Sex and estrous cycle differences in immediate early gene activation in the hippocampus and the dorsal striatum after the cue competition task

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Abstract

The hippocampus and dorsal striatum are important structures involved in place and response learning strategies respectively. Both sex and estrous cycle phase differences in learning strategy preference exist following cue competition paradigms. Furthermore, significant effects of sex and learning strategy on hippocampal neural plasticity have been reported. However, associations between learning strategy and immediate early gene (IEG) expression in the hippocampus and dorsal striatum are not completely understood. In the current study we investigated the effects of sex and estrous cycle phase on strategy choice and IEG expression in the hippocampus and dorsal striatum of rats following cue-competition training in the Morris water maze. We found that proestrous rats were more likely to choose a place strategy than non-proestrous or male rats. Although male cue strategy users travelled greater distances than the other groups on the first day of training, there were no other sex or strategy differences in the ability to reach a hidden or a visible platform. Female place strategy users exhibited greater zif268 expression and male place strategy users exhibited greater cFos expression compared to all other groups in CA3. Furthermore, cue strategy users had greater expression of cFos in the dorsal striatum than place strategy users. Shorter distances to reach a visible platform were associated with less activation of cFos in CA3 and CA1 of male place strategy users. Our findings indicate multiple differences in brain activation with sex and strategy use, despite limited behavioral differences between the sexes on this cue-competition paradigm.

Keywords: cue competition, hippocampus, adult neurogenesis, dentate gyrus, sex differences, immediate early genes, learning strategy, zif268, c-Fos, estrous cycle

1. Introduction

Sex differences in cognition can be a hotly contested topic; however meta-analyses of human and rodent studies indicate male advantages in spatial reference and working memory (Jonasson, 2005; Maeda and Yoon, 2013; Voyer et al., 1995). Significant sex differences favoring males exist in mental rotation tasks, spatial perception tasks and virtual water maze tasks in humans (Dabbs et al., 1998; Galea and Kimura, 1993; Kaufman, 2007; Woolley et al., 2010) and radial arm maze and Morris water maze tasks in rats (Endo et al., 1994; Markowska, 1999; Perrot-Sinal et al., 1996). Furthermore, sex differences in the preference of learning strategies have been reported, where males prefer hippocampus-dependent place strategies and females prefer striatum-dependent cue strategies (Cherney, Brabec and Runco, 2008; Silverman and Choi, 2006; Korol, 2004; Hawley et al., 2012). Interestingly, ovarian hormones may play a role as female rats in proestrus tend to prefer a place strategy over a cue strategy (Warren and Juraska, 1997). The cue strategy relies more on goal oriented landmark cues such as a visible platform in the Morris water maze, while the place strategy relies more on allocentric cues such as distal spatial cues on the walls of the maze room (Morris et al., 1982; Packard and McGaugh, 1996).

McDonald and White (1993) used the cue competition version of the Morris water maze task to examine contributions of the hippocampus and the dorsal striatum to a learning strategy choice of rats. In short, rats were trained in the Morris water maze to find a visible or

a hidden platform in the same location throughout training. In males, hippocampal lesions caused a shift in strategy choice toward a cue strategy, whereas dorsal striatum lesions shifted strategy choice towards a place strategy. Studies with intact males have shown the two memory systems work cooperatively and interchangeably depending on the availability of spatial cues (Devan and White, 1999; McDonald and White, 1994).

Differences in learning strategy preference suggest that the neural circuits for spatial versus cued navigation may differ by sex, or that the sexes have differential reliance on neural circuits for different strategies. As estrous cycle phase also influences strategy choice in females, ovarian hormones likely affect neural activation in the hippocampus and dorsal striatum differently. Neural activation can be studied using immediate early genes (IEG) such as *zif268* and *cFos*. IEG encode transcription factors that regulate gene expression which can modify neuronal plasticity in response to behavioral experience (Sheng and Greenberg, 1990; Guzowski et al., 2001; Jones et al., 2001). Therefore, IEG expression is useful to examine possible sex, strategy and estrous stage differences in neural activation of different memory systems in response to spatial learning.

To date there have been only a few studies examining IEG expression in the hippocampus and the dorsal striatum comparing males and females (Mendez-Lopez et al., 2009; Chow et al., 2012; Yagi et al., 2015), and the effects of sex on IEG expression in response to spatial navigation remains to be resolved. Furthermore, associations between IEG

expression and spatial learning ability specifically assessing sex and strategy have not been well studied. Studying sex differences in cognition and the underlying mechanisms is important, as the knowledge may help elucidate why sex differences exist in the incidence and severity of disorders associated with cognitive deficits such as Alzheimer's disease, schizophrenia, and depression (Gutierrez-Lobos et al., 2002; Beinhoff et al., 2008).

