Sex differences in adult hippocampal neurogenesis and the influence of estradiol

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

The Faculty of Graduate and Postdoctoral Studies

(Neuroscience)

The University of British Columbia

(Vancouver)

February 2023

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Sex differences in adult hippocampal neurogenesis and the influence of estradiol

submitted by Shunya Yagi in partial fulfilment of the requirements for

the degree of Doctor of Philosophy

in Neuroscience

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Abstract

Sex differences exist in hippocampal plasticity, cognition, and in various brain diseases that target the integrity of the hippocampus. Females are more likely to be diagnosed with stress and anxiety related disorders and have a greater lifetime risk for Alzheimer's disease than males. Females are more likely to experience greater cognitive decline in Alzheimer's disease and cognitive symptoms in depression and post-traumatic stress disorders, and these diseases characterized by hippocampal dysfunction. The hippocampus is a highly plastic structure, important for processing higher order information, and is sensitive to intrinsic and extrinsic factors such as gonadal hormones and cognitive training. The hippocampus retains the ability to produce new neurons in the dentate gyrus, and new neurons play an important role in pattern separation, a process of separating similar inputs and making distinct neural representations during memory encoding. This thesis investigated sex differences in and the effects of estrogens on basal characteristics of neurogenesis, and in neural network of activated neurons following pattern separation. In Chapter 2, I found that adult-born neurons matured faster in males compared to females, whereas males showed a greater reduction in neurogenesis between one week and two weeks after mitosis. The faster maturation and greater attrition of new neurons in males compared to females suggests greater potential for neurogenesis to respond to external stimuli in males. In Chapter 3, I found that females showed greater contextual pattern separation compared to males, with females and males employing different brain networks during fear memory retrieval. In Chapter 4, I found that estradiol and estrone initially enhanced production of immature neurons whereas long exposure to estrogens eliminated the enhancing effects on neurogenesis. In Chapter 5, I found that a chronic high dose of estradiol reduced adult

neurogenesis in females, but also enhanced functional connectivity in the hippocampus during spatial pattern separation.

Overall, these results highlight the importance of studying sex differences and hormonal regulation of hippocampal neuroplasticity and the contribution of adult neurogenesis to hippocampus-dependent cognition. Therefore, attention to these factors in research will lead to a better understanding how sex contributes to the susceptibility to hippocampus-related diseases.

Lay Summary

There are sex differences not only in the prevalence of diseases, such as Alzheimer's Disease and depression, but also in the manifestation of these diseases. The hippocampus is a very plastic brain region with the presence of new brain cells produced in the adult, called adult neurogenesis. Studying sex differences in adult neurogenesis is important to our understanding of how these new neurons might work to regulate memory and mood. I investigated sex differences in adult neurogenesis and how new neurons are related to memory in males and females. I found sex differences in new cell production and survival and how they are integrated into the brain. Furthermore, I found that estrogens play important regulatory roles for neurogenesis in females which depend on type and timing of estrogens. These findings encourage future studies to explore sex and hormonal state differences in treatment for hippocampus-related diseases.

Preface

Portions of **Chapter 1** and **Chapter 6** has been published in a review paper and a book chapter: 1) Yagi, S., Galea, L.A.M. (2019). Sex differences in hippocampal cognition and adult neurogenesis. *Neuropsychopharmacology* 44(1):200-213. This review was conceived and planned by Shunya Yagi and Dr. L.A.M. Galea, and Shunya Yagi wrote the review with supervision and feedback from Dr. L.A.M. Galea. 2) Yagi, S., Mahmoud, R., Qiu, W., Duarte-Guterman, P., Galea, L.A.M. (2020). Estrogenic regulation of hippocampal neurogenesis throughout the lifespan. In K. Frick (ed), Estrogens and Memory: Basic Research and Clinical Implications. Oxford University Press, New York, NY. This book chapter was conceived, planned, and written by all five authors.

Chapter 2 has been published as: Yagi, S., Splinter, J.E.J., Tai, D., Wong, S., Wen, Y., Galea, L.A.M. (2020). Sex differences in maturation and attrition of adult neurogenesis in the hippocampus. eNeuro, 7(4): eNeuro 0468-19.2020. This experiment was conceived and designed by S. Yagi and Dr. L.A.M. Galea. S. Yagi executed all animal work and collected data with the assistance of S. Wong, D. Tai, Y. Wen and J. Splinter assisted with the codes of Matlab automated cell counting program. S. Yagi performed all statistical analyses and wrote the manuscript with supervision and feedback from Dr. L.A.M. Galea. All other authors provided feedback and suggested edits prior to manuscript submission.

Chapter 3 has been published as: Yagi, S., Lee, A., Truter, N., Galea, L.A.M. (2022). Sex differences in contextual pattern separation, neurogenesis, and functional connectivity within the limbic system. Biology of Sex Differences, 13(42): s13293-022-00450-2. This experiment was conceived and designed by S. Yagi and Dr. L.A.M. Galea. S. Yagi executed all animal work and

collected data with the assistance of A. Lee, and N. Truter assisted with the codes of Matlab automated cell counting program. S. Yagi performed all statistical analyses and wrote the manuscript with supervision and feedback from Dr. L.A.M. Galea. All other authors provided feedback and suggested edits prior to manuscript submission.

Chapter 4 has been available on biorxiv as: Yagi, S., Wen, Y., Galea, L.A.M. Estrogens dynamically regulate neurogenesis in the dentate gyrus of adult female rats. This experiment was conceived and designed by S. Yagi and Dr. L.A.M. Galea. S. Yagi executed all animal work and collected data with the assistance of Y. Wen. Yagi performed all statistical analyses and wrote the manuscript with supervision and feedback from Dr. L.A.M. Galea. All other authors provided feedback and suggested edits prior to manuscript submission.

Chapter 5 has been available on biorxiv as: Yagi, S., Lieblich, S.E., Galea, L.A.M. High Estradiol reduces adult neurogenesis but strengthens functional connectivity within the hippocampus during spatial pattern separation in adult female rats. This experiment was conceived and designed by S. Yagi and Dr. L.A.M. Galea. S. Yagi executed all animal work and collected data with the assistance of S.E. Lieblich. Yagi performed all statistical analyses and wrote the manuscript with supervision and feedback from Dr. L.A.M. Galea. All other authors provided feedback and suggested edits prior to manuscript submission.

All animal studies presented in this thesis were conducted in accordance with ethical guidelines set by the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of British Columbia (certificates A20-0147).

Table of Contents

Abstract ..... **iii**

Lay Summary ..... **v**

Preface ..... **vi**

Table of Contents ..... **viii**

List of Tables ..... **xviii**

List of Figures ..... **xix**

List of Symbols ..... **xxi**

List of Abbreviations ..... **xxii**

Acknowledgements ..... **xxvii**

Dedication ..... **xxviii**

Chapter 1: Introduction **1**

1. 1 Sex differences in hippocampus related diseases and importance of studying sex differences in the hippocampus 1

1. 2 Anatomy of the hippocampus 4

1. 3 The Function of the Hippocampus 5

1. 3. 1 Pattern separation and pattern completion 5

1. 3. 2 The hippocampus and learning strategy use 7

1. 4 Dorsal versus ventral hippocampus 10

1. 5 Plasticity of the Hippocampus 14

1. 5. 1 Adult neurogenesis in the hippocampus..... 15

1. 5. 2 Functional role of adult neurogenesis in learning and memory 19

1. 5. 3 Dorsal/ventral regional differences in neurogenesis 21

1. 6 Sex differences in hippocampus-dependent cognition 23

1. 6. 1 Sex differences in learning strategy 23

1. 6. 2 Sex differences in learning and memory 24

1. 6. 3 Sex differences in contextual fear conditioning..... 26

1. 7 Sex differences in the morphology, activation, and connectivity of the hippocampus: Findings from human studies..... 28

1. 8 Sex differences in morphology, electrophysiological properties and activation of hippocampal neurons: Findings from rodent studies 30

1. 9 Sex differences in adult neurogenesis in the hippocampus 35

1. 9. 1 Sex differences in basal adult neurogenesis..... 36

1. 9. 2 Sex differences in adult neurogenesis after learning..... 36

1. 10 Hormone therapy and hippocampal cognition 38

1. 11 Estrogen manipulation and hippocampus dependent cognition..... 39

1. 12 Estrogen manipulation and adult hippocampal neurogenesis 40

1.13 Thesis overview and objectives..... 44

Chapter 2: Sex differences in maturation and attrition of adult neurogenesis in the hippocampus 47

2. 1 Introduction 47

2. 2 Materials and methods 50

2. 2. 1 Subjects 50

2. 2. 2 Experimental design 52

2. 2. 3 Radioimmunoassay for 17β-estradiol and testosterone 53

2. 2. 4. Estrous cycle stage determination 54

2. 2. 5. Immunohistochemistry 54

2. 2.6 Cell counting 56

2. 2. 7 Statistical analyses 58

2. 3 Results 58

2. 3. 1 Males had larger dorsal dentate gyrus volumes compared to females. 58

2. 3. 2 Male rats, compared to female rats, had a greater density of Sox2-ir cells in the dorsal dentate gyrus. Females had greater density of Sox2-ir cells in the ventral compared to dorsal region. 59

2. 3. 3 Males had greater levels of cell proliferation (Ki67) compared to females. 62

2. 3. 4 Males, but not females, show greater attrition of BrdU-ir cells from 1 week to 2 weeks after mitosis 64

2. 3. 5 Male adult-born neurons mature faster compared to female adult-born neurons. 67

2. 3. 6 Males have a greater density of BrdU/Sox2-ir cells in the dorsal DG

at 2h compared to females. 71
2. 3. 7. The percentage of BrdU/Sox2 co-expressing cells decreased dramatically over time in both sexes 71
2. 4 Discussion 74
2. 4. 1 Male adult-born dentate granule cells mature faster compared to female adult-born dentate granule cells 74
2. 4. 2 Males had more neural stem cells than females, whereas females showed a regional difference with more neural stem cells in the ventral, compared to dorsal, dentate gyrus. 75
2. 4. 3 The neural progenitor cell-type composition changes after mitosis with sex-dependent manner 76
2. 4. 4 Neurogenesis in males has a different trajectory compared to females ... 77
2. 4. 5 Males, compared to females, had greater cell proliferation in the dentate gyrus 77
2. 5 Conclusion 78
Chapter 3: Sex differences in contextual pattern separation, neurogenesis, and functional connectivity within the limbic system 80
3. 1 Introduction 80
3. 2 Material and methods 83
3. 2. 1 Subjects 83

3. 2. 2 Apparatus 83

3. 2. 3 Experimental timeline 84

3. 2. 4 Behavioral testing for contextual pattern separation 84

3. 2. 5 Activation trial and perfusion 85

3. 2. 6 Tissue processing 85

3. 2. 7 Immunohistochemistry 87

3. 2. 8 Cell counting 89

3. 2. 9 Estrous-cycle determination 92

3. 2. 10 Statistical analyses 92

3. 3 Results 93

3. 3. 1 Females, but not males, discriminated shock-paired contexts from neutral contexts 93

3. 3. 2 There were more 4-week-old cells in the dorsal compared to the ventral dentate gyrus 94

3. 3. 3 Females had a greater percentage of IdU/zif268-ir cells in the dentate gyrus compared to males 96

3. 3. 4 Females had greater zif268 immunoreactivity than males in the frontal cortex and dorsal CA1 region of the hippocampus in response to a shocked-paired context 99

3. 3. 5 The density of zif268-ir cells in the dorsal CA1 was positively correlated with amount of freezing during memory recall in males, but not

in females 100

3. 3. 6 Neural activation of adult-born cells in the dentate gyrus was associated with the ability for pattern separation in males but not females. 102

3. 3. 7 Males and females showed distinct patterns of significant inter-regional correlations of the density of zif268-ir cells 102

3. 3. 8 Principal component analyses on the density of zif268-ir cells..... 107

3. 4 Discussion 109

3. 4. 1 Females are better at fear-associated contextual pattern separation compared to males 109

3. 4. 2 Females show greater neuronal activation of young granule cells in the dorsal DG compared to males..... 111

3. 4. 3 Females show greater neuronal activation in the frontal cortex and dorsal CA1 in response to fear memory retrieval 112

3. 4. 4 Males and females show distinct patterns of functional connectivity between frontal cortex, hippocampus and amygdala. 113

3.4.5 Perspectives and Significance 114

3.5 Conclusion 114

Chapter 4: Estrogens dynamically regulate neurogenesis in the dentate gyrus of adult female rats..... 116

4. 1 Introduction 116

4. 2 Materials and methods 118

4. 2. 1 Subjects 118

4. 2. 2 Experimental timeline 119

4. 2. 3 Perfusion and tissue processing 119

4. 2. 4 Immunohistochemistry 120

4. 2. 5 Cell counting 122

4. 2. 6 Statistical analyses 123

4. 3 Results 125

4. 3. 1 Estradiol reduced the density of Sox2-ir cells in the dorsal DG, whereas estrone reduced the density of Sox2-ir cells in the ventral DG compared to vehicle-treated females 125

4. 3. 2 Estradiol increased, whereas estrone reduced, the density of Ki67-ir cells in the DG compared to vehicle-treated females after one week of hormone exposure 126

4. 3. 3 Estrone and estradiol-treated females had a greater density of BrdU-ir cells and BrdU/DCX-ir cells compared to vehicle-treated females one week after cell division/ exposure to estrogens 128

4. 3. 4 Estradiol-treated female rats showed greater percentage of BrdU/DCX-ir cells compared to vehicle-treated female rats two weeks after cell division and exposure to estrogens 131

4. 4 Discussion 133

4. 4. 1 Estrone and estradiol reduce expression of neural stem/progenitor

cells in the DG 133

4. 4. 2 Estradiol enhances, whereas estrone reduces, cell proliferation in the DG one week after treatment 134

4. 4. 3. Estrone and estradiol increase the density of one-week old neurons after one week of exposure and increase the attrition of new neurons between one and two weeks. 135

4. 4. 4 Estrogens' ability to increase maturation of new neurons is diminished after sustained exposure 136

4. 4. 5 Implications 136

4. 5 Conclusion 138

Chapter 5: High Estradiol reduces adult neurogenesis but strengthens functional connectivity within the hippocampus during spatial pattern separation in adult female rats 139

5. 1 Introduction 139

5. 2 Materials and methods 139

5. 2. 1 Subjects 139

5. 2. 2 Animal husbandry 140

5. 2. 3 Apparatus 140

5. 2. 4 Experimental design 140

5. 2. 5 Immunohistochemistry 144

5. 2. 6 Cell counting 146

5. 2. 7 Statistical analyses 148
5. 3 Results 151
5. 3. 1 There was no significant effect of estradiol on strategy use or on the ability for separating either adjacent or distinct patterns 151
5. 3. 2 High estradiol reduced the density of BrdU/NeuN-ir cells in the dDG 151
5. 3. 3 zif268 expression in the vCA1 was greater in High estradiol-treated rats than the other groups 151
5. 3. 4 High estradiol-treated rats had distinct inter-regional correlations compared to the other groups 152
5. 4 Discussion 154
5. 4. 1 High levels of estradiol decreased adult-born neurons in the dDG 154
5. 4. 2 Estradiol altered patterns of neural activation and functional connectivity in the hippocampus 155
5. 4. 3 Strategy use was not altered by estradiol dose 156
5. 5 Conclusion 157
Chapter 6: General discussion 158
6. 1 Summary of experimental findings 158
6. 2 The maturation of new neurons in neurodegenerative diseases 159
6. 3 How can the overall neurogenesis be increased in males and females? 160
6. 4 Estrogenic regulation of neurogenesis as a potential mechanism of sex differences in the maturation and trajectory of new neurons 162

6. 5 Sex differences in the functional connectivity and cognition..... 163

6. 6 Effects of stress on hippocampal cognition and adult neurogenesis in males and females 164

6. 7 Species differences in adult neurogenesis such as maturation time course 166

6. 8 Ovariectomized rats with exogenous estrogens and naturally cycling estrogens 170

6. 9 Conclusion..... 170

Bibliography..... 172

Appendices 209

Appendix A Supplemental Figures 209

A. 1 Supplemental Fig. 3.1..... 209

A. 2 Supplemental Fig. 3.2..... 209

A. 3 Supplemental Fig. 5.1..... 210

Appendix B Supplemental Tables..... 211

B. 1 Supplemental Table 3.1..... 211

B. 2 Supplemental Table 3.2..... 211

B. 3 Supplemental Table 3.3..... 211

List of Tables

Table 2.1. Mean (±SEM), minimum and maximum concentration of serum testosterone in males (ng/ml) and estradiol in females (pg/ml). 54

Table 2.2. Mean (±SEM) volume of the dorsal and ventral dentate gyrus in male and female rats (mm^3)..... 59

List of Figures

Fig. 1.1. Schematic illustration for pattern separation in the hippocampus 7

Fig. 1.2. Schematic illustrations for spatial learning strategies in the Morris Water Maze and Dual T-Maze Task. 9

Fig. 1.3. Schematic illustration for hippocampus-related neural network 11

Fig. 1.4. Three-dimensional model of the hippocampus and coronal section rat brains, and dorsoventral differences 13

Fig. 1.5. Schematic image of the dorsal hippocampus and dentate adult neurogenesis 18

Fig. 1.6. Effects of acute estrogen treatment on adult neurogenesis in the dentate gyrus 43

Fig. 1.7. Effects of chronic administration of estrogens on adult neurogenesis and activation of new neurons in female rats 44

Fig. 2.1. Schematic illustrations for the experimental procedures 51

Fig. 2.2. Sex differences in neural stem cells (Sox2-ir) 61

Fig. 2.3. Sex differences in proliferating cells (Ki67-ir) in the dentate gyrus 63

Fig. 2.4. Sex differences in the trajectory of adult-born BrdU-ir cells 66

Fig. 2.5. Sex differences in the maturation rate of adult-born neurons in the dentate gyrus (BrdU/NeuN) 69

Fig. 2.6. Sex differences in the maturation rate of adult-born neurons in the dentate gyrus (BrdU/DCX) 70

Fig. 2.7. Sex differences in BrdU/Sox2-ir cells across timepoints 73

Fig. 3.1. Experimental design 86

Fig. 3.2. Sex differences in contextual fear discrimination and Zif268 activation of dentate adult-born cells 95

Fig. 3.3. Sex differences in zif268 activation of dentate adult-born cells 97

Fig. 3.4. Sex differences in zif268 activation in the brain, and correlations with fear-response 101

Fig. 3.5. Sex differences in inter-regional correlations of zif268-ir cell density 106

Fig. 3.6. Sex difference in the principal component analysis (PCA) 108

Fig. 4.1. Experimental design 124

Fig. 4.2. Estrogenic regulation for the neural stem/progenitor cells and cell proliferation ... 127

Fig. 4.3. Estrogenic regulation for the trajectory of new cells in the dentate gyrus 130

Fig. 4.4. Estrogen treatment and the maturation rate of new neuron 132

Fig. 5.1. Experimental design of the spatial pattern separation task in ovariectomized rats . 149

Fig. 5.2. Estrogenic regulations for adult neurogenesis and inter-regional correlations in the hippocampus 153

Fig. 6.1. Schematic images for species difference in expression of immature and mature neuronal markers (DCX: orange, NeuN; blue) 168

List of Symbols

α: Alpha

β: Beta

η: Eta

List of Abbreviations

ACC	anterior cingulate cortex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AN	anterior nucleus
ANOVA	analysis of variance
AR	androgen receptor
BOLD	blood-oxygen- level dependent
BrdU	5-bromo-2-deoxyuridine
BLA	basolateral amygdala
CA1	cornu ammonis 1
CA3	cornu ammonis 3
CeA	central amygdala
CIHR	Canadian Institutes for Health Research
CldU	5-chloro-2-deoxyuridine
CUS	chronic unpredictable stress
DAB	diaminobenzidine
DCX	doublecortin

DG	dentate gyrus
DNA	deoxyribonucleic acid
EB	estradiol benzoate
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GCL	granule cell layer
GPER	G-coupled protein receptor
GR	glucocorticoid receptor
H.M.	Henry Molaison
HPA	hypothalamic-pituitary-gonadal
HPC	hippocampus
Htr1a	hydroxytryptamine receptor 1a
Htr3a	hydroxytryptamine receptor 3a
IdU	5-iodo-2-deoxyuridine
IEG	immediate early gene

IL	infralimbic cortex
I.p.	intraperitoneal
IPC	intermediate neural progenitor cell
Ir	immunoreactive
LA	lateral amygdala
lDS	lateral dorsal striatum
LTD	long-term depression
LTP	long-term potentiation
MAM	methylazoxymethanol
mDC	medial dorsal striatum
ML	molecular layer
NAc	nucleus accumbens
NDS	normal donkey serum
NeuN	neuronal nuclei
NIH	National Institutes for Health Research
NMDA	N-methyl-D-aspartate
NSC	neural stem cell

OVX	ovariectomy
PBS	phosphate buffered saline
PCA	principal component analysis
PET	positron emission tomography
PFA	paraformaldehyde
PFC	prefrontal cortex
PTSD	post-traumatic stress disorder
PrL	prelimbic cortex
PSA-NCAM	polysialylated neural cell adhesion molecule
PVA-DABCO	polyvinyl alcohol-1,4-Diazabicyclo[2.2.2]octane
RGL	radial glia-like neural stem cell
RIA	radioimmunoassay
SABV	sex as a biological variable
SEM	standard error of the mean
SGBA	sex and gender based analysis
S.q.	subcutaneous
TBS	tris buffered saline

Trhr	thyrotropin releasing hormone receptors
VTA	ventral tegmental area
WHI	Women's Health Initiative

Acknowledgements

I would like to acknowledge that all the works in this dissertation was conducted at the University of British Columbia Point Grey campus that is located on the traditional, ancestral, and unceded territory of the Musqueam people.

I'd like to thank my supervisor, Dr. Liisa Galea, for her insight, support, and mentorship. She has made it very enjoyable for me to be engaged in academic research.

Thank you, also, to my past and present lab members in the Galea lab for their friendly and helpful advice: Dr. Paula Duarte Guterman, Dr. Rand Eid, Dr. Steve Wainwright, Dr. Dannia Islas, Dr. Travis Hodges, Dr. Paul Sheppard, Dr. Wansu Qiu, Dr. Jennifer Richard, Kim Go, Muna Ibrahim, Rebecca Rechlin, Tallinn Splinter, Jared Splinter, Yanhua Wen, Stephanie Lieblich, Bonnie Lee, Tanvi Puri, Amanda Namchuk, Samantha Blankers, Romina Garcia de leon, Nadine Truter, Ulrike Mayer, Daria Tai and Amanda Lee. This project could not have been completed without their help. A special thank you to Stephanie Lieblich and Yanhua Wen for their support with incredible lab techniques and for their insights. I also would like to thank Alice Chan and Anne Cheng for their assistance with this work.

Thank you to all my friends for their kindness and for always being interested in my research. A special thank you to Brian Smith and Yukari Smith for their warm support.

Finally, I'd like to thank my wife, Jacqueline Yvette Spisa-Yagi, for always understanding me and for her incredible support.

This thesis is dedicated to my children, Finn Tatsuto Yagi, Lennard Sota Yagi and Kelo Yagi.

Chapter 1: Introduction

1.1 Sex differences in hippocampus related diseases and importance of studying sex differences in the hippocampus

Studying sex differences in various diseases and disorders has become increasingly important as there are sex differences not only in the prevalence of certain diseases but also manifestation of the disease and in treatment efficacy (Brookmeyer et al., 1998; Gutiérrez-Lobos et al., 2002; Irvine et al., 2012; McPherson et al., 1999). The inclusion of sex as a biological variable (SABV) at federal funding agencies, such as National Institutes for Health Research (NIH) and sex and gender based analysis (SGBA) at Canadian Institutes for Health Research (CIHR) has highlighted the need for more research on disease prevalence, manifestation, and treatment outcomes in men, women and gender diverse individuals (Liu and Dipietro Mager, 2016; Nolan and Nguyen, 2013). Nonetheless while the use of males and females has been mandated in NIH-sponsored clinical trials since 1993 (NIH, 1993), only approximately 20% of these trials analyse with sex as a factor (Geller et al., 2018). Furthermore, this sex-inclusion mandate does not extend to clinical trials that are not sponsored by NIH, and these trials form the majority of clinical trials listed on clinicaltrials.gov (over 85%). In addition, only 19% of papers in the major journals in neuroscience and psychiatry reported the appropriate sample size, and analyses from both sexes in 2019 (Rechlin et al., 2022). Although the percentage of studies that reported both sexes increased from 38% in 2009 to 68% in 2019, only 5% of studies in 2019 analyzed data with sex as a discovery variable (Rechlin et al., 2022). Thus, there is a lack of substantial progress in how biological sex may interact with treatment and disease. This exclusion has likely contributed to greater adverse side effects of drugs seen in human females compared to males (Rademaker, 2001; Seeman, 2021, 2009). Here in this thesis, I focus on the

importance of studying sex differences in cognition with an eye toward a better understanding of the notable sex differences in the prevalence and severity of memory symptoms in a number of brain diseases that favour either sex. This research is important as cognitive deficits with neurological disease show significant sex differences (Cereda et al., 2016; Han et al., 2012; McPherson et al., 1999; Nicoletti et al., 2017).

In both Alzheimer's Disease and depression, females show greater prevalence of disease and steeper declines in memory or more severe cognitive symptoms (Brookmeyer et al., 1998; Gutiérrez-Lobos et al., 2002; Hy and Keller, 2000; McPherson et al., 1999). On the other hand, men with schizophrenia or Parkinson's Disease show greater cognitive impairments compared to women with these same diseases (Cereda et al., 2016; Han et al., 2012; Leung and Chue, 2000; Mossaheb et al., 2018; Nicoletti et al., 2017; Szewczyk-Krolikowski et al., 2014). Although underlying biological mechanisms of these sex differences in cognitive impairment with brain diseases are not completely elucidated, there are sex and sex hormones differences in hippocampus structure and plasticity that may contribute to the greater vulnerability within each sex by disease type. Understanding sex differences in the contribution of hippocampal plasticity to cognition can give us important clues on the underlying mechanisms of disorders that involve disruptions to cognition and hippocampal integrity in an effort to lead to better tailored treatments for patients with such disorders.

There are sex differences in neurodegeneration noted in Alzheimer's disease. The CA1 region of the hippocampus is one of the first sites of integrity loss in Alzheimer's disease (Padurariu et al., 2012). Sex differences exist in the areas that correlate with Alzheimer's disease progress and neuronal density in the hippocampus (Martínez-Pinilla et al., 2016). Negative associations are seen in Alzheimer's patients between Braak stage and CA1 neuronal density in

females, but with CA3 neuronal density in males (Martínez-Pinilla et al., 2016). Furthermore, studies suggest that females are more likely to show associations of progression to Alzheimer's disease with changes in hippocampal volume and amyloid burden, whereas white matter hyperintensities are more related to disease progression in males (Burke et al., 2018; Caldwell et al., 2017). The present work is an important step to determine whether sex differences can aid in our understanding of disease progression and possible treatment strategies that may need to differ by sex.

The hippocampus is a brain region of interest as it is a highly plastic structure due in part to the presence of adult neurogenesis in the dentate gyrus (Christie and Cameron, 2006; Eriksson et al., 1998; Neves et al., 2008), fluctuations in dendritic spine/synapse density, dendritic arbourization (McEwen, 2018), and electrophysiological plasticity with long-term potentiation (LTP) and long-term depression (LTD) (Artola et al., 2006; Whitlock et al., 2006). This plasticity is modified in a sex-dependent manner either under normal conditions or can manifest after exposure to learning experiences. The circuitry, (discussed below), and receptor characteristics of the hippocampus are well known, but an important caveat is that we know little of how these characteristics may differ between males and females. Indeed, there are sex and age differences in the level of mRNA and protein expression of the hormone receptors in the hippocampus (discussed below). Therefore, understanding sex differences in the contribution of hippocampal plasticity to cognition can give us important clues on the underlying mechanisms of disorders that involve disruptions to cognition and hippocampal integrity in an effort to lead to better treatments for patients with such disorders.

1.2 Anatomy of the hippocampus

The hippocampus receives major input through the perforant path from the superficial layers of entorhinal cortex. In addition to perforant path, the pyramidal neurons in the entorhinal cortex layer II send axons to pyramidal neurons in the CA3, and furthermore, layer III neurons connect directly to the CA1. These inputs are highly processed sensory information from many cortical, subcortical, and brainstem structures (Burwell and Amaral, 1998; Insausti et al., 1997). The trisynaptic circuit includes perforant path projection from the pyramidal neurons in the entorhinal cortex to granule neurons in the dentate gyrus, the axons of dentate granule neurons, called mossy fibers, send output to CA3 pyramidal neurons, and these CA3 neurons then project axons to CA1 pyramidal neurons through Schaffer collaterals. Indeed, the CA1 region sends the information back to the deep layers of entorhinal cortex, whereas CA3 pyramidal neurons also send projections to CA3 and the dentate gyrus. These projections called recurrent collaterals are proposed to be critical for some forms of hippocampal functions such as pattern completion (Marr, 1971). The hippocampus also sends major outputs to prefrontal cortex, and various other brain regions such as retrosplenial cortex, amygdala and hypothalamus (Leary and Cryan, 2014). In addition, the CA1 receives modulatory input of serotonin, dopamine, and GABA from various brain regions. Importantly, the hippocampus contains a large concentration of glucocorticoid and mineralcorticoid receptors compared to other regions, which makes the hippocampus more vulnerable to chronic glucocorticoid exposure via stress (Aronsson et al., 1988; McEwen et al., 1968; Mocuilewsky and Raynaud, 1980). Again whereas, the structural and receptor characteristics of the hippocampus are well known, an important caveat is that we know little of how these characteristics may differ between males and females. Therefore, we need to

acknowledge that connectivity patterns and receptor density characteristics within the hippocampus may differ between the sexes.

1.3 The Function of the Hippocampus

The hippocampus is a central brain structure implicated in contextual, declarative, episodic and spatial memory (Eichenbaum, 2004; Fanselow and Dong, 2010). The CA1 region of the hippocampus is one of the first sites of damage in Alzheimer's disease (Tang et al., 2015). Atrophy of the CA1 region in Alzheimer's disease and other forms of dementia is associated with memory loss and spatial disorientation during early stages of the disease (Henneman et al., 2009; Kerchner et al., 2012; Sabuncu and Desikan, 2011). The H.M. case and other studies suggested that the hippocampus is particularly important for transition from a short-term memory to a long-term memory and consolidation of memory as hippocampal lesions lead to anterograde amnesia but intact, or less affected, short-term memory and previously acquired memories (Scoville and Milner, 1957; Young et al., 1994; Zola-Morgan et al., 1986). Furthermore, retrograde amnesia is reported from patients with hippocampal lesions and animal models, which is dependent on the severity and extent of the lesions (For review see Nadel l. and Moscovitch M., 1997). These findings suggest that the hippocampus is also important structure needed to retrieve memory. The integrity of the hippocampus is also important for encoding of memory as is discussed below.

1.3.1 Hippocampal function for pattern separation and pattern completion

Anatomical analyses indicate that up to 200,000 pyramidal neurons in the entorhinal cortex send projections to up to 1,000,000 dentate granule neurons, which project to up to 160,000 CA3 pyramidal neurons (Marr, 1971). This structural dispersion of neural projections

from the entorhinal cortex to the dentate gyrus via the perforant path reduces the probability of activating the same set of neurons in the dentate gyrus and the CA3 region in response to different external stimuli (see Fig. 1.1). This process of separating overlapping information or similar input patterns at the dentate gyrus during memory encoding is called pattern separation, and it was proposed one of the primary functions of the hippocampus (Kesner et al., 1987; Marr, 1971; McClelland et al., 1995; Olton and Papas, 1979; Rolls, 1996). On the other hand, the direct path from the entorhinal cortex to the CA3 and CA1 regions, and recurrent collaterals are proposed to play an important role for pattern completion (Marr, 1971; Yassa and Stark, 2011). Pattern completion is a process to recall a learned pattern or a previously acquired memory from degraded or partial cues during memory retrieval. When a subset of previously activated pyramidal neurons in the CA3 region is reactivated by input through the direct path from the entorhinal cortex, the input through the direct path can compete with the input from mossy fibers. Then, recurrent collaterals from the reactivated neurons in the CA3 region modulate the mossy fiber signal as well as CA3 neurons. Studies demonstrated that young adult-born neurons in the dentate gyrus play an important role for pattern separation but not for pattern completion in both males and females (Clelland et al., 2009; Nakashiba et al., 2012).

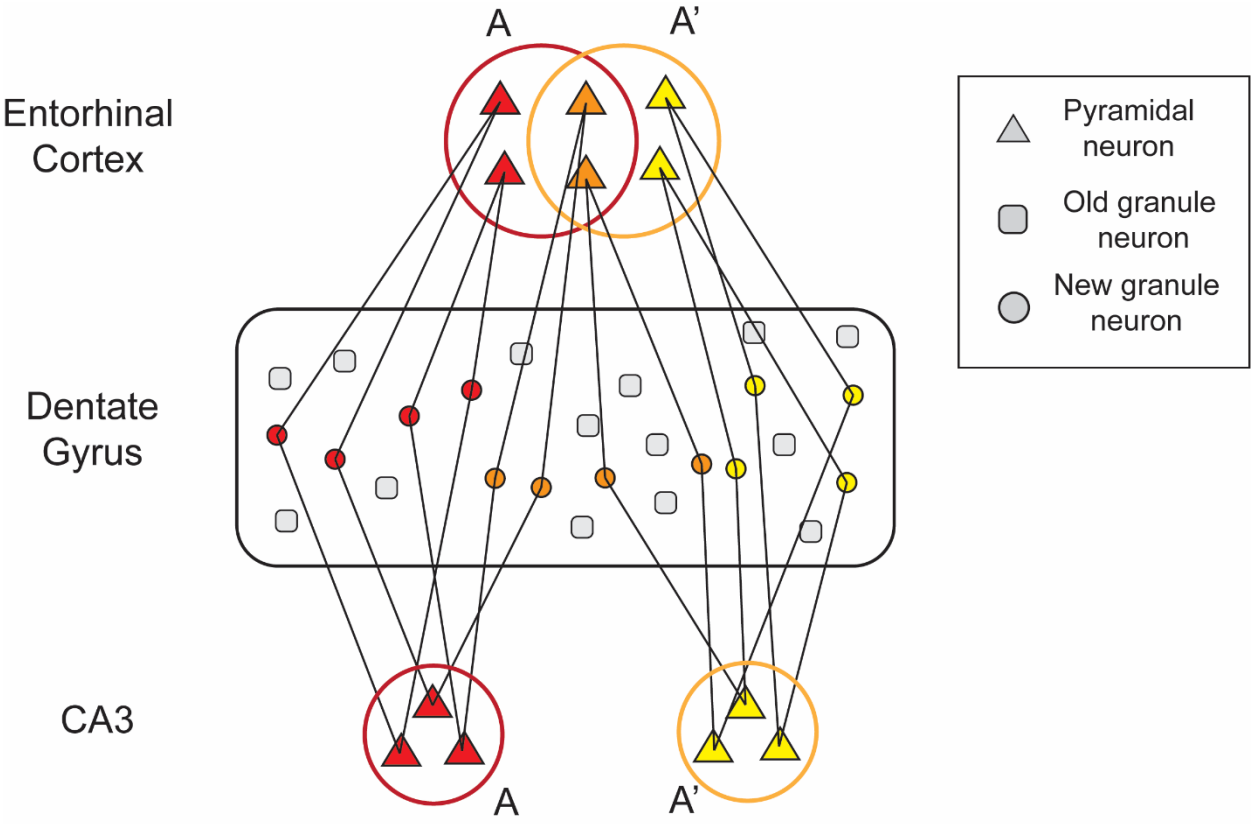


Fig. 1.1. Schematic illustration for pattern separation in the hippocampus. Overlapping neural stimuli from the entorhinal are separated via dispersion of dentate granule neurons and make neural representation in the CA3. Adult-born new neurons in the dentate gyrus play critical roles for pattern separation (Kesner et al., 1987; Marr, 1971; McClelland, 1995; Olton and Papas, 1979; Rolls, 1991; Clelland et al., 2009).

1.3.2. The hippocampus and learning strategy use

The hippocampus is also engaged when using place strategies to find a goal location, while the striatum is engaged when using a response strategy (Iaria et al., 2003; Maguire et al., 2017; McDonald and White, 1993). The place strategy relies on knowledge of one's position and a goal location using geometric spatial cues (Morris et al., 1982), whereas the response strategy relies on proprioceptive or landmark cues (Cook and Kesner, 1988; see Fig. 1.2). Place strategies are more likely to be used during the acquisition phase of learning, while the response strategies

are more common once repetitive rules or landmark cues are learned (Chang and Gold, 2003; Iaria et al., 2003; Poldrack et al., 2001). The two learning strategies work concurrently so that a more efficient strategy can compensate for the less efficient strategy. Indeed, inactivation of striatum leads to a shift in preference to the place strategy, while inactivation of hippocampus leads to a shift in preference of the response strategy in rodents (Packard and McGaugh, 1996). Human studies using fMRI or PET imaging demonstrate that subjects using a place strategy had greater activation in the right hippocampus, while subjects using a response strategy had greater activation of the caudate nucleus (Iaria et al., 2003; Maguire et al., 2017). Recent studies have demonstrated that there are sex differences in learning strategy choice, which is discussed in Section 1.6.1 (Juraska et al., 1985; Maren et al., 1994; Scheinost et al., 2015; Zhang et al., 2016).

