT) in female spatial strategy users. Thus, sex differences in the patterns of IEG expression and correlations of IEG expression with performance varies based on location, type of pattern separation and strategy use. Together these results demonstrate that male spatial strategy users show superior performance and greater neurogenesis in the dentate gyrus than females, but exhibit fewer associations with IEG expression and performance. These findings collectively suggest that there are sex differences that extend beyond performance level and are reflected in the neural response to pattern separation training.

4. 1. Male spatial strategy users are better at separating similar patterns than female strategy users

In the present study, males outperformed females during similar (ADJACENT) pattern trials, but not during distinct (SEPARATE) pattern trials. Because extramaze cues overlap more in the ADJACENT condition versus the SEPARATE condition, this results in more similar patterns in ADJACENT versus distinct patters in SEPARATE arm pairs, thus greater pattern separation ability is needed in ADJACENT trials. These results indicate that males perform better than females on the ability to separate similar, but not distinct, patterns. This finding is consistent with previous studies showing that sex differences favoring males in spatial learning are more

prominent when spatial cues overlap to greater degrees – indicating the need for better pattern separation under similar conditions (van Haaren et al., 1987; Williams et al., 1990; Chamizo et al., 2011). Furthermore, our findings that the sex differences were only observed in spatial strategy users are somewhat consistent with Chow et al. (2013), who showed sex differences, favoring males, in acquisition of the spatial Morris water maze only among spatially-trained, but not cue-trained, rats.

In the present study, there was no significant sex difference in strategy choice. This finding is inconsistent with previous findings that males rely more on hippocampus dependent spatial strategies while females rely more on striatum dependent response strategies (Hawley et al., 2012). Nevertheless my findings are consistent with another study demonstrating that both males and females do not show preference of strategy choice when spatial and response strategies are equally effective to reach a goal location (van Gerven et al., 2012). Indeed in the present study spatial users were not more effective than idiothetic users overall, indicating that both strategies were equally effective. However, although not statistically significant more proestrous females were spatial strategy users than idiothetic strategy users, which is consistent with previous studies that proestrous females rely more on hippocampus dependent spatial strategies (Korol et al., 2004; Rummel et al., 2010). Collectively these findings suggest that because sex differences in spatial learning and pattern separation were limited to hippocampal-based strategy

users that any sex differences in hippocampal neurogenesis may tightly link to spatial pattern separation.

4. 2. Male spatial strategy users showed greater cell survival of adult-born neurons in the dorsal dentate gyrus than all other groups.

The present study found greater neurogenesis (27 day old neurons) in the dorsal dentate gyrus of male spatial strategy users compared to all other groups. This finding is partially consistent with a previous finding showing that males that had undergone spatial training where more likely to show greater neurogenesis than females that had undergone spatial training (Chow et al., 2013). The greater levels of neurogenesis in male spatial strategy users could be due to either a consequence of the pattern separation training or may be better related to better innate ability in these males. It is difficult to tease these explanations apart with the paradigm we have used. In the latter explanation, subjects with greater adult neurogenesis may be able to recruit new neurons to perform pattern separation or may be better at hippocampus-dependent strategy compared to striatum-dependent strategies. Consistent with this explanation, a previous study showed that inactivation of hippocampus results in shifts from spatial strategy to response strategy (Packard and McGaugh, 1996). However it is also possible that 14 testing days using a spatial strategy enhanced the survival of new neurons in the dorsal dentate gyrus in males. This is partially consistent with studies showing that exposure to spatial training 6-10 days after a BrdU

injection enhances survival of immature neurons in male but not in female rats (Chow et al. 2013) in the Morris water maze. More studies have been done in males and spatial training 6-10 (Epp et al., 2007) or 7-14 days (Gould et al., 1999) after a BrdU injection increases neurogenesis in male rats. However, there are studies that failed to find enhancement of cell survival by spatial training in either males or females (Van der Borght et al., 2005; Mohapel et al., 2006; Epp et al., 2007; Barha and Galea, 2013). These inconsistencies may be due to the use of different tasks, food restriction requirements, different timing of BrdU to training, and/or duration of spatial learning (see Epp et al., 2013 for review). For instance, there is no significant effect of spatial learning on survival of BrdU-ir cells when rats received spatial training in the Morris water maze either 1-5, 7-9, 7-11 or 11-15 days after a BrdU injection (Epp et al., 2007; Mohapel et al., 2006; Van der Borght et al., 2005). Furthermore, BrdU-ir cell survival decreases with longer exposure to spatial learning in the Morris water maze 1-14 days after a BrdU injection in male rats (Mohapel et al., 2006), and in the radial arm maze 1-33 days after a BrdU injection in ovariectomized female rats (Barha and Galea, 2013). This reduction with prolonged duration of spatial learning may be due to task associated stress and task difficulty. It has been reported that chronically high levels of corticosterone or chronic stress reduces cell survival (Pham et al., 2003; Brummelte and Galea, 2010) and increasing difficulty in spatial learning tasks also reduces cell survival (Epp et al., 2010). In the present study, rats were exposed to two weeks of spatial