The present study aimed to determine whether there are sex and/or learning strategy differences in IEG activation within the hippocampus or dorsal striatum using the cue competition task. Furthermore, we aimed to determine whether estrous cycle affects strategy choice and neural activation. Male and female rats were tested in the cue competition version of Morris water maze task for nine days followed by one day of probe trials. Zif268 and cFos expression in the dentate gyrus, CA3 and CA1 region of hippocampus and the dorsal striatum were examined by immunohistochemistry to measure neural activation in response to cue competition. We expected to find sex, estrous cycle, and strategy differences in hippocampal and dorsal striatal activation after the cue competition task.

2. Methods

2. 1. Subjects

Sixty-nine Sprague-Dawley rats (thirty three females and thirty six males) were purchased from Charles River (St. Constant, Quebec, Canada). All rats were approximately

7-8 months old at the time of testing. Rats were double-housed in standard cages with *ad libitum* access to water and lab chow (Purina Lab Diet) and were kept on a 12/12 h light/dark cycle with lights turned on at 07:00. All experiments were carried out in accordance with the ethical guidelines of the Canada Council for Animal Care and were approved by the local 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 Morris water maze was a white circular pool, 180 cm in diameter and filled to a depth of 30 cm with room temperature water (approximately 20-22 °C). White non-toxic paint was added to render the water opaque. A camera centered above the pool was connected to a computer running ANY-maze video tracking system (Stoelting; Wood Dale, IL, USA) in order to measure latency, distance and average speed taken by rats to reach the platform in the pool. The pool was surrounded by large distinctive distal black cues and remained constant throughout the experiment.

2. 3. Procedure

2. 3. 1. Behavioral training

The cue competition version of the Morris water maze task was used to assess the ability of rats to learn a spatial location and to determine their preferred learning strategy (place vs. cue) (McDonald and White, 1994; Epp and Galea, 2009). Rats were exposed to 9

daily training sessions followed by two probe trials on the 10th day (see Figure 1A and B). A platform 10 cm in diameter was placed in the center of the northeast quadrant of the pool and it remained in the same position throughout the entire experiment, except during the probe trials. On the first two days of training, the platform extended 2 cm above the surface of the water (visible session). On the third day the platform was submerged 2 cm below the surface of the water (hidden session). The training sessions (two visible and one hidden) were repeated three times so that there were a total of six days of visible sessions and three days of hidden sessions. Each session was comprised of four trials and one session was conducted per day. For each trial, rats started from each of the four cardinal compass points in a pseudo-random order. Rats were allowed 60s to reach the platform and remained on the platform for 10s prior to being removed from the maze. If the rat was unable to find the platform within the 60s time limit, the rat was led to the platform by the investigator.

2. 3. 2. Probe trial

On day 10, the platform was moved to the middle of the southwest quadrant and was extended above the surface of the water. Two probe trials were conducted, during which the rats were released from two points equidistant from the original platform location and the new platform location. The two probe trials were given approximately two minutes apart. Rats were classified as preferring the cue strategy or place strategy based on their swim paths during the probe trials. Rats that swam within 10 cm of the original platform location on one

of the two probe trials were considered place strategy users as previously done (Devan and White, 1999; Epp and Galea, 2009). Those that made a direct path toward the visible platform in its new location were considered cue strategy users (see Figure 1C). Rats were perfused ninety minutes after the second probe trial and thirty-two (sixteen males and sixteen females) subjects were used for immunohistochemistry.

2. 4. Vaginal Lavage

Vaginal cells suspended in saline were obtained using a glass pipette, transferred onto microscope slides, stained with Cresyl Violet (Sigma), and analyzed using a 20× objective. Proestrous stage was determined when 70% of the cells were nucleated epithelial cells. Daily lavage samples were taken from all females immediately after water maze training. Estrous cycle determination was done as the estrous cycle stage can affect strategy use (Korol et al., 2004) and excitability of the hippocampus (Warren et al., 1995).

2. 5. Tissue processing

Rats were administered euthanyl (Bimeda-MTC Animal Health Inc, Cambridge, ON, Canada) and perfused transcardially with 60 ml of 0.9% saline followed by 120 ml of 4% paraformaldehyde (Sigma-Aldrich). The extracted brains were 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 dorsal striatum, and stored in anti-freeze solution consisting of ethylene glycol, glycerol and 0.1M PBS at -20°C.

2. 5. 1. Zif268/cFos immunohistochemistry

Brain tissue was rinsed overnight with 0.1 M PBS at 4 °C. The tissue was incubated in 0.6% H₂O₂ 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. Slides were dehydrated, cleared with xylene and cover-slipped with Permount (Fisher Scientific; Ottawa, ON, Canada).

2. 6. Cell counting

Optical density of zif268 and c-Fos expression in the dentate gyrus, CA1, CA3 and dorsal striatum were analyzed as an estimate of the proportion (%) of immunoreactive cells in the subregions (see figure 5A-D). Images were acquired at 40× magnification from three

sections from the dorsal hippocampus (4.5-6.5mm from the interaural line) and three sections from the dorsal striatum (9.0-10.5mm from the interaural line) for each rat on a Nikon E600 light microscope. 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 (Hartig, 2013; Selinummi et al., 2005). The threshold was set to 2.5 times above the background gray levels (Yagi et al., 2015). The background gray levels were the mean gray values that were obtained from three randomly selected areas in the corpus collosum without immunoreactivity. Then, we traced the corresponding subregions on ImageJ. The areas that expressed gray levels above the threshold were highlighted and using ImageJ we obtained the summed area of these highlighted regions as well as the total area of corresponding subregions (DG, CA3, CA1 and DS) in pixels. Optical density for each brain was then calculated as the total summed immunoreactive highlighted area as a percentage of the total area of corresponding subregions of each brain.