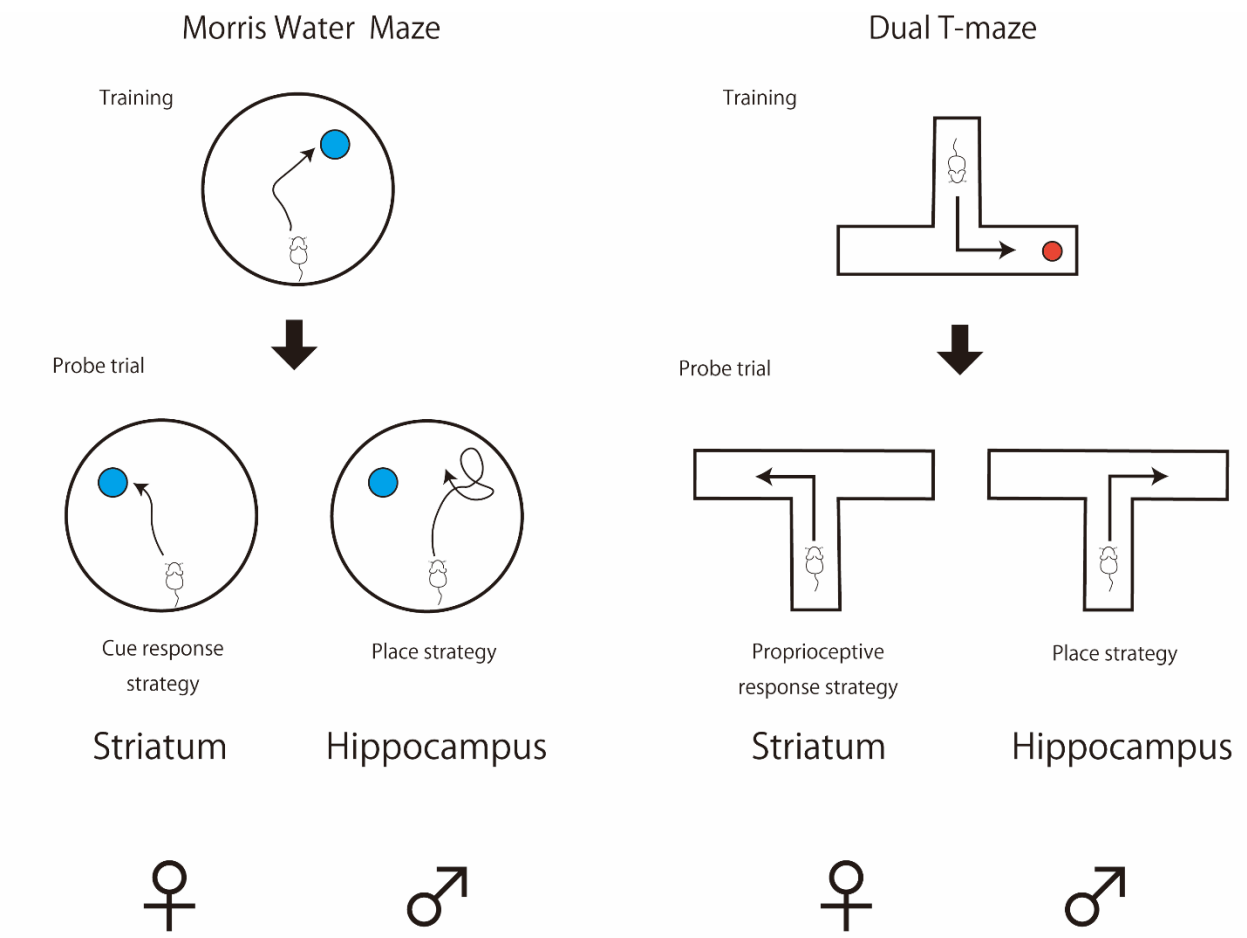


Fig. 1.2. Schematic illustrations for spatial learning strategies in the Morris Water Maze and Dual T-Maze Task. The hippocampus-dependent place strategy relies on knowledge of one's position and a goal location using geometric spatial cues (Morris et al., 1982), whereas the striatum-dependent response strategy relies on proprioceptive or landmark cues (Cook and Kesner, 1988). Males are more likely to use geometric cues (place strategy) and females are more likely to use landmark cues (cue response strategy) or proprioceptive cues (proprioceptive response strategy) to reach a destination (Andersen et al., 2012; Galea and Kimura, 1993; Williams et al., 1990; Galea and Kimura, 1993; Cherney et al., 2008; Grissom et al., 2013)

1.4. Dorsal versus ventral hippocampus

The hippocampus can be divided into two different regions along its longitudinal axis (rostral/dorsal and caudal/ventral regions). Dorsal, but not ventral, hippocampal lesions impair spatial working and reference memory, while ventral, but not dorsal, hippocampus lesions hamper stress response (negative feedback inhibition), non-spatial working memory and anxiety-like behaviours in male rats (Hauser et al., 2020; Henke, 1990; Kjelstrup et al., 2002; Potvin et al., 2006; Weeden et al., 2014). These findings collectively add support to the idea that the dorsal hippocampus is important for spatial reference memory, while the ventral hippocampus serves to regulate emotion/anxiety and the stress response (Henke, 1990; Kjelstrup et al., 2002; Moser et al., 1995). Indeed, the dorsal hippocampus has a greater number of place cells (cells which are more responsive to the environmental location) compared to the ventral hippocampus in male rats (Jung et al., 1994). Furthermore, increased spatial memory in London cab drivers is linked specifically to increased volume of the posterior hippocampus (akin to the dorsal hippocampus in rodents). Anatomically, these two dorsal ventral regions have distinct neural connectivity (Swanson and Cowan, 1977). For example, CA3 pyramidal neurons in the ventral, but not dorsal, hippocampus have direct projections to the dentate granule neurons (Li et al., 1994). The dorsal hippocampus sends projections from the CA1 to structures processing visuospatial information such as the retrosplenial cortex, whereas the ventral hippocampus is primarily connected to areas implicated in regulating anxiety and the stress response including the amygdala and hypothalamus (Leary and Cryan, 2014). The ventral, but not dorsal, hippocampus is connected directly to the basolateral amygdala (Herry et al., 2008; Xu et al., 2016; see fig. 1.3). Furthermore, the dorsal hippocampus projects to the anterior cingulate cortex while the ventral hippocampus projects to the infralimbic cortex and prelimbic cortex (Cenquizca and Swanson,

2007; Chiba, 2000). Again, though the anatomical connectivity of the hippocampus is well known, an important caveat is that we know little of how these connections may differ between males and females. Therefore, we need to acknowledge that connectivity patterns between the hippocampus and other brain regions may differ between the sexes.

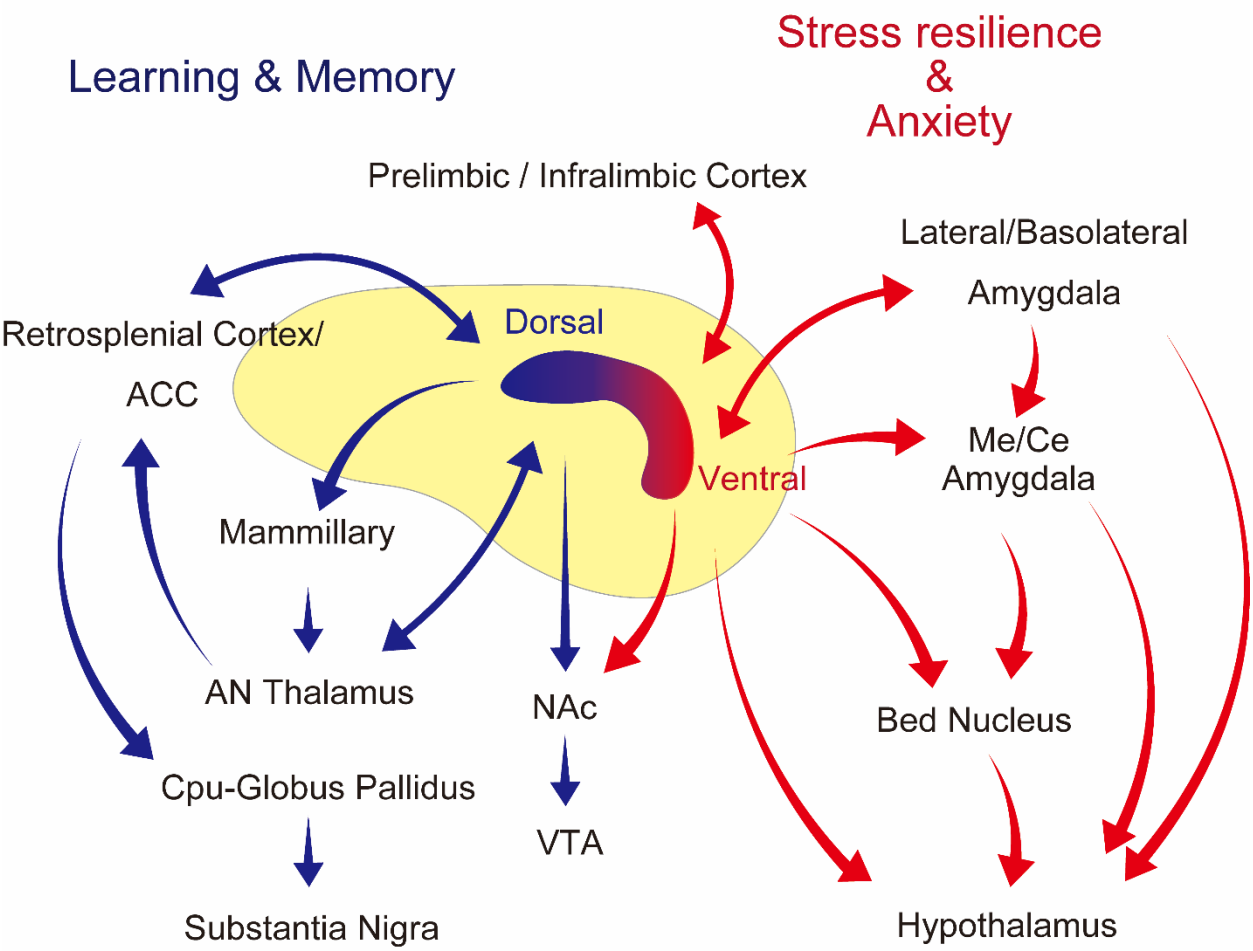
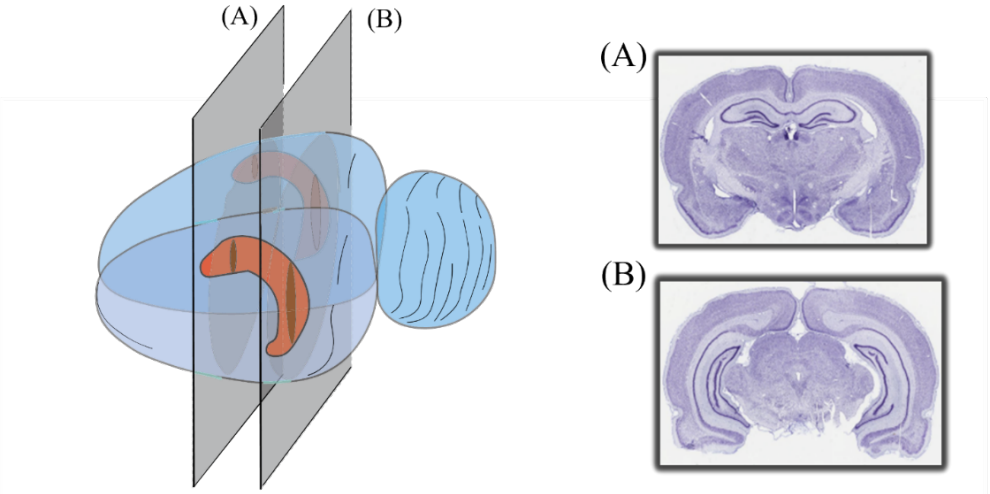


Fig. 1.3. Schematic illustration for hippocampus-related neural network. The dorsal and ventral hippocampus has a distinct neural network, where the dorsal hippocampus sends projections to the retrosplenial cortex and the anterior cingulate cortex (ACC) whereas the ventral hippocampus is primarily connected to the prelimbic/infralimbic cortex, amygdala and hypothalamus (O'Leary and Cryan, 2014; Herry et al., 2008; Xu et al., 2016). AN: anterior neucleus, Me: medial, Ce: central, ACC: anterior cingulate cortex, NAc: neucleus accumbens, VTA: ventral tegmental area, Cpu: caudate-putamen.

The functional differences between the dorsal and ventral regions may be due in part to the differences in gene expression for a specific receptor composition, such as 5-hydroxytryptamine (serotonin) receptor 3a (*Htr3a*), serotonin receptor 1a (*Htr1a*) and thyrotropin releasing hormone receptors (*Trhr*) gene. Htr3a and Htr1a are expressed in the ventral dentate gyrus to a greater extent compared to the dorsal dentate gyrus (Christensen et al., 2010; Tanaka et al., 2012), while *Trhr* is expressed specifically in the ventral dentate gyrus (Manaker et al., 1985) in male rats. As serotonin and thyrotropin releasing hormones are associated with regulation of stress response and depression (reviewed in Owens and Nemeroff, 1994; Porter et al., 2004), the longitudinal variations of gene expression of these receptors may attribute to the functional difference of the dorsal and ventral hippocampus. While most studies have been conducted in males, *Htr1a* knockout female, but not male, mice show decreased level of BDNF specifically in the ventral hippocampus (Wu et al., 2012; summarized in Fig. 1.4). However, in this study, while males and females were used they were not analyzed together, complicating the interpretation of these differences between males and females. Furthermore, prenatal stress decreased binding affinity of 5-HT receptor1a in the ventral hippocampus only in male rat offspring (Van den Hove et al., 2006). These two studies show there may be sex differences in the dorsal/ventral expression of 5-HT receptor genes. Studies examining transcriptomic analyses in the hippocampus are scarce but do indicate that there are sex differences in gene expression of the hippocampus (Bundy et al., 2017; Vied et al., 2016). More studies directly comparing males and females are essential for further understanding of underlying mechanisms of sex differences in hippocampal function.



	(A) Dorsal	(B) Ventral
Major connections	Anterior cingulate cortex, Retrosplenial cortex, Ventral tegmental area, Lateral/medial mammillary nucleus	Prelimbic/Infralimbic cortex, Bed nucleus of the stria terminalis, Nucleus accumbens, Amygdala, Hypothalams, Ventral tegmental area
Function	Spatial learning and memory Spatial/contextual pattern separation	Emotion, stress response Reward-related early learning, Olfactory pattern separation
Receptor genes expression (DG)	Inositol 1,4,5-triphosphate receptor 1	Sstr1 Trhr Chrm4 ERα 5HT1a/3a ERβ
Neurogenesis	Greater and faster maturation	Greater stress-induced decrease

Fig. 1.4. Three-dimensional model of the hippocampus and coronal section rat brains, and dorsoventral differences. Major differences along dorsoventral axis of the hippocampus in its connections, functions, gene expression and adult neurogenesis. The dorsal hippocampus is connected to structures that play roles in spatial learning and visuospatial processing while the ventral hippocampus is connected to structures that regulates stress hormones, reward processing and anxiety. The dorsal dentate gyrus (DG) shows greater expression of Inositol 1,4,5-triphosphate receptor1 while the ventral dentate gyrus shows greater expression of somatostatin receptor 1 (Sstr1), cholinergic receptor muscarinic 4 (Chrm4), serotonin receptor 1a and 3a (5HT1a, 5HT3a), thyrotropin releasing hormone receptor (Trhr) and estrogen receptor α and β (ERα, ERβ). Adult neurogenesis in the dorsal dentate gyrus is more numerous and faster maturation compared to the ventral dentate gyrus.

1.5 Plasticity of the Hippocampus

The hippocampus is a highly plastic structure due in part to the presence of adult neurogenesis in the dentate gyrus (Christie and Cameron, 2006; Eriksson et al., 1998; Neves et al., 2008). Although the functional roles of adult neurogenesis are not fully understood, adult-born young neurons in the dentate gyrus may be critical for pattern separation, cognitive flexibility and stress tolerance (Clelland et al., 2009; Epp et al., 2016; Mateus-Pinheiro et al., 2013; Nakashiba et al., 2012; Snyder et al., 2011).

Along with neurogenesis, the hippocampus displays large fluctuations in dendritic spine density and dendritic arborization, typically in response to hormonal fluctuations, environmental enrichment, and/or stress (McEwen, 2018). Furthermore, principal cells of the hippocampus contain place cells, in which the place fields change with experience with the environment indicating its importance in spatial navigation (Frank et al., 2006; O'Keefe et al., 1998; O'Keefe and Burgess, 1996). In addition, the hippocampus shows significant synaptic plasticity with the discovery of long-term potentiation (LTP) and long-term depression (LTD). LTP is considered the cellular mechanism behind learning and memory (reviewed in Bliss and Collingridge, 1993). Glutamate receptors (NMDA and AMPA) are the main contributors for inducing and maintaining LTP (McHugh et al., 1996; McHugh and Tonegawa, 2009). The distribution and density of NMDA and AMPA receptors are plastic in the hippocampus in response to stress and sex hormonal fluctuations (Mikasova et al., 2017; Palomero-Gallagher et al., 2003). There are potential sex and sex hormones differences in the hippocampal plasticity so that I will investigate sex differences in one form of hippocampal plasticity, adult neurogenesis, in Chapter 2 and estrogenic regulation of neurogenesis in Chapter 4.

1.5.1. Adult neurogenesis in the hippocampus

The dentate gyrus in the hippocampus is one of few brain regions where adult neurogenesis exists in all mammalian species studied to date (Christie and Cameron, 2006; Eriksson et al., 1998). Adult neurogenesis refers to the ability of the brain to produce new neurons in adulthood (Christie and Cameron, 2006; Eriksson et al., 1998). Adult neurogenesis in the dentate gyrus plays important roles for some forms of learning and stress resilience (Anacker et al., 2018; Clelland et al., 2009; Nakashiba et al., 2012; Planchez et al., 2021; Snyder et al., 2011). Although a few studies indicate little to no neurogenesis in adult humans (Dennis et al., 2016; Sorrells et al., 2018), several other studies indicate that neurogenesis does exist in humans using multiple methods (Epp et al., 2013a; Eriksson et al., 1998; Knoth et al., 2010; Moreno-jiménez et al., 2019; Spalding et al., 2013; Terreros-Roncal et al., 2021). Given the diversity of markers and techniques used to label neurogenesis and the diversity of age, sections counted, postmortem interval, cause of death, sex, and other factors it is clear more research needs to be done with respect to which protein markers are the best to examine neurogenesis in humans. While there are relatively few studies, there are sex differences in the production and survival of adult-born neurons in response to stress and hippocampus dependent learning (Falconer and Galea, 2003; Tanapat et al., 1999; Westenbroek et al., 2004; Yagi et al., 2016).

Adult neurogenesis in the dentate gyrus consists of cell proliferation, migration, differentiation and survival into a neuron or glia cell (see Fig. 1.5). Net increases or decreases in the amount of neurogenesis are determined by changes in any one of these components independently or in orchestration together. For example, chronic antidepressants increase neurogenesis via an increase in cell proliferation independent of any changes in survival of new neurons in male rats (Malberg et al., 2000). On the other hand, testosterone increases

neurogenesis via an increase in cell survival independent of any changes in cell proliferation in male rats (Hamson et al., 2013; Spritzer and Galea, 2007; Swift-Gallant et al., 2018). Finally, prenatal alcohol exposure decreases neurogenesis in female, but not male, rats via reduction in the ratio of new cells differentiating into neurons without affecting cell proliferation or survival (Uban et al., 2010). Theoretically modification of one of the four stages may lead to changes in the function of the dentate gyrus. It is important to note that early in the development of adult-born neurons, these new immature neurons are excited by the GABAergic input, and this input may play an important role for the survival of these young adult-born neurons (Ge et al., 2006; Jagasia et al., 2009). There are likely optimum levels of the hippocampal neurogenesis for optimal function of the hippocampus. For example, increased neurogenesis via epileptic seizures contributes to the disruption of hippocampal cognition in male and female rodents following seizures (Botterill et al., 2015; Cho et al., 2015; Jessberger et al., 2007a, 2007b). This may be due in part to the ectopic connections of the new neurons created under seizures. In order for the hippocampus to function appropriately, new neurons must integrate appropriately into the molecular layer, not into the hilus of dentate gyrus. Studies show that epileptic seizures enhanced the integration of newborn neurons into the hilus, which increases neural activity in the CA3 and dentate gyrus of male rats (Cameron et al., 2011; Parent et al., 1997).

Characteristics of adult neurogenesis can be studied along with stage specific endogenous cell markers such as Ki67 (proliferating cells) or doublecortin (DCX; immature neurons), or exogenous thymidine analog markers such as bromodeoxyuridine (BrdU). BrdU is generally injected intraperitoneally and is incorporated into the DNA of mitotic cells during the synthesis-phase of cell cycle. BrdU is active for a period of 2 hours after its injection into the system (Nowakowski et al., 1989). Thus, BrdU is often used for birth dating and monitoring the fate of

divided cells (for discussion see, Taupin, 2007). However, BrdU does not exclusively label neural precursor cells but also other cell types such as dividing glial cells (Taupin, 2007). Therefore, it is necessary to co-label BrdU with endogenous cell-specific markers such as DCX and neuronal nuclei (NeuN), protein markers for immature or mature neurons, respectively. DCX is a microtubule binding protein expressed from 0 to 3 weeks of age in newly produced neurons in the rat (Brown et al., 2003; Rao and Shetty, 2004). NeuN is expressed in neurons and expression in new neurons begins about one week after mitosis in rats and two weeks after mitosis in mice (Brown et al., 2003; Snyder et al., 2009). A number of studies have used multiple injections of BrdU, or oral dosing of BrdU across days, but the issue with this approach is that this will label a heterogenous population of cells (both dividing and survival of the divided cells), thereby obstructing whether a treatment affects cell proliferation or survival of new neurons. Thus, it will not be possible to determine whether the effects of a treatment are on proliferation independent of survival of new cells. BrdU is rapidly integrated into dividing cells and has the advantage of long-lasting stability in DNA (Cameron and Mckay, 2001). There are two other thymidine analogs that are used, CldU and IdU. Among these thymidine analogs, BrdU has greater probability to be integrated into DNA during the synthesis phase of mitosis (Leuner et al., 2009). However, a combination of the two thymidine analogs allows researchers to label proliferating cells at multiple time points which is only feasible using CldU and IdU (W. R. Hawley et al., 2012; Mcavoy et al., 2016). Care must be taken when designing any experiment investigating neurogenesis to take advantage of the best marker for the question, timeline, and species being investigated.

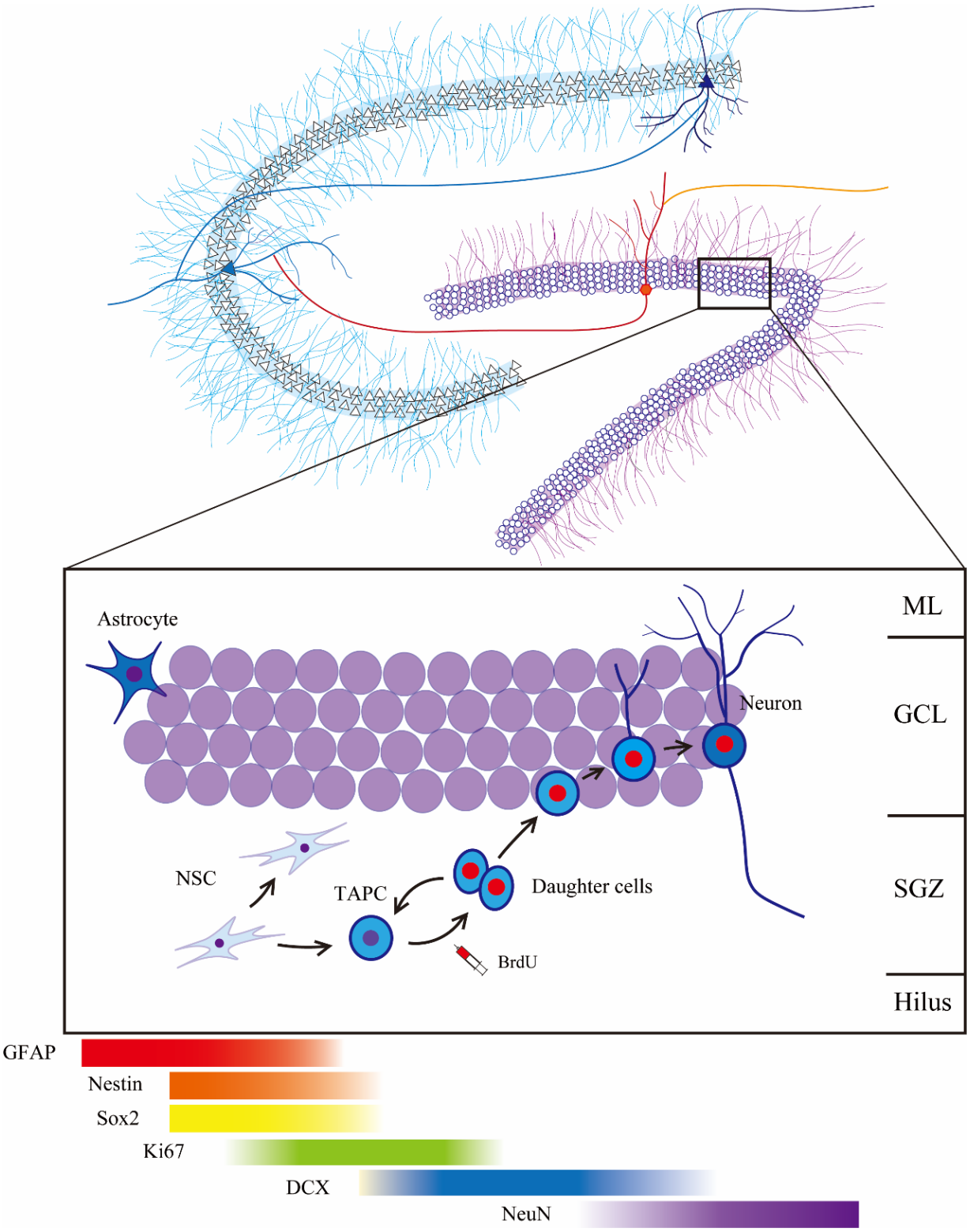

Astrocyte
Neuron
ML
GCL
SGZ
Hilus
NSC
TAPC
Daughter cells
BrdU
GFAP
Nestin
Sox2
Ki67
DCX
NeuN

Fig. 1.5. Schematic image of the dorsal hippocampus and dentate adult neurogenesis. A neural stem cell (NSC) can give rise to a transient amplifying progenitor cell (TAPC), which is divided into two neural progenitor cells (NPC) and migrate in the granular cell layer of dentate gyrus and differentiate into a neuron and start to send dendrites to the molecular layer (Braun and Jessberger, 2014). Neural stem cells express glia cell marker, glial fibrillary acidic protein (GFAP), neural stem cell marker, Nestin and Sox2(Braun and Jessberger, 2014). Ki67 is a nuclear protein which is expressed during all active phase of cell cycle and as such is a marker of cell proliferation (Kee et al., 2002). Doublecortin (DCX) is a microtubule associated protein expressed in the cytoplasm of dividing neural progenitor cells and immature neurons (Brown et al., 2003). NeuN is expressed in new neurons beginning about one week after mitosis in rats and two weeks after mitosis in mice and used as a nuclear marker for mature neurons (Snyder et al., 2009).

1.5.2. Functional role of adult neurogenesis in learning and memory

Adult neurogenesis in the dentate gyrus plays important roles for some forms of learning and stress response (Clelland et al., 2009; Kjelstrup et al., 2002; Nakashiba et al., 2012; Snyder et al., 2011). For instance, reduction of hippocampal adult neurogenesis leads to an impairment in trace eye blink conditioning, pattern separation, contextual fear conditioning task (Denny et al., 2012; Drew et al., 2010), and proactive interference (Akers et al., 2014; Epp et al., 2016; Feng et al., 2001; Kitamura et al., 2009), and long-term, but not short-term, spatial memory in male rats (Madsen et al., 2003; Tracey J. Shors et al., 2001; Snyder et al., 2005) and reversal learning both in male and female mice (Garthe et al., 2016; Kalm et al., 2013), although for the most part in these studies sex differences were not explicitly tested.

A number of correlational studies indicate that an increase of adult neurogenesis by environmental enrichment or exercise leads to improved spatial memory (For review see Kempermann, 2002) or reversal learning in the Morris water maze in both sexes (Epp et al., 2016; Garthe et al., 2016). Furthermore, suppression of adult neurogenesis via x-ray irradiation or transgenic knock out leads to impairments in reversal learning both in male and female mice

(Garthe et al., 2014; Kalm et al., 2013). However, inhibition of adult neurogenesis does not always lead to impairment in hippocampus-dependent learning. For example, Shors et al. (2002) failed to demonstrate impairment in contextual fear conditioning, or in the Morris water maze two weeks after reduction in neurogenesis via exposure to an antimitotic agent, methylazoxymethanol (MAM) in male Sprague-Dawley rats. However, Winocur et al. (2006) demonstrated that reducing proliferation of subgranular zone precursor cells by gamma irradiation led to an impairment in contextual fear conditioning four weeks after irradiation in male Long-Evans rats(Shors et al., 2002; Winocur et al., 2006). Furthermore, one study has noted that the ablation of adult neurogenesis did not influence memory for novel object placement task in both male and female rats (Seib et al., 2018). Therefore, it is likely that adult neurogenesis plays a key role for a specific stage of hippocampus-dependent learning and memory at specific times during maturation of new neurons.

As noted earlier, pattern separation is a process during memory encoding to separate similar patterns in the environment, or discriminate small changes in the environment, and to store the encoded patterns as more distinct from each other. Clelland et al. (2009) ablated adult neurogenesis which impaired pattern separation as there was an impairment in the ability to separate similar patterns but no impairments in separating distinct patterns in female mice. Nakashiba et al. (2012) showed that ablated adult neurogenesis led to impaired pattern separation, whereas older adult-born neurons (greater than four weeks of age) or developmentally produced granule neurons played a critical role for rapid pattern completion in male mice. Furthermore, they demonstrated that reducing neural activity of older granule neurons led to enhanced ability for pattern separation (Nakashiba et al., 2012). According to

these results, young granule neurons, which exhibit lower activation thresholds for action potentials (Schmidt-Hieber et al., 2004), play a critical role for pattern separation.

In addition to pattern separation, adult-born young neurons play a key role for proactive interference and forgetting (Akers et al., 2014; Epp et al., 2016; Feng et al., 2001; Kitamura et al., 2009). Akers et al. (2014) demonstrated that increasing neurogenesis by voluntary wheel running, or using the proneurogenic drug memantine, after fear conditioning resulted in reduced memory of the previously acquired fear-conditioned context. Furthermore, reducing neurogenesis after fear conditioning promoted the stability of the previously acquired fear responses, indicating that new neurons were important for forgetting in both male and female mice (Akers et al., 2014). These results may seem counterintuitive to the well-known effects of exercise to promote cognition in both human and rodent studies (Colcombe and Kramer, 2003; Fordyce and Wehner, 1993; Van der Borght et al., 2007; Vaynman et al., 2004). However, further studies indicate that increasing neurogenesis by voluntary running promoted the acquisition of new memory (reversal learning) but reduced memory for older (pre-running) learning in both male and female mice (Epp et al., 2016; Garthe et al., 2016). These results suggest that neurogenesis can promote the acquisition of new memories but also reduce proactive interference via the forgetting of older memories. Further research is needed to identify the role of adult neurogenesis on aspects of hippocampus-dependent learning. Further research is needed to identify the role of adult neurogenesis on different aspects of hippocampus-dependent learning and whether there are sex differences in these tasks.

1.5.3 Dorsal/ventral regional differences in neurogenesis

In addition to the anatomical neural connectivity, hippocampal adult neurogenesis is differently regulated depending on the location of the neural stem/progenitor cells along the dorsoventral axis. Adult neurogenesis is present at higher levels in the dorsal hippocampus, and adult-born neurons in the dorsal hippocampus mature faster compared to the ventral hippocampus (Piatti et al., 2011; Snyder et al., 2012; summarized in Fig. 1.4). Furthermore, cognitive training, environmental factors and sex, mediate neurogenesis differently in the dorsal or ventral hippocampus. For example, Yagi et al. (2016) reported that spatial pattern separation enhances survival of adult-born neurons specifically in the dorsal, but not ventral, dentate gyrus of male rats, but this is not seen in female rats. Furthermore, pharmacological antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs) increase neurogenesis in the ventral dentate gyrus to a greater extent compared to the dorsal dentate gyrus in males (Banasr et al., 2006; Jayatissa et al., 2006; Mahmoud et al., 2016). Stress and/or chronic exposure to corticosterone act on hippocampal function and adult neurogenesis with dorsoventral region specific and sex dependent manner (Brummelte and Galea, 2010; Donley et al., 2005). For example, chronic unpredictable stress (CUS) decreased proliferation and survival of adult-born neurons to a greater extent in the ventral compared to the dorsal hippocampus in male rats (D. F. Hawley et al., 2012; Jayatissa et al., 2006; Tanti et al., 2012). Furthermore, chronic corticosterone treatment decreases adult-born neurons only in the ventral but not dorsal hippocampus in female rats, whereas neurogenesis in both regions decreased in male rats to a greater extent in males compared to females (Brummelte and Galea, 2010). Furthermore, administration of glucocorticoid receptor (GR) antagonist into the ventral, but not dorsal, hippocampus interferes with contextual fear memory in male rats (Donley et al., 2005). Collectively these studies suggest that neurogenesis is regulated differently dependent on the

dorsal or ventral region of the dentate gyrus and it is critical for future research to explore these regional differences.

1.6. Sex Differences in hippocampus-dependent cognition

1.6.1 Sex differences in learning strategy

Previous studies in both humans (Dabbs et al., 1998; Lawton, 1994; Silverman and Choi, 2006) and rodents (Grissom et al., 2013; W. R. Hawley et al., 2012; Korol et al., 2004) demonstrate that males and females differentially rely on two different learning strategies during spatial navigation tasks (Fig. 1.3). Place strategy engages the hippocampus, while response or cue strategy engages the striatum (Iaria et al., 2003; Maguire et al., 2017; McDonald and White, 1994). Men are more likely to use geometric cues and women are more likely to use landmark cues to reach a destination (Andersen et al., 2012; Galea and Kimura, 1993). In rodents, female rats preferentially use response strategies when ovarian hormones are low, while male rats preferentially use the place strategy to solve the same tasks (Cherney et al., 2008; Galea and Kimura, 1993; Grissom et al., 2013; Williams et al., 1990). Studies have found that strategy choice varied in part by testosterone levels in male rats and ovarian hormones in female rats. Removal of testicular hormones by castration slightly reduced the preference for a place learning strategy (W. R. Hawley et al., 2012) and low testosterone increased the use of a response strategy while high testosterone led to a preference for a place strategy in dual-solution water maze (Spritzer et al., 2013). In contrast, female rats relied more on landmark cues during non-proestrous phases (lower levels of estradiol), and a place strategy during the proestrous phase (higher estradiol levels; Keeley et al., 2013). This is consistent with other studies showing that higher levels of estradiol are associated with a place strategy and lower levels of estradiol with response strategies in female rats (Korol et al., 2004; Moradpour et al., 2013; Rummel et al.,

2010; Yagi et al., 2017). These sex differences in the preference of the two learning strategies suggest that males and females may rely on different brain regions during spatial training, and may contribute to sex differences in performance during spatial navigation.

1.6.2 Sex differences in spatial learning and memory

Meta-analyses indicate that males show higher scores on tasks of spatial navigation and working memory compared to females, in both human and rodent studies (Jonasson, 2005; Linn and Petersen, 2016; Voyer et al., 1995). For instance, Galea and Kimura (1993) demonstrated that men made fewer errors to recall a learned route on a map, but intriguingly women remembered more landmarks along the route than men, indicating differential attention to cues in men versus women while route learning. In agreement with human studies, male rodents show higher score compared to female rodents in a variety of spatial navigation and working memory tasks (Jonasson, 2005). However, a number of studies have failed to demonstrate sex differences in spatial navigation tasks. This inconsistency may be due to types of spatial tasks, strategy use, stress exposure, and/or hormone levels. As described in the previous section, males and females have different tendencies to rely on the hippocampus-dependent place strategy or the striatum-dependent response strategy. Indeed, a sex difference favouring males exists in the standard reference memory version of the Morris water maze task; but there is no sex difference observed during a cue competition task in which subjects can use both the place and response strategy to solve the task (Yagi et al., 2017). Furthermore, the environmental cues within the task can dictate whether sex differences in performance are seen. Men perform more accurately to find a platform location than women when more geometric spatial cues exist in the virtual water maze, whereas there is no sex difference when more landmark cues exist (Chamizo et al., 2011). These findings imply that males and females use different strategies, or exhibit differential cue use, to

solve the same maze. Indeed, males attend to geometric cues while females attend to landmark and visual cues to solve spatial tasks in both humans and rodents (Galea and Kimura, 1993; Williams et al., 1990; see Fig. 1.2). Thus, it is important to ensure that cues are considered when using both males and females in spatial tasks, as these may significantly influence whether sex differences will be seen.