pattern separation task in the radial arm maze and experienced three weeks of food restriction. It is possible that two weeks of spatial pattern separation task could rescue the reduction of cell survival due to food restriction and/or stress of task performance only in male spatial strategy users, but not in the other groups. It is also possible that male spatial strategy users are more likely to have higher levels of neurogenesis innately. However, in another study, hippocampus-dependent strategy users were more likely to show lower levels of cell proliferation and no significant differences were observed in neurogenesis levels (Epp and Galea, 2009), contrary to the findings of the present study. With the current design of this experiment it is not possible to distinguish between the two possible explanations of why male strategy users have greater levels of neurogenesis (innate difference or difference as a result of testing). However it is clear that males that use a spatial strategy not only perform better when distinguishing between similar patterns but also have greater levels of neurogenesis in the dentate gyrus than females or males that use an idiothetic strategy.

4. 3. There were very few BrdU labeled cells co-expressing zif268.

In the present study, there were very few (1.5%) cells that co-expressed BrdU and zif268, partially inconsistent with previous studies which showed 2-7% co-labelling (Snyder et al., 2009; Chow et al., 2013; McClure et al., 2013). This inconsistency may be because of age of neurons, type of task, or presence of food restriction. In the present study we examined 27 d old BrdU-ir

cells after food restriction in the pattern separation task while after water maze training Chow et al. (2013) and McClure et al. (2013) examined zif268 expression in 20 day old BrdU-ir cells and Snyder et al. (2009) examined zif268 expression in 14-28 day old BrdU-ir cells in rats. It is also possible that new neurons are recruited at different times for pattern separation and we were too late for these BrdU-ir cells to be recruited for pattern separation. A recent study is consistent with this interpretation as only young adult-born neurons are recruited for pattern separation while 6 weeks or older adult-born and developmental-born neurons do not play a role for pattern separation in mice (Nakashiba et al., 2012). The maturation timeline of adult-born neurons differs between mice and rats, as there is a faster maturation rate of newborn neurons in rats compared to mice (Snyder et al., 2009). Therefore, 4 weeks old dentate granule neurons in rats in the present study may not play a large role for pattern separation. More research examining shorter timelines for BrdU/zif268 co-expression after a pattern separation task is needed to determine the timing of when new neurons are activated in response to pattern separation performance.

4. 4. Immediate early gene expression in the dorsal CA3 was differently activated in males and females.

In the present study zif268 expression was greater in females compared to males in the dorsal CA3 after the spatial pattern separation task. To my knowledge, this is the first study that

demonstrates a sex difference in zif268 expression in the CA3. However, I also found sex differences in c-Fos expression in the opposite direction. c-Fos expression was greater in males than females in the dorsal CA3, which is inconsistent with a previous study (Méndez-López et al., 2009). Méndez-López et al.(2009) showed that c-Fos expression was greater in the CA1 and CA3 regions in females but not in males after the Morris water maze task. This inconsistency may be because due to task differences between the radial arm maze and the Morris water maze. The differences observed between c-Fos and zif268 expression in the current study may also be due to differences between zif268 and c-Fos function. For example, *zif268* is rapidly induced in association with long term potentiation (LTP) (Abraham et al., 1993; Jones et al., 2001), while LTP is not always required for *c-fos* induction (Demmer et al., 1993; Douglas et al., 1988; Wisden et al., 1990). However, further research is needed to determine the underlying mechanisms of different patterns of IEG induction between sexes. Recently, immediate early gene Arc has received considerable attention because it is suggested tight association with the induction of synaptic plasticity (Guzowski et al., 1999). However, there is not a study examining sex differences of Arc expression after pattern separation tasks and it is important to examine Arc expression in future studies.

4. 5. Adult neurogenesis in the ventral dentate gyrus was tightly linked to the ability to separate similar patterns in female spatial strategy users.