2. 7. Data analyses

Data were analyzed using the software program Statistica (Statsoft, Tulsa, OK, USA). A chi-square analysis was performed on the frequency of strategy choice (place, cue) by sex (females, males) or estrous cycle phase (proestrus, non-proestrus). Latency and distance to reach the platform were each analyzed for both hidden and visible platform training sessions using repeated measures analysis of variance (ANOVA) with sex (male or female) and

strategy choice (place or cue) as the between-subject factors and session (days 3, 6 and 9 for hidden platform training or days 1, 2, 4, 5, 7, 8 for visible platform training) as the within-subject factor. Swim speed was analyzed using repeated measures ANOVA with sex and strategy choice as the between-subject factors and training type (visible or hidden) as the within-subject factor. A one way ANOVA was used to analyze the optical density of zif268 and c-Fos in each region (dentate gyrus, CA1, CA3 and dorsal striatum) with sex or estrous cycle stage and strategy choice as the between-subject factor. Post hoc tests were performed with the Neuman-Keuls procedure. A priori tests were subjected to Bonferroni corrections. Pearson product-moment correlations were calculated to examine the relationship between performance and density of zif268 expression or c-Fos expression. Effect sizes were calculated for any significant effects (η_p^2 and Cohen's d). For all statistical analyses, $\alpha = 0.05$.

3. Results

3. 1. Behavioral testing

3. 1. 1. More proestrous females chose a place strategy than non-proestrous females but there were no sex differences in strategy choice.

Nineteen males (53%) chose a place strategy and seventeen males (47%) chose a cue strategy, while eighteen females (55%) chose a place strategy and fifteen females (45%) chose a cue strategy. Two rats that swam directly toward the new visible platform (cue strategy)

during the first probe trial were ambiguous during the second probe trials. However, the swim path of these two rats during the first probe trial clearly satisfied the criteria as a cue strategy. Therefore, these two subjects were categorized as cue strategy users. In rats that swam directly to the old platform location (place strategy) during the first probe trial, ten males and fourteen females chose a cue strategy on the second probe trial. As there was not a platform in the original quadrant (northeast) during the probe trials, place strategy users needed to adopt a cue strategy after exploring the original platform location to escape from the water. Therefore, we considered rats that used a place strategy on the first probe trial and used either cue strategy or place strategy on the second probe trial as place strategy users, using the same criteria as McDonald and White (1994). There was no significant sex effect on the frequency of strategy choice [$\chi^2(1) = 0.022$, $p = 0.89$; see Figure 2A]. Seven females in proestrus (78%) chose a place strategy and two females in proestrus (22%) chose a cue strategy, while eleven females in non-proestrus (46%) chose a place strategy and thirteen females in non-proestrus (54%) chose a cue strategy. More proestrous females chose a place strategy than cue strategy, while less non-proestrous females chose a place strategy than a cue strategy [$\chi^2(1) = 2.69$, $p = 0.05$; see Figure 2 B].

3. 1. 2. Male cue strategy users took longer and swam greater distances to reach the visible platform on the first day of acquisition.

Analysis of swim distance and latency to reach the visible platform revealed that

male cue strategy users swam significantly greater distance and swam longer than the other groups on day 1 {both p 's < 0.01: interaction effect of day by strategy choice by sex on swim distance [$F(5, 320) = 2.45$; $p = 0.034$; $\eta_p^2 = 0.037$; see Figure 3D-F], and latency [$F(5, 320) = 3.38$; $p = 0.0054$; $\eta_p^2 = 0.05$; see Figure 3A-C]}. There were also significant interaction effects and a significant main effect for latency only [strategy: $F(1, 64) = 6.89$; $p = 0.011$; $\eta_p^2 = 0.096$; day by sex: $F(5, 320) = 2.73$; $p = 0.020$; $\eta_p^2 = 0.041$; day by strategy: $F(5, 320) = 2.27$; $p = 0.047$; $\eta_p^2 = 0.034$]. Furthermore, males swam slower to reach the visible platform compared to females [session by sex: $F(1, 65) = 10.22$; $p = 0.0022$; see Table 1].

3. 1. 3. There were no significant differences in performance between sexes or strategy users to reach the hidden platform.

There was a trend for day by sex on swim latency [day by sex: $F(2, 130) = 2.39$; $p = 0.096$] to reach the hidden platform (see Figure 4A-F) and no other significant main or interaction effects (all p 's > 0.15). There were no significant main or interaction effects on swim speed during the hidden sessions (see Table 1).