Reviews of power analyses in neuroscience suggest that studies are underpowered to detect sex differences (Button et al., 2013). However, this review fails to appreciate that conditions of testing are important in the outcome of sex differences on learning such as cue availability affecting strategy use, housing, hormone levels, and temperature of the water, that all contribute to whether sex differences in spatial acquisition are seen or not. Indeed, Button et al. (2013) suggests that sample sizes of 68-134 are needed to demonstrate a sex difference. However, the sample size needed to detect a sex difference in spatial performance is much lower if attention is paid to parameters such as cue choice. The heterogeneity in findings on sex differences in spatial ability, are more likely due to variability in protocols and experimental conditions, affecting effective strategy use rather than whether a true sex difference in spatial ability exists. Variability in cognitive testing has lead more than one researcher to suggest the use of standardized protocols/testing (Hvoslef-Eide and Oomen, 2016) and new efforts are underway to share and compare data via platforms such as http://mousebytes.ca/home to improve our understanding of biological various that influence learning.

While there are fewer studies, studies examining the ability to perform pattern separation indicate that males perform more accurately in challenging spatial pattern separation tasks compared to females (Clelland et al., 2009; Nakashiba et al., 2012; Yagi et al., 2016). For example, comparing different studies where sex was not compared statistically, male mice were

able to distinguish two adjacent arms in the radial 8-arm maze while females failed to distinguish the two arms in this same task (Clelland et al., 2009; Nakashiba et al., 2012). However, both sexes performed equally well in distinguishing between distant arms in that task (Clelland et al., 2009; Nakashiba et al., 2012). Yagi et al. (2016) found sex differences in pattern separation performance based on strategy use in rats (see Fig. 1.2). Male place strategy users outperformed female place strategy users when separating similar patterns during a pattern separation task, whereas there were no significant sex differences among response strategy users (Yagi et al., 2016). This study also demonstrated that male place strategy users had greater number of adult-born neurons in the dorsal dentate gyrus than all other groups. Furthermore, the number of new neurons in the ventral dentate gyrus was more strongly associated with the ability to distinguish two adjacent arms (similar patterns) in female place strategy users. These results suggest that new neurons in the dorsal hippocampus are more responsive to enhancing effect of spatial learning on survival of new neurons in males. Whereas the relationship of neurogenesis in the ventral hippocampus in females with performance suggests that female performance is more sensitive to stress and/or that different connectivity patterns may exist between males and females in the dorsal versus ventral hippocampus, an effect detected in humans (Persson et al., 2014). With this in mind, this thesis will examine sex differences in basal characteristics of neurogenesis (Chapter 2), and sex differences in functional connectivity of newborn neurons after fear memory recall following pattern separation (Chapter 3).

1.6.3 Sex differences in contextual fear conditioning

The hippocampus is also important for contextual fear learning and memory. Women show a greater prevalence of posttraumatic stress disorder (PTSD) with more generalization of fear memories compared to men (Breslau et al., 1997; Kessler et al., 2012). Furthermore, women

show greater pain sensitivity and anxiety within the context of previously experienced painful event (Meulders et al., 2012). Female rats demonstrated greater generalization of fear related contextual memory and prolonged fear memory compared to male rats (Keiser et al., 2017). examined fear memory generalization using two different contexts: one with electric foot shock and the other without foot shock. They examined passive avoidance of fear (foot shock) related context 1d, 3d, 5d, and 7d after conditioning. Females gradually showed increased fear related response in the neutral context while male rats did not show such an increase and even at 7d after conditioning, male rats showed the ability to discriminate between the two contexts. These studies and others (Gresack et al., 2009) demonstrate that females may have more fear generalization than males which may contribute to the findings that women are more susceptible to fear generalization disorders such as PTSD.

Females show less extinction to contextual fear conditioning than males (Matsuda et al., 2015; Voulo and Parsons, 2017), but males show stronger retention and more freezing to the conditioned stimulus especially after pre-exposure (Gresack et al., 2009; Keiser et al., 2017; Sliwowska et al., 2010). Males are typically found to have stronger or greater long-term retention of contextual fear conditioning (Gresack et al., 2009), which coincided with increased levels of phosphorylated ERK in the ventral hippocampus of males but not females. Using a context pre-exposure paradigm that relies on the integrity of the dentate gyrus, gonadectomized males and intact males were found to have stronger contextual fear conditioning than females (Barker and Galea, 2010; Keiser et al., 2017). A caveat with respect to these findings is that researchers have traditionally used freezing as an index of fear memory. However, females show more active patterns of fear expression termed ‘darting’ (Gruene et al., 2015), and thus care must be taken in fear conditioning paradigms to assess the appropriate behaviours in males and females.

These sex differences in conditioned fear responses may be related to sex differences in activation with greater neural activation (cFos) in the basal amygdala of females and in the dorsal hippocampus of males (Chen et al., 2014; Keiser et al., 2017). Keiser et al (2017) suggest this may have to do with the competition between the amygdala and hippocampus and it is also possible that different IEGs or different time points may find a different pattern of activation after fear conditioning. Sex differences in neural patterns may be seen even when there are no sex differences in conditioning strength, as work from the Shansky laboratory as noted sex differences in the prefrontal cortex to amygdala circuits, even when there were no overt sex differences in behavior (Gruene et al., 2015). It is clear that care must be taken when studying sex differences in fear conditioning, including type of conditioning (cue, contextual), behaviour measured (freezing, darting), and pre-exposure to the context.

1.7 Sex differences in the morphology, activation, and connectivity of the hippocampus: Findings from human studies

Human males typically are reported to have larger volumes of both the left and right hippocampus than females (Ruigrok et al., 2014) but once total brain volume or intracranial volume is used as a correction factor, there is no longer a significant sex difference (Tan et al., 2016). Recent studies show no sex differences in hippocampal volume across development (Tamnes et al., 2018). One caveat in these studies is that a number of factors that influence hippocampal volume are not often documented including early adversity (reductions are often seen in men but not in females: Colle et al., 2017), phase of menstrual cycle (Lisofsky et al., 2015), parity status (Hoekzema et al., 2017), hormone therapy (Wnuk et al., 2012), menopausal status (Goto et al., 2011), genotype (Everaerd et al., 2012), and testosterone levels in males

(Lord et al., 2008). Researchers are cautioned that these variables need to be considered to obtain an accurate understanding of sex differences in the volume of the hippocampus.

Whereas whole hippocampal volume may not show a sex difference, regional differences within the hippocampus and connectivity to the hippocampus do exist between the sexes (Persson et al., 2014; Sacher et al., 2013). For example, females have larger posterior hippocampus than males (Persson et al., 2014), with differences between the sexes in structural covariance and functional connectivity, indicating differences in connectivity. Resting-state functional magnetic resonance image (rs-fMRI) measures spontaneous fluctuations of blood-oxygen- level dependent (BOLD) without externally prompted tasks so that the synchronized BOLD activation can indicate the functional organization of the brain. Studies measuring rs-fMRI reported sex differences in functional brain connections, with females have more inter-hemispheric connections compared to males, while males have stronger intra-hemispheric connection compared to females (Ingalhalikar et al., 2014; Scheinost et al., 2015; Zhang et al., 2016). Furthermore, Filippi et al. (2013) showed females have greater intra-connectivity within the temporal lobe compared to males. One study has argued that because there is no true distinct dichotomy between the sexes in terms of MRI measures of volume and connectivity that this suggest there is no true 'male' or 'female' typical brain (Joel et al., 2015). But other researchers have argued that the lack of a dichotomy (an extreme form of sex difference) does not preclude the idea that there are patterns of connectivity, and structural differences on a continuum that relate to a male-typical or female-typical brain (see Glezerman, 2016; Gobinath et al., 2017 for more discussion). fMRI studies in language processing show females have greater bilateral activation of BOLD in the temporal and frontal lobes in comparison to males (Baxter et al., 2003; Kansaku et al., 2000). Furthermore, females have greater activation in regions other than

the hippocampus compared to males during a virtual water maze task, such as prefrontal cortex (Gron et al., 2000; Sneider et al., 2011). This is particularly intriguing as it matches animal data indicating a greater involvement of the prefrontal cortex in female rats compared to male rats during the Morris water maze task (Kolb and Stewart, 1995). These types of studies are important to consider when examining activation patterns without regard to stratifying the data by sex.

1.8. Sex differences in morphology, electrophysiological properties and activation of hippocampal neurons: Findings from rodent studies

Sex differences exist in the morphology and electrophysiological properties of hippocampal neurons. However, researchers need to be aware that estrous cycle significantly influences a number of parameters in which sex differences are seen such as cell proliferation (Rummel et al., 2010; Tanapat et al., 1999), hippocampal volume (Qiu et al., 2013), LTP and LTD (Good et al., 1999; Warren et al., 1995), CA1 apical spine density (Woolley et al., 1990), and hippocampal AMPA receptor stoichiometry (Tada et al., 2015). Thus, if estrous phase is not taken into consideration this may affect magnitude and appearance of sex differences in these measures. Importantly, sex differences in the hippocampus, at least in part, relate to steroid hormone manipulations and it is important to acknowledge that the hippocampus contains sex hormone receptors such as androgen receptors (AR), and estrogen receptors (ER)-α, β and G-coupled protein receptor (GPER). The relative receptor density differs by sex in a region-specific way as the CA3 and CA4 regions of adult female rats contain a greater amount of ER-β compared to male rats (Zhang et al., 2002) whereas there are greater levels of AR in male compared to female rats, dependent on estrous cycle phase in the CA1 and dentate gyrus (Feng et

al., 2010). Furthermore, there are notable alterations in females in the distribution of ERα and β and progesterone receptors across the estrous cycle (Mitterling et al., 2010).

Studies have reported that there are sex differences in the morphology of granule neurons and CA3 pyramidal neurons (Elizabeth Gould et al., 1990; Juraska et al., 1989; Mendell et al., 2017). Juraska et al. (1989) found that male rats had greater dendritic intersections in granule neurons of the dentate gyrus compared to female rats. Galea et al. (1997) indicated that female rats had greater branch points in the basal dendrites of CA3 pyramidal neurons compared to male rats. While Gould et al. (1990a) found that females had more primary dendrites in CA3 than males, and that males had more thorny excrescences than females. Another study found more dendritic spines in the CA3 pyramidal neurons in females than in males although the sexes were not directly compared statistically in that study (Mendell et al., 2017). These results suggest basal sex differences in the morphology of granule and pyramidal neurons in the rat hippocampus.

Gonadal hormone manipulations, estrous cycle phase and exposure to stress can also reveal sex and hormone differences in hippocampal morphology (Galea et al., 1997; T J Shors et al., 2001; Woolley et al., 1990). For example, proestrous female rats have greater apical dendritic spine density in CA1 pyramidal neurons in compared to males and diestrous females (Woolley et al., 1990). Acute stress increases apical dendritic CA1 spine density in males but decreases in females, dependent on estrous cycle phase (T J Shors et al., 2001). Many of the structural differences in the dentate gyrus and CA3 regions show sex differences in response to gonadectomy, with mossy fibers innervations increased in castrated males but not in ovariectomized females (Mendell et al., 2017). In addition, castrated males have longer dendrites and larger mossy fibers, while ovariectomy decreases dendritic spine density in the CA1 (E

Gould et al., 1990) and the CA3 region (Mendell et al., 2017). Acute administration of estradiol and progesterone prevents the effect of ovariectomy in the CA1 region (E Gould et al., 1990). Chronic stress decreases apical dendritic complexity in male rats, and basal dendritic complexity in female rats in the CA3 region of hippocampus (Galea et al., 1997). In the dentate gyrus, granule neurons have greater dendritic intersections in single-housed males than single-housed females, whereas females raised in an enriched environment have larger dendritic trees compared to males raised in an enriched environmental (Juraska et al., 1985). Intriguingly, much like work in humans, early life adversity reduces hippocampal volume, neurogenesis and impairs spatial memory in male rodents (Oomen et al., 2010; reviewed in Maccari et al., 2014) but separate studies indicate that females show resilience after early life adversity with little effect on the hippocampus (Loi et al., 2017; Oomen et al., 2011). Even though these studies did not directly examine sex differences, a number of studies have corroborated these findings (reviewed in Maccari et al., 2014). There are sex differences in the manifestation of early life adversity on the hippocampus and amygdala (Guadagno et al., 2018) with males, but not females, showing more morphological changes after various forms of early life stress (Guadagno et al., 2018). These results collectively suggest that sex differences in the morphology of hippocampal subregions exist under basal conditions and that other sex differences are unveiled after environmental or gonadal hormone perturbations.

In addition to the morphological differences, there are sex differences in LTP in the hippocampus. Males exhibit larger early and late-LTP compared to females when a high frequency stimulus is introduced in the dentate gyrus, CA3 and CA1 regions (Harte-Hargrove et al., 2015; Maren et al., 1994; Monfort et al., 2015; Yang et al., 2004). Interestingly, proestrous females showed greater magnitude of early-LTP compared to diestrous females through the

perforant path (Qi et al., 2016). It is important to note however, that during proestrus, seizure threshold is also decreased (Tan and Tan, 2001) and as such, it becomes more difficult to find an appropriate tetanus (Warren et al., 1995), indicating greater excitability in the female hippocampus during proestrus. Furthermore, the composition of AMPA/NMDA receptors of CA1 pyramidal neurons is different between males and females, as females show greater AMPA/NMDA ratio than males (Monfort et al., 2015; Qi et al., 2016). Oberlander and Woolley (2016) demonstrated that estradiol enhances presynaptic and postsynaptic potentials in the CA1 pyramidal neurons in both males and females. However, postsynaptic sensitivity of male CA1 pyramidal neurons is potentiated by ERβ while female CA1 pyramidal neurons are potentiated by GPER1 (Oberlander and Woolley, 2016). In addition to sex differences in the CA1 region of hippocampus, there are sex differences in the neural plasticity at DG-CA3 synapses (see Scharfman and MacLusky, 2017 for review). In short, mossy fibers evoke larger population spikes in CA3 pyramidal neurons in females during proestrus and estrus relative to males, while male mossy fibers have stronger synaptic connections to CA3 neurons than females (Scharfman, 1997; Scharfman et al., 2003). However, these two studies did not compare males and female directly. These anatomical and electrophysiological findings suggest that intrahippocampal circuitry and ER mechanisms are differently organized between males and females. Furthermore, the female hippocampus is dynamic across the estrous cycle and through reproductive experience (for review, see Duarte-Guterman et al., 2015). Further research is needed to elucidate the underlying cellular mechanisms of sex differences in hippocampal physiology under basal and environmental perturbations.

Cellular activity in response to spatial memory can be examined by quantifying expression of IEG proteins such as zif268, cFos and Arc, which are rapidly induced after

learning and regulate learning-related neural plasticity (Guzowski et al., 2001; Jones et al., 2001). Although Long-term potentiation (LTP) is not required for *c-fos* induction (Douglas et al., 1988; Wisden et al., 1990), the IEG z*if268* encodes the zinc finger transcription factors zif268/Egr1 (early growth response protein1) which, plays a critical role in the maintenance of LTP in the hippocampus and in the consolidation of long-term memory (Jones et al., 2001; Penke et al., 2014; Petersohn et al., 1995). Only a few studies have examined sex differences in response to spatial and non-spatial learning on IEGs. Yagi et al., (2017) found that in the CA3 male place learners showed greater activation of cFos, while female place learnings showed greater activation of zif268. Intriguingly, there were sex by strategy use differences in activation with female place strategy users showing greater zif268 expression but less cFos expression in the CA3 compared to cue strategy users. This same relationship in the CA3 region was seen after pattern separation with greater zif268 expression in females, but more cFos expression in males (Yagi et al., 2016). These sex differences in the expression of IEGs were only seen in the dorsal hippocampus, which is intriguing as the dorsal area is more tightly linked to spatial reference memory (Moser et al., 1995). Furthermore, none of these same sex differences in IEG expression were seen in the CA1 or DG. Intriguingly, changes in cFos expression after spatial memory retrieval are seen across the estrous cycle with proestrous rats having greater cFos expression in the dentate gyrus than females not in proestrus (Yagi et al., 2017). These results suggest that greater number of zif268-expressing CA3 pyramidal neurons in response to memory retrieval in female compared to male rats, may help recruit LTP, as noted above zif268 is strongly associated with LTP. While a few studies have examined sex differences in the expression of cFos and zif268, no studies have been conducted using Arc to determine underlying mechanisms of sex differences in spatial learning ability, or Arc expression of newly produced neural dendrites in

the dentate gyrus, which is a clear gap in the literature. Researchers should also be aware that production of these immediate early genes proteins after learning has different time courses dependent on the IEG (Barros et al., 2015; Lonergan et al., 2010). For instance, zif268 protein expression reaches peak levels 60 minutes after learning (Lonergan et al., 2010), whereas, albeit to different stimuli (pentylenetetrazol-induced seizures), cFos protein expression reaches its peak at 60-120 minutes after the seizure (Barros et al., 2015). These differences in induction time courses must be carefully considered when comparing the different immediate early gene expression after learning. These sex differences in IEG expression seen after learning may be due not only to different IEGs being recruited during different tasks, but also different regions may show different IEG expression responses, and there may be different timing in IEG expression between the sexes that contribute to these differences.

1.9. Sex differences in adult neurogenesis in the hippocampus

There are sex differences in the basal production of new neurons (Galea and McEwen, 1999; Tanapat et al., 1999) and in the production and survival of adult-born neurons in response to stress and hippocampus dependent learning (Falconer and Galea, 2003; Tanapat et al., 1999; Westenbroek et al., 2004; Yagi et al., 2016). Net increases or decreases in the amount of neurogenesis are determined by changes in cell proliferation, migration, differentiation and survival either independently or in orchestration together. For example, sex differences are seen some of these responses to manipulations as prenatal alcohol exposure decreases neurogenesis in female, but not male, rats via reduction in the ratio of new cells differentiating into neurons without affecting cell proliferation or survival, although the sexes were not directly compared across studies (Sliwowska et al., 2010; Uban et al., 2010).

1.9.1 Sex differences in basal adult neurogenesis

Some studies find basal sex differences in cell proliferation but not in survival of new neurons in the dentate gyrus. In Sprague Dawley rats and meadow voles, females have greater levels of cell proliferation compared to males that may depend on phase of estrous cycle or season (Galea and McEwen, 1999; Spritzer et al., 2017; Tanapat et al., 1999). Galea and McEwen (1999) showed that female wild meadow voles have greater cell proliferation in the dentate gyrus only during the non-breeding season compared to males. Furthermore, Tanapat et al. (1999) showed that female Sprague Dawley rats during proestrus have greater cell proliferation compared to non-proestrous females and males. Estrous cycle effects on cell proliferation are more equivocal as some studies have failed to find sex differences in cell proliferation in mice (Amrein et al., 2004; Lagace et al., 2007), but others show the same estrous cycle effect seen in rats (Tzeng et al., 2014). In contrast to cell proliferation, most studies indicate that there are no significant sex differences in the survival of new neurons in rats, mice or voles (Barker and Galea, 2008; Lagace et al., 2007; Lee et al., 2014; Spritzer et al., 2017; Tanapat et al., 1999) though (Dalla et al., 2009) found sex differences favouring male rats. Some studies do show sex differences, favouring males, in immature neurons in adulthood (Hillerer et al., 2013). Sex hormones such as androgens and estrogens are potent modulators of adult neurogenesis in the hippocampus (see below). Briefly, estrogens modulate neurogenesis in females but to a lesser extent in males (Barker and Galea, 2008). Androgens modulate neurogenesis in males (Hamson et al., 2013; Swift-Gallant et al., 2018) but do not modulate neurogenesis in females (Duarte-Guterman et al., 2019).

1.9.2 Sex differences in adult neurogenesis after learning

Adult neurogenesis is modulated by learning differently based on sex, the direction of which is modified by sex differences in task performance (Chow et al., 2013; Dalla et al., 2009; Yagi et al., 2016). Chow et al. (2013) found that male rats outperformed female rats in acquiring the spatial version of the Morris water maze, which resulted in an enhancement of new neuron survival in the dentate gyrus of males but not females. Conversely, Dalla et al. (2009) showed that diestrous female rats outperformed male rats in the trace eyeblink conditioning, which resulted in learning-enhanced survival of new neurons in female, but not male, rats in the ventral dentate gyrus. Yagi et al. (2016) demonstrated that male place-strategy learners showed greater spatial pattern separation and greater adult neurogenesis in the dorsal dentate gyrus than female rats. In female place learners, greater pattern separation was associated with enhanced neurogenesis in the ventral dentate gyrus, an effect that failed to reach significance in males (Yagi et al., 2016). Interestingly, male place learners with poor learning ability are more likely to show enhanced neurogenesis in response to spatial training compared to better learners (Epp et al., 2007). Collectively, these findings indicate that task difficulty may be differently related to the neurogenic response by sex and/or that sex differences in the functional incorporation and integration of new neurons into the existing circuitry may be differently related to the degree of contribution of the hippocampus for learning.

One study has reported sex differences in activation of adult-born cells in the dentate gyrus relating to performance (Chow et al., 2013). In that study, zif268 protein expression was examined after memory retrieval in the Morris water maze 20 days after cell birth. Spatially trained rats showed greater activation than cue-trained rats with no overall sex differences in expression of activated new neurons. However, greater activation of new neurons was strongly associated with less distanced travelled in female rats but not in male rats. This sex difference

may be due to sex differences in excitability of 20 days old neurons, in the timing of expression of zif268, and/or the maturation rate of new neurons and further research is encouraged in these areas. Thus, in this thesis, Chapter 3 will explore sex differences in zif268 protein expression of different ages of new neurons in response to fear memory retrieval after contextual pattern separation.

1.10 Hormone therapy and hippocampal cognition

The effects of estrogen on cognition have been investigated for decades. There are four types of estrogens, estrone, estradiol, estriol and estetrol. Estrone and estradiol are the two most abundant of the estrogens. Estradiol binds with greater affinity to estrogen receptors (ERs) and is present at higher levels than estrone before menopause whereas estrone is present at higher levels than estradiol after menopause in human females (Rannevik et al., 1995). Menopause commonly occurs between the age of 45 and 55 years (Dratva et al., 2009; McKinlay et al., 1992). Menopause is characterized by permanent cessation of menstrual cycles which can be associated with some cognitive symptoms (Carlson et al., 2001) and vasomotor symptoms. Hormone therapy has been commonly used in order to mitigate these symptoms. Cross-sectional studies and meta-analyses have given evidence that hormone therapy enhanced memory performance and decreased the risk of developing dementia (Sherwin, 2003; Zandi et al., 2022). However, the 2002 Women's Health Initiative (WHI) reported that a specific hormone therapy, Premarin, comprised mainly of estrone, impaired memory and increased the risk of developing dementia after the age of 65 years (Resnick et al., 2006). One explanation for these conflicting findings is that there is a limited window of opportunity for hormone therapy efficacy (Erickson et al., 2010) or the effects of hormone therapy are dependent on the duration and composition of hormone therapy. For example, short duration of hormone therapy increases hippocampal

volume (Boyle et al., 2021; Erickson et al., 2010; Lord et al., 2008), whereas longer duration of hormone therapy is associated with smaller hippocampal volume in postmenopausal females (Erickson et al., 2007; Lord et al., 2008). Furthermore, estradiol-based hormone therapy improves verbal memory in post-menopausal females, whereas conjugated estrone-based hormone therapy has detrimental (or no significant) effects on verbal memory (Joffe et al., 2006; Linzmayer et al., 2001; Maki et al., 2007; Phillips and Sherwin, 1992; Ryan et al., 2012; Shaywitz et al., 2003). The timing of initiation of hormone therapy relative to menopause also plays important roles for the effects of hormone therapy as early initiation after menopause enhances cognitive performance, whereas late initiation after menopause leads to poorer performance (MacLennan et al., 2006). Furthermore, early initiation relative to menopause of hormone therapy increases hippocampal volume, whereas late treatment initiation relative to menopause has no such beneficial effects on hippocampal volume (Erickson et al., 2010). Therefore, different estrogens modulate hippocampal plasticity and cognition depending on initiation of treatment relative to menopause/ovariectomy, on the type and duration of hormone therapy (Boccardi et al., 2006; Maki et al., 2007; Phillips and Sherwin, 1992; Resnick et al., 2009; Shaywitz et al., 2003).

1.11 Estrogen manipulation and hippocampus-dependent cognition

Estrogens modulate hippocampus-dependent cognition, which depends on dose and duration of estrogen, and on type of memory task (Duarte-Guterman et al., 2015; Mahmoud et al., 2016). Low doses of acute or chronic estradiol improve, whereas high doses of estradiol impair, spatial working memory in young ovariectomized rats and in female meadow voles (Galea et al., 2002, 2001; Holmes et al., 2002). Furthermore, medium-high doses of estradiol improve spatial reference memory in young or middle-aged rats (Kiss et al., 2012; Talboom et

al., 2008). In contrast, a low dose of exogenous estrogen increases spatial memory retention in middle-aged, whereas a high dose of estradiol increases the retention in aged rats (Foster et al., 2003). In contextual fear memory, a single dose of low estradiol enhances, whereas high doses of estradiol impair acquisition of contextual fear conditioning in young ovariectomized rats (Barha et al., 2010). Furthermore, a high dose of estradiol enhances performance in the novel object recognition task, which is coincident to enhanced LTP at CA3-CA1 synapses (Vedder et al., 2013). In addition, type of estrogen also plays a key role for estrogenic regulation of hippocampus dependent cognition. Chronically administered high, but not low, dose of estrone impairs spatial working memory in middle-aged ovariectomized rats (Engler-Chiurazzi et al., 2012). However, a chronically administered high dose of estrone does not affect on spatial reference memory in ovariectomized young adult rats (McClure et al., 2013). Chronic administration of Premarin, the conjugated equine estrogen (estrone sulfate), impairs both spatial working and reference memory in ovariectomized young adult rats (Barha and Galea, 2013). These studies demonstrate that estrone can negatively affect hippocampus dependent cognition, which is at least in partially associated with modulatory effects of estrogens on hippocampal plasticity.

1.12 Estrogen manipulation and adult hippocampal neurogenesis

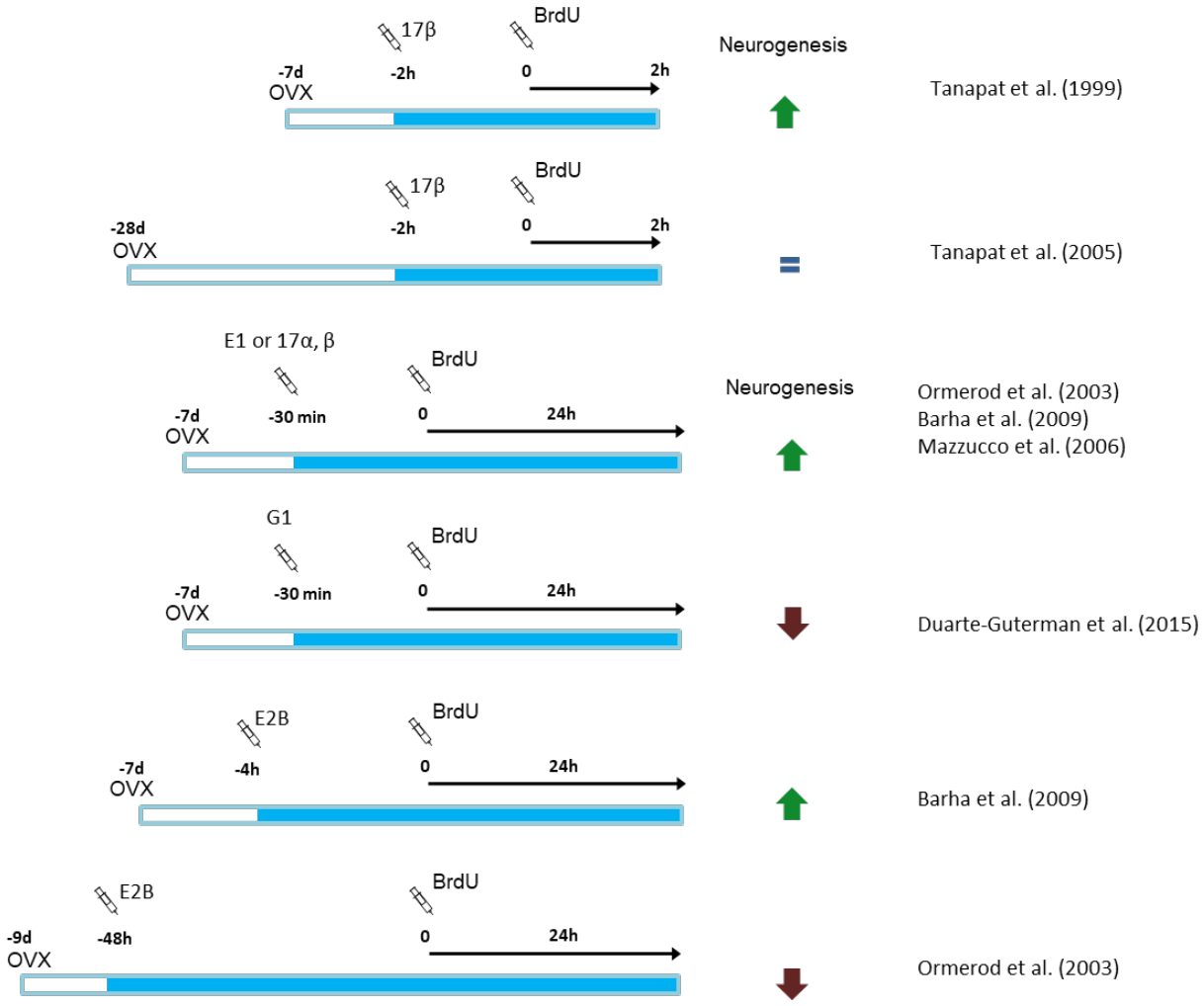
Estrogens regulate hippocampal neurogenesis in young adult females modulating both the level of proliferation of neural precursor cells and survival of new neurons depending on duration, type, and dose of estrogens, timing of treatment initiation since ovariectomy. A single dose of diestrous and proestrous levels of 17β-estradiol rapidly enhances the proliferation 30 min and 2 h, but not 4 h, after injection in ovariectomized young adult rats (Barha et al., 2009; Tanapat et al., 2005, 1999; see Fig. 1.6). A single dose of estrone also increases the proliferation

after 30 min in ovariectomized young rats (Barha et al., 2009). There is a bi-phasic effect of estradiol on cell proliferation as a single dose of estradiol benzoate (EB) increases cell proliferation after four hours but decreases cell proliferation 48 hours after exposure in female voles and rats (Ormerod et al., 2003b; Ormerod and Galea, 2001). The decrease is due to estradiol's influence to upregulate the HPA system (Ormerod and Galea, 2001). In terms of cell proliferation, estradiol also influences neurogenesis after chronic exposure as 16 days of high dose EB treatment enhanced the proliferation (Barker and Galea, 2008) whereas 22 days of EB treatment had no effect (Chan et al., 2014) in female rats, indicating a potential loss of proliferating effects of estradiol with time. Therefore, effects of estrogens on cell proliferation in the dentate gyrus depend on dose and type of estrogens, and timing of treatment (Fig. 1.7 A).

Much like the effects of acute administration of estrogens, the effects of chronic administration of estrogens on adult hippocampal neurogenesis are dependent on timing, duration, dose, type of estrogens, and whether animals underwent behavioral testing during the treatment (see Fig. 1.7 B). Daily administration of estradiol benzoate (EB) for 29 days in ovariectomized rats without any behavioral testing decreased survival of new neurons that were labelled one day prior to EB administration (Barker and Galea, 2008; Chan et al., 2014). However, the influence of EB or 17β-estradiol to suppress survival of new neurons are limited to those produced prior to EB treatment. McClure et al. (2013) demonstrated that 20 days of chronic treatment with high of 17β-estradiol increased the number of new neurons that had been produced one day after the initiation of 17β-estradiol treatment. Chronic effects of estrone on neurogenesis are opposite to those of estradiol, as 20 days of high dose of estrone reduced the survival of new neurons (McClure et al., 2013). This is intriguing because both doses of estradiol and estrone increase cell proliferation, (Barha et al., 2009), yet the new neurons surviving in an

estrone-enriched environment were less likely to survive than those in an estradiol-enriched environment (McClure et al., 2013). An important caveat to these findings of McClure et al. (2013) is that all females underwent five days of Morris water maze training during 12-15 days post cell division. This period is the axon extension period and the critical period for spatial training to inhibit the survival of new neurons in male rats (Epp et al., 2013b). Thus, chronic administration of 17β-estradiol, but not estrone, with spatial training, during 12-15 days post cell division in ovariectomized rats, enhanced survival of new neurons (McClure et al., 2013). Furthermore, Eid et al. (2020) demonstrated that 33 days of 17β-estradiol treatment enhanced adult neurogenesis in female mice with behavioural testing. Premarin, which is mainly comprised of estrone, increased the survival of new neurons in young adult female rats that had undergone the radial arm maze testing but not in cage controls (Barha and Galea, 2013) indicating the importance of the effect of spatial training on the influence of estrogens. Premarin also has variable effects on survival of new neurons depending on dorsoventral region as Premarin enhanced survival of new neurons in the dorsal, but not ventral region of the dentate gyrus in middle aged female rats (Galea et al., 2018). Therefore, the effects of estrogens on adult neurogenesis in the hippocampus are highly dependent on type, dose, experience and duration of treatment.

Various kinds of estrogens have been used for the hormone therapy for women after menopause. However, it is critical to take into account the dose, type, and duration of estrogens given as these can change the direction of the effect on neuroplasticity. Thus, in Chapter 4, I will explore effects of estrogens on basic characteristics of adult neurogenesis.



Fi. 1.6. Effects of acute estrogen treatment on adult neurogenesis in the dentate gyrus. Effects of acute estrogenson adult neurogenesis of adult female rats vary with duration of estrogen deprivation after ovariectomy (OVX) and timing of BrdU injection after administration of estrogen. 17β-estradiol (17β) or estrone (E1) rapidly increases cell proliferation after 30 min and 2 h, but not after 4 h, while estradiol bensoate (E2B) increases cell proliferation after 4 h, but decreases after 48 h. However, 17β has no effect 28 days after

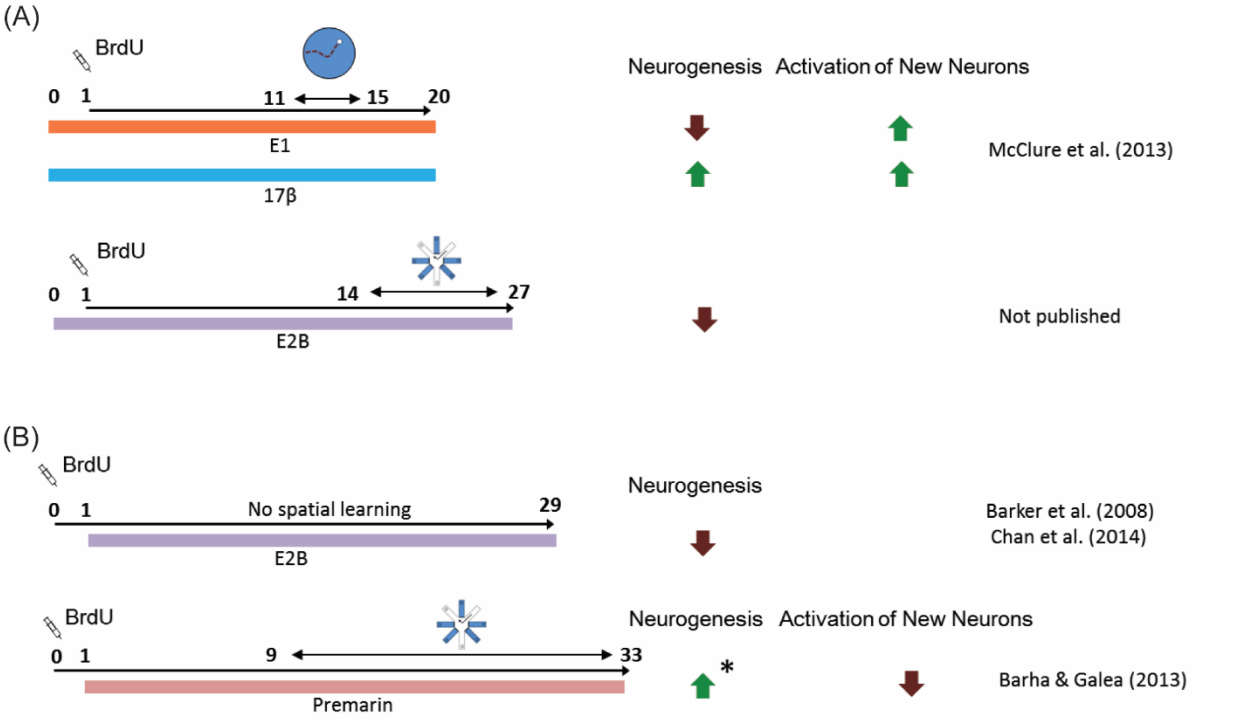


Fig. 1.7. Effects of chronic administration of estrogens on adult neurogenesis and activation of new neurons in female rats. (A) Chronic administration of estrone (E1) for 20days with spatial training in the Morris water maze on days 11-15 decrease the survival of new neurons but increase with 17β-estradiol (17β). Both E1 and 17β increase the activation of new neurons on day 20. 28 days of estradiol benzoate (E2B) with spatial training in the radial arm maze (RAM) decreases the survival of new neurons. BrdU is given on the day following initiation of treatment. (B) Chronic administration of E2B without spatial learning decreases the survival of new neurons. 33 days of Premarin treatment with RAM training on days 9-33 increase the survival but decrease the activation of new neurons. BrdU is given 1 day prior to the initiation of treatment.