In the present study, there was a significant positive correlation for spatial strategy users showing better performance on similar pattern trials (ADJACENT) being associated with greater neurogenesis in the dorsal DG. However when broken down by sex, correlations only remained for the ventral DG as the proportion of correct choices during ADJACENT trials was positively correlated with neurogenesis in female spatial strategy users and negatively correlated with neurogenesis in male idiothetic strategy users. The dorsal hippocampus is thought to be more responsible for spatial learning while the ventral hippocampus is thought to be more responsible for stress and anxiety (Moser et al., 1993; Kjelstrup et al., 2002). However, these results suggest that ventral dentate gyrus may play an important role for spatial pattern separation in females. Studies demonstrate that the ventral hippocampus plays a crucial role for spatial working memory (Moser et al, 1993; Nott and Levin, 2006), during early stages of a goal oriented task in males (Ruediger et al., 2012) and for pattern separation using odor discrimination (Weeden et al., 2012). These studies suggest that adult neurogenesis in the ventral dentate gyrus may play a role for spatial pattern separation either directly or indirectly in females. Further studies are necessary to determine contributions of adult-born neurons in the dorsal and ventral dentate gyrus to spatial pattern separation in both males and females.

4. 6. Zif268 activation in the dentate gyrus in male striatum-dependent learners, and c-Fos activation in the CA1 region in female hippocampus-dependent learners may interfere with pattern separation.

In the present study, greater zif268 expression in the ventral dentate gyrus and in the CA1 region was associated with poorer performance of pattern separation only in male idiothetic strategy users. This result suggests that greater zif268 activation in the hippocampus may interfere with performance of spatial pattern separation in male striatum-dependent learners and partially supports the interpretation that only dentate granule cells in a particular age play a role for pattern separation (Nakashiba et al., 2012). Greater c-Fos expression in the CA1 region was associated with poorer performance in spatial pattern separation in female spatial strategy users, in this study. This suggests that greater c-Fos activation may interfere with spatial pattern separation in female spatial strategy users. Thus future studies need to determine interactions among hippocampal subregions during pattern separation and pattern completion in both males and females.

4. 7. Future studies

In the present study, there was sex difference in the ability of pattern separation in young adult Sprague-Dawley rats. The difference may be because due to the different hormonal states

between males and females, such as higher androgen levels and lower estrogen levels in males compared to females. For future studies, hormone influences on the ability of pattern separation and pattern completion are needed to understand the possible hormone mechanisms underlying the sex differences observed in this study. Furthermore, it is important to examine sex differences in the ability of pattern separation and influences of sex hormones on neural plasticity in the hippocampus because it allows us to understand sex differences in the response to cognitive training. Cognitive training is touted as a possible therapy for dementia (Ball et al., 2002). Furthermore there are sex differences in the development and severity of dementia with neurodegenerative disease such as Alzheimer's disease (AD) and these types of studies may inform the research community on the potential mediators surrounding these sex differences in vulnerability. Studies report that women have a higher risk of developing AD, somewhat independent of their greater longevity (Fratiglioni et al., 1997). Furthermore it has been reported that larger differences in input are required for elderly adults to encode new information (Yassa et al., 2011; Toner et al., 2009). However, to date, there have been no studies examining sex differences in pattern separation with aged rats. Finally, the present study examined immediate early gene expression, zif268 and c-Fos. However, it is important to examine the expression of other IEGs such as Arc because Arc expression is more tightly linked to memory consolidation

and further there are reductions in Arc expression observed with age, which may attribute to cognitive decline with age (Guzowski et al., 2001; Penner et al., 2011).

CHAPTER 5: Conclusion

The results from this study found sex differences, favouring males, in pattern separation ability for similar patterns and adult neurogenesis in the dentate gyrus in a learning strategy dependent manner. These findings highlight the importance of biological sex on hippocampal function and neural plasticity. There were significant associations between adult neurogenesis and performance of pattern separation stratified by sex and strategy use. The findings of low activation of new neurons, despite these new neurons being required for pattern separation (Clelland et al, 2009) suggest that there may be a specific earlier timeline for adult-born neurons to be recruited during pattern separation task. These findings further emphasize the need for more research in examining the underlying mechanisms for sex differences in hippocampus function and the time/age-dependent role of adult-born neurons in pattern separation. It is vital to study sex differences in hippocampal plasticity in response to hippocampus-dependent training as these findings may provide important information for understanding the mechanisms for sex differences in cognitive training to reduce the severity and incidence of cognitive impairment in neuropsychiatric and neurodegenerative disorders.

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