3. 2. Female place strategy users had greater zif268 expression in the CA3 region than all other groups.

Female place strategy users had greater zif268 expression in the CA3 region compared to the other groups [all p 's < 0.0495; sex by strategy: $F(1, 28) = 4.68$, $p = 0.039$; $\eta_p^2 = 0.143$, Cohen's $d = 1.11, 1.06, 1.23$, between female place and male place, male cue or

female cue strategy users respectively; see Figure 5E]. There were no other significant main or interaction effects of sex or strategy use on zif268 expression in the dentate gyrus, CA1 and dorsal striatum (p 's > 0.13 ; see Table 2). There were no significant main or interaction effects of estrous cycle on zif268 expression in the dentate gyrus, CA3, CA1 and dorsal striatum (p 's > 0.103). We also investigated whether zif268 expression pattern differed in the suprapyramidal and infrapyramidal blade of the dentate gyrus, as previous studies have shown this (Chawla et al., 2005; Satvat et al., 2011), and IEG expression in the two blades may have different functional roles (Schmidt, Marrone and Markus, 2012). In the current study, zif268 expression in the infrapyramidal blade of dentate gyrus was greater than in the suprapyramidal blade [main effect of region: $F(1, 28) = 12.13$, $p = 0.0016$; $\eta_p^2 = 0.30$, Cohen's $d = 0.23$] but there were no other significant effects (all p 's > 0.13).

3. 3. cFos expression

3. 3. 1. Male place strategy users had greater cFos expression in the CA3 region than all other groups, and cue strategy users had greater cFos expression in the dorsal striatum than place strategy users.

Male place strategy users exhibited significantly greater cFos expression in the CA3 region than all other groups [all p 's < 0.0493 ; sex by strategy: $F(1, 27) = 6.83$, $p = 0.014$; $\eta_p^2 = 0.20$; Cohen's $d = 0.909, 1.74, 1.12$, between male place and male cue, female place or female cue strategy users respectively; see Figure 5F]. A priori analyses found that male place

strategy users had significantly greater CA3 cFos expression than female place strategy users ($p = 0.02$; see Figure 5F).

Cue strategy users had greater cFos expression in the dorsal striatum than place strategy users [main effect of strategy: $F(1, 28) = 8.46$, $p = 0.007$; $\eta_p^2 = 0.23$, Cohen's $d = 1.03$; see Figure 5G].

In contrast to zif268, cFos expression in the suprapyramidal blade of the dentate gyrus was greater than in the infrapyramidal blade [main effect of region: $F(1, 26) = 20.04$, $p = 0.0001$; $\eta_p^2 = 0.44$, Cohen's $d = 0.67$].

3. 3. 3. cFos expression in the dentate gyrus was greater in proestrous females than non-proestrous females.

Proestrous females had greater cFos expression in the dentate gyrus than non-proestrous females [main effect of estrous cycle: $F(1, 11) = 10.18$, $p = 0.0086$; $\eta_p^2 = 0.48$, Cohen's $d = 1.08$; see Figure 6A]. There were no other significant main or interaction effects with strategy choice and estrous cycle on cFos expression (p 's > 0.1 ; see Table 3).

3. 4. Correlations

3. 4. 1. Better performance during visible sessions was associated with more zif268 expression in the dorsal striatum, but less cFos expression, in the CA1 or CA3.

In the dorsal striatum, total swim distance during visible sessions was negatively correlated to zif268 expression in place strategy users [$r(14) = 0.714$, $p = 0.004$; see figure

6B]. When broken down by sex, total swim distance during visible sessions was negatively correlated to zif268 expression in the dorsal striatum in female place strategy users [$r(9) = -0.760$, $p = 0.017$;], but not in male place strategy users ($p > 0.61$).

Total swim distance during visible sessions was positively correlated to cFos expression in the CA3 [$r(5) = 0.966$, $p = 0.008$; see Figure 6C], in the CA1 [$r(5) = 0.983$, $p = 0.003$; see Figure 6D]. There was no other significant association of performance during visible and hidden sessions with zif268 and cFos expression after correcting for alpha (p 's > 0.017). All significant correlations were summarized in Table 4 and 5.

4. Discussion

The results of the present study demonstrate that proestrous rats preferentially chose a place strategy to a cue strategy, while non-proestrous rats preferentially choose a cue strategy over a place strategy, with no sex differences in overall strategy choice. Proestrous rats also exhibited greater cFos expression in the dentate gyrus. Female place strategy users expressed greater zif268 expression than the other groups in the CA3 region of hippocampus. However, male place strategy users showed greater overall cFos expression in the CA3 than all other groups. Furthermore, cFos expression in the dorsal striatum was greater in cue strategy users than in place strategy users. Lastly, while there were no significant associations between IEG expression and learning performance in a specific sex and learning strategy

manner, less cFos expression in the CA3 and CA1 regions of hippocampus, but more zif268 expression in the CA3, were associated with better performance in visible sessions. These data add to the growing literature on sex differences in activation of IEGs after spatial and cued training in the Morris water maze.