1.13 Thesis overview and objectives

The overall objective of this dissertation research is to elucidate sex differences and effects of estrogens on basal characteristics of neurogenesis, and in functional connectivity following pattern separation. Specifically, the following experimental chapters outline studies investigating sex differences and effects of estrogens in the amount of neural stem cells, the

maturation rate and trajectory of adult-born neurons in the dentate gyrus, and sex differences in the ability for contextual pattern separation and functional brain connectivity of adult-born neurons for fear memory. My central hypothesis is that there are different characteristics of maturation and attrition of new neurons and in pattern separation between the sexes, that are modulated by estrogens in females.

Chapter 2: Are there sex differences in the trajectory and maturation rate of adult-born neurons in the dentate gyrus of hippocampus of young adult rats? A thymidine analogue labelling approach was used to label proliferating neural progenitors and their progeny in male and female rats and examine changes in maturation stage specific protein expression with time. I hypothesized that adult-born neurons in male rats would mature faster compared to females, and there would be sex differences in trajectory of adult-born neurons.

Chapter 3: Are there sex differences in the ability for fear-associated contextual pattern separation and functional brain connectivity of adult born neurons for fear memory recall? A fear-associated contextual discrimination task was used to assess sex differences in the ability for contextual pattern separation. Moreover, a combination of thymidine analogue labelling and immediate early gene imaging was used to examine the coordinated neuronal activation among anatomically remote brain regions as a measure of functional connectivity. I hypothesize that male rats would have greater ability for separating two similar contexts compared to females, and males and females show distinct interregional neuronal activation patterns following pattern separation and that new neurons will show disparate functional connectivity patterns between the sexes.

Chapter 4: Do estrogens modulate the maturation and trajectory of adult-born neurons in the dentate gyrus of hippocampus? Ovariectomized young rats were subcutaneously injected with estradiol or estrone daily to assess the modulatory effects of estrogens on adult neurogenesis. A thymidine analogue labelling approach was used to label proliferating neural progenitors and their progeny, and changes in developmental stage specific protein expression and trajectory of new neurons with estrogen exposure time were examined. I hypothesized that estradiol and estrone differentially modulate the trajectory and maturation rate of new neurons based on the duration of exposure to estrogens.

Chapter 5: Do estrogens modulate the ability for spatial pattern separation, neurogenesis in the dentate gyrus and hippocampal neural network?

Ovariectomized young rats were subcutaneously injected with low (0.32μg) or high (5μg) dose of estradiol benzoate daily to assess the modulatory effects of estrogens on spatial pattern separation and adult neurogenesis. A thymidine analogue labelling approach was used to label proliferating neural progenitors and their survival with estradiol exposure and the spatial pattern separation task in the radial arm maze. I hypothesized that estradiol differentially modulates the survival of new neurons depending on their strategy use and dose of estradiol.

Chapter 2: Sex Differences in Maturation and Attrition of Adult Neurogenesis in the Hippocampus[1]

2.1 Introduction

Adult neurogenesis in the dentate gyrus (DG) has been observed in all mammalian species studied including primates (Boldrini et al., 2018; Briley et al., 2016; E Gould et al., 1999; Knoth et al., 2010; Kornack and Rakic, 1999; Kuhn et al., 1996; Moreno-jiménez et al., 2019). Despite two papers indicating a lack of neurogenesis in humans (Dennis et al., 2016; Sorrells et al., 2018), recent studies have definitively shown adult neurogenesis exists in humans and is modulated by disease, age, and perhaps sex in response to antidepressants (Cipriani et al., 2018; Epp et al., 2013a; Moreno-jiménez et al., 2019; Sorrells et al., 2018; Tobin et al., 2019). Adult hippocampal neurogenesis arises from the radial glia-like neural stem cells (RGLs: type1; See Fig. 2.1) in the subgranular zone of the DG, which express stage specific proteins such as Sox2. Sox2 plays a critical role maintaining pluripotency of RGLs (Amador-Arjona et al., 2015; Bonaguidi et al., 2011; Encinas et al., 2011; Micheli et al., 2018; Steiner et al., 2006; see Fig. 2.1 A for a summary). The RGLs undergo asymmetrical cell division and generate one RGL and either an astroglia or a transiently amplifying intermediate neural progenitor cell (IPC: type2). The IPCs can undergo multiple symmetrical or asymmetrical cell divisions but generally daughter cells differentiate into neurons (Bonaguidi et al., 2011; Cameron et al., 1993; Encinas et al., 2011; Kempermann, 2003; Steiner et al., 2006). Previous studies show that adult-born cells in the DG divide multiple times, increasing the number of daughter cells which peaks one week

[1] Yagi, S., Splinter, J.E.J., Tai, D., Wong, S., Wen, Y., Galea, L.A.M. (2020). Sex differences in maturation and attrition of adult neurogenesis in the hippocampus. eNeuro, 7(4): eNeuro 0468-19.2020.

after initial mitosis in male rats (Cameron et al., 1993) and perhaps earlier in mice (undisclosed sex: Amador-Arjona et al., 2015). Adult-born cells in the DG start to die off and show a rapid decrease in the number of new cells between one week and three weeks after the initial cell division in male rodents (Cameron et al., 1993; Encinas et al., 2011; Snyder et al., 2009). A subset of IPCs (type2b), neuroblasts (type3) and immature neurons transiently express a microtubule-associated protein, doublecortin (DCX) for up to three weeks, and new neurons start to express a neuronal nuclear protein, NeuN, approximately one week after mitosis in rats (Brown et al., 2003; Snyder et al., 2009) or two weeks after mitosis in mice (Snyder et al., 2009). Surviving new neurons integrate into the existing neural circuitry, and play an important role in pattern separation and stress resilience (Clelland et al., 2009; França et al., 2017; Hill et al., 2015; Snyder et al., 2011). However, whereas there are species differences in the maturation rate of adult born neurons (Snyder et al., 2009), as of yet no studies to our knowledge have explored sex differences in the maturation rate of adult born neurons.

It is important to acknowledge that most of our information about the trajectory and timeline of maturation of new neurons comes from data in male rodents (Cameron et al., 1993; Snyder et al., 2009), with one study in female rodents (Brown et al., 2003). Thus, to our knowledge no study has directly compared female to male rats on trajectory and maturation timeline of new neurons in the DG. Previous studies demonstrate notable sex differences in the regulation of adult neurogenesis in response to stress, estrogens, androgens, or cognitive training in the DG (Barker and Galea, 2008; Chow et al., 2013; Duarte-Guterman et al., 2019; Falconer and Galea, 2003; Hillerer et al., 2013; Yagi et al., 2016). For instance, acute stress suppresses adult neurogenesis in male rats, but not in female rats (Falconer and Galea, 2003; Hillerer et al., 2013). Furthermore, spatial navigation tasks or spatial pattern separation tasks enhance adult

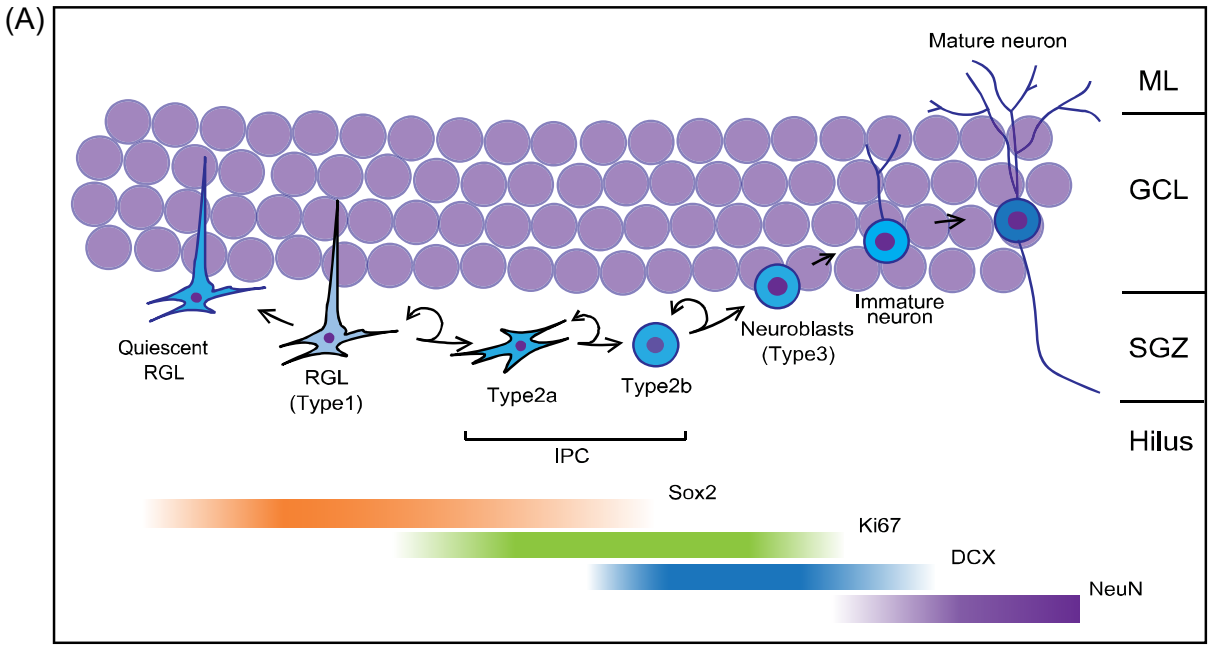
neurogenesis in male rats but not in female rats (Chow et al., 2013; Yagi et al., 2016). The enhancing effect of cognitive training on adult neurogenesis in male rats has a critical period, in which cognitive training must occur 6-10 days after cell birth (Epp et al., 2011), which is curiously the same time that 17β-estradiol also increases neurogenesis in the male meadow vole (Ormerod et al., 2004). The sex differences in the ability of cognitive training to enhance neurogenesis in males but not females suggest one of three scenarios: 1) neurogenesis in the hippocampus is not important for cognitive training in females; 2) the neural activity in the hippocampus may not be as active in females; or 3) there are sex differences in the maturation rate of neurogenesis. Either of these scenarios would lead to the inability of cognitive training to boost survival of new neurons in females in response to spatial training. However, evidence suggests neither of the first two scenarios are correct. Adult DG neurogenesis is associated with less distance travelled in females (Chow et al., 2013; Yagi et al., 2016) and females show increased zif268 expression in the CA3 after training compared to males (Yagi et al., 2017, 2016). Collectively, these findings suggest sex differences following cognitive training may be due to differences in the maturation rate and perhaps trajectory of adult-born neurons in the DG.

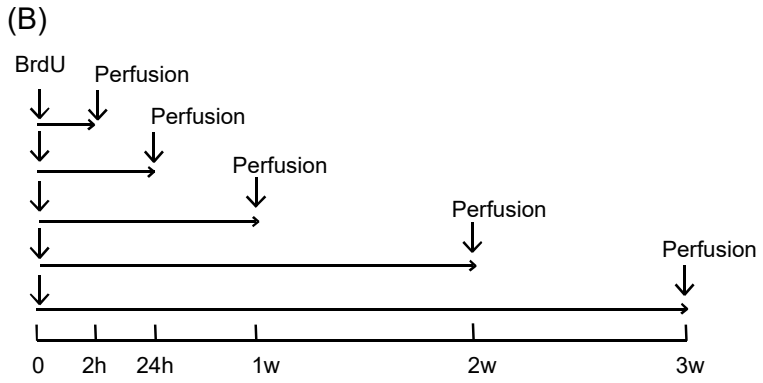
Therefore, the present study aimed to elucidate whether there were sex differences in the maturation and attrition of the new neurons as well as the number of neural stem cells in the dorsal versus ventral DG. A single injection of bromodeoxyuridine (BrdU) was used for birth-dating of adult-born new cells in male and female rats, and brains were immunohistochemically stained for BrdU and endogenous cell-stage-specific protein makers such as Sox2, Ki67, doublecortin (DCX) and NeuN. Given the work above, we expected sex differences in the maturation rate of new neurons with males showing a faster maturation rate than females.

2.2 Materials and methods

2.2.1 Subjects

Forty-four age-matched (two-month old) *Sprague-Dawley* rats were bred at the University of British Columbia and used in this study (n=22 per sex). All subjects were same-sex pair-housed in opaque polyurethane bins (48 × 27 × 20 cm) with paper towels, polyvinylchloride tube, cedar bedding, under a 12h light/dark cycle with 7 am lights-on. Food and water were provided *ad libitum*. Females weighed 240-280g and males weighed 315-355g. All animals were handled every day for two minutes for one week prior to the beginning of the experiment. 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.


(A)
Mature neuron
ML
GCL
SGZ
Hilus
Quiescent RGL
RGL (Type1)
Type2a
Type2b
Neuroblasts (Type3)
Immature neuron
IPC
Sox2
Ki67
DCX
NeuN


(B)
BrdU
Perfusion
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0
2h
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1w
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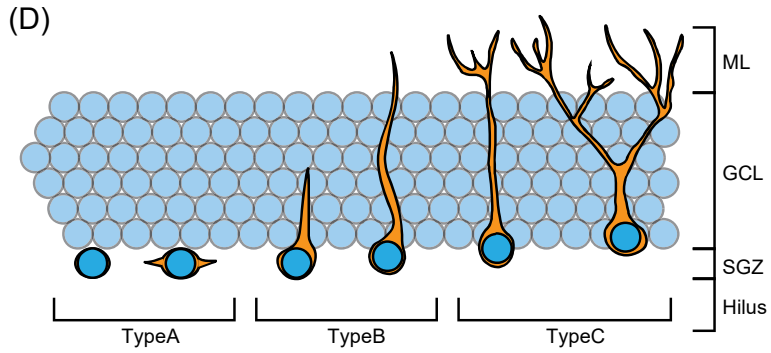
3w


(D)
ML
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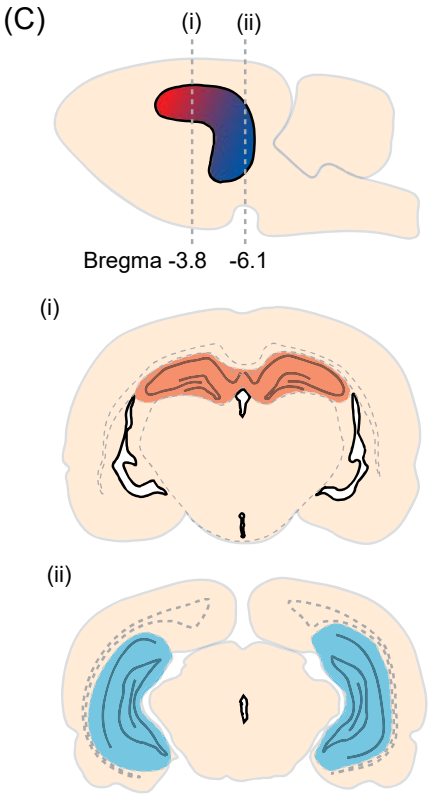

(C)
(i)
(ii)
Bregma -3.8
-6.1
(i)
(ii)

Fig. 2.1. Schematic illustrations for the experimental procedures. (A) Schematic illustrations for the timeline of neural stem cell lineage with expression of stage-specific proteins (Amador-Arjona et al., 2015; Bonaguidi et al., 2011; Encinas et al., 2011; Micheli et al., 2018; Steiner et al., 2006). (B-D) Schematic illustrations for the experimental design: (B) The experimental timeline, all animals were age-matched and received BrdU injection at 10 weeks. (C) examples of the dorsal (section (i): red; Bregma -3.8mm), and ventral (section (ii): blue; Bregma -6.8mm) hippocampus (numbers represent mm from the bregma) and (D) morphological phenotypes of DCX-ir cells. H- hours, w- weeks, BrdU- bromodeoxyuridine, DCX- doublecortin, GCL- granule cell layer, IPC- intermediate proliferating cell, ML- molecular layer, RGL- radial glial cell, SGZ- subgranular zone

2.2.2 Experimental design

One intraperitoneal (i.p.) injection of BrdU (200mg/kg) was given to all rats between 11am-12 pm. Rats were perfused either two hours (2h), 24 hours (24h), one week (1w), two weeks (2w) or three weeks (3w) after the BrdU injection, but otherwise were left undisturbed except for weekly cage changes (see Fig. 2.1 B). On the day of perfusion, rats were administered an overdose of sodium pentobarbital (500mg/kg, i.p.). Blood samples were collected from the chest cavity, and rats were 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 30 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 until immunostaining. Complete series of sections were immunohistochemically stained for BrdU/DCX and BrdU/NeuN to examine sex differences in the maturation timeline of new

neurons, for Sox2 to examine the number of neural stem cells, and for Ki67 to examine actively dividing progenitor cells. In addition, the brain sections were double-stained for BrdU/Sox2 to examine changes of Sox2 expression over the three weeks after BrdU injection.

2.2.3 Radioimmunoassay for 17β-estradiol and testosterone

Previous studies reported that 17β-estradiol increases cell proliferation in females but not males (Barker and Galea, 2008; Tanapat et al., 1999). Androgens increase survival of new neurons in males but not in females, but do not influence cell proliferation in either sex (Duarte-Guterman et al., 2019; Spritzer and Galea, 2007).Thus, we examined serum levels of 17β-estradiol and testosterone in females and males of the 1w, 2w and 3w groups, respectively. Blood samples were stored at 4℃ overnight and centrifuged at 10g for 15 minutes to collect serum. Serum 17β-estradiol levels in female rats and serum testosterone levels in male rats were assayed using commercially available radioimmunoassay (RIA) kits from Beckman Coulter (Brea, USA) or MP Biomedicals (Santa Ana, USA) respectively. The sensitivity of the RIA kits was 0.75 ng/mL for 17β-estradiol and 0.03ng/mL for testosterone. The intra- and inter-assay coefficient of variation were <8.9% and <12.2% respectively for 17β-estradiol and <8.2% and <13.2% for testosterone. For females with 50 pg/ml or higher serum estradiol levels were considered to be in proestrus (Cameron et al., 2008). Based on estradiol levels, none of the females in the 1w, 2w and 3w groups were in proestrus at the time of sacrifice (see Table 2.1).

Table 2.1

Mean (±SEM), minimum and maximum concentration of serum testosterone in males (ng/ml) and estradiol in females (pg/ml). SEM – standard error of the mean n=13 per group

	Min	Max	Mean±SEM
Male (Testosterone)	0.37	4.46	1.067±0.43
Female (Estradiol)	10.99	21.08	14.41±1.30

2.2.4 Estrous cycle stage determination

As the estrous cycle phase can influence early timepoints of neurogenesis, estrous cycle stages of the 2h and 24h groups were determined with vaginal lavage samples. Vaginal cells suspended in water were obtained using a glass pipette, transferred onto a microscope slide and stained with cresyl violet (Sigma-Aldrich). Proestrus was determined when 70% of the cells were nucleated epithelial cells. Two females (one each in the 2h and 24h groups) were in proestrus at the time of sacrifice.

2.2.5 Immunohistochemistry

BrdU/NeuN, BrdU/DCX or BrdU/Sox2 double-staining

The exogenous DNA synthesis marker, 5-bromo-2'-deoxyuridine (BrdU) is incorporated into DNA during the synthesis phase of the cell cycle (Kee et al., 2002; Miller et al., 2018). BrdU is a thymidine analogue which is active for two hours after injection in rats (Cameron and Mckay, 2001). Briefly our protocol was as follows: sections were prewashed three times with 0.1

M TBS and left overnight at 4 °C. Sections were then incubated in a primary antibody solution containing 1:250 mouse anti-NeuN (Millipore; MA, USA), 1:200 goat anti-DCX(Santa Cruz Biotechnology; Dallas, Texas, USA) or 1:500 mouse anti-Sox2 (Santa Cruz Biotechnology; Dallas, Texas USA), 0.3% Triton-X, and 3% normal donkey serum (NDS; Vector Laboratories) in 0.1 M TBS for 24 hours at 4 °C. Next, sections were incubated in a secondary antibody solution containing 1:250 donkey anti-mouse ALEXA 488 (Invitrogen, Burlington, ON, Canada) or donkey anti-goat ALEXA 488 (Invitrogen, Burlington, ON, Canada) in 0.1 M TBS, for 18 hours at 4 °C. After being rinsed three times with TBS, sections were washed with 4% paraformaldehyde for 10 minutes, and rinsed twice in 0.9% NaCl for 10 minutes, followed by incubation in 2N HCl (Fisher Scientific, Waltham, Massachusetts, USA) for 30 minutes at 37 °C. Sections were then rinsed three times in TBS for 10 minutes each and 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 TBS for 24 hours at 4 °C. A further incubation of sections commenced in a secondary antibody solution containing 1:500 donkey anti-rat ALEXA 594 (Invitrogen, Burlington, ON, Canada) in 0.1 M TBS for 24 hours at 4 °C. Following three final rinses with TBS, the sections were mounted onto microscope slides and cover-slipped with PVA DABCO.

Ki67 or Sox2 immunofluorescent staining

Ki67 is expressed in actively dividing cells (all stages of the cell cycle except G_0) and therefore is expressed at higher levels than BrdU 24 h after injection (Kee et al., 2002). Randomly selected brain sections were also immunohistochemically stained with anti-Ki67 or anti-Sox2 (n=8 per sex). Brain sections were prewashed with 0.1 M PBS and left to sit overnight at 4 °C. The next day, sections were incubated in 10mM sodium citrate buffer for 45 minutes at

90 °C to retrieve antigens of Ki67 and blocked with 3% NDS and 0.3% Triton-X in 0.1M PBS, followed by incubation in primary antibody solution made with 1:1000 mouse anti-Sox2 (Santa Cruz Biotechnology; Dallas, Texas USA) or 1:250 mouse anti-Ki67 (Leica Biosystems; Newcastle, UK), 1% NDS, and 0.3% Triton-X in 0.1 M PBS for 24 hours at 4 °C. Then the sections were incubated in secondary antibody solution, consisting of 1:500 Donkey anti-Mouse ALEXA 488 for Sox2 (Invitrogen, Burlington, ON, Canada) and 1:500 Donkey anti-mouse ALEXA 594 for Ki67 (Invitrogen, Burlington, ON, Canada), 1% NDS, and 0.3% Triton-X in 0.1 M PBS, for 18 hours at 4 °C. After three rinses with PBS, sections were incubated in 1:5000 DAPI in PBS for 3 mins and mounted onto slides and cover-slipped with PVA DABCO.

2.2.6 Cell counting

All counting was conducted by an experimenter blind to the group assignment of each animal using an Olympus epifluorescent microscope and confocal microscope. Location of immunoreactive cells was examined in the dorsal or ventral DG using the criterion defined by Banasr et al. (2006) with sections 7.20-4.48mm from the interaural line (Bregma -1.80 to -4.52mm) defined as dorsal and sections 4.48-2.20 mm from the interaural line (Bregma -4.52 to -6.80mm) as ventral (Banasr et al., 2006; see Fig. 2.1 C). Cells were counted separately in each region because the different regions are associated with different functions (reviewed in Fanselow and Dong, 2010) and possibly different maturation timelines (Snyder et al., 2012). The dorsal hippocampus is associated with spatial learning and memory, whereas the ventral hippocampus is associated with stress and anxiety (Kjelstrup et al., 2002; Moser et al., 1993).

BrdU and Ki67

Ki67-ir and BrdU-ir cells were counted under a 100x oil immersion objective lens (Fig. 2.3 A, 2.5 A). Every 10th section of the granule cell layer (GCL) that includes the subgranular zone on one half of each brain were counted. An estimate of the total number of cells was calculated by multiplying the aggregate by 10 (Ngwenya et al., 2015; Snyder et al., 2005; Workman et al., 2015). Density of BrdU-ir or Ki67-ir cells was calculated by dividing the total estimate of immunoreactive cells in the GCL by volume of the corresponding region. Volume of the DG was calculated using Cavalieri's principle (Gundersen and Jensen, 1987) by multiplying the summed areas of the DG by thickness of the section (300μm). Area measurements for the DG were obtained using digitized images on the software ImageJ (NIH).

Percentage of BrdU/NeuN, BrdU/DCX and BrdU/Sox2 co-expression

The percentages of BrdU/NeuN and BrdU/DCX-ir cells were obtained by randomly selecting 50 BrdU-labeled cells and calculating the percentage of cells that co-expressed DCX, NeuN or Sox2 (Fig. 2.5 A, 2.6 A and 2.7 A; method used by Banasr et al., 2006). The percentage of BrdU/DCX-ir cells was also categorized into the three morphology types using the criteria used by (Plümpe et al., 2006). Briefly, stages were defined as type-A proliferative: neurons with no or short plump processes, type-B intermediate: neurons possess medium-length processes or apical dendrites that reach the molecular layer, and type-C postmitotic: neurons possess apical dendrites with at least one branching into the molecular layer (see Fig. 2.1 D). The density of BrdU-ir cells was multiplied by the percentage of BrdU-ir cells that expressed DCX or Sox2.

Sox2

Photomicrographs of the DG were obtained with a 20x objective lens of an Olympus confocal microscope (three images from three sections each from the dorsal and ventral DG; Fig.

2.1 C and 2.2 A). Immunoreactive cells were counted automatically using a code developed by JEJS from the digitized images using MATLAB (MathWorks; Natick, Massachusetts, USA). The code is available by contacting the author.

2.2.7 Statistical analyses

All analyses were conducted using STATISTICA (Statsoft Tulsa, OK). The density of BrdU-ir cells, BrdU-ir/DCX-ir, or the percentage of BrdU-ir cells that express Sox2 or DCX were each analyzed using repeated-measures analysis of variance (ANOVA), with maturation time (2h, 24h, 1w, 2w, 3w) and sex (male, female) as between-subject variables and with hippocampal region (dorsal, ventral) as the within-subject variable. The percentage of BrdU-ir cells that express NeuN was analyzed using a repeated-measures ANOVA, with maturation time (1w, 2w, 3w) and sex (male, female) as between-subject variables and with hippocampal region (dorsal, ventral) as the within-subject variable. Repeated-measures ANOVAs were used to each analyze the density of Ki67-ir and Sox2-ir cells with sex as between subject factor and with hippocampal region as the within-subject factor. Pearson product-moment correlations were calculated to examine the relationship between dependent variables of interest. Furthermore, the percentage of BrdU/DCX-ir cells expressing type-C morphology was analyzed using repeated-measures ANOVA with sex as between-subject variables and with maturation time and hippocampal region as within-subject variables. Post-hoc tests utilized the Neuman-Keuls procedure. A priori comparisons were subjected to Bonferroni corrections. Significance was set to $\alpha=0.05$ and effect sizes are given with Cohen's d or partial η^2.

2.3 Results

2.3.1 Males had larger dorsal dentate gyrus volumes compared to females

As expected, males had significantly greater volume of dorsal DG compared to females and as such cell density was used for direct comparison between the sexes for all analyses [$p = 0.012$; region by sex interaction: $F(1,22) = 4.61$, $p = 0.043$, Cohen's $d = 1.26$; see Table 2.2]. In addition, the ventral DG was larger than the dorsal DG, as expected [main effect of region: $F(1,22) = 36.19$, $p < 0.0001$].

Table 2.2

Mean (±SEM) volume of the dorsal and ventral dentate gyrus in male and female rats (mm^3). Females had a smaller dorsal dentate gyrus volume. SEM standard error of the mean, n= 42 (20 males and 22 females)

	Dorsal	Ventral
Male	0.905±0.056	1.334±0.083
Female	0.688±0.043	1.593±0.195

2.3.2 Male rats, compared to female rats, had a greater density of Sox2-ir cells in the dorsal dentate gyrus. Females had greater density of Sox2-ir cells in the ventral compared to dorsal region

To examine sex differences in neural stem cells, we investigated the expression of Sox2. Sox2 is a transcriptional factor that plays a role in maintaining self-renewal of neural stem cells and is considered a neural stem cell marker. Male rats had a greater density of Sox2-ir cells compared to female rats in the dorsal DG ($p = 0.024$, Cohen's $d = 1.39$; sex by region [$F(1,16) = 6.34$ $p = 0.023$, see Fig. 2.2 B). Females had a greater density of Sox2-ir cells in the ventral DG

compared to the dorsal DG ($p = 0.005$, Cohen's $d = 1.10$) whereas this regional difference was not observed in males ($p = 0.74$). There were trends for a main effect of sex [$F(1, 16) = 3.67$, $p = 0.074$] and region [$F(1,16) = 4.20$, $p = 0.057$].

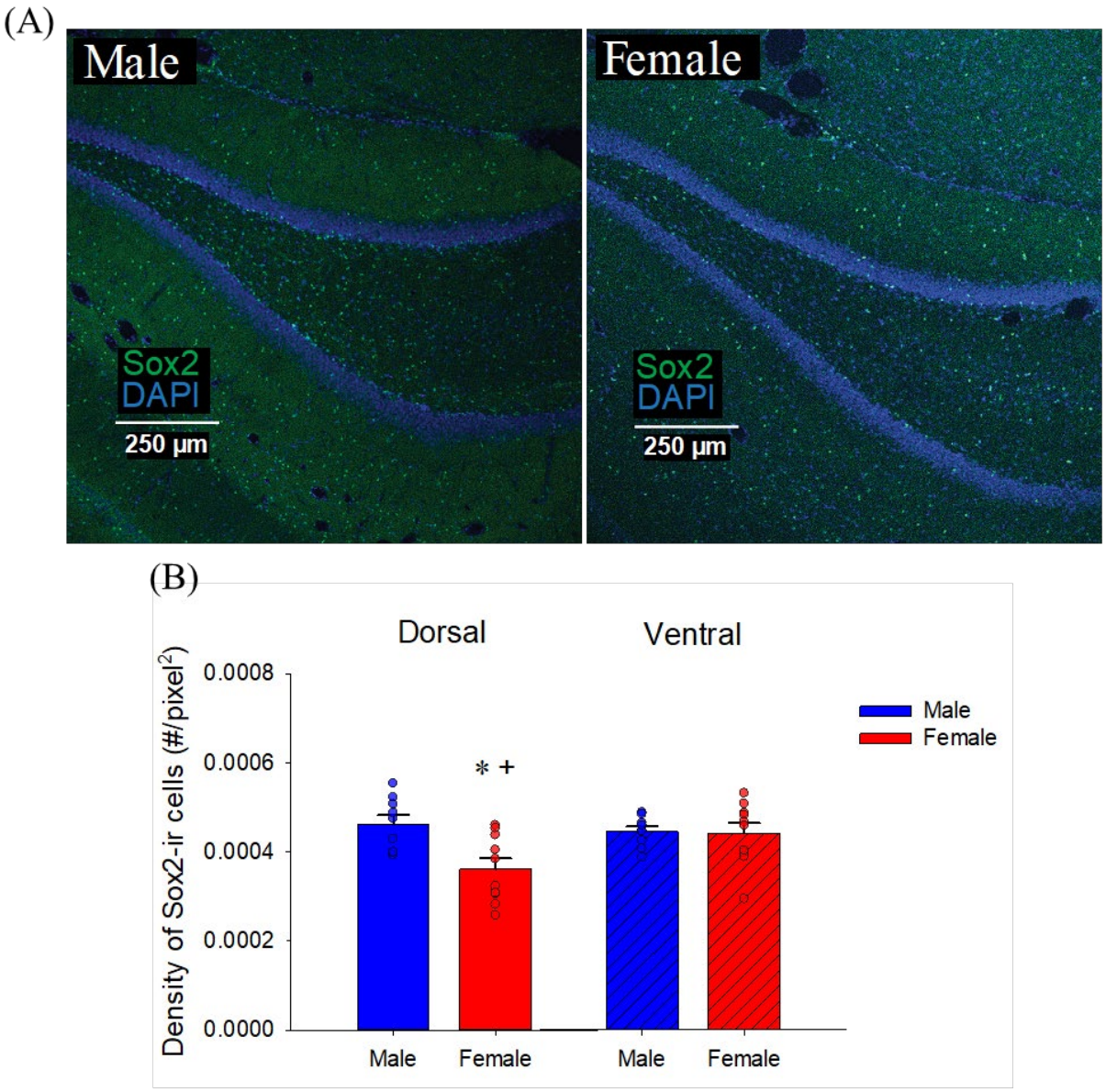


Fig. 2.2. Sex differences in neural stem cells (Sox2-ir). (A) Photomicrographs of Sox2 (green) with DAPI (blue) taken with 10x objective lens from a male (left) and female (right) young adult rat (11 weeks old) in the dorsal dentate gyrus. (B) Mean (+SEM) density of Sox2-ir cells: Males, compared to females, had a greater density of Sox2-ir cells in the dorsal dentate gyrus. The ventral dentate gyrus of females, but not males, had a greater density of Sox2-ir cells compared to the dorsal dentate gyrus. * indicates a significant sex differences and + indicates significant a regional difference ($p<0.05$). ir- immunoreactive. All animals were age-matched.

2.3.3 Males had greater levels of cell proliferation (Ki67) compared to females

To examine potential sex differences in cell proliferation, we used Ki67, which labels all cells undergoing mitosis. Males had a greater density of Ki67-ir cells compared to females [main effect of sex: $F(1,15) = 13.90$, $p = 0.002$, Cohen's $d = 1.80$; see Fig. 2.3 B]. There was also a trend of main effect of region [$F(1, 15) = 3.44$, $p = 0.083$, partial $\eta^2 = 0.187$], but no significant interaction ($p=0.11$). Because previous studies have observed the rats in proestrus have higher levels of cell proliferation (Rummel et al., 2010; Tanapat et al., 1999), we also examined the relationship between the density of Ki67-ir cells and the levels of 17β-estradiol in females, or testosterone in males, but none was observed (all ps' > 0.268).

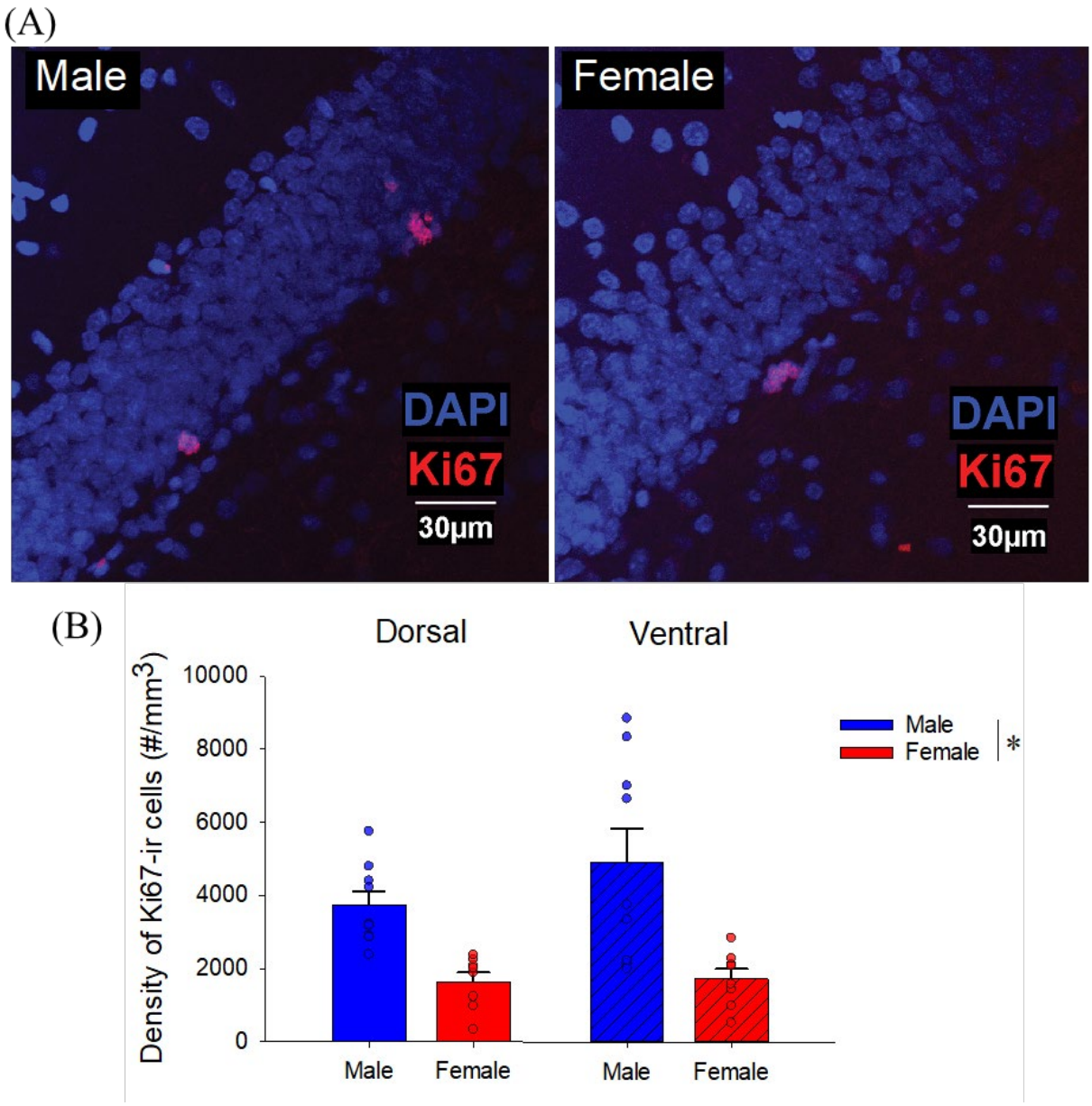


Fig. 2.3. Sex differences in proliferating cells (Ki67-ir) in the dentate gyrus. (A) Photomicrographs of Ki67 (Red) with DAPI (blue) taken with x40 objective from a male (left) and female (right) young adult rat (11 weeks old) in the dorsal dentate gyrus. (B) Mean (±SEM) density of Ki67-ir cells: Males had a greater density of Ki67-ir cells compared to females. * indicates a significant difference ($p<0.05$). ir- immunoreactive. All animals were age-matched.