4.1. Estrous cycle phase affected strategy choice preference and cFos expression in the dentate gyrus

Consistent with other studies (Korol et al., 2004; Warren et al., 1997), in the present study, we found that proestrous rats chose a place strategy preferentially over a cue strategy. In a previous study, Rummel et al. (2010) found that rats in proestrus were more likely to be place strategy users than rats in non-proestrus within the cue-competition version of the Morris water maze task. Using the dual-solution T-maze task, proestrous female rats have also been shown to prefer a place strategy over a response strategy (Korol et al., 2004). These findings, across two different paradigms, show that proestrous rats are more likely to choose a place strategy than a cue strategy. During proestrus, the circulating level of 17β -estradiol rises to the highest point and then falls quickly after the peak (Butcher et al., 1974). Higher levels of 17β -estradiol increase neural excitability in the dentate gyrus (Warren et al., 1995), which is consistent with our finding that cFos expression in the dentate gyrus was greater in proestrous rats than non-proestrous rats. Furthermore, a high dose of 17β -estradiol increases zif268 expression of adult-generated young neurons in the dentate gyrus in response to the

Morris water maze task (McClure et al., 2013). Thus, the increase of neural excitability in the dentate gyrus with proestrus is associated with biasing place strategy in proestrous females. In addition to increased neural excitability in the dentate gyrus, proestrous rats showed increased hippocampal cell proliferation in the dentate gyrus (Rummel et al., 2010; Tanapat et al., 1999). Collectively these studies indicate that the proestrous phase is associated with increased spatial strategy use corresponding with increased cell proliferation and cFos activation in the dentate gyrus. In the present study there were no significant effects of estrous phase on zif268 expression in the hippocampus or dorsal striatum. Warren et al. (1995) demonstrated that higher levels of estradiol lowered the threshold to induce LTP in anesthetized rats. Zif268 expression is induced by LTP and plays a critical role for the maintenance of LTP (Jones et al., 2001; Petersohn et al., 1995). Thus, our finding of no significant effects of estrous cycle phase on zif268 expression in the dentate gyrus is partially inconsistent with the findings of Warren et al. (1995). However, the means were in the predicted direction with greater zif268 expression in the dentate gyrus during proestrus compared with non-proestrus. Our lack of a statistically significant finding may be due to sample size, timing, or possibly because the induction of zif268 expression requires specific stimuli in addition to that typically required to induce LTP (200 Hz for 0.5 s). It is also possible that compensatory regulations exist in awake animals during spatial learning.

The present study failed to find a sex difference in strategy choice, contradicting a

previous study using the dual-solution T-maze (Hawley et al., 2012) but consistent with a pattern separation study with two weeks of training sessions (Yagi et al., 2015). However, it is important to note that in the same cue competition task used in this present study, with the same latency of training sessions, approximately 50% of intact male rats chose cue strategy (Devan and White, 1999; McDonald and White, 1994), which is consistent with our finding. These results suggest that 9 days of spatial training with both strategies may lead rats to equal strategy preference. Indeed strategy choice is associated with task difficulty (Andersen et al., 2012; Roof and Stein, 1999) and may explain why the present study failed to demonstrate the bias in strategy choice in males and non-proestrous rats. Ten male place strategy users and fourteen female place strategy users changed their learning strategy to a cue strategy during the second probe trial. Although we failed to demonstrate a sex difference in strategy choice, more female place strategy users than male place strategy users adopted the cue strategy on the second probe trial. This suggests that female place strategy users may rely more weakly on extramaze spatial cues or may be more flexible with their strategy choice than male place strategy users.

4.2. No sex differences were observed in performance of the cue competition task

Despite the fact that male cue strategy users spent more time to find a visible platform than the other groups during the first day of memory acquisition, there were no other significant sex differences in performance. This result is partially inconsistent with previous

studies using the spatial reference memory version of the Morris water maze task: the reference memory (Roof and Havens, 1992; Chow et al., 2012). However, our lack of sex differences after training, with both visual and hidden platform trials, is consistent with other studies that pre-trained rats in the Morris water maze with a visible platform (Beiko et al., 2004; Bucci et al., 1995; Warren et al., 1997). Perrot-Sinal et al., (1996) found that male rats outperformed female rats to reach a hidden platform in the Morris water maze, but the male advantage was eliminated when rats received training prior to testing. The female advantage over males in cue strategy users' ability to reach a visible platform is partially consistent with some studies that found that males swim longer distances to reach a hidden platform than female rats on the first day in the Morris water maze (Frick et al., 2000; Warren and Juraska, 1997). This may be due to anxiety induced by the novel environment or a sex difference in the response to acute stress associated with the paradigm influencing spatial learning on the first day. In short, while sex differences favouring males have been observed in a number of studies using spatial memory paradigms, it is not consistently found when using more pre-training or visual to hidden platform training. The differences in paradigms are important to keep in mind as these subtle differences can have important implications for the expression of sex differences in spatial ability. Indeed, males pay more attention to the geometrical cues in the room while females pay more attention to the landmarks or visual cues surrounding the maze (Williams, Barnett and Meck, 1990). It is therefore important to note that the

environment (salience of cues in room) and training sessions (number, massed versus distributed) will influence the expression of sex differences and may be the reason why power calculations suggest using very high numbers of animals are required to obtain sex differences in spatial tasks (Button et al., 2013).