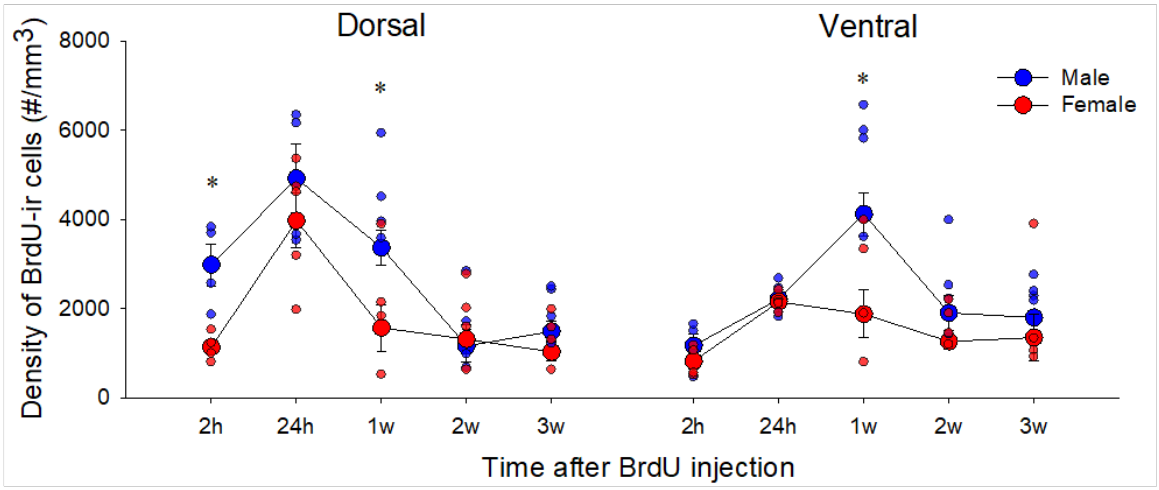
2.3.4 Males, but not females, show greater attrition of BrdU-ir cells from 1 week to 2 weeks after mitosis

To determine whether there were sex differences in the trajectory of new neurons across weeks we examined the density of BrdU-ir cells at various time points after BrdU injection (2h, 24h, 1w, 2w, and 3w). Using the same timeline with ^{3}H-thymidine, males show an increase ^{3}H-thymidine-labelled cells after 24 hours and a large attrition rate of ^{3}H-thymidine-labelled from one week to three weeks after injection (Cameron et al., 1993). Consistent with past research (Cameron et al., 1993), males had a greater density of 1w old BrdU-ir cells compared to 2h, 24h, 2w and 3w after BrdU injection (p's < 0.001; interaction effect of sex by time [$F(4,31) = 2.95$, $p = 0.035$, partial $\eta^2 = 0.276$; see Fig. 2.4 A]). However, females did not show appreciable differences in the density of BrdU-ir cells across any time points (all p's > 0.147) except between 2h and 24h ($p = 0.156$). Furthermore, males had a greater density of BrdU-ir cells than females at the 1w timepoint ($p = 0.0003$, Cohen's $d = 2.26$) but not at any other timepoint (all ps' > 0.308). Given our findings with Ki67, we also examined sex differences at the 2h and 24h timepoints and saw males had more BrdU-ir cells in the dorsal region only at 2h (priori: p=0.009, Cohen's d = 2.64) which failed to reach significance at 24 h (p=0.15) compared to females. There were main effects of sex [$F(1, 31) = 17.57$, $p < 0.002$, Cohen's $d = 0.746$], time [$F(4, 31) = 11.78$, $p < 0.0001$, partial $\eta^2 = 0.603$] and region [$F(1, 31) = 4.43$, $p = 0.044$, Cohen's $d = 0.254$] and an interaction effect of region by time [$F(4, 31) = 12.21$, $p < 0.0001$, partial $\eta^2 = 0.639$] was noted but no other significant interactions (p's > 0.125).

Complementing the attrition rate in BrdU-ir cells across weeks in males, we found that males had a greater density of BrdU/DCX-ir cells than females only at the 1w time point (p=0.00036, Cohen's $d = 2.61$) but not at any other timepoint (all p's > 0.130 [interaction effect

of sex by time: $F(4, 29) = 4.04$, $p = 0.0101$, partial $\eta^2 = 0.358$; see Fig. 2.4 B]. Given our findings with Ki67, we also examined the 2h and 24h timepoint and found that males had a greater density of BrdU/DCX-ir cells compared to females in the dorsal dentate gyrus at 2h ($p = 0.005$, Cohen's $d = 3.18$). There were also main effects of sex [$F(1,29) = 11.71$, $p = 0.0047$, Cohen's $d = 0.320$], time [$F(4, 29) = 29.31$, $p < 0.0001$, partial $\eta^2 = 0.802$] and region [$F(1, 29) = 8.66$, $p = 0.0063$, partial $\eta^2 = 0.230$] and an interaction effect of region by time [$F(4, 29 = 12.86$, $p < 0.0001$), partial $\eta^2 = 0.639$] but no other significant interactions were noted (p's > 0.269).

(A)



(B)

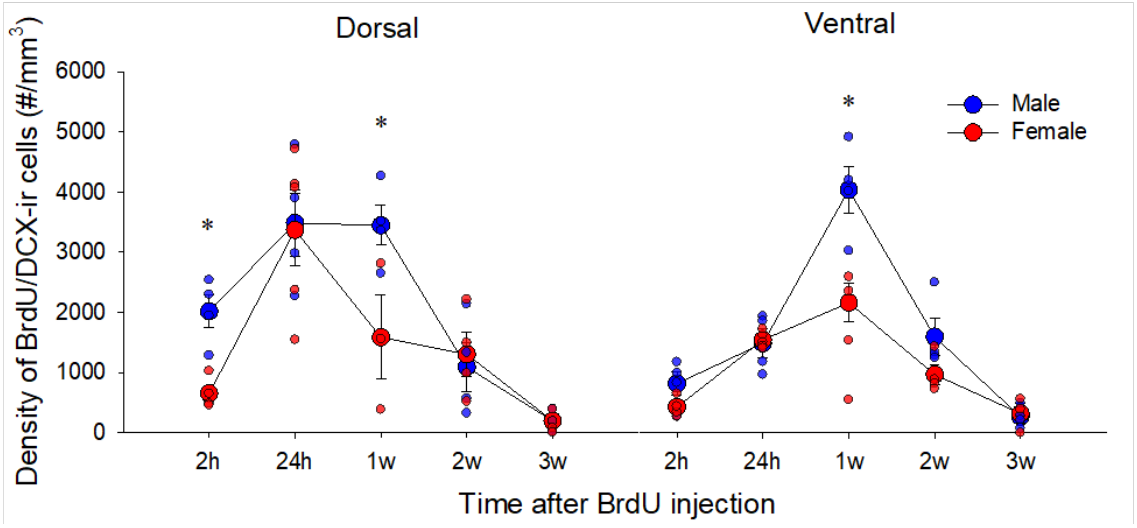


Fig. 2.4. Sex differences in the trajectory of adult-born BrdU-ir cells. (A) Mean (±SEM) density of BrdU-ir cells. Male adult rats had a greater density of BrdU-ir cells at 2h and 1w compared to female adult rats and showed a greater reduction in density between 1w and 2w after BrdU injection. (B) Mean (±SEM) density of BrdU/DCX-ir cells. Males had a greater density of BrdU-ir cells that express DCX cells at 2h and 1w. * indicates a significant sex difference ($p<0.05$). h-hours, w-weeks, BrdU- bromodeoxyuridine, DCX-doublecortin. All animals were age-matched and received BrdU injection at 10 weeks old.

2.3.5 Male adult-born neurons mature faster compared to female adult-born neurons

We then examined whether there are sex differences in maturation rate of adult-born neurons by examining the percentage of BrdU-ir cells expressing maturation stage specific neuronal markers, immature neurons (DCX) and mature neurons (NeuN) across the three weeks. Males, compared to females, had a greater percentage of BrdU-ir cells that expressed NeuN 2w ($p = 0.003$, Cohen's $d = 2.14$) but not 1w ($p=0.99$) or 3w ($p=0.54$) after BrdU injection (interaction effect of sex by time [$F(2, 17) = 3.52$, $p = 0.05$, partial $\eta^2 = 0.293$; see Fig. 2.5 B]). There were also main effects of sex: [$F(1, 17) = 7.14$, $p = 0.016$, partial $\eta^2 = 0.296$] and time [$F(2, 16) = 41.92$, $p < 0.00001$, partial $\eta^2 = 0.834$] but no other significant main or interaction effects (all p's > 0.24). The percentage of BrdU-ir cells that expressed NeuN by three weeks after BrdU injection in both males and females was approximately 90% and did not significantly differ between the sexes ($p = 0.583$).

As expected, in both sexes across both regions, the percentage of BrdU-ir cells that also express DCX decreased significantly as time progressed with the least co-expression at 3w compared to all other time points (all p's <0.002). Furthermore, the 2h timepoint had lower co-expression than all other earlier timepoints (all p's <0.024) except 2w ($p=0.34$) and 3 w [main effect of time: $F(4, 30) = 63.69$, $p < 0.0001$; partial $\eta^2 = 0.895$; see Fig. 2.6 B]. Females had greater percentage of BrdU-ir cells that co-expressed DCX in 24h group compared to 2h group (a priori: $p = 0.0003$, Cohen's $d = 6.68$; see Fig. 2.6 B), which was not seen in males ($p = 0.895$; sex by time interaction ($p = 0.086$)). There were no other significant main or interaction effects on the percentage of BrdU-ir cells that co-express DCX (p's > 0.12). Given the findings showing that new neurons expressed NeuN faster in males compared to females, we also examined BrdU/DCX-ir cells by maturation stage, which we classified using morphology (Plümpe et al.,

2006). Consistent with our BrdU/NeuN findings, males had a greater percentage of BrdU/DCX-ir cells expressing type-C morphology compared to females at 2w in the dorsal DG [a priori: $p = 0.017$, Cohen's $d = 1.84$; effect of time: $F(2, 18) = 5.39$, $p = 0.015$, partial $\eta^2 = 0.37$; see Fig. 2.6 C) but not at 1w ($p = 0.95$) or 3w ($p = 0.84$) after BrdU injection.

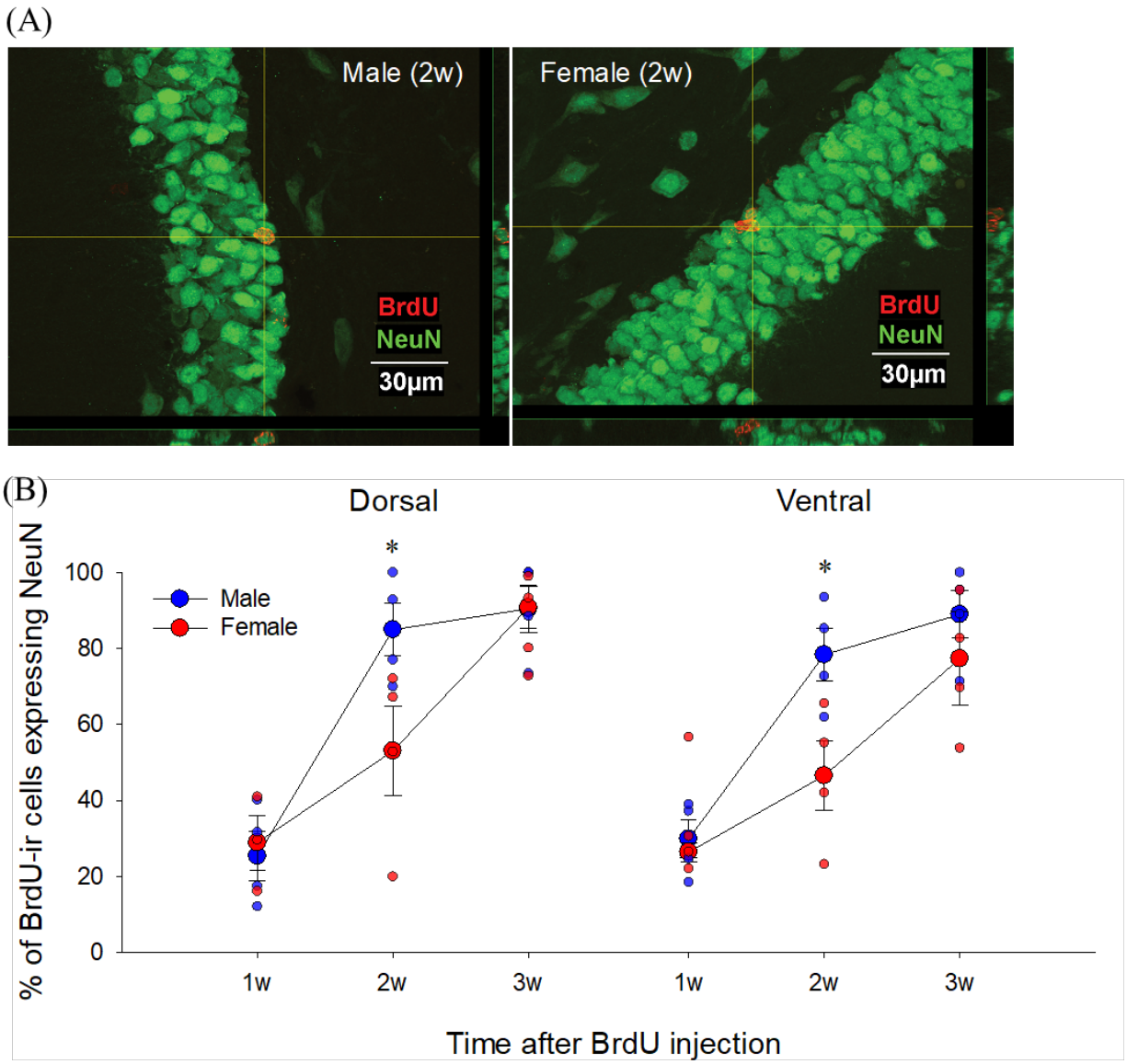


Fig. 2.5. Sex differences in the maturation rate of adult-born neurons in the dentate gyrus (BrdU/NeuN). (A) Photomicrographs of BrdU (red)/NeuN (green) taken with 60x objective lens from a male (left) and female (right) young adult rats in the 2w group. (B) Mean (±SEM) percentages of BrdU-ir cells that express NeuN. Male young adult rats had a greater percentage of BrdU-ir cells that express NeuN at 2w in the dorsal and ventral dentate gyrus. * indicates a significant sex difference ($p<0.05$). w-weeks, BrdU-bromodoxyuridine, ir- immunoreactive. All animals were age-matched and received BrdU injection at 10 weeks old.

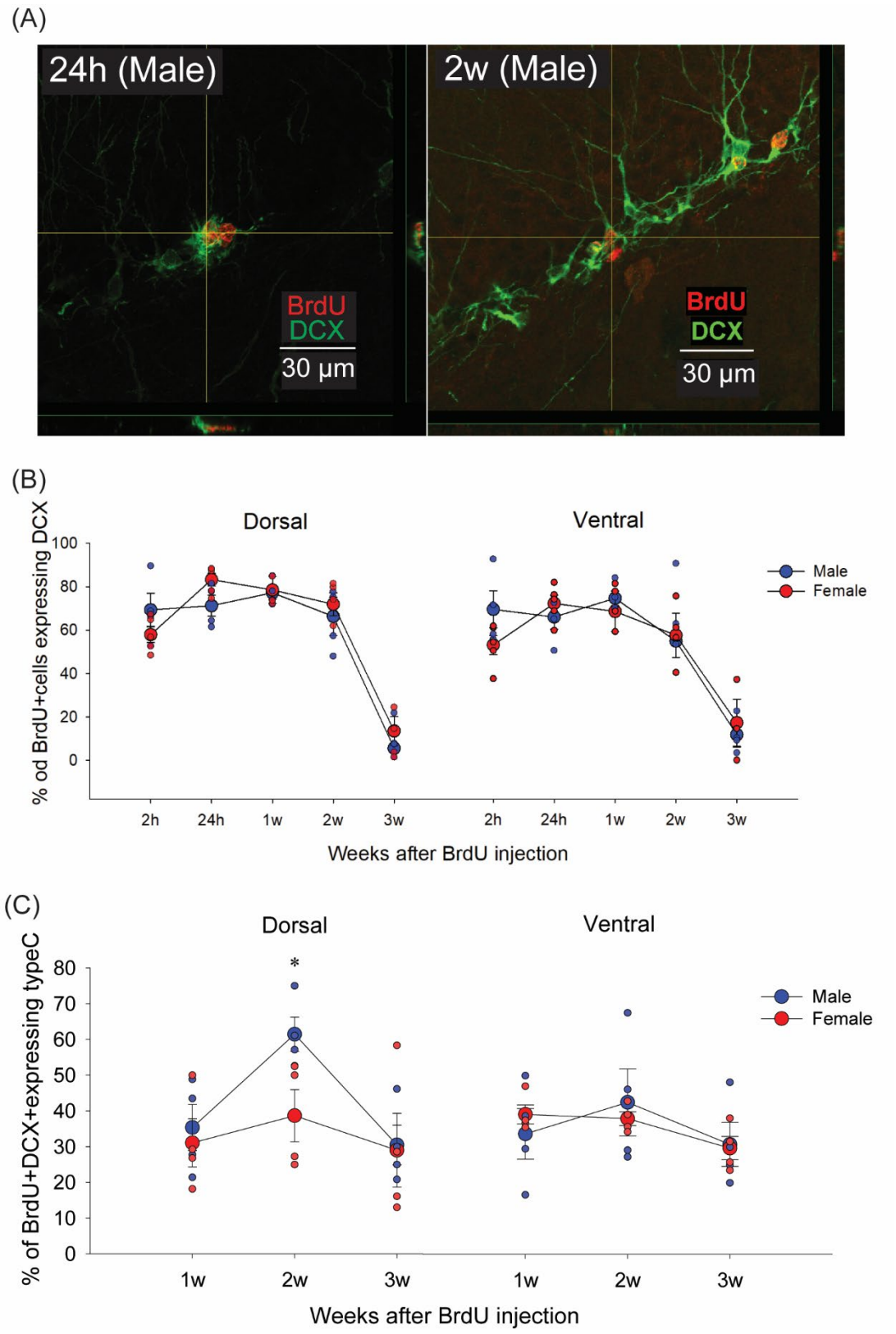

(A)
24h (Male)
BrdU
DCX
30 μm
2w (Male)
BrdU
DCX
30 μm
(B)
Dorsal
Ventral
% od BrdU+cells expressing DCX
100
80
60
40
20
0
2h
24h
1w
2w
3w
Male
Female
Weeks after BrdU injection
(C)
Dorsal
Ventral
% of BrdU+DCX+expressing typeC
80
70
60
50
40
30
20
10
0
*
1w
2w
3w
Male
Female
Weeks after BrdU injection

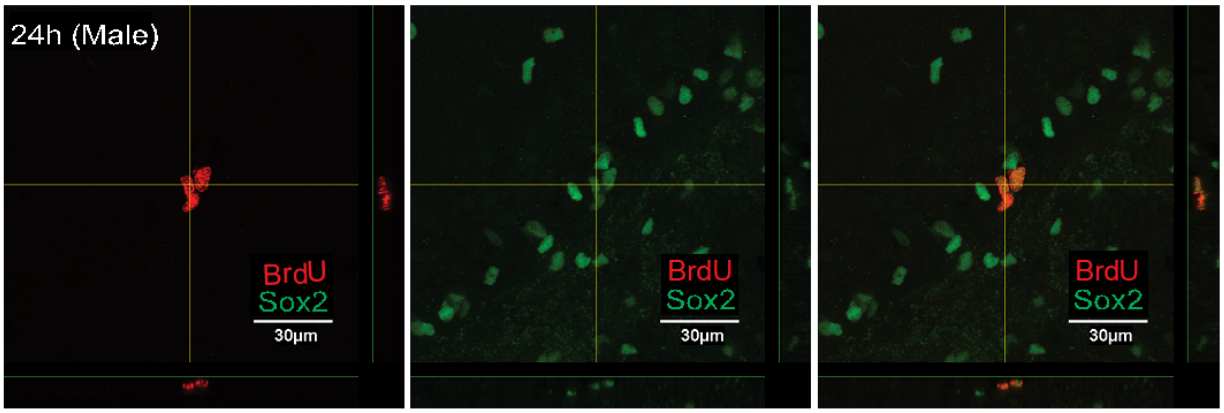
Fig. 2.6. Sex differences in the maturation rate of adult-born neurons in the dentate gyrus (BrdU/DCX). (A) Photomicrographs of BrdU (Red)/DCX (Green) taken from male young adult rat at 24h (left: 60x objective lens) and 2w (right: 40x objective lens) group. (B) Mean (±SEM) percentages of BrdU-ir cells that express DCX. There was no significant sex difference in the percentage of BrdU-ir cells that co-express DCX (C) Mean (±SEM) percentages of BrdU/DCX-ir cells that had a type-C morphological phenotype. A priori comparisons showed that male adult rats had a greater percentage of BrdU/DCX-ir cells that showed the type-C morphological phenotype at 2w compared to female adult rats in the dorsal dentate gyrus. * indicates a significant sex difference ($p<0.05$). h-hours, w-weeks, BrdU- bromodeoxyuridine, DCX- doublecortin, ir- immunoreactive. All animals were age-matched and received BrdU injection at 10 weeks old.

2.3.6 Males have a greater density of BrdU/Sox2-ir cells in the dorsal DG at 2h compared to females

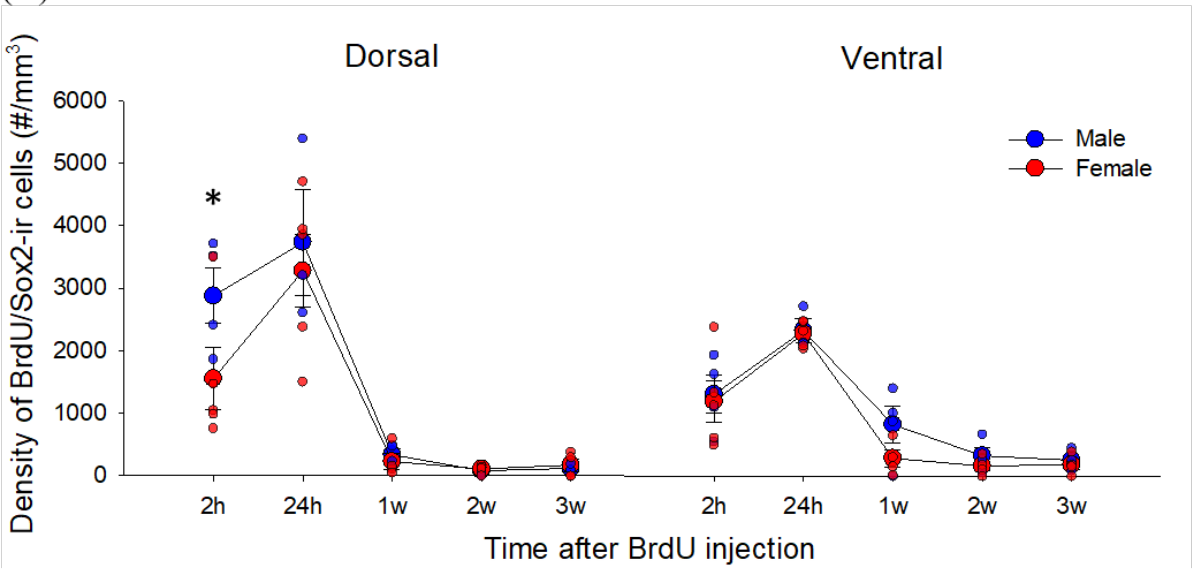
To understand if there are differences between sexes in the time course of neural stem cell marker expression after mitosis, we examined the density of BrdU/Sox2-ir cells at 2h, 24h, 1w, 2w and 3w after BrdU injection. Males had a greater density of BrdU/Sox2-ir cells compared to females in the dorsal dentate gyrus at 2h but not at any other timepoint [a priori: $p = 0.0019$; see Fig. 2.7 B]. In addition, the dorsal dentate gyrus had a greater density of BrdU/Sox2-ir cells at 2h and 24h than the ventral dentate gyrus compared to all other timepoints (all p's <0.0003; interaction of region by time $F(4, 31) = 11.66$, $p < 0.0001$, partial $\eta^2 = 0.601$). There were also significant main effects of time ($F(4, 31) = 40.46$, $p < 0.0004$, partial $\eta^2 = 0.84$) and region ($F(1, 31) = 20.50$, $p < 0.0001$, partial $\eta^2 = 0.398$) but no other main or interaction effects (both p's >0.109).

2.3.7 The percentage of BrdU/Sox2 co-expressing cells decreased dramatically over time in both sexes

As expected, the percentage of BrdU-ir cells expressing Sox2 decreased across time, with the highest levels at the 2h and 24h timepoints in the dorsal and ventral region (all p's < 0.0002), with the 2h timepoint having higher levels than 24h in the dorsal dentate gyrus only ($p = 0.003$; interaction effect of region by time: $F(4, 31) = 4.25$, $p = 0.007$, partial $\eta^2 = 0.354$; main effect of region: $F(1, 31) = 5.37$, $p = 0.027$, partial $\eta^2 = 0.148$; main effect of time: $F(4, 31) = 640.85$, $p < 0.001$, partial $\eta^2 = 0.988$; see Fig. 2.7 C]. There was a trend for an interaction effect of region by sex [$F(1, 31) = 3.77$, $p = 0.061$, partial η^2 0.108]. There were no other significant main or interaction effects on the percentage of BrdU-ir cells expressing Sox2 ($p > 0.317$).


24h (Male)
BrdU
Sox2
30μm
BrdU
Sox2
30μm
BrdU
Sox2
30μm

(B)


Dorsal
Ventral
Density of BrdU/Sox2-ir cells (#/mm3)
6000
5000
4000
3000
2000
1000
0
*
Male
Female
2h
24h
1w
2w
3w
2h
24h
1w
2w
3w
Time after BrdU injection

(C)

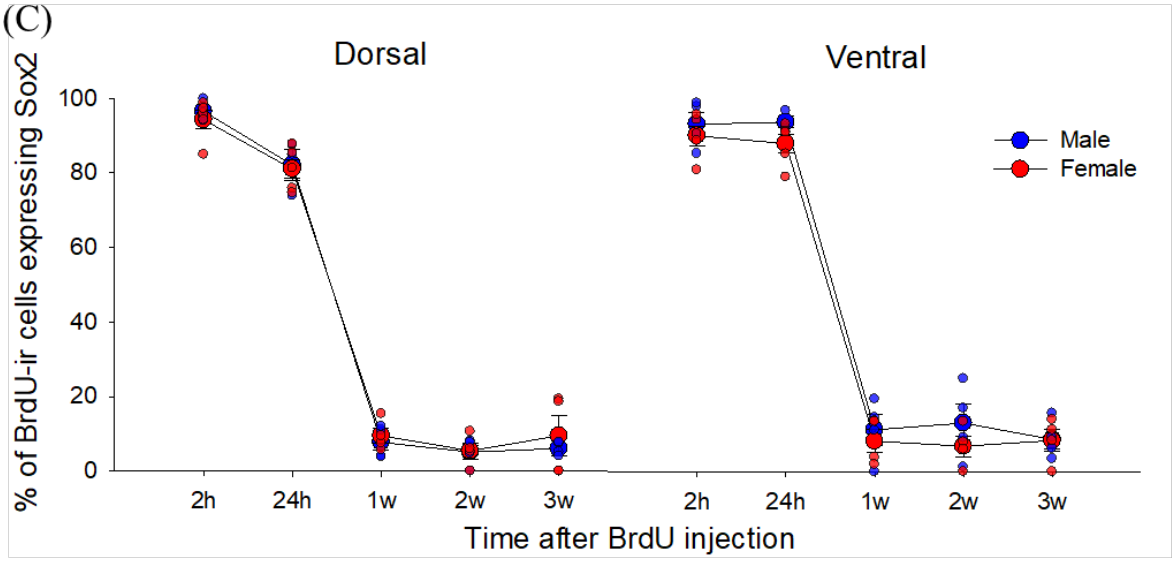

Dorsal
Ventral
% of BrdU-ir cells expressing Sox2
100
80
60
40
20
0
Male
Female
2h
24h
1w
2w
3w
2h
24h
1w
2w
3w
Time after BrdU injection

Fig. 2.7. Sex differences in BrdU/Sox2-ir cells across timepoints. (A) Photomicrographs of BrdU (left: red) /Sox2-ir (center: green) cells and merged images (right), taken from a male young adult rat in 24h group. (B) Mean (±SEM) density of BrdU-ir cells that express Sox2. A priori comparisons showed that male, compared to female, young adult rats had a greater density of BrdU-ir cells that co-expressed Sox2 in the dorsal dentate gyrus at 2h after BrdU injection. * indicates a significant sex difference ($p<0.05$). BrdU- bromodeoxyuridine, ir- immunoreactive. All animals were age-matched and received BrdU injection at 10 weeks old.

2.4 Discussion

Our findings indicate that adult-born neurons mature faster in males compared to females. We also found notable sex differences in the attrition or survival rate of BrdU-ir cells across weeks, with males showing reductions across time, and females showing no appreciable reduction in the density of BrdU-ir cells across the three weeks. Furthermore, males had a higher density of dorsal neural stem cells (Sox2) and cell proliferation (Ki67) compared to females. There were notable differences in early expression of DCX in females, but not in males, showing a greater percentage of BrdU-ir cells expressing DCX at 24h compared to 2h. Intriguingly, the density of BrdU-ir cells 2 weeks after production was comparable between males and females. Although a tremendous amount of research has unveiled the characteristics of neurogenesis in the adult hippocampus, these findings underscore that we cannot assume that the same characteristics will be similar in females as they are in males.

2.4.1 Male adult-born dentate granule cells mature faster compared to female adult-born dentate granule cells

We found that adult born neurons mature faster in males than in females, with males showing a rapid increase in the percentage of BrdU-ir cells that expressed NeuN at 2 weeks.

Although previous studies did not directly compare the sexes, they are consistent with our results (Brown et al., 2003; Snyder et al., 2009). These studies showed that in male rats 65-75% of BrdU-ir cells expressed NeuN two weeks after BrdU injection (Snyder et al., 2009), whereas a separate study found in female rats less than 10% of BrdU-ir cells expressed NeuN at two weeks after BrdU injection (Brown et al., 2003). Sex differences in the maturation time course of new neurons may be due to sex differences in the neural activity of the hippocampal network. Maturation of adult-born neurons is accelerated by electrophysiological activity in the hippocampus (Piatti et al., 2011), and cFos expression in the dorsal CA3 of hippocampus is greater in males compared to females in response to a Morris water maze task and radial arm maze task (Yagi et al., 2017, 2016). However, in the same studies, females show greater activation of zif268 in the dorsal CA3 compared to males, which is inconsistent with the interpretation of greater activity in the hippocampus accounting for the sex differences in maturation timelines. However, it is possible that genetic or physiological differences between two sexes modulate the maturation time course of adult-born neurons.

2.4.2 Males had more neural stem cells than females, whereas females showed a regional difference with more neural stem cells in the ventral, compared to dorsal, dentate gyrus

In the present study, males had a greater density of Sox2-ir cells in the dorsal DG compared to females. We also found that females had a greater density of Sox2-ir cells in the ventral compared to the dorsal region, that was not observed in males. To our knowledge, neither of these findings have been reported previously. These findings suggest that within females, there is more chance of maintaining pluripotency in the ventral compared to the dorsal DG. How this might be reflected in sex differences in the functions attributed to the dorsal versus ventral hippocampus remains to be determined. However, there are some intriguing possibilities as

males generally show higher scores in spatial learning (Jonasson, 2005; Voyer et al., 2017), whereas females show different stress reactions compared to males (Young and Korszun, 2010). Indeed, one study has shown that classical conditioning using shock as the unconditioned stimulus, did increase neurogenesis in the ventral DG of females but not males (Dalla et al., 2009). Our results emphasize the importance of further investigation of sex differences in the preservation of neural stem cells in the hippocampus is a potential treatment (Briley et al., 2016).

2.4.3 The neural progenitor cell-type composition changes after mitosis with sex-dependent manner

Consistent with past studies, we found similar percentages of Sox2-ir cells and DCX-ir cells in the progenitor proliferating pool in male rodents (Nickell et al., 2017; Sibbe et al., 2015). However, we found that females had a greater increase in the percentages of DCX-ir cells between two and 24 hours after mitosis (greater percentage of BrdU/DCX-ir at 24h compared to 2h) whereas males did not exhibit any significant change from two hours to 24 hours. This finding suggests that the neural progenitor cell-type composition within the actively dividing pool in females changes after each cell division more so than in males. It also suggests that early on in division, the daughter cells proceed more rapidly through the neuronal cell lineage in females compared to males. This finding may in part explain the ability of females to compensate for the lower levels of proliferation of new cells to end up with a similar number of new neurons at two weeks compared to males. More studies are needed to examine sex differences in the timeline and mechanism of the transition of proliferating progenitors to new neurons for a comprehensive understanding of the regulation of neural progenitor cell pool in males and females.

2.4.4 Neurogenesis in males has a different trajectory compared to females

The present study found that males, but not females, showed substantial changes in the density of BrdU-ir cells across timepoints with an early increase from 24 hours to one week followed by a substantial decrease from one to two weeks. The decrease was notable such that despite the fact that males showed greater density of one week old BrdU-ir cells than females, but there was no sex difference in density of older (two-three week) old BrdU-ir cells. Our findings are consistent with previous studies that demonstrating the same trajectory in male Sprague Dawley rats (Cameron et al., 1993; Snyder et al., 2012, 2009) and no significant sex difference in the amount of two week or three week old BrdU-ir cells in cage controls (Tanapat et al., 1999; Barha et al., 2011; Chow et al., 2013 but see Lee et al., 2014). Collectively these results suggest that males and females regulate adult neurogenesis differently as males produce more new cells and show greater attrition of these new cells, whereas females produce fewer new cells but these cells are preserved across maturation. These findings may explain why spatial learning and or estrogens given during the first week of new neuron development increases the survival of new neurons in males, but not in females (Chow et al., 2013; Epp et al., 2007; Ormerod et al., 2004; Yagi et al., 2016). Taken together, these results suggest that spatial training between one week and two weeks after production of new neurons can prevent the attrition of adult-born neurons in males but perhaps not in females.

2.4.5 Males, compared to females, had greater cell proliferation in the dentate gyrus

Males had a greater density of Ki67-ir cells in the DG compared to females, consistent with findings in meadow voles (Galea and McEwen, 1999). In contrast a number of other studies have not found sex differences in cell proliferation in the DG (Barha et al., 2011; Brummelte and

Galea, 2010; Lagace et al., 2007; Spritzer et al., 2017). However, these inconsistences may be related to estrous cycle, as only proestrous females show greater cell proliferation than male rats (Tanapat et al., 1999), although this effect has not always been noted (Lagace et al., 2007). None of the females in the Ki67 analysis were in proestrus and thus, we would expect lower levels of cell proliferation in these females. Consistent with our Ki67 results we also see increased BrdU-ir cells at 2h in males compared to females, but no differences at 24h, which likely has to do with the population that Ki67 labels versus the pulsatile BrdU (Kee et al., 2002).

2.5 Conclusion

In the present study, sex differences are noted in the neural stem cell population, cell proliferation, maturation rate and the attrition rate of adult-born neurons in the hippocampus. Males had a higher density of dorsal neural stem cells and cell proliferation compared to females. However, males showed reductions across time in the density of adult-born dentate granule cells, whereas females showed no appreciable reduction. Furthermore, adult-born new neurons mature faster in males compared to females. The trajectory of new neuron survival is dramatically different in males compared to females suggesting that the ability to influence neurogenesis within each sex may be due to the existing differences in timing and/or maturation of new neurons. Therefore, future studies manipulating adult neurogenesis should consider the sex differences in the trajectory and the maturation time course of adult-born new neurons as the intervention can bear very different outcomes between the two sexes. Future studies should also target mechanisms of these sex differences in adult neurogenesis as there are likely multiple factors involved that could profoundly affect these sex differences such as genetic (four core genotypes; 66), epigenetic (Sase et al., 2019) and mitochondrial functions (Biala et al., 2011) that differ between the sexes. These findings have profound implications for our understanding of

adult neurogenesis in the DG, the use of therapeutics that modulate neurogenesis in the general population and underscore the need to include both sexes in research on hippocampal neurogenesis.

Chapter 3: Sex differences in contextual pattern separation, neurogenesis, and functional connectivity within the limbic system[2]

3.1 Introduction

Females are more likely to present with anxiety disorders such as post-traumatic stress disorder (PTSD) compared to males (Kessler et al., 2012, 1995), disorders which are associated with disrupted hippocampal integrity (Campbell et al., 2004; O'Doherty et al., 2015). The hippocampus plays important roles for pattern separation and pattern completion (Marr, 1971; Yassa and Stark, 2011). Pattern separation is a major component of episodic memory, which refers to the process of forming distinct representations of similar inputs during memory encoding (Yassa and Stark, 2011). Impairments in pattern separation are involved in overgeneralization of fear memory among patients with PTSD (Lange et al., 2017; O'Doherty et al., 2015).

Adult hippocampal neurogenesis is required for pattern separation and for stress resilience, as rodents with ablation of adult neurogenesis show impairments during pattern separation tasks and reduced stress resilience (Anacker et al., 2018; Clelland et al., 2009). Sex differences have been noted in the ability for pattern separation and the neurogenic response to pattern separation (Yagi et al., 2016). But curiously, these sex differences can show either a male advantage or female advantages in context discrimination tasks in rodents (Day et al., 2016; Foilb et al., 2018) and in emotional episodic tasks in humans (Asperholm et al., 2019; Filippi et al., 2013; Reber and Tranel, 2017). These differences in finding may be due to the availability of allocentric cues and egocentric cues in the context, as there are sex differences in preferential cue

[2] Yagi, S., Lee, A., Truter, N., Galea, L.A.M. (2022). Sex differences in contextual pattern separation, neurogenesis, and functional connectivity within the limbic system. Biology of Sex Differences, 13(42): s13293-022-00450-2.

and strategy use in both humans (Galea and Kimura, 1993) and rodents (Williams et al., 1990). Curiously, new neurons are required for pattern separation in both males and females (Clelland et al., 2009; Nakashiba et al., 2008), but pattern separation using the delayed non-match to sample radial arm maze, increased hippocampal neurogenesis in male but not female rats (Yagi et al., 2016). Although no sex differences were observed in activation of new neurons in response to spatial learning (Chow et al., 2013; Yagi et al., 2016), to our knowledge no studies have examined activation of new neurons in response to fear memory after a contextual pattern separation task between the sexes. Given that there are sex differences in the timing of maturation of new neurons in the hippocampus (Yagi et al., 2020: Chapter 2) it is important to determine whether different ages of neurons are active in response to fear memory after a contextual pattern separation task.

Studies demonstrate that hippocampus-amygdala-frontal cortex connectivity plays a critical role for long-term fear memory in humans (Hermans et al., 2017; Shvil et al., 2013) and there are sex differences in the resting-state functional connectivity within this circuit (Engman et al., 2016). In rodents, functional connectivity has been investigated via immediate early gene (IEG) mapping (Tanimizu et al., 2017; Wheeler et al., 2013). IEGs such as *zif268* are genes that are rapidly induced in response to neuronal stimulation and IEG proteins play an important role in neural plasticity and memory (Guzowski et al., 2001, 2000; Jones et al., 2001). Brain-wide IEG imaging in rodents can detect coordinated activation with high spatial resolution, which is useful to describe functional connectivity (Wheeler et al., 2013). New neurons contribute to contextual fear memory (Huckleberry et al., 2018), with younger new neurons more likely to play a critical role for pattern separation (Clelland et al., 2009; Nakashiba et al., 2018). However, there are no studies, to our knowledge, examining possible sex differences in patterns of

activation with new neurons of different ages. Furthermore, it remains to be determined whether new neurons in the dentate gyrus (DG) are activated in a coordinated fashion with other brain regions, and whether sex modulates the functional connectivity of adult-born neurons during recall of fear memory.

Therefore, we examined sex differences in contextual pattern separation and functional connectivity among 16 different limbic and reward regions during fear memory retrieval. A fear conditioning context discrimination task was used to assess sex differences in the ability to discriminate between two contexts in male and female rats. As younger new neurons contribute to pattern separation more so than older new neurons (Nakashiba et al., 2018), and there are sex differences in the timing of maturation of new neurons (Yagi et al., 2020: Chapter 2), we capitalized on different methods to examine the activity of 2-week, 3-week or 4-week old new neurons in response to fear memory. We used two different thymidine analogues that can be used in concert, along with an endogenous marker of immature neurons, to understand how different ages of new neurons responded to fear memory retrieval. Furthermore, we examined the activation of these different ages of new neurons in combination with IEG imaging and examined the coordinated neuronal activation of adult-born dentate granular cells (DGC)s with other brain regions. We hypothesized that there would be sex differences in context discrimination and activity of new neurons dependent on age of the new neuron. Furthermore, we also predicted that males and females would show distinct patterns of coordinated neuronal activation of different brain regions (hippocampus, amygdala, frontal cortex and striatum) during fear memory retrieval and that new neurons would show disparate patterns of functional connectivity between the sexes.

3.2 Methods

3.2.1 Subjects

Sixteen 8-week-old Sprague Dawley rats (males: n = 8; females: n = 8) were purchased from Charles River Canada (St-Constant, QC, Canada). Rats were pair-housed in opaque polysulfone bins (432 mm × 264 mm × 324 mm) with paper towels, a single polycarbonate hut, virgin hardwood chip bedding, and free access to food and water. Males and females were housed in separate colony rooms that were maintained under a 12:12-h light/dark cycle (lights on at 07:00 h). All animals were handled every day for two minutes beginning one week after arrival for two weeks. All experiments were carried out in accordance with the 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.

3.2.2 Apparatus

Behavioral testing for all experiments was conducted in four operant chambers (30.5 × 24 × 21 cm; Med-Associates, St Albans, VT) enclosed in sound-attenuating boxes. The boxes were equipped with a fan to provide ventilation and to mask extraneous noise. All behaviors were monitored and recorded by a single video camera mounted on the ceiling of each box. The chambers were equipped with a single 100-mA house light located in the top center of a wall and the chamber floor consisted of 23 metal grid bars (0.4 cm in diameter) that ran parallel to the shorter wall of the chamber, which connected to a shock generator. Two chambers had wide vertical black (18 mm width) and white (12 mm width) stripe patterns on the walls and wiped with vinegar before and after each animal. The other two chambers had narrow vertical black (12 mm width) and white (12 mm width) stripe patterns on the walls and wiped with 70%

isopropanol before and after each animal (see Fig. 3.1). All the chambers were connected to a computer through a digital interface that recorded all experimental settings.

3.2.3 Experimental timeline

Subjects received one injection of 5-chloro-2'-deoxyuridine (CldU:171 mg/kg; intraperitoneal (i.p.), MP Biomedicals, Santa Ana, CA, USA) on Experimental Day 1 and one injection of 5-iodo-2'-deoxyuridine (IdU: 56.75 mg/kg; i.p., Cayman Chemical, Ann Arbor, MI, USA) on Experimental Day 8, thus ensuring that we examined 3 week old and 4 week old cells, respectively. A previous study demonstrates that adult-born neurons reach the full maturation four weeks after BrdU injection in rats (Snyder et al., 2009), the present study examined zif268 expression of 4-week-old neurons as activation of fully-matured neurons. Thymidine analogs, IdU and CldU, incorporate into DNA during synthesis phase of cell proliferation, which can be distinguished from one another using respective antibodies (Kee et al., 2002; Leuner et al., 2009; Llorens-Martín and Trejo, 2011; Miller et al., 2018). Subjects were tested in the contextual pattern separation task (modified from (Mchugh et al., 2007)) for 12 days (Experimental Days 16--28, which are referred to as Trial Days 1-12), followed by a day of activation test trial that is described below (Experimental Day 29; see Fig. 3.1 A).

3.2.4 Behavioral testing for contextual pattern separation

Subjects were exposed daily for five minutes each to two different contexts (4-5 hours interval between contexts), a shock-paired context (Context A) and a neutral context (Context A'), for a total of 12 days. The contexts for Context A trials and Context A' trials were counterbalanced across subjects and remained the same for each subject throughout the entire experiment. During the shock-paired trial in Context A, subjects were allowed to explore the chamber for three minutes followed by three one-second foot shocks (0.6 mA) with 30 second

intervals between each shock. The subjects returned to their home cage one minute after the third shock. During the neural trial in Context A', the subjects explored a different context from Context A for five minutes without receiving a foot shock and returned to their home cage. The order of two contexts that subjects were exposed each day for the first six days followed AA' - A'A - A'A - AA' - AA' - A'A design, and the order was reversed for the remaining days (Mchugh et al., 2007: see Fig. 3.1A).

The duration of freezing during the first three minutes of each trial (prior to any shocks) was examined as the conditioned fear response, and the percentage of freezing was calculated by dividing the duration of freezing by 180 seconds. A discrimination index (DI) was calculated with the following formula on the last two days of training:

$$\mathrm{DI} = \frac{(\text{freezing time in Context A} - \text{freezing time in Context A'})}{(\text{freezing time in Context A} + \text{freezing time in Context A'})}.$$

As a previous study found sex differences in darting, an active fear response, in a cued fear conditioning task (Gruene et al., 2015), darting behavior was also recorded.

3.2.5 Activation trial and perfusion

On the day after Training Day 12, the Activation Test Trial was conducted to examine fear memory. Subjects were exposed to the Context A for five minutes without a foot shock and returned to their home cage. Video recordings were analyzed for active fear behavior (darting), passive fear behavior (freezing), or other behaviors (rearing, grooming and non-specific behaviors; see supplemental). However, no darting in our paradigm was observed. Ninety minutes after the Activation trial, subjects were administered an overdose of sodium pentobarbitol (500 mg/kg, i.p.) and perfused transcardially with 60 mL of 0.9% saline followed by 120 mL of 4% paraformaldehyde (Sigma-Aldrich).

3.2.6 Tissue processing

Extracted brains were postfixed in 4% paraformaldehyde overnight, then transferred to 30% sucrose (Fisher Scientific, Ottawa, ON, Canada) solution for cryoprotection and remained in the solution until sectioning. Brains were sliced into 30-µm coronal sections using a Leica SM2000R microtome (Richmond Hill, ON, Canada). Sections were collected in series of 10 throughout the entire rostral-caudal extent of the forebrain (Bregma 5.64 to -7.56 mm) and stored in antifreeze solution consisting of ethylene glycol, glycerol, and 0.1 M PBS at -20°C.

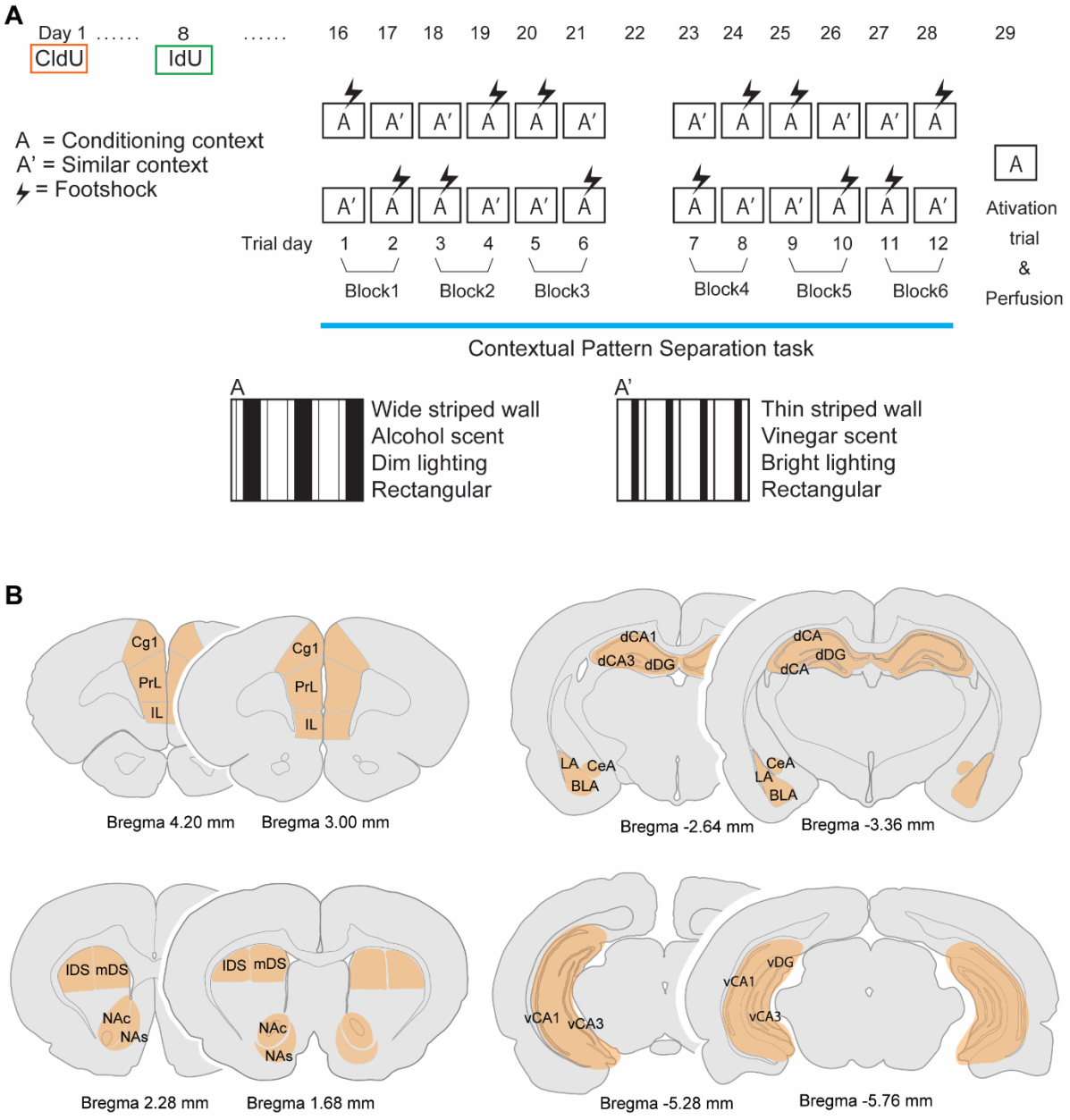


Fig. 3.1. Experimental design. (A) Schematic illustration of experimental timeline: Subjects received one intraperitoneal injection of 5-chloro-2'-deoxyuridine on Experimental Day 1 and one intraperitoneal injection of 5-iodo-2'-deoxyuridine on Experimental Day 8. Then, subjects were tested in the contextual pattern separation task for a total of 12 days (Experimental Day 16-28), followed by an activation trial in which the rats were placed in the context previously paired with shock but received no shock (Experimental Day 29). During the contextual pattern separation task, subjects were exposed to two different contexts each day; context A a shock-paired context (context paired with foot shocks) and context A' a neutral context (context with no foot-shock). (B) Brain regions that were examined for functional connectivity using zif268. ACC: cingulate cortex (Cg1); PrL: prelimbic cortex; IL: infralimbic cortex; lDS: lateral dorsal striatum ; mDS : medial dorsal striatum ; NAc : nucleus accumbens core; NAs: nucleus accumbens shell; LA: lateral amygdala; BLA: basolateral amygdala; CeA: central amygdala; dDG: dorsal dentate gyrus; vDG: ventral dentate gyrus; dCA1: dorsal cornu ammonis 1; vCA1: ventral cornu ammonis 1; dCA3: dorsal cornu ammonis 3; vCA3: ventral cornu ammonis 3.

3.2.7 Immunohistochemistry

Brain tissue was double-stained for the immature neuronal protein, doublecortin (DCX), and the immediate early gene, zif268 (Fig. 3.2 A). A majority (70% or more) of adult-born granule cells express DCX within 24 hours after mitosis for up to two weeks, with maximal expression at 4 days after mitosis, and that DCX expression is rapidly reduced three weeks after mitosis (less than 20%) in both male and female rats (Brown et al., 2003; Snyder et al., 2009; Yagi et al., 2020: Chapter 2). Therefore, we used DCX to examine a cell population of new neurons that were larger 2 weeks old or younger. In addition, tissue was triple-stained for IdU, CldU, and zif268 to examine neural activation of 3-week-old (IdU) cells and 4-week-old (CldU) cells in the dentate gyrus (Fig. 3.2 B and 3.2 C; see supplemental Table 3.1 and 3.2 for a list of antibodies and reagents used).

Doublecortin/zif268 double labeling

Free-floating sections were prewashed three times for 10 minutes with 0.1 M tris buffer saline (TBS; Sigma-Aldrich, Oakville, ON, Canada). Sections were then incubated in a primary

antibody solution containing 1:500 rabbit anti-zif268 (Santa Cruz Biotechnology, Dallas, TX, USA), 1:500 goat anti-doublecortin (Santa Cruz Biotechnology, Dallas, TX, USA) 0.3% Triton-X (Sigma-Aldrich) and 3% normal donkey serum (NDS; MilliporeSigma, Burlington, MA, USA) in 0.1 M TBS for 24 hours at 4 °C. Sections were washed three times for 10 minutes in TBS and a further incubation of sections commenced in a secondary antibody solution containing 1:500 donkey anti-rabbit ALEXA 594 (Invitrogen, Burlington, ON, Canada), 1:500 donkey anti-goat ALEXA 488 (Invitrogen, Burlington, ON, Canada), 3% NDS and 0.3% Triton-X in 0.1 M TBS for 24 hours at 4 °C. Following three final rinses with TBS, the sections were mounted onto microscope slides and cover-slipped with PVA DABCO.

IdU/CldU/zif268 triple labeling

Two different thymidine analogues (CldU and IdU) were visualized with CldU-specific (rat monoclonal, clone BU1/75) and IdU-specific (mouse monoclonal, clone B44) antibodies (Podgorny et al., 2018), coupled with labelling using the immediate early gene, zif268 antibody (rabbit polyclonal). Briefly our protocol was as follows: free-floating sections were prewashed three times for 10 minutes with 0.1 M TBS. Sections were then incubated in a primary antibody solution containing 1:500 rabbit anti-zif268 (Santa Cruz Biotechnology, Dallas, TX, USA), 0.3% Triton-X (Sigma-Aldrich) and 3% NDS in 0.1 M TBS for 24 hours at 4 °C. Next, sections were incubated in a secondary antibody solution containing 1:250 donkey anti-rabbit ALEXA 647 (Invitrogen, Burlington, ON, Canada), 0.3% Triton-X, and 3% NDS in 0.1 M TBS, for 18 hours at 4 °C. After being rinsed three times for 10 minutes with TBS, sections were washed with 4% paraformaldehyde for 10 minutes, and rinsed twice in 0.9% NaCl for 10 minutes, followed by incubation in 2N HCl (Fisher Scientific, Waltham, Massachusetts, USA) for 30 minutes at 37 °C. Sections were then rinsed three times in TBS for 10 minutes each and incubated in a CldU

primary antibody solution consisting of 1:1000 rat anti-BrdU (BU1/75; Abcam; Toronto, ON, Canada), 3% NDS, and 0.3% Triton-X in 0.1 M TBS for 24 hours at 4 °C. Sections were then incubated in an IdU primary antibody solution consisting of 1:500 mouse anti-BrdU (B44; BD Biosciences, San Jose, CA, USA), 0.3% NDS, and 0.3% Triton-X in 0.1 M TBS for 24 hours at 4 °C. Sections were then washed twice for 10 minutes each in a high stringency wash solution consisting of 32mM tris buffer, 50mM NaCl and 0.5% tween (pH 8.0) at 37 °C. Following three washes in TBS, sections were incubated in a secondary antibody solution containing 1:500 donkey anti-rat ALEXA 594 (Invitrogen, Burlington, ON, Canada), 1:500 donkey anti-mouse ALEXA 488 (Invitrogen, Burlington, ON, Canada), 3% NDS and 0.3% Triton-X in 0.1 M TBS for 24 hours at 4 °C. Following three final rinses with TBS, the sections were mounted onto microscope slides and cover-slipped with PVA DABCO.

3.2.8 Cell counting

All counting was conducted by an experimenter blind to the group assignment of each animal using an Olympus FV1000 confocal microscope and/or Zeiss Axio Scan.Z1 (Carl Zeiss Microscopy, Thornwood, NY, USA). Density of immunoreactive cells was calculated by dividing the total immunoreactive (ir) cells by volume (mm^3) of the corresponding region. Volume estimates were calculated by multiplying the summed areas by thickness of sections (0.03 mm, using Cavalieri's principle (Gundersen and Jensen, 1987)). Area measurements for the region of interest were obtained using digitized images on Zen 3.0 software (blue edition; Carl Zeiss Microscopy, Thornwood, NY, USA).

Brain regions were defined according to a standard rat brain atlas (Paxinos and Watson, 2004). Location of immunoreactive cells in the hippocampus was examined in the dorsal or ventral dentate gyrus using the criterion defined by Banasr et al. (2006) with sections 7.20-4.48mm from the interaural line (Bregma -1.80 to -4.52mm) defined as dorsal and sections 4.48-

2.20 mm from the interaural line (Bregma -4.52 to -6.80mm) as ventral (Banasr et al., 2006). Cells were counted separately in each region because the different regions are associated with different functions (reviewed inFanselow and Dong, 2010) and different maturation timelines of neurogenesis (Snyder et al., 2012; Yagi et al., 2020: Chapter 2). The dorsal hippocampus is associated with spatial reference memory, whereas the ventral hippocampus is associated with working memory, stress and anxiety (Kjelstrup et al., 2002; Moser et al., 1993).

IdU and CldU counting

Thymidine analogue immunoreactive (IdU-ir and CldU-ir) cells were counted under a 40x objective lens using Olympus FV1000 confocal microscopy. Every 20th section of the granule cell layer (GCL) that includes the subgranular zone (SGZ) was counted. The SGZ was defined as a narrow layer of cells within 30μm (equivalent to the width of three granule cell bodies) away from the innermost edge of GCL (Redila and Christie, 2006).

The percentages of IdU/zif268-ir and CldU/zif268-ir cells were obtained by randomly selecting 200 IdU-ir or 200 CldU-ir cells (100 cells from dorsal and 100 cells from ventral DG) and calculating the percentage of cells that were double-labelled with zif268 under a 40x objective lens using Olympus FV1000 confocal microscopy. Density of DCX/zif268-ir, IdU/zif268-ir or CldU/zif268-ir cells were calculated by multiplying the density of IdU-ir or CldU-ir cells by the percentage of double-labelled cells.

Doublecortin counting

Doublecortin immunoreactive (DCX-ir) cells were counted on digitized images on Zen 3.0 software (blue edition). Photomicrographs were taken from four dorsal and four ventral hippocampi using a ZEISS Axio Scan.Z1 slidescanner with a 40x objective lens. The percentages of DCX/zif268-ir cells were obtained by randomly selecting 200 DCX-ir cells (100

cells from dorsal and 100 cells from ventral DG) and calculating the percentage of cells that were double-labelled with zif268 on Zen 3.0 software. Density of DCX/zif268-ir cells were calculated by multiplying the density of DCX-ir cells by the percentage of DCX/zif268-ir cells.

Estrous Cycle Determination. Vaginal cells suspended in water 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 (Hubscher et al., 2005).

zif268 counting

Photomicrographs of coronal sections containing the frontal cortex, amygdala, hippocampus, striatum, nucleus accumbens were obtained from ZEISS Axio Scan.Z1 slidescanner with a 20x objective lens (four images from each region of interest: see Fig. 3.1 B). Zif268-ir cells in the infralimbic cortex (IL), prelimbic cortex (PrL), anterior cingulate cortex (ACC: Cg1), medial part of dorsal striatum (mDS), lateral part of dorsal striatum (lDS), nucleus accumbens core (NAc), nucleus accumbens shell (NAs), central nucleus of amygdala (CeA), basaolateral nucleus of the amygdala (BLA), lateral nucleus of the amygdala (LA), dorsal(d) hippocampus (dCA1, dCA3, dDG) and ventral(v) hippocampus (vCA1, vCA3, vDG) were counted automatically from the digitized images using a code developed by JEJS (see Yagi et al., 2020: Chapter 2 for details) on MATLAB (MathWorks; Natick, Massachusetts, USA).

3.2.9 Estrous-cycle determination

Daily lavage samples were taken from all females after behavioral procedures (see methods section in Chapter 2). Estrous cycle determination was done as the estrous cycle stage can affect long term potentiation and IEG expression in the hippocampus (Warren et al., 1995;

Yagi et al., 2017). There was one female in the proestrous stage during Activation Trial in the present study. Thus, estrous cycle phase was used as a covariate for all analyses.

3.2.10 Statistical analyses

All analyses were conducted using Statistica (Statsoft Tulsa, OK) unless otherwise stated, and significance level was set at $\alpha = 0.05$. Repeated-measures or factorial analysis of variance (ANOVA), with sex (male and female) as between-subject variables were conducted on our variables of interest (freezing, zif268 expression). Post-hoc tests used the Newman-Keuls procedure. A priori comparisons were subjected to Bonferroni corrections. Effect sizes are given with Cohen's d or partial η^2. Pearson product-moment calculations and principal component analyses on zif268 expression across regions were also performed.

The percentage of freezing during the Context A trials and Context A' trials in the contextual pattern separation task was analyzed using repeated-measures analysis of variance (ANOVA), with sex (male and female) as between-subject variables and context (Context A and Context A') and trial day (1^{st} – 12^{th} day) as within-subject factors. The discrimination index of the last trial block and percentage of freezing during the activation trial were analyzed using one-way ANOVA with sex as between-subject variable. The density of adult-born cells (DCX-ir, IdU-ir or CldU-ir cells) and those double-labelled with zif268 in the dentate gyrus were each analyzed using repeated-measures ANOVA with sex as between-subject variable and region (dorsal and ventral) as within-subject variable. The density of zif268-ir cells in each region (frontal cortex, dorsal striatum, nucleus accumbens, amygdala) was analyzed separately using repeated-measures ANOVA with sex as between-subject variables and subregions (frontal cortex: IL, PrL, ACC; dorsal striatum: lateral, medial; nucleus accumbens: core, shell; amygdala:

central, lateral, basal; hippocampus: dorsal and ventral CA1, CA3 and DG) as within-subject variables.

Pearson product-moment correlations between the percentage of freezing and the density of zif268-ir cells were calculated in the regions of interest. For functional connectivity, Pearson product-moment correlations were calculated with the density of zif268-ir cells between each brain region. To examine the functional connectivity of adult-born cells in the dentate gyrus with the other brain regions, correlations were also calculated between the density of IdU/zif268-ir, CldU/zif268-ir or DCX/zif268-ir cells and the density of zif268-ir cells in each region. Inter-regional correlations were compared between the two sexes (male and female) using the single-sided observed Fischer z-test statistic.

Principal component analyses were conducted to assess brain networks that explain variances of zif268-ir cell density in the regions of interest. PCA data analyses were conducted using Statistica and R (3.4.3) statistical analysis software with the "FactoMineR" package. Horn's parallel analysis was used to determine which component factors were retained for further analyses (Franklin et al., 1995). Horn's parallel analysis was conducted using R (3.4.3) statistical analysis software with the "psych" package. Following the PCA, repeated-measures ANOVA was conducted to analyze principal component scores for individual samples with sex (male, female) and factors (1^{st}, 2^{nd}, 3^{rd}) as the within-subject variable and sex (male, female) as the between-subject variable.

3.3 Results

3.3.1 Females, but not males, discriminated shock-paired contexts from neutral contexts

Male and female rats were exposed to 12 days of the contextual pattern separation task to examine the ability for discriminating a shock-paired context from a neutral context. Females exhibited a significantly greater percentage of freezing in the shock-paired context (Context A) than in the neutral context (Context A') on two days: Trial Day 9 ($p < 0.02$, Cohen's $d = 1.114$), and Trial Day 12 ($p < 0.0001$, Cohen's $d = 1.696$), whereas there were no significant differences between the contexts on any day in males (Fig. 3.2 A and 3.2 B; interaction effect of sex by day by context [$F(11, 154) = 2.25$, $p = 0.014$, $\eta_p^2 = 0.139$)]). There was also a significant interaction effect of day by context [$F(11, 154) = 6.26$, $p < 0.0001$, $\eta_p^2 = 0.309$] and a main effect of day [$F(11, 154) = 21.04$, $p < 0.0001$, $\eta_p^2 = 0.600$]. Consistent with these findings, the discrimination index (DI) of the last two days (Trial Day 11 and 12 or Block 6) indicated that females showed a greater DI compared to males [$F(1, 14) = 5.81$, $p = 0.030$, Cohen's $d = 1.205$; Fig. 3.2 C].

On the activation trial day, all subjects were exposed to the conditioning context A (on the day after the 12 training Trials) without any shock to assess fear memory. There was no significant sex difference in the percentage of freezing during the activation trial ($p = 0.932$, Cohen's $d = 0.045$; Fig. 3.2 D), indicating the memory strength for the shock-paired context was equivalent between the sexes, despite the greater discrimination learning in females. As noted earlier, no darting behavior was observed throughout the experiment.

3.3.2 There were more 4-week-old cells in the dorsal compared to the ventral dentate gyrus

IdU and CldU were injected three weeks and four weeks, respectively, before perfusion. The density of CldU-ir cells was greater in the dDG compared to vDG [main effect of region: $F(1, 11) = 6.50$, $p = 0.027$, Cohen's $d = 1.104$; see supplemental Fig. 3.1]. There were no other significant main or interaction effects on the density of DCX-ir, CldU-ir or IdU-ir cells (p's > 0.283).

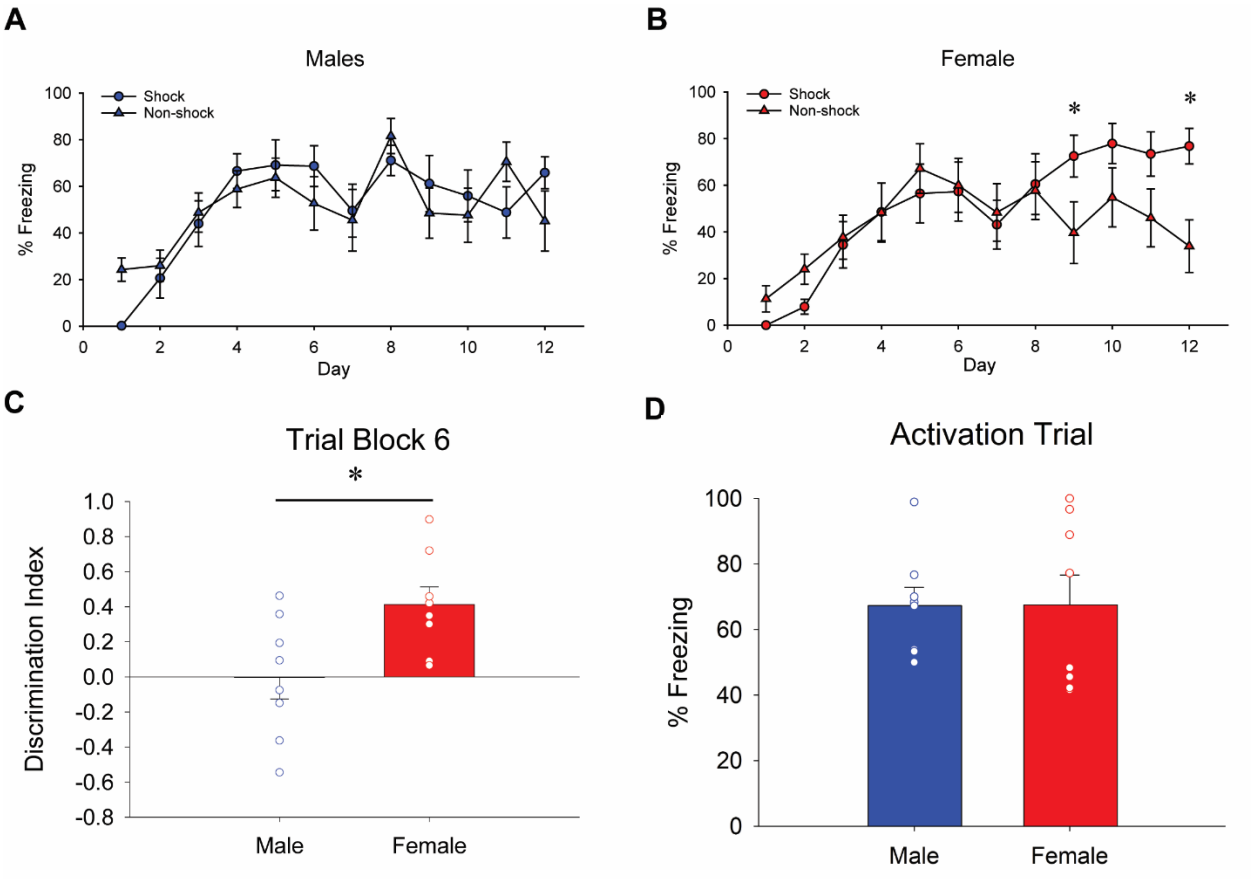


Fig. 3.2. Sex differences in contextual fear discrimination and Zif268 activation of dentate adult-born cells. (A-B) Mean (±SEM) percentage of freezing in Context A (shock) and Context A' (non-shock) in males (A) and females (B). (C) Mean (±SEM) discrimination index of the trial block 6 (Trial Day 11+12). (D) Mean (±SEM) percentage of freezing in males and females during the activation trial on Day 29. Females exhibited significantly greater percentage of freezing in the shock-paired context (Context A) than in the neutral context (Context A') on Trial Day 9 and Trial Day 12, whereas there was no significant difference in percentage of freezing between the two contexts in any days in males. Furthermore, females showed greater discrimination based on the index on the last trial block (Trial Day 11+12) compared to males. There was no significant sex difference in the percentage of freezing during the Activation Trial. * indicates $p < 0.05$.

3.3.3 Females had a greater percentage of IdU/zif268-ir cells in the dentate gyrus compared to males

The percentage of DCX-ir, IdU-ir, or CldU-ir cells that were double-labelled with zif268 was measured to examine neural activation of adult-born cells in the DG. Females, compared to males, had a greater percentage of IdU/zif268-ir cells in the DG [main effect of sex: $F(1, 12) = 4.59$, $p = 0.05$, Cohen's $d = 1.539$; Fig. 3.3 D and 3.3 E] but no other main or interaction effects for IdU/zif268-ir cells. Females, compared to males, had greater percentage of CldU/zif268-ir cells in the dDG ($p = 0.047$, Cohen's $d = 1.538$), whereas, males, compared to females, had greater percentage of CldU/zif268-ir cells in the vDG ($p = 0.015$, Cohen's $d = 1.317$) [interaction effect of region by sex: $F(1, 10) = 19.53$, $p = 0.001$, $\eta_p^2 = 0.661$; Fig. 3.3 D and 3.3 E]. There were no significant main effects (all p's > 0.46) for CldU/zif268-ir cells. There were no significant effects of DCX/zif268-ir cells (p's > 0.66).

Along with the percentage of double-labelled cells we also examined the density of DCX-ir, IdU-ir, and CldU-ir cells that were double-labelled with zif268. Females, compared to males, had greater density of CldU/zif268-ir cells in the dDG ($p = 0.033$), whereas males, compared to females, had greater density of CldU/zif268-ir cells in the vDG ($p = 0.023$) [interaction effect of region by sex: $F(1, 10) = 19.34$, $p = 0.001$, $\eta_p^2 = 0.659$; Fig. 3.3 F and 3.3 G]. There were no main or interaction effects for the density of IdU/zif268-ir cells or DCX/zif268-ir cells (all p's > 0.106).