4.3. Sex and strategy use matters for immediate early gene expression in the CA3 and dorsal striatum after cue competition training in the Morris water maze.

In the present study, zif268 expression in the CA3 of female place strategy users was greater than all other groups after cue-competition training and probe trial testing with the platform in a new location. This is partially consistent with our previous studies showing that zif268 expression in the CA3 was greater in female rats compared to male rats (Yagi et al., 2015). However, in our previous study zif268 expression was greater regardless of the strategy use chosen by the females (Yagi et al., 2015), while in the present study females showed greater zif268 expression in CA3 than males only in place strategy users. This partial inconsistency may be due to differences in the behavioral paradigms, as in the previous study rats were tested with a delayed non-match to place pattern separation task in the radial arm maze, and zif268 expression was measured in response to exploration of a goal arm that was located in a different spatial position from the sample phase regardless of strategy choice. In the current study, the platform location was located in a different quadrant from the quadrant that place strategy users first explored during the trial. Taken together with the previous study,

the present result suggests that females show greater zif268 expression in the CA3 when they explore a different spatial location to reach their goal.

In the present study, male place strategy users showed greater cFos expression in the CA3 region than the other groups (an opposite pattern to what was seen with zif268 expression pattern). This contradiction between cFos and zif268 expression in the CA3 may be due to the differences in the role of cFos and zif268 for memory acquisition in the Morris water maze. The proto-oncogene *c-fos* is induced in response to various stimuli such as increase levels of stress, neural depolarization and epileptic seizures (Labiner et al., 1993; Nakajima et al., 1989). cFos proteins play an important role for many cell functions including cell growth, cell proliferation, apoptosis, and immune response (Herrera and Robertson, 1996; Hu et al., 1994), and can be used as a neural metabolic marker (Sagar et al., 1988). In contrast, zif268 is induced with LTP-related neural activity, which regulates the expression of synapsin and plays an important role for maintaining LTP (Abraham et al., 1993; Petersohn et al., 1995; Williams et al., 2000). Therefore, the current results suggest that female place strategy users may require greater LTP-related neural activities in the CA3, while male place strategy users may require greater general, but not necessarily LTP-related neural activities, in the CA3 during spatial navigation after ten days of training in the same Morris water maze.

Furthermore, it should be noted that there is a different time course between zif268 and cFos protein induction at least in male rats (Zangenehpour and Chaudhuri, 2002). Zangenehpour

and Chaudhuri (2002) reported that zif268 protein increases rapidly to reach its peak level 90 minutes after stimulation, while cFos protein increases gradually to reach its peak level 120 minutes after stimulation followed by relatively rapid decrease. Furthermore, DNA binding activity of zif268 is more rapid compared to cFos in male rats (Williams et al., 2000). In the present study, rats were perfused 90 minutes after the last behavioral testing. This time course is the optimal for maximizing zif268 protein induction (Mello and Ribeiro, 1998), but too short for maximizing cFos protein induction (Ivashkina et al., 2015). This experimental timeline may attribute to the different induction patterns between cFos and zif268 proteins, however it has not yet been established if there are sex differences in the timeline for induction of IEG activation which could be an area of future investigation.

In the present study, cue strategy users showed greater cFos expression in the dorsal striatum than place strategy users. Our results support the idea that the dorsal striatum is a crucial region for stimuli-response cue learning (McDonald and White, 1994). Lesions and pharmacological silencing of the dorsal striatum lead to impaired stimuli-response learning (McDonald and White, 1994; Packard and McGough, 1996). Non-invasive studies, such as immunohistochemically examining neural activity like done in the present study, also showed associations between the dorsal striatum and stimuli-response cue learning (Colombo et al., 2003; Miranda et al., 2006). Miranda et al. (2006) examined the expression of cytochrome c-oxidase, required for cellular oxidative metabolism in mitochondria, in the dorsal striatum

after either place or cue training. They found that oxidative metabolism in the dorsal striatum was higher in cue-trained rats than place-trained rats in response to the Morris water maze task, which is partially consistent with current findings. These findings collectively suggest higher activity (cFos, oxidative metabolism) in cue-trained rats compared to place-trained rats in the dorsal striatum in both males and females.

4.4. Better performance was associated with less cFos expression in the CA3 and CA1 region in male place strategy users.

Better performance of male place strategy users during visible sessions was associated with less cFos expression in the CA3 and CA1 region of the hippocampus in the present study. This result suggests that better ability of male place strategy users for using both landmark and distal spatial cues to reach a visible platform was associated with more efficient use of the CA3 and CA1 regions to find a new platform location during probe trials. This interpretation is supported by previous studies using functional MRI with human subjects that show that oxidative blood flow in the hippocampus increased when subjects learned spatial relations in novel environments and it reduced with training in the same environment (Köhler et al., 2005; Astur et al., 2005). However, it is important to note that only five male place strategy users were examined; IEG expression in the present study and further studies with larger sample sizes are needed to definitively conclude the association between IEG expression and the performance in the Morris water maze.