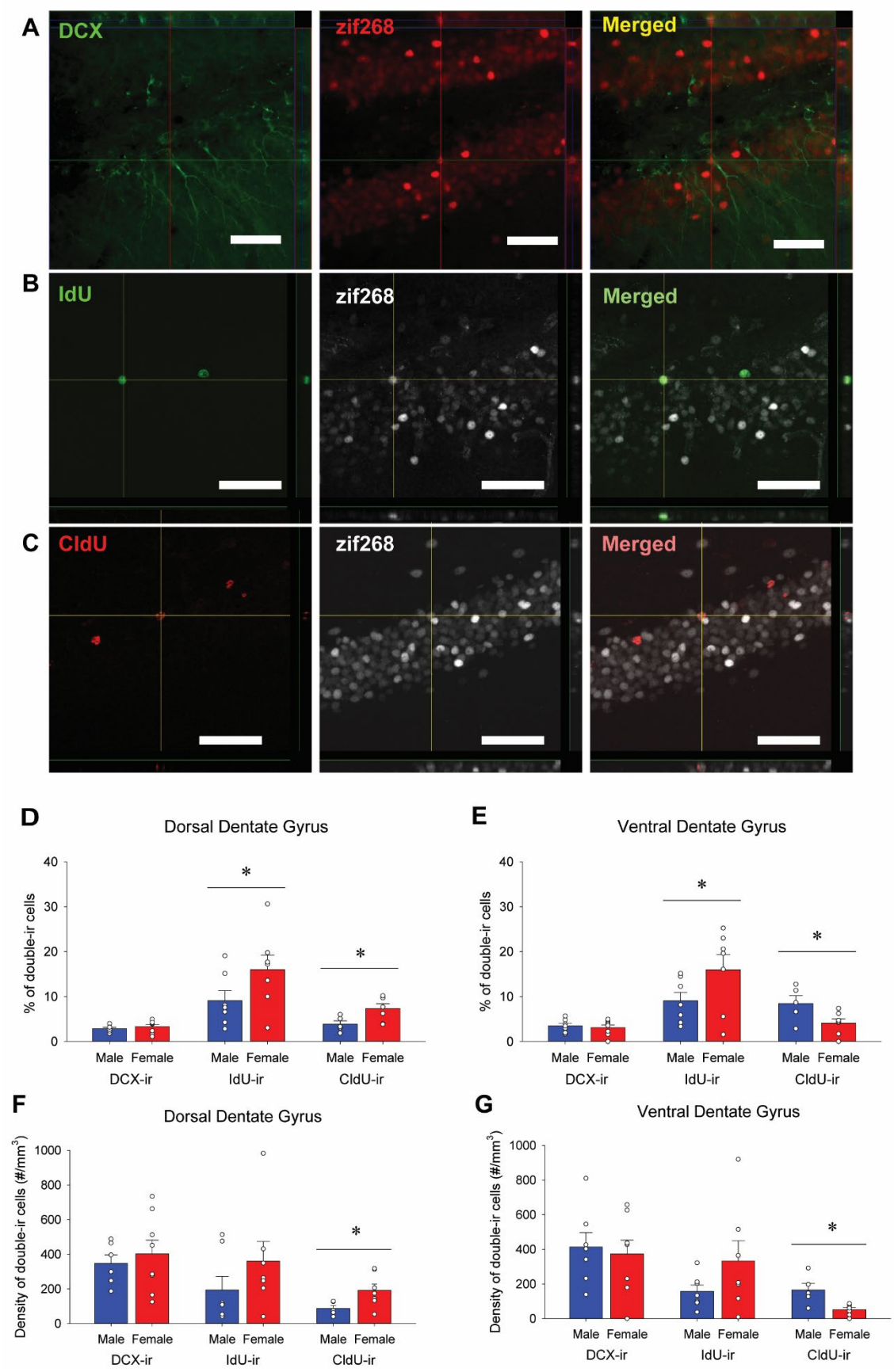

A
DCX
zif268
Merged
B
IdU
zif268
Merged
C
CldU
zif268
Merged
D
Dorsal Dentate Gyrus
% of double-ir cells
Male Female
DCX-ir
IdU-ir
CldU-ir
E
Ventral Dentate Gyrus
F
Dorsal Dentate Gyrus
Density of double-ir cells (#/mm3)
G
Ventral Dentate Gyrus

Fig. 3.3. Sex differences in zif268 activation of dentate adult-born cells. (A) Photomicrographs of doublecortin-immunoreactive (ir) cells (DCX-ir: green) and zif268-ir cells (red) were taken under Zeiss Axio Scan.Z1 with 40x objective lens. (B) Photomicrographs of IdU-ir cells (green) and zif268-ir cells (white) were taken under Olympus FV1000 confocal microscope with 40x objective lens. (C) Photomicrographs of CldU-ir cells (red) and zif268-ir (white) cells were taken under Olympus FV1000 confocal microscope with 40x objective lens. Scale bars indicate 50 μm. (D-E) Mean (±SEM) percentage of double-labelled cells in the dorsal (D) and ventral (E) dentate gyrus. Females, compared to males, had greater percentage of IdU/zif268-ir cells in the dorsal and ventral dentate gyrus, whereas there was a significant interaction effect of sex by region for the percentage of CldU/zif268-ir cells. (F-G) Mean (±SEM) density of double-labelled cells in the dorsal (F) and ventral (G) dentate gyrus. There was a significant interaction effect of sex by region for the percentage of CldU/zif268-ir cells. * indicates $p < 0.05$.

3.3.4 Females had greater zif268 immunoreactivity than males in the frontal cortex and dorsal CA1 region of the hippocampus in response to a shocked-paired context

The density of zif268-ir cells was measured to examine neural activation in subregions of the frontal cortex, dorsal striatum, nucleus accumbens, hippocampus and amygdala in response to exposure to the shocked-paired context. Females, compared to males, showed greater density of zif268-ir cells across the different regions of the frontal cortex [main effect of sex: $F(1, 12) = 10.14$, $p = 0.008$, $\eta_p^2 = 0.458$], as well there was greater density of zif268-ir cells in the ACC and PrL compared to IL [main effect of subregion: $F(2, 24) = 34.40$, $p < 0.001$, $\eta_p^2 = 0.741$; post-hoc: all p's < 0.001; Fig. 3.4 C].

Furthermore, females showed greater density of zif268-ir cells in the dorsal CA1 compared to males [$p < 0.001$, Cohen's $d = 2.957$; interaction effect of sex by subregion by dorsoventral axis: $F(2, 26) = 17.56$, $p < 0.001$; Fig. 3.4 D]. In both males and females, the density of zif268-ir cells in the dCA1 is greater than dDG and dCA3, and the density of zif268-ir cells in the vCA1 is greater than vCA3 (all p's < 0.01). There were also significant interaction effects of sex by subregion [$F(2, 26) = 10.36$, $p < 0.001$, $\eta_p^2 = 0.444$], sex by dorsoventral axis [$F(1, 13) = 10.82$, $p = 0.006$, $\eta_p^2 = 0.454$] and subregion by dorsoventral axis [$F(2, 26) = 1028.60$, $p < 0.001$, $\eta_p^2 = 0.988$], and main effects of sex [$F(1, 13) = 21.53$, $p < 0.001$, $\eta_p^2 = 0.624$], subregion [$F(2, 26) = 819.06$, $p < 0.001$, $\eta_p^2 = 0.984$] and dorsoventral axis [$F(1, 13) = 690.17$, $p < 0.001$, $\eta_p^2 = 0.982$].

There were no significant main or interaction effects involving sex in activation in the amygdala, striatum or the nucleus accumbens, but there were significant regional differences within these areas. The lDS had greater density of zif268-ir cells compared to the mDS [main effect of subregion: $F(1, 12) = 6.88$, $p = 0.022$, $\eta_p^2 = 0.364$; supplemental Fig. 3.2 A]. In the

amygdala, the LA had greater density of zif268-ir cells compared to the CeA ($p = 0.007$) and the BLA ($p < 0.001$), and the CeA had greater density of zif268-ir cells compared to the BLA ($p < 0.001$) [main effect of subregion: $F(2, 24) = 28.45$, $p < 0.001$, $\eta_p^2 = 0.703$; Supplemental Fig. 3.2 B]. In the nucleus accumbens, the density of zif268-ir cells was significantly greater in the shell compared to the core [main effect of subregion: $F(1, 12) = 22.22$, $p < 0.001$, $\eta_p^2 = 0.649$; Supplemental Fig. 3.2 C]. There were no significant main or interaction effects of sex for the density of zif268-ir cells in any of these regions ($P > 0.111$).

3.3.5 The density of zif268-ir cells in the dorsal CA1 was positively correlated with amount of freezing during memory recall in males, but not in females

Pearson product-moment correlations were calculated between the percentage of freezing during the activation trial and the density of zif268-ir cells in the 16 brain regions and the six different populations of adult-born cells (dorsal or ventral DCX/IdU/CldU co-expressing zif268; see Fig. 3.4 E). There was a significant positive correlation between the density of zif268-ir cells and the percentage of freezing in males in the dCA1 [$r (7) = 0.907$, $p = 0.005$; Fig. 3.4 F]. There were no other significant correlations between the density of zif268-ir cells and the percentage of freezing during the activation trial after Bonferroni corrections (p's > 0.037). Sex differences in the correlations were noted, with males having positive correlations and females having negative correlations, between the percentage of freezing and the density of zif268 cells in the dDG ($p = 0.041$), dCA1 ($p = 0.006$; Fig. 3.4 F), PrL ($p = 0.040$) and lDS ($p = 0.005$; Fig. 3.4 G). See Supplemental Results for details.

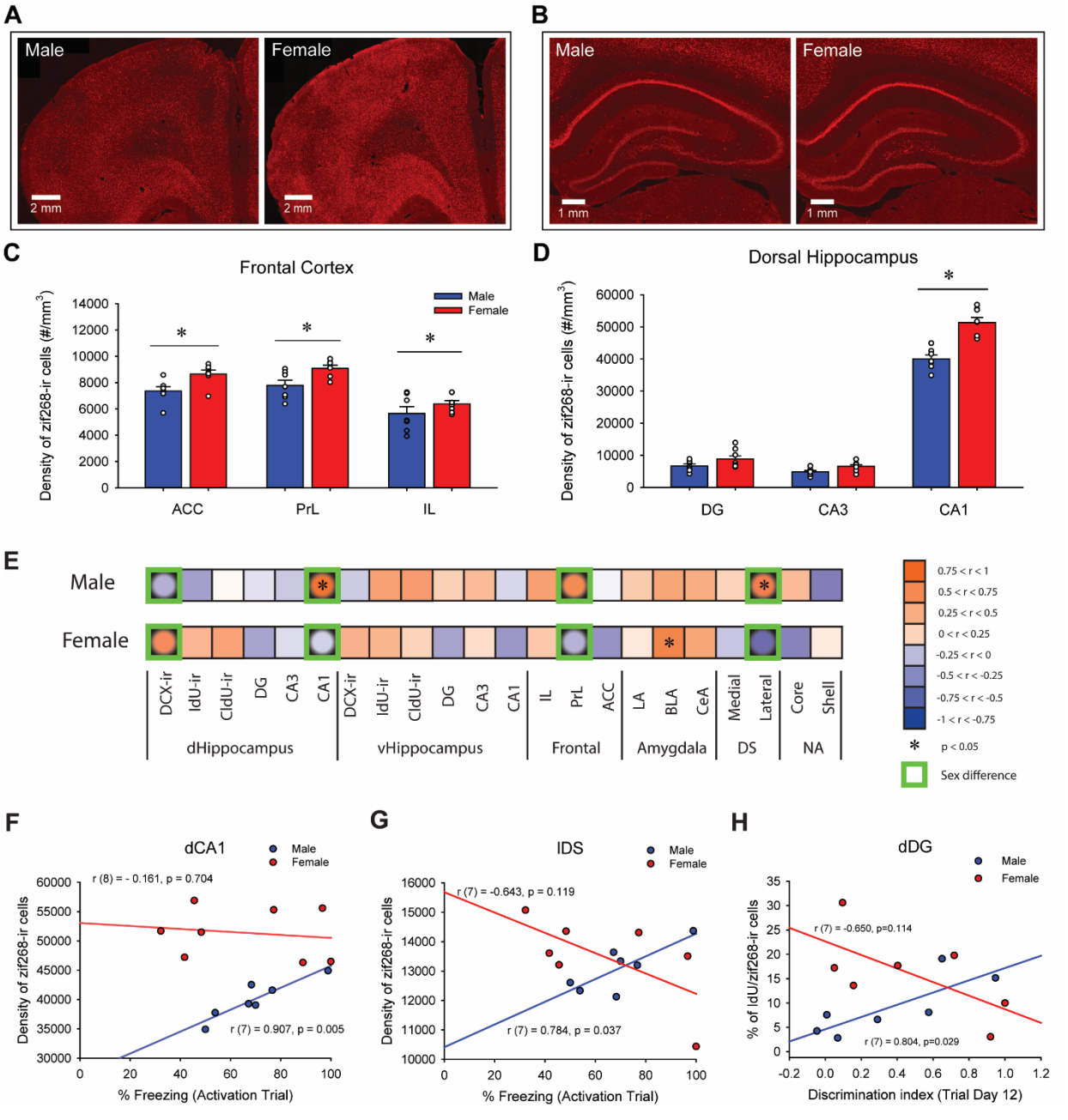

A
Male
Female
2 mm
2 mm
B
Male
Female
1 mm
1 mm
C
Frontal Cortex
Density of zif268-ir cells (#/mm3)
Male
Female
ACC
PrL
IL
D
Dorsal Hippocampus
Density of zif268-ir cells (#/mm3)
DG
CA3
CA1
E
Male
Female
DCX-ir
IdU-ir
CldU-ir
DG
CA3
CA1
dHippocampus
vHippocampus
IL
PrL
ACC
Frontal
LA
BLA
CeA
Amygdala
Medial
Lateral
DS
Core
Shell
NA
0.75 < r < 1
0.5 < r < 0.75
0.25 < r < 0.5
0 < r < 0.25
-0.25 < r < 0
-0.5 < r < -0.25
-0.75 < r < -0.5
-1 < r < -0.75
p < 0.05
Sex difference
F
dCA1
Male
Female
r (8) = - 0.161, p = 0.704
r (7) = 0.907, p = 0.005
Density of zif268-ir cells
% Freezing (Activation Trial)
G
IDS
Male
Female
r (7) = -0.643, p = 0.119
r (7) = 0.784, p = 0.037
Density of zif268-ir cells
% Freezing (Activation Trial)
H
dDG
Male
Female
r (7) = -0.650, p=0.114
r (7) = 0.804, p=0.029
% of IdU/zif268-ir cells
Discrimination index (Trial Day 12)

Fig. 3.4. Sex differences in zif268 activation in the brain, and correlations with fear-response. (A-B) Photomicrographs of zif268 immunoreactivity in the frontal cortex (A) and in the dorsal hippocampus (B) in male (left) and female rats (right). (C-D) Mean (±SEM) density of zif268-ir cells in the frontal cortex (C) and in the dorsal hippocampus (D). Females, compared to males, had greater density of zif268-ir cells in the anterior cingulate cortex (ACC), in the prelimbic cortex (PrL) and in the dorsal CA1. * indicates $p < 0.05$. (E) Heat maps generated based on correlations coefficients. Males and females had significant correlations of zif268-ir cell density in different brain regions with the percentage of freezing (* indicates correlations with $p < 0.05$). There were significant sex differences in the correlations between the percentage of freezing and the density of zif268-ir cells in four different brain regions (Green boxes indicates significant sex differences with $p < 0.05$). (F-G) Scatter plots for correlations between the percentage of freezing and the density of zif268-ir cells in the dorsal CA1 (dCA1) (F) or in the lateral dorsal striatum (lDS) (G), and correlations between the discrimination index on the last trial day and the percentage of IdU/zif268-ir cells in the dorsal dentate gyrus (H) in males (blue) and females (red).

3.3.6 Neural activation of adult-born cells in the dentate gyrus was associated with the ability for pattern separation in males but not females

Pearson product-moment correlations were calculated between the percentage of DCX, IdU or CldU-ir cells that were double-labelled with zif268 and the discrimination index (DI) on the last trial day. The percentage of IdU-ir cells that were double-labelled with zif268 in the dDG was significantly correlated with DI in males [$r(7) = 0.804$, $p = 0.029$], but not in females [$r(7) = -0.650$, $p = 0.114$; Fig. 3.4 H], which was significantly different between the sexes ($p = 0.004$). There were no other significant correlations between the percentage of DCX, IdU or CldU-ir cells that were double-labelled with zif268 and the DI (all p's > 0.127).

3.3.7 Males and females showed distinct patterns of significant inter-regional correlations of the density of zif268-ir cells

Pearson product-moment correlations were calculated with the density of zif268-ir cells between 16 brain regions and six different populations of adult-born cells (double-labelled with

zif268 and DCX, IdU, or CldU in the dorsal or ventral DG) to examine functional connectivity between these regions to activated new neurons of different ages (see Fig. 3.5A). As can be seen in Figure 3.5B and 3.5C there were mainly positive correlations between activation of new neurons of different ages within the hippocampus in males (19), with much fewer seen in females (4). In addition, there were more correlations of activated new neurons with regions outside the hippocampus in females (7, with only 2 significant to the amygdala) than in males (4 with none significant) (see Supplemental Table 3.3 for the detailed statistical data). The Fischer z-test statistic revealed significant sex differences in the 27 inter-regional correlations.

Inter-regional Correlations

In males, within the hippocampus, there were significant correlations between activation of new neurons and activation of subregions of the hippocampus between vDG IdU/zif268-ir cells and vCA3 zif268-ir cells [$r(7) = 0.801$, $p = 0.030$], between dDG CldU/zif268-ir cells and dDG zif268-ir cells [$r(5) = 0.9926$, $p = 0.001$], and between vDG CldU/zif268-ir cells and dCA3 zif268-ir cells [$r(5) = -0.915$, $p = 0.029$]. In females, within the hippocampus, there were significant correlations between the density of dDG IdU/zif268-ir cells and either the vDG DCX/zif268-ir cells [$r(7) = 0.848$, $p = 0.016$] or the dCA3 zif268-ir cells [$r(7) = -0.794$, $p = 0.033$] and between the density of dDG DCX/zif268-ir cells and the vDG zif268-ir cells [$r(8) = -0.745$, $p = 0.034$]. Within females there were also significant correlations between activated new neurons and brain regions outside of the hippocampus (that did not exist in males) between dDG DCX/zif268-ir cells and BLA zif268-ir cells [$r(7) = 0.860$, $p = 0.013$] and between vDG IdU/zif268-ir cells and LA zif268-ir cells [$r(6) = -0.936$, $p = 0.006$].

Correlations between the 15 different brain regions in males were between the BLA and LA [$r(7) = 0.922$, $p = 0.003$], between BLA and mDS [$r(7) = 0.789$, $p = 0.035$], between CeA

and LA [r(7) = 0.780, p = 0.039] between CeA and IL [r(7) = 0.779, p = 0.039], and between NAc and NAs [r(7) = 0.755, p = 0.050]. In females, between BLA and CeA [r(7) = 0.860, p = 0.013], between BLA and vDG [r(7) = -0.785, p = 0.037], between lDS and NAc [r(7) = 0.910, p = 0.004], between lDS and ACC [r(7) = 0.939, p = 0.002], between lDS and IL [r(7) = -0.760, p = 0.047], between ACC and NAc [r(7) = 0.953, p = 0.001], between PL and dCA1 [r(7) = 0.888, p = 0.008], between dDG and vDG [r(8) = 0.937, p = 0.001], between dDG and dCA1 [r(8) = 0.718, p = 0.045], and between dCA3 and dCA1 [r(8) = 0.790, p = 0.020].

Sex differences in inter-regional correlations

The Fischer z-test statistic revealed significant sex differences in correlations between activation of new neurons and activation of subregions of the hippocampus between the density of vDG IdU/zif268-ir cells and vDG DCX/zif268-ir cells [Z(13) = 1.652, p = 0.049], between dDG DCX/zif268-ir cells and vDG zif268-ir cells [Z (13) = 1.723, p = 0.042], vDG DCX/zif268-ir cells and dDG zif268-ir cells [Z(14) = 2.427, p = 0.008], vDG DCX/zif268-ir cells and vDG zif268-ir cells [Z(14) = 2.061, p = 0.020], vDG DCX/zif268-ir cells and vCA1 zif268-ir cells [Z(14) = 1.995, p = 0.023], vDG IdU/zif268-ir cells and vCA3 zif268-ir cells [Z(13) = 1.669, p = 0.048], and dDG CldU/zif268-ir cells and dDG zif268-ir cells [Z(11) = 3.276, p = 0.001]. In addition, there were also significant correlations between activated new neurons and brain regions outside of the hippocampus, between dDG IdU/zif268-ir cells and LA zif268-ir cells [Z(12) = 1.715, p = 0.043], vDG IdU/zif268-ir cells and LA zif268-ir cells [Z(12) = 2.873, p = 0.002].

Furthermore, there were significant sex differences in the correlations between the 15 different brain regions including between LA and BLA [Z(13) = 1.724, p = 0.042], BLA and NAc [Z(13) = 1.765, p = 0.039], lDS and NAc [Z(13) = 2.177, p = 0.015], mDS and NAs [Z(13)

= 1.701, p = 0.044], lDS and ACC [Z(13) = 2.696, p = 0.004], NAc and ACC [Z (13) = 2.089, p = 0.018], PL and IL [Z(13) = 2.184, p = 0.014], vDG and BLA [Z(13) = 2.115, p = 0.017], vDG and CeA [Z(13) = 2.133, p = 0.016], vDG and IL [Z(13) = 1.651, p = 0.049], vCA3 and IL [Z(13) = 1.907, p = 0.028], dCA1 and IL [Z (13) = 2.143, p = 0.016], dCA3 and dCA1 [Z (14) = 2.202, p = 0.014], CeA and vCA1 [z(13) = 1.748, p = 0.040], lDS and vCA1 [Z(13) = 1.712, p = 0.043], NAc and vCA1 [Z(13) = 1.967, p = 0.025], IL and vCA1 [Z(13) = 1.881, p = 0.030].

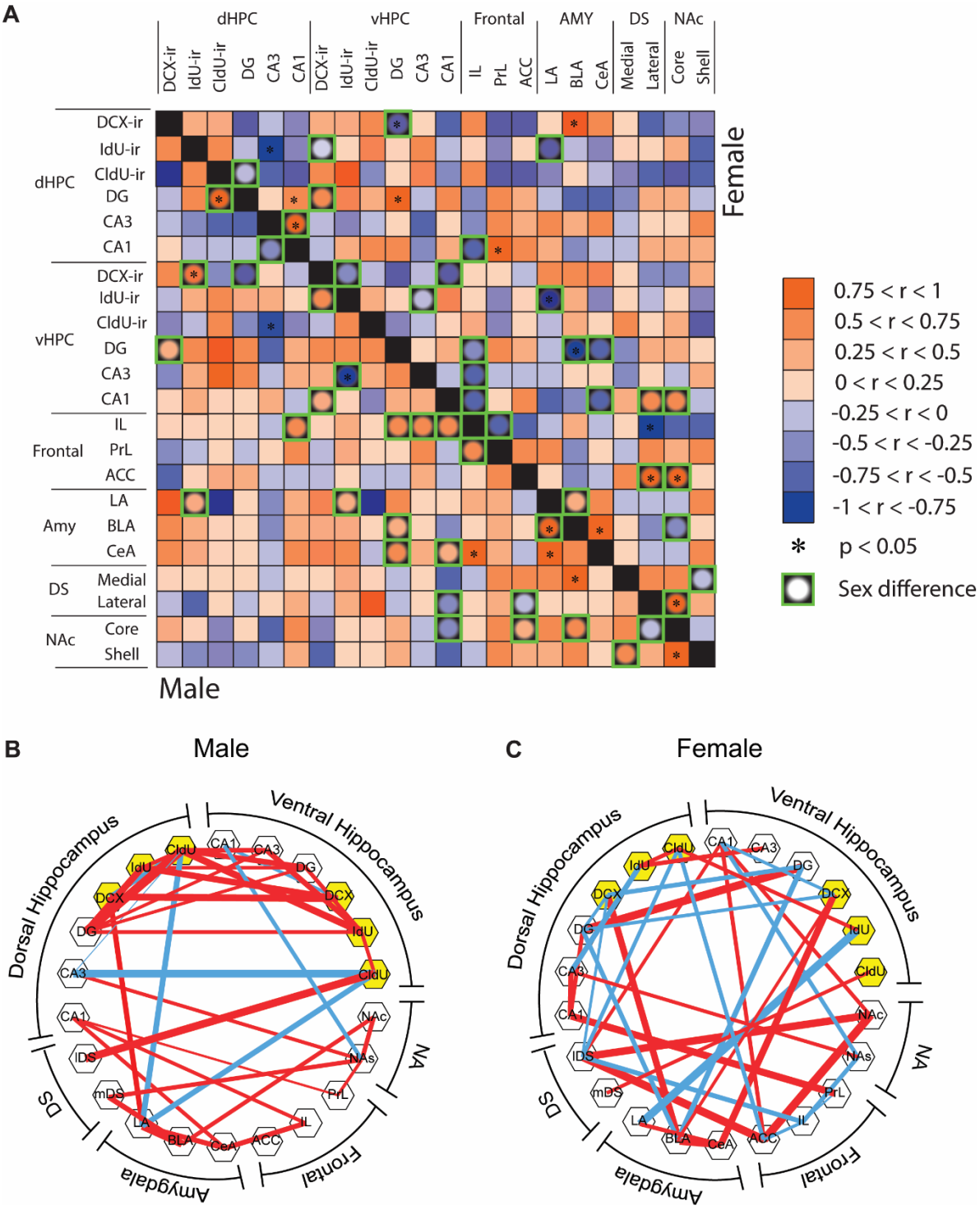

A
dHPC
vHPC
Frontal
AMY
DS
NAc
DCX-ir
IdU-ir
CldU-ir
DG
CA3
CA1
IL
PrL
ACC
LA
BLA
CeA
Medial
Lateral
Core
Shell
Female
Male
0.75 < r < 1
0.5 < r < 0.75
0.25 < r < 0.5
0 < r < 0.25
-0.25 < r < 0
-0.5 < r < -0.25
-0.75 < r < -0.5
-1 < r < -0.75
p < 0.05
Sex difference
B
Male
C
Female
Dorsal Hippocampus
Ventral Hippocampus
DS
NA
Amygdala
Frontal
CldU
IdU
DCX
NAc
NAs
IDS
mDS
PrL
IL
ACC
CeA
BLA

Fig. 3.5. Sex differences in inter-regional correlations of zif268-ir cell density. (A) A heatmap showing correlation coefficients (r) of the density of zif268-ir cells between each brain region in males and females. Males and females showed distinct patterns of significant inter-regional correlations of zif268-ir cell density. * indicates significant correlations ($p < 0.05$) and green boxes indicate sex differences between the correlations ($p < 0.05$). (B) Brain network maps were generated with correlations with coefficients larger than 0.67 or smaller than -0.67 in males (left) and females (right) with $p<0.1$. Red lines indicate positive correlations with wider lines indicating larger coefficients and blue lines indicate negative correlations with wider lines indicating smaller coefficients.

3.3.8 Principal component analyses on the density of zif268-ir cells

Principal component analyses (PCA) were conducted with the density of zif268-ir cells in the 16 brain regions. PCA demonstrated that the first three principal components factors accounted for 70.45% of the variance with PC1 explaining 37.50% of the variance, PC2 explaining 18.57% and PC3 explaining 14.38% of the variance (see Fig. 3.6 B). PC1 included significant positive loading on the density of zif268-ir cells in all of the hippocampus, most of the frontal cortex and the amygdala (except the IL and BLA) and included the mDS (see Fig. 3.6 A). A repeated-measures ANOVA on the principal component scores revealed that females showed significantly greater positive scores compared to males in PC1 [interaction effect of sex by factor: $F(2, 24) = 9.11$, $p = 0.001$; post-hoc: $p = 0.031$; see Fig. 3.6 C], indicating that females had greater activation of zif268 among these regions compared to males.

A

Regions	Factor 1	Factor 2	Factor 3
BLA	0.335	** 0.825	-0.113
LA	* 0.569	* 0.603	-0.093
CeA	* 0.590	** 0.636	-0.411
mDS	* 0.610	0.299	0.376
lDS	0.489	-0.257	* 0.577
Nac	0.318	0.202	** 0.760
Nas	0.07	0.495	** 0.666
Cg1	** 0.810	-0.174	0.284
PrL	** 0.895	0.103	0.136
IL	0.423	0.124	* -0.503
dDG	* 0.589	** -0.643	-0.055
vDG	** 0.691	-0.447	-0.113
dCA3	** 0.644	0.051	-0.317
vCA3	** 0.695	-0.128	-0.351
dCA1	** 0.910	-0.068	-0.113
vCA1	* 0.559	* -0.624	0.099

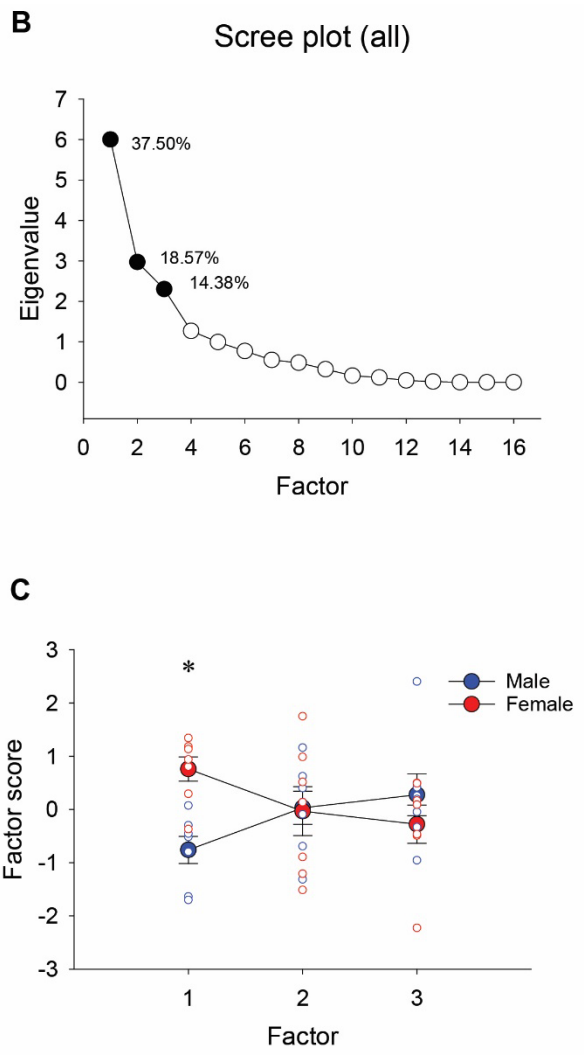


Fig. 3.6. Sex difference in the principal component analysis (PCA). (A) Factor coordinates of the variables in the first three factors identified using principal component analysis (PCA) for zif268 activation. (B) Scree plot for eigenvalues with the total percentage of variance for the first three factors. Eigenvalues for the first three factors were significant based on Horn's parallel analysis. (C)The first three factors explained 70.45% of the variances. A graph showing sex difference in the factor scores of individual samples. ANOVA and post-hoc revealed males and females showed significant sex difference in the first factor. * indicates $p < 0.05$ and ** indicates $p < 0.01$.

3.4 Discussion

We found that female rats showed greater contextual pattern separation and had greater neural activation in the frontal cortex, dorsal CA1 region and in adult-born DGCs, in response to fear memory compared to males. Furthermore, we found distinct sex differences in functional connectivity in both direction (positive, negative) and in activation patterns between limbic regions, as males had more positive correlations among these regions than females. Intriguingly, we saw that activation of new neurons of different ages were intercorrelated among new neurons, which correlated with different regions in the hippocampus in males but not females. However, females, but not males, showed significant correlations between activated new neurons and the amygdala during fear memory retrieval. These results demonstrate that males and females employ different brain networks during fear memory retrieval. These findings highlight the importance of studying sex differences in fear memory and the contribution of adult neurogenesis to the neuronal network. They also have implications for targeting treatment of fear-related disorders between males and females.

3.4.1. Females show greater fear-associated contextual pattern separation compared to males

Females showed greater discrimination of the two contexts compared to males on the last trial days using the discrimination index. This result is consistent with previous studies that reported a female advantage in fear-conditioning context discrimination tasks in rodents (Day et al., 2016; Foilb et al., 2018) and in performance in emotional episodic memory tasks in humans (Andreano and Cahill, 2009; Gavazzeni et al., 2012; Naveh-benjamin et al., 2004). However, others have found the opposite, with a male advantage in fear-conditioning context

discrimination that used different spatial configurations for the contexts (Keiser et al., 2017). Indeed, in our own previous work, we found a male advantage in the ability for spatial pattern separation in rats (Yagi et al., 2016), that was observed only among rats that rely more on allocentric (geometric) spatial cues (Yagi et al., 2016). The inconsistency between findings across studies may be due to sex differences in learning strategies and the types of cues between the paradigms. Males rely preferentially on spatial strategies whereas females rely more idiothetic strategies in human and rodents (Barkley and Gabriel, 2007; Chai and Jacobs, 2010; Sandstrom et al., 1998; Williams et al., 1990), which may explain why a male advantage was found when using different geometry (or shapes) of the conditioning chambers (Keiser et al., 2017). In contrast, the two contexts in the present study shared the same geometric cues, making it more difficult for rats relying on geometric cues to discriminate between the two contexts. Together these results suggest that males and females process contextual information differently and/or males and females rely on different learning strategies during a fear-conditioning context discrimination task so that the availability of favored memory cues influences their performance during a given task (Chen et al., 2021; Tronson, 2018).

In addition to the potential sex differences in learning strategies, the sex differences favouring females in the present contextual pattern separation task may be due to learned context discrimination. In our protocol, we gave 12 days of continual exposure to the two contexts but other protocols using fear context discrimination or generalization of fear use fewer trials (Keiser et al., 2017; Lynch et al., 2013) which may also affect the results. In addition, previously learned memory can interfere with learning of new memories (reviewed in (Yassa and Reagh, 2013)) and intriguingly, neurogenesis minimizes proactive interference (Epp et al., 2016). Indeed, pre-exposure to a conditioning context enhances the ability for contextual discrimination in females,

but not in males (Keiser et al., 2017). Therefore, it is also plausible that previous experience (foot shocks) in the other context may affect the ability for contextual discrimination differently between the sexes in the present study. Further research is needed to determine whether there are sex differences in strategy use during a contextual learning and to determine what types of memory cues and protocol differences might contribute to the sex difference in the ability for contextual pattern separation.

3.4.2 Females show greater neuronal activation of young granule cells in the dorsal DG compared to males

We found that 3-week-old adult-born DGCs showed greater neuronal activation in females compared to males in response to fear memory retrieval. Our previous work demonstrates that adult-born DGCs in male rats mature faster than in females (Yagi et al., 2020: Chapter 2). Therefore, it is possible that female 3-week-old adult-born DGCs are more immature and highly excitable in response to fear memory retrieval compared to males, however then we might have expected to see a sex difference favoring males in activation of the mostly younger DCX-ir cells which was not the case. Another possible explanation for the sex difference in neural activation of 3-week-old adult-born DGCs is that pattern separation circuits are differently recruited during re-exposure to familiar environment in female compared to male rats. Indeed, we did see different patterns of activation of activated new neurons in females compared to males, with females showing coordinated activation of new neurons with the amygdala whereas in males there were more intercorrelations within the hippocampus. Previous studies demonstrated that adult-born DGCs play different roles depending on the age of DGCs, as younger DGCs play a role for pattern separation while older DGCs play a role for pattern completion (Clelland et al., 2009; Nakashiba et al., 2012). Therefore, greater neural activation of

younger DGCs in females during memory retrieval may indeed be the reason for superior pattern separation performance by female rats compared to male rats in this task.

In addition to 3-week-old DGCs, we found sex differences in neural activation of 4-week-old DGCs depending on its location along the longitudinal axis. Females exhibited greater neural activation of 4-week-old adult-born DGCs in the dorsal DG whereas males exhibited greater neural activation of 4-week-old adult-born DGCs in the ventral DG compared to the opposite sex. This result suggests that 4-week-old adult-born DGCs play different functional roles in the dorsal and ventral DG between males and females, or that males and females differently recruit 4-week-old adult-born DGCs in the dorsal and ventral DG during contextual fear conditioning paradigms. The dorsal hippocampus plays an important role for spatial learning and memory, and the ventral hippocampus is important for regulation of stress (Henke, 1990; Kjelstrup et al., 2002; Moser et al., 1993; Pothuizen et al., 2004). However, sex differences in the contribution of adult-born DGCs depending on its location along the longitudinal axis and depending on maturity of DGCs to the hippocampal cognition have yet to be determined.

3.4.3 Females show greater neuronal activation in the frontal cortex and dorsal CA1 in response to fear memory retrieval

We found that females, compared to males, showed greater neural activation in the frontal cortex despite there being no significant sex difference in fear memory during the activation trial. Previous studies have demonstrated that females have greater reliance on the frontal cortex (PrL, IL) to auditory fear memory acquisition, extinction and recall (Baran et al., 2010; Fenton et al., 2016, 2014; Kirry et al., 2019). Collectively, these studies suggest that females rely on the frontal cortex to maintain fear memory.