Conclusion

We found no evidence for a sex difference in strategy use in the cue competition paradigm. We found that the proestrous stage was associated with greater IEG expression in the dentate gyrus and increased preference of a place strategy. This emphasizes the importance of considering estrous cycle phase when examining a learning strategy choice or activation in the hippocampus of female subjects. Furthermore, our findings indicate that there are sex differences in activation of different regions of the hippocampus and striatum even when no overt sex differences in learning outcome was seen (acquisition or strategy use). These findings highlight the idea that neural manifestations of acquisition and strategy use can be different in males and females without behavioural differences (Becker and Koob, 2016).

Contributors

Shunya Yagi wrote the manuscript and all authors approved the final manuscript.

Shunya Yagi analyzed the data with assistance of LAMG. Shunya Yagi, Dimka Drewczynski and Olivia Hershorn performed experiment with assistance of Cindy Barha and Steven Wainwright. Shunya Yagi, Dimka Drewczynski, Steven Wainwright, Cindy Barha and Liisa Galea designed the study.

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FIGURES

Figure 1. Experimental design of cue competition version of Morris water maze task. Time line of cue competition learning and probe trials (A). Behavioral training consisted of three repeat of two days of visible session and one day of hidden session (B). Rats were classified as a cue learner or a place learner based on their swim path during the probe trials (C).

Figure 2. The proportion of strategy choice in males and females (A), and proestrous females and non-proestrous females (B). There was a significant effect of estrous cycle phase. *indicates $p < 0.05$.

Figure 3. Mean latency to the visible platform in all groups (A), in place learners (B) and in cue learners (C). Male cue learners spent longer time than the other groups to reach the platform on the first day, and female place learners and female cue learners spent more time than male cue learners and place learners. Mean distance to the hidden platform in all groups (D), in place learners (E) and in cue learners (F). There was a trend that male cue learners swam longer distance than the other groups. Error bars represent $\pm$ SEM. *indicates $p < 0.05$.

Figure 4. Mean latency to the hidden platform in all groups (A), in place learners (B) and in cue learners (C). There was a trend that males spent more time to reach the platform than females on the sixth day. Mean distance to the visible platform in all groups (D), in place learners (E) and in cue learners (F). There was a trend that male cue learners swam longer distance than the other groups. Error bars represent $\pm$ SEM. *indicates $p < 0.05$.

Figure 5. Photomicrograph of cFos expression in the hippocampus (A) and dorsal striatum (B), and zif268 expression in the hippocampus (C) and dorsal striatum (D). Mean value of optical density of zif268 expression in the CA3 (E), cFos expression in the CA3 (F), and mean value of optical density of cFos expression in the dorsal striatum (G). Female place learners had greater zif268 expression in the CA3 than the other groups, males had greater cFos expression in the CA3 than females, and cue learners had greater cFos expression in the dorsal striatum than place learners. Error bars represent $\pm$ SEM. *indicates $p < 0.05$.

Figure 6. Mean value of optical density of cFos expression in the dentate gyrus (DG) between proestrous (P) and non-proestrous (NP) rats (A). Correlations between total swim distance during visible sessions and zif268 expression in the dorsal striatum (DS) in place learners (B), cFos expression in the CA3 in place learners (C), and cFos expression in the CA1 in place learners (D).

TABLES

Table 1.

Group means ($\pm$ standard error of the mean) for swim speed across sex and strategy use (m/s). Females swam significantly slower than males during visible sessions.

Sex	Strategy	Visible session	Hidden session
Male	Place	0.295 $\pm$ 0.0057	0.335 $\pm$ 0.0044
	Cue	0.292 $\pm$ 0.0059	0.326 $\pm$ 0.0054
Female	Place	0.308 $\pm$ 0.0071	0.329 $\pm$ 0.0066
	Cue	0.292 $\pm$ 0.0079	0.328 $\pm$ 0.0047

Table 2.

Group means ($\pm$ standard error of the mean) for zif268 expression across sex and strategy use in the dentate gyrus (DG), CA3, CA1 and dorsal striatum (DS). (number) indicates the number of subjects in the group. *indicates $p < 0.05$.

zif268		Strategy	DG	CA3	CA1	DS
Male		Place (6)	10.5 $\pm$ 1.1	0.747 $\pm$ 0.17	11.6 $\pm$ 0.70	40.8 $\pm$ 1.2
		Cue (10)	9.32 $\pm$ 1.2	0.832 $\pm$ 0.085	12.6 $\pm$ 0.68	41.8 $\pm$ 2.2
		Place+Cue	9.75 $\pm$ 0.86	0.800 $\pm$ 0.081	12.2 $\pm$ 0.50	41.4 $\pm$ 1.4
Female	Non-proestrus (NP)	Place (5)	8.04 $\pm$ 0.97	1.60 $\pm$ 0.28	12.1 $\pm$ 1.2	42.3 $\pm$ 4.4
		Cue (6)	9.21 $\pm$ 1.4	0.762 $\pm$ 0.11	11.8 $\pm$ 1.2	46.3 $\pm$ 2.5
		Place+Cue	8.68 $\pm$ 0.84	1.14 $\pm$ 0.19	11.9 $\pm$ 0.81	44.5 $\pm$ 2.4
	Proestus (P)	Place (4)	10.5 $\pm$ 1.6	0.964 $\pm$ 0.17	12.6 $\pm$ 0.74	36.4 $\pm$ 2.3
		Cue (1)	4.5	0.784	8.62	44.1
		Place+Cue	9.38 $\pm$ 1.7	0.928 $\pm$ 0.14	11.8 $\pm$ 0.98	37.9 $\pm$ 2.3
	P + NP	Place (9)	9.13 $\pm$ 0.94	1.32 $\pm$ 0.20 *	12.3 $\pm$ 0.73	39.7 $\pm$ 2.7
		Cue (7)	8.61 $\pm$ 1.3	0.765 $\pm$ 0.091	11.3 $\pm$ 1.1	45.9 $\pm$ 2.1
		Place+Cue	8.90 $\pm$ 0.75	1.08 $\pm$ 0.13	11.9 $\pm$ 0.62	42.4 $\pm$ 1.9

Table 3.

Group means ($\pm$ standard error of the mean) for cFos expression across sex and strategy use in the dentate gyrus (DG), CA3, CA1 and dorsal striatum (DS). (number) indicates the number of subjects in the group. *indicates $p < 0.05$.

cFos	Strategy	DG	CA3	CA1	DS
Male	Place (6)	1.23 ± 0.10	0.739 ± 0.14 *	0.191 ± 0.038	1.69 ± 0.16
	Cue (10)	1.46 ± 0.17	0.455 ± 0.093	0.243 ± 0.071	2.24 ± 0.14
	Place+Cue	1.38 ± 0.11	0.569 ± 0.085	0.223 ± 0.046	2.03 ± 0.12
	Non-proestrus (NP)	Place (5)	0.994 ± 0.084	0.309 ± 0.059	0.174 ± 0.035
		Cue (6)	1.29 ± 0.23	0.432 ± 0.053	0.191 ± 0.016
		Place+Cue	1.15 ± 0.13	0.376 ± 0.042	0.183 ± 0.017
	Proestrus (P)	Place (4)	1.20 ± 0.33	0.293 ± 0.015	0.180 ± 0.073
		Cue (1)	2.6	0.552	0.387
		Place+Cue	1.76 ± 0.33	0.345 ± 0.053	0.222 ± 0.070
Female	P + NP		1.18 ± 0.13		
		Place (9)		0.302 ± 0.032	0.177 ± 0.035
		Cue (7)	1.47 ± 0.27	0.449 ± 0.048	0.219 ± 0.031
	P + NP	Place+Cue	1.26 ± 0.15	0.366 ± 0.033	0.195 ± 0.024
					2.06 ± 0.18

Table 4.

Significant correlations ($p < 0.05$) of swim distance during visible and hidden sessions with IEG expressions in place strategy users (male and female combined) and in cue strategy users (male and female combined). (+) indicates a positive correlation and (-) indicates a negative correlation. *indicates $p < 0.017$.

Region	IEG	Place ($\varnothing + \sigma^7$)	Cue ($\varnothing + \sigma^7$)	Male (Place+Cue)	Female (Place+Cue)
DG	zif268	-	-	-	Hidden (-0.59)*
	cFos	-	-	-	-
CA3	zif268	-	-	-	-
	cFos	-	-	-	-
CA1	zif268	-	Visible (+0.67)*	Visible (+0.54)	-
	cFos	-	Hidden (+0.53)	-	-
DS	zif268	Visible (-0.71)*	-	-	-
	cFos	-	-	-	-

Table 5.

Significant correlations ($p < 0.05$) of swim distance during visible and hidden sessions with IEG expressions in males (place learners, cue learners and the two strategies combined) and in females (place learners, cue learners and the two strategies combined). *indicates $p < 0.017$.

Region	IEG	Males		Females	
		Place	Cue	Place	Cue
DG	zif268	Visible (-0.89)	-	-	-
	cFos	-	-	-	-
CA3	zif268	-	-	-	-
	cFos	Visible (+0.97) *	-	Hidden (-0.74)	-
CA1	zif268	-	Visible (+0.66)	-	-
	cFos	Visible (+0.98) *	-	-	Hidden (+0.77)
DS	zif268	-	-	Visible (-0.76) *	-
	cFos	Visible (+0.91)	-	-	Hidden (+0.77)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

(A)

(B)

(C)

(D)

(E)

(F)

(G)

Figure 6