Females also showed greater neural activation in the dorsal CA1 in response to fear memory than in males, consistent with other studies in contextual fear retrieval (Colon and Poulos, 2020). The CA1 in the hippocampus plays important roles for pattern completion during memory retrieval (Hunsaker and Kesner, 2013). Further research is warranted to elucidate sex differences in the functional roles of dorsal CA1 during various memory tasks.

3.4.4 Males and females show distinct patterns of functional connectivity between frontal cortex, hippocampus and amygdala

The present study indicates significant sex differences in functional connectivity between the amygdala (LA, CeA), hippocampus (all subregions), dorsal striatum (mDS) and frontal cortex (PrL, ACC) where females show stronger positive connectivity among the regions in response to fear memory retrieval. This finding is consistent with resting-state functional connectivity and BOLD-signal changes in response to fear conditioning in humans (Engman et al., 2016; Kogler et al., 2016; Lebron-Milad et al., 2012) and with functional connectivity in rats (Worley et al., 2020). Human females have greater resting-state functional connectivity between the amygdala, frontal regions and the hippocampus than human males (Engman et al., 2016; Kogler et al., 2016) and show greater BOLD-signal changes in the amygdala, and anterior cingulate cortex compared to males to fear-conditioned stimuli in humans (Lebron-Milad et al., 2012). Furthermore, we found sex differences in patterns of associations between neural activation in adult-born DGCs and neural activation in other brain regions, with females showing correlations to the amygdala that were not seen in males. To our knowledge, this is the first study demonstrating sex differences in functional connectivity of adult-born DGCs and other brain regions. Overall, our study indicated significant involvement of the hippocampus in the functional connectivity in the present study, more so in females compared to males.

3.4.5 Perspectives and Significance

The present study found that female rats acquired pattern discrimination faster than males in a contextual pattern separation task. However, despite similar fear memory, females showed greater activation of new neurons in the dorsal dentate gyrus in response to fear memory. Furthermore, males and females showed distinct functional connectivity between limbic regions and activated new neurons during fear memory retrieval, with more correlations between activated new neurons of different ages in males but more correlations with activated new neurons to other limbic regions in females. These data suggest that the functional contribution of adult neurogenesis to pattern separation and pattern completion may be via different pathways in males and females. To our knowledge, the dynamics of the functional connectivity with activated new neurons has not been recorded previously and the functional significance of these sex differences remains to be determined. Future studies using manipulating the activity of dentate adult-born neurons are needed to determine how adult-born young neurons contribute to the functional connectivity of long-term fear memory. Furthermore, ovarian hormones may play an important role for modulating the sex difference in prevalence of PTSD as postmenopausal females show decreased prevalence of PTSD (Creamer and Parslow, 2008). Therefore, further studies examining how estradiol and age modulate the ability for pattern separation are needed for elucidating the mechanisms underlying the sex difference in the prevalence of PTSD.

3.5 Conclusion

Our data demonstrate that females, compared to males, show greater context discrimination, greater activation of 3-week-old adult-born DGCs in response to memory retrieval, and strong functional connectivity in the frontal cortex, the hippocampus, dorsal striatum and the amygdala

during fear memory retrieval. Our findings indicate that sex differences exist in the underlying neural mechanisms and network activation even when no significant sex difference is observed in fear memory retrieval. Our work highlights the importance of elucidating sex-specific neural connections that may contribute to differences in susceptibility to fear related disorders such as PTSD. It also underscores that any treatments for fear-related disorders will need to consider sex as very different neural mechanisms may be underlying fear memory.

Chapter 4: Estrogens dynamically regulate neurogenesis in the dentate gyrus of adult female rats

4.1 Introduction

Hippocampal integrity is compromised in diseases such as Alzheimer's disease and depression (Scheff et al., 2006; Selden et al., 1991). A unique characteristic of the hippocampus is its ability to generate new neurons in adulthood. Adult neurogenesis in the hippocampus plays important roles for pattern separation during memory encoding and in stress resilience (Anacker et al., 2018; Clelland et al., 2009; Tobin et al., 2019). New neurons in the adult hippocampus are produced from neural stem/progenitor cells in the subgranular zone of dentate gyrus (DG). Developing new neurons express stage-specific endogenous markers such as Sox2 in neural stem/progenitor cells, Ki67 in proliferating cells, doublecortin (DCX) in immature neurons, and neuronal nuclei (NeuN) in mature neurons (Lugert et al., 2010). Although there are no sex differences in the number of new three-week old neurons in rats, there are sex differences in the maturation pathways for adult neurogenesis (Chapter 2: Yagi et al., 2020). Male rats have a greater density of neural stem/progenitor cells, greater cell proliferation, and faster maturation of new neurons than female rats (Chapter 2: Yagi et al., 2020). However, male rats also have greater attrition of immature neurons between one and two weeks after production compared to female rats (Chapter 2: Yagi et al., 2020). These findings suggest it may be fruitful to determine whether ovarian hormones, such as estrogens, regulate the maturation of neurogenesis in female rodents.

There are four types of estrogens, estrone, estradiol, estriol and estetrol. Estrone and estradiol are the two most abundant of the estrogens. Estradiol binds with greater affinity to estrogen receptors (ERs) and is present at higher levels than estrone before menopause whereas

estrone is present at higher levels than estradiol after menopause in human females (Rannevik et al., 1995). Previous studies demonstrate that estrogens modulate cell proliferation in the dentate gyrus which depends on both the type of estrogens and duration of exposure (Barha et al., 2009; Mazzucco et al., 2006; Ormerod et al., 2003b; Tanapat et al., 2005, 1999). A single dose of 17β-estradiol or estradiol benzoate (EB) rapidly increases cell proliferation but this effect depends on the duration of exposure and formulation of estradiol (Barha et al., 2009; Mazzucco et al., 2006; Ormerod et al., 2003b; Tanapat et al., 1999). However, repeated administration of estradiol or EB for three weeks had no significant effect on cell proliferation (Chan et al., 2014; McClure et al., 2013; Tanapat et al., 2005). Collectively these studies suggest that the duration of exposure to estradiol can dramatically influence the effects on cell proliferation in the dentate gyrus.

Estrone treatment has different effects on neurogenesis in the hippocampus and contextual fear conditioning than estradiol in female rats (Barha and Galea, 2010; McClure et al., 2013). Although both estrogens increase cell proliferation (Barha et al., 2009), estradiol enhances, whereas estrone decreases, survival of three-week old new neurons in rats that also underwent cognitive training (McClure et al., 2013). In addition, acute exposure to estradiol enhances, whereas estrone impairs, contextual fear conditioning in adult female rats (Barha and Galea, 2010). These studies suggest that these different estrogens have differential effects on different aspects of neurogenesis and hippocampus-dependent function.

Estrogens also influence cognition and hippocampal volume in humans depending on the type, timing and duration of hormone therapy (reviewed in (Maki and Sundermann, 2009; Wnuk et al., 2012)). For instance, short duration of hormone therapy increases hippocampal volume (Boyle et al., 2021; Erickson et al., 2007), whereas longer than ten years of hormone therapy decreases hippocampal volume in postmenopausal females (Erickson et al., 2007). Furthermore,

estradiol-based hormone therapy improves verbal memory in post-menopausal females, whereas conjugated estrone-based hormone therapy has detrimental (or no significant) effects on verbal memory (Joffe et al., 2006; Linzmayer et al., 2001; Maki et al., 2007; Phillips and Sherwin, 1992; Ryan et al., 2012; Shaywitz et al., 2003). The timing of initiation of hormone therapy relative to menopause also plays important roles for the effects of hormone therapy as early initiation after menopause enhances cognitive performance, whereas late initiation after menopause leads to poorer performance (MacLennan et al., 2006), an effect mirrored in animal models (reviewed in (Daniel and Bohacek, 2010)).

To date, duration-dependent changes in the effects of estrogens have not been investigated on the characteristics of neurogenesis in the DG. Thus, we aimed to elucidate effects of estrone and estradiol on neural stem/progenitor cells, maturation rate of new neurons, and the trajectory (attrition) of new neurons. We hypothesized that estradiol and estrone would differentially modulate the trajectory and maturation rate of new neurons based on the duration of exposure to estrogens.

4.2 Materials and Methods

4.2.1 Subjects

Thirty-six female Sprague-Dawley rats (four females each treatment and each maturation time courses) obtained from our breeding colony at University of British Columbia (Vancouver, BC, Canada) were used in this study. Rats were weaned at postnatal day 21 and housed with same-sex siblings until puberty. Rats were then pair-housed until the end of the study in opaque polysulfone bins (432 mm × 264 mm × 324 mm) with paper towels, a single polycarbonate hut, virgin hardwood chip bedding, and free access to food and water. The colony room was

maintained under a 12:12-h light/dark cycle (lights on at 07:00 h). All experiments were carried out in accordance with the 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.

4.2.2 Experimental timeline

Rats were handled for 2 minutes every day beginning at the age of ten weeks for two weeks. Rats received ovariectomy bilaterally at the age of twelve weeks and rats were randomly assigned into three treatment groups. Following one week of recovery period, rats started to receive 5 μg Estrone in 0.1 ml sesame oil, 5 μg of 17β in 0.1 ml sesame oil or 0.1 ml sesame oil (vehicle) via subcutaneous injection. Daily subcutaneous injections of 5 μg of 17β results in serum concentrations of estradiol equivalent to the levels of estradiol during proestrous phase (Becker and Rudick, 1999). Each group received the same treatment every day (approximately 9-11 am) until the end of experiment. On the next day, all rats received one injection of bromodeoxyuridine (BrdU; 200 mg/kg i.p.) one hour after hormone or vehicle treatment. Rats were perfused one, two or three weeks after BrdU injection (Fig. 4.1 A). Serum estradiol levels were 1.59x times higher in the estradiol group versus the estrone group which were 50x and 20x higher than the oil injected groups (verified via a multiplex electrochemiluminescence immunoassay kit (Custom Steroid Hormone Panel, Human/Mouse/Rat) from Meso Scale Discovery (Rockville, MD, USA)).

4.2.3 Perfusion and tissue processing

Rats were administered an overdose of sodium pentobarbitol (500 mg/kg, i.p.) and perfused transcardially with 60 ml of 0.9% saline followed by 120 ml of 4% formaldehyde

(Sigma-Aldrich). Brains were extracted and post-fixed in 4% formaldehyde overnight, then transferred to 30% sucrose (Fisher Scientific) solution for cryoprotection and remained in the solution until sectioning. Brains were sliced into 30 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.

4.2.4 Immunohistochemistry

Brain sections were stained for Sox2 (Fig. 4.1 B) and Ki67 (Fig. 4.1 E) to examine the density of neural stem/progenitor cells or the density of proliferating cells in the DG, respectively. Furthermore, brain sections were double-stained for BrdU/DCX (Fig. 4.2 A-C) and BrdU/NeuN (Fig. 4.2 D-F) to examine the maturation time course of new cells. Furthermore,

BrdU/NeuN or BrdU/DCX double-labelling

Brain sections were 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 (Milli- pore; MA, USA) or 1:200 goat anti-DCX (Santa Cruz Biotechnology, CA, USA), 0.3% Triton-X, and 3% normal donkey serum (NDS; Vector Laboratories) in 0.1 M PBS for 24 hours at 4 °C. Following three rinses in 0.1 M PBS, sections were incubated in a secondary antibody solution containing 1:200 donkey anti-mouse Alexa Fluor 488 (Invitrogen, Burlington, ON, Canada) or 1:200 donkey anti-goat Alexa Fluor 488 (Invitrogen, Burlington, ON, Canada) in 0.1 M PBS, for 18 hours at 4 °C. After rinsing three times with PBS, the sections were washed with 4% formaldehyde, and rinsed twice in 0.9% NaCl, followed by incubation in 2N HCl for 30 minutes at 37 °C. Following three rinses in 0.1 M PBS, the sections were then incubated in a

BrdU primary antibody solution consisting of 1:1000 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. Sections were 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 rinsed with PBS, the sections were mounted onto microscope slides and cover-slipped with PVA DABCO.

Sox2

Brain sections were prewashed with 0.1 m PBS and left to sit overnight at 4°C. The next day, sections were washed in 0.1M PBS for 10 min each and blocked with 3% NDS and 0.3% Triton X-100 in 0.1 M PBS, followed by incubation in primary antibody solution made with 1:1000 mouse anti-Sox2 (Santa Cruz Biotechnology), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS for 24 h at 4°C. Then the sections were incubated in secondary antibody solution, consisting of 1:500 donkey anti-mouse Alexa Fluor 594 (Invitrogen), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS, for 18 h at 4°C. After three rinses with PBS, the sections were incubated in 1:5000 DAPI in PBS for 3 min. Followed by three rinses, tissues were mounted onto slides and cover-slipped with PVA DABCO.

Ki-67

Brain sections were prewashed with 0.1 M PBS and left to sit overnight at 4°C. The next day, sections were incubated in 10 mM sodium citrate buffer for 30 min at 90°C to retrieve antigens of Ki67 and blocked with 3% NDS and 0.3% Triton X-100 in 0.1 M PBS. Tissue was then incubated in primary antibody solution made with 1:250 mouse anti-Ki67 (Leica Biosystems), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS for 24 h at 4°C. Following three washes in 0.1 M PBS, brain sections were incubated in secondary antibody solution, consisting

of 1:500 donkey anti-mouse Alexa Fluor 488 (Invitrogen), 1% NDS, and 0.3% Triton X-100 in 0.1 M PBS, for 18 h at 4°C. After three rinses with PBS, sections were incubated in 1:5000 DAPI in PBS for 3 min. Followed by three rinses, tissue was mounted onto slides and cover-slipped with PVA DABCO.

4.2.5 Cell counting

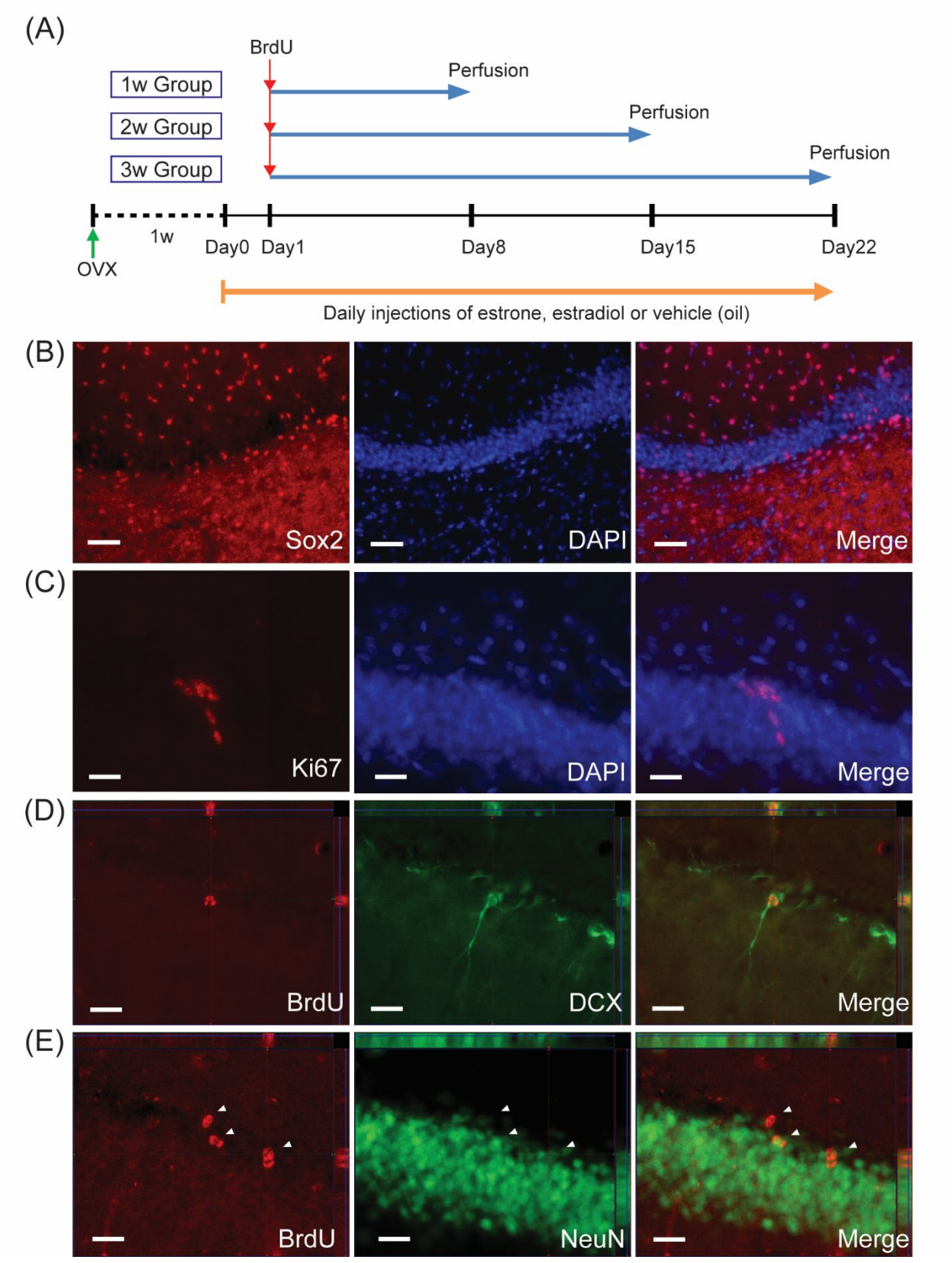
All counting was conducted by an experimenter blind to the group assignment of each animal using an Olympus FV1000 confocal microscope and/or Zeiss Axio Scan.Z1 (Carl Zeiss Microscopy, Thornwood, NY, USA). Density of immunoreactive (ir) cells was calculated by dividing the total number of ir cells by volume (mm^3) of the corresponding region. Volume estimates were calculated by multiplying the summed areas by thickness of sections (0.03 mm, using Cavalieri's principle; (Gundersen and Jensen, 1987)). Area measurements for the region of interest were obtained using digitized images on Zen 3.0 software (blue edition; Carl Zeiss Microscopy, Thornwood, NY, USA). Cells were categorized as to whether they were in the dorsal or ventral DG using the criterion defined by Banasr and others (2006), with sections 6.20-3.70 mm from the interaural line defined as dorsal and sections 3.70-2.28 mm from the interaural line as ventral. Cells were counted separately in each region because the dorsal hippocampus is associated with spatial learning and memory, whereas the ventral hippocampus is associated more with stress and anxiety (Kjelstrup et al., 2002; Moser et al., 1993).

BrdU-ir cells were counted under a 60x oil immersion objective lens using an Olympus epifluorescent microscope and the percentages of BrdU/NeuN-ir cells were obtained by randomly selecting 50 BrdU-ir cells and calculating the percentage of cells that double-labelled-ir with NeuN under 40x objective lens using an Olympus FV1000 confocal microscope

(Olympus, Richmond Hill, ON, Canada). The percentages of BrdU/DCX-ir cells were obtained by randomly selecting 50 BrdU-ir cells and calculating the percentage of cells that double-labelled-ir with DCX on digitized images acquired under 40x objective lens using Axio Scan.Z1 slidescanner with Zen 3.0 software (blue edition; Carl Zeiss Microscopy, Thornwood, NY, USA). Ki67-ir cells were counted on digitized images every twentieth section. Photomicrographs for Ki67-ir cells were taken with a 40x objective lens on a Axio Scan.Z1 slidescanner with Zen 3.0 software (Carl Zeiss Microscopy, Thornwood, NY, USA). Photomicrographs for Sox2-ir cells were taken from four dorsal and three ventral hippocampi using a 40x objective lens on Axio Scan.Z1 slidescanner, and optical density of Sox2-ir cells were measured on digitized images using ImageJ (NIH, Bethesda, MD, USA).

4.2.6 Statistical analyses

All analyses were conducted using STATISTICA (Statsoft Tulsa, OK). Repeated-measures ANOVAs were used to each analyze the density of Ki67-ir and Sox2-ir cells with exposure time (1w, 2w, 3w) and hormone (estrone, estradiol, vehicle) as between subject factor and with hippocampal region (dorsal, ventral) as the within-subject factor. The density of BrdU-ir cells or the percentage of BrdU/DCX-ir cells or BrdU/NeuN-ir cells were each analyzed using repeated-measures analysis of variance (ANOVA), with week (1w, 2w, 3w) and hormone (estrone, estradiol, vehicle) as between-subject variables and with hippocampal region (dorsal, ventral) as the within-subject variable. Post-hoc tests utilized the Neuman-Keuls procedure. A priori comparisons were subjected to Bonferroni corrections. Significance was set to $\alpha=0.05$ and effect sizes are given with Cohen's d or partial η^2.


(A)
BrdU
1w Group
2w Group
3w Group
Perfusion
Perfusion
Perfusion
1w
OVX
Day0
Day1
Day8
Day15
Day22
Daily injections of estrone, estradiol or vehicle (oil)
(B)
Sox2
DAPI
Merge
(C)
Ki67
DAPI
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(D)
BrdU
DCX
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(E)
BrdU
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Fig. 4.1. Experimental design. (A) Schematic illustration for the experimental timeline. (B-E) Photomicrographs for (B) Sox2-ir cells (red) with DAPI (blue), (C) Ki67-ir cells (red) with DAPI (blue), (D) BrdU-ir cells (red) with DCX-ir cells (green) and (E) BrdU-ir cells (red) with NeuN-ir cells (green). Scale bars in (B) indicate 50 μm and scale bars in (C)-(E) indicate 20 μm. All photomicrographs were taken by Zeiss Axio Scan.Z1 with 20x (B) or 40x (C-E) objectives.

4.3 Results

4.3.1 Estradiol reduced the density of Sox2-ir cells in the dorsal DG, whereas estrone reduced the density of Sox2-ir cells in the ventral DG compared to vehicle-treated females

Both estrogens reduced the density of Sox2-ir cells dependent on region but not on exposure time. Estradiol-treated females had a lower density of Sox2-ir cells in the dorsal DG compared to vehicle-treated females ($p = 0.021$, Cohen's $d = 1.222$), whereas estrone-treated females had a lower density of Sox2-ir cells in the ventral DG compared to both groups (vehicle-treated: $p = 0.006$, Cohen's $d = 1.410$; estradiol-treated: $p = 0.002$, Cohen's $d = 1.000$) [interaction effect of region by hormone: $F(2, 25) = 5.60$, $p < 0.01$, partial $\eta^2 = 0.309$: Figure 2A and 2B]. These differences with estrogens appeared to differ by week as estradiol-treated females had a lower density of Sox2-ir cells in the dorsal DG after one week after hormone treatment ($p = 0.0064$, Cohen's $d = 1.580$), whereas estrone-treated females had a lower density of Sox2-ir cells in the ventral DG after two weeks ($p = 0.0051$, Cohen's $d = 2.102$: not significant with Bonferroni corrections) compared to vehicle-treated females, but these failed to reach significance after Bonferroni corrections (Figure 2A). Furthermore, there was a greater density of Sox2-ir cells in the ventral DG compared to the dorsal DG in estradiol-treated ($p < 0.001$, Cohen's $d = 1.909$) and vehicle-treated females ($p = 0.01$, Cohen's $d = 0.959$) but not in estrone-

treated females (p=0.53). There was also a main effect of hormone [$F(1, 25) = 4.19$, $p = 0.027$, partial $\eta^2 = 0.251$] and region as expected [$F(1, 25) = 29.36$, $p < 0.001$, partial $\eta^2 = 0.540$]. There were no other significant main or interaction effects on the density of Sox2-ir cells ($p > 0.180$).

4.3.2 Estradiol increased, whereas estrone reduced, the density of Ki67-ir cells in the DG compared to vehicle-treated females after one week of hormone exposure

After one week of hormone treatment, estradiol-treated females had a greater density of Ki67-ir cells compared to both groups (vehicle-treated: $p < 0.001$, Cohen's $d = 2.864$; estrone-treated: $p < 0.001$, Cohen's $d = 5.239$), whereas estrone-treated females had a lower density of Ki67-ir cells compared to both groups (vehicle-treated: $p = 0.026$, Cohen's $d = 1.883$) [interaction effect of exposure time by treatment: $F(4, 26) = 10.453$, $p < 0.001$, partial $\eta^2 = 0.617$: Fig. 4.2 C and 4.2 D]. Furthermore, all groups had a reduction in the density of Ki67-ir cells between one and two weeks of exposure to hormones or vehicle [estrone-treated ($p = 0.028$, Cohen's $d = 2.265$), estradiol-treated ($p < 0.001$, Cohen's $d = 8.756$) and vehicle-treated females ($p < 0.001$, Cohen's $d = 2.742$)]. There were also a significant interaction effect of region by exposure time [$F(2, 26) = 3.406$, $p = 0.049$, partial $\eta^2 = 0.208$] and main effects of hormone [$F(2, 26) = 4.061$, $p = 0.029$, partial $\eta^2 = 0.238$], week [$F(2, 26) = 70.034$, $p < 0.001$, partial $\eta^2 = 0.843$] and region [$F(1, 26) = 18.033$, $p < 0.001$, partial $\eta^2 = 0.410$]. There were no other significant main or interaction effects on the density of Ki67-ir cells (all p's > 0.608).

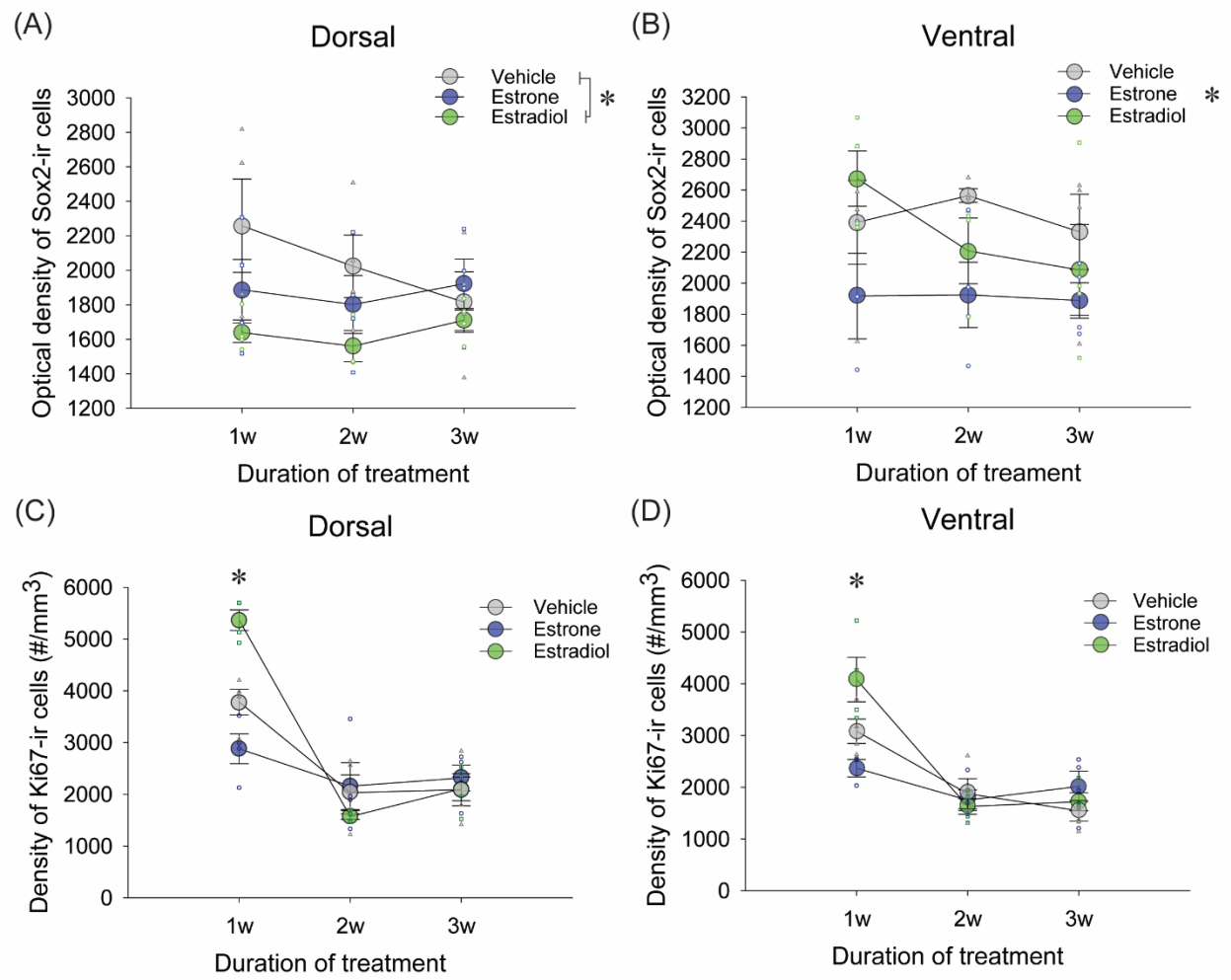

Fig. 4.2. Estrogenic regulation for the neural stem/progenitor cells and cell proliferation. (A-B) Mean (±SEM) density of Sox2-ir cells in the dorsal dentate gyrus (A) and the ventral dentate gyrus (B). Vehicle-treated females had a greater density of Sox2-ir cells compared to estradiol-treated females in the dorsal dentate gyrus, and estradiol-treated and vehicle-treated females had a greater density of Sox2-ir cells in the ventral dentate gyrus. (C-D) Mean (±SEM) density of Ki67-ir cells in the dorsal dentate gyrus (C) and the ventral dentate gyrus (D). Estradiol-treated females showed greater density of Ki67-ir cells compared to vehicle-treated females and compared to estrone-treated females, and vehicle-treated females showed greater density of Ki67-ir cells compared to estrone-treated females after one week of hormone exposure. * indicates $p < 0.05$. SEM-standard error of the mean.

4.3.3 Estrone and estradiol-treated females had a greater density of BrdU-ir cells and BrdU/DCX-ir cells compared to vehicle-treated females one week after cell division/ exposure to estrogens

After one week of exposure to estrogens, estrone-treated ($p = 0.002$, Cohen's $d = 2.655$) and estradiol-treated females ($p = 0.004$, Cohen's $d = 1.868$) had a greater density of one-week old BrdU-ir cells compared to vehicle-treated females [interaction effect of week by hormone: $F(4, 24) = 3.120$, $p = 0.034$, partial $\eta^2 = 0.342$: Fig. 4.3 A], but there were no significant differences between treatment groups after two or three weeks in BrdU-ir cells (p's > 0.184). In both estrone and estradiol-treated females, there was a significantly greater density of BrdU-ir cells at one week compared to two weeks (estrone: $p = 0.004$, Cohen's $d = 3.955$; estradiol: $p = 0.001$, Cohen's $d = 2.980$) but no significant difference between two weeks and three weeks (p's > 0.118). In vehicle-treated females, there were no significant differences in the density of BrdU-ir cells between any of the weeks (p's > 0.302). There were also significant main effects of hormone [$F(2, 24) = 7.550$, $p = 0.003$, partial $\eta^2 = 0.342$], week [$F(2, 24) = 30.114$, $p < 0.001$, partial $\eta^2 = 0.715$] and region [$F(1, 24) = 4.284$, $p = 0.049$, partial $\eta^2 = 0.151$], but no other significant interaction effects on the density of BrdU-ir cells (p's > 0.288).

One week of exposure to estrogens also increased the density of BrdU/DCX-ir cells (estrone: $p < 0.001$, Cohen's $d = 2.529$; estradiol ($p = 0.001$, Cohen's $d = 1.903$) compared to vehicle-treated females [interaction effect of hormone by week: $F(4, 24) = 4.109$, $p = 0.011$, partial $\eta^2 = 0.406$: Fig. 4.3 B]. Treatment with estrogens decreased the density of BrdU/DCX-ir cells with each week of exposure [one to two weeks: p's < 0.001, Cohen's $d = 3.777$(estrone), Cohen's $d = 3.026$ (estradiol); two to three weeks: p's < 0.002, Cohen's $d = 8.906$ (estrone), Cohen's $d = 3.430$ (estradiol)]. This same pattern was not seen in vehicle-treated females (with no significant difference in density of BrdU/DCX-ir cells between one to two weeks ($p = 0.103$)

but a decrease from two to three weeks of treatment ($p = 0.019$, Cohen's $d = 2.403$). There was also a significant main effect of hormone [$F(2, 24) = 10.249$, $p < 0.001$, partial $\eta^2 = 0.461$] and week [$F(2, 24) = 101.756$, $p < 0.001$, partial $\eta^2 = 0.895$]. There were no other significant main or interaction effects on the density of BrdU/DCX-ir cells ($p > 0.167$).

In terms of the density of mature new neurons over the weeks, it was only the vehicle-treated groups that showed greater increase in the density of BrdU/NeuN-ir cells across time with an increase between one and three weeks ($p=0.018$, Cohen's $d = 3.974$) which was not seen in the groups treated with estrogens regardless of region ($p> 0.401$; interaction effect of hormone by week: $F(4, 24) = 2.963$, $p = 0.040$, partial $\eta^2 = 0.331$; Fig. 4.3 C). Furthermore, estradiol-treated and estrone-treated females had a trend for a greater density of BrdU/NeuN-ir cells compared to vehicle-treated females after one week (estradiol: $p = 0.089$, Cohen's $d = 3.225$; estrone: $p = 0.095$). There were also main effects of hormone [$F(2, 24) = 3.932$, $p = 0.033$, partial $\eta^2 = 0.247$] and week [$F(2, 24) = 4.963$, $p = 0.016$, partial $\eta^2 = 0.293$]. There were no other main or interaction effects on the density of BrdU/NeuN-ir cells (all p's > 0.208).

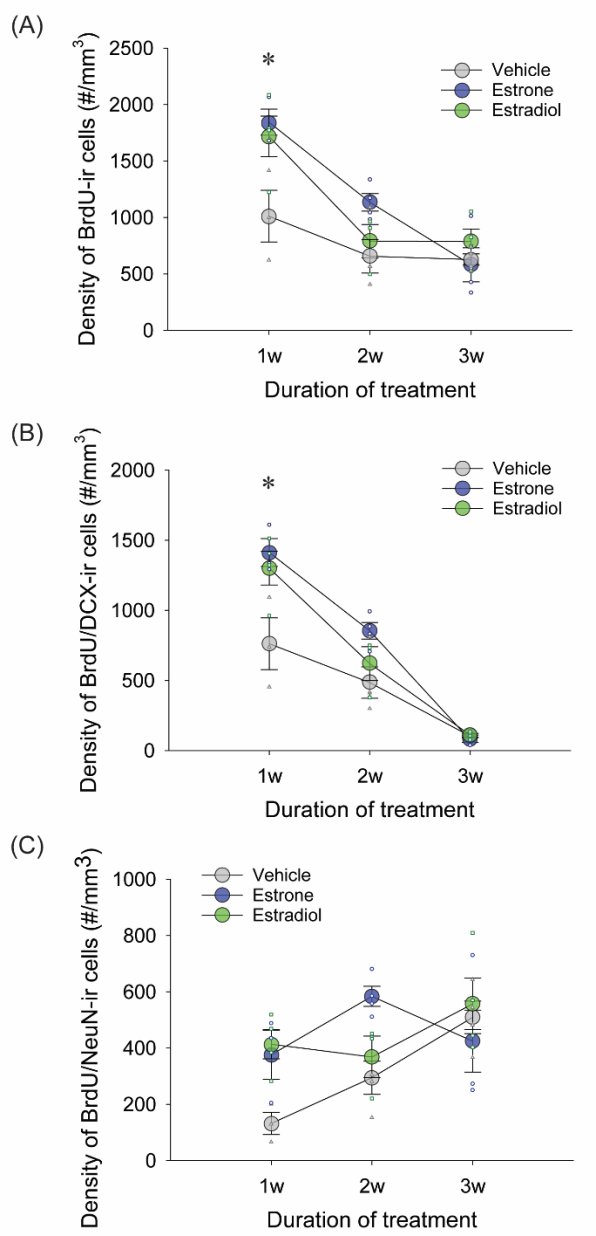

Fig. 4.3. Estrogenic regulation for the trajectory of new cells in the dentate gyrus. (A) Mean (±SEM) density of BrdU-ir cells in the dentate gyrus. (B) Mean (±SEM) density of BrdU/DCX-ir cells in the dentate gyrus. Estrone or estradiol-treated females had a greater density of BrdU-ir cells and BrdU/DCX-ir cells in the ventral dentate gyrus compared to vehicle-treated females one week after BrdU injection and exposure to hormones. (C) Mean (±SEM) density of BrdU/NeuN-ir cells in the dentate gyrus. Estradiol-treated females had a trend of greater density of BrdU/NeuN-ir cells compared to vehicle-treated females one week after BrdU injection and exposure to hormones. * indicates $p < 0.05$.

4.3.4 Estradiol-treated female rats showed greater percentage of BrdU/DCX-ir cells compared to vehicle-treated female rats two weeks after cell division and exposure to estrogens

Estradiol-treated females tended to have a greater percentage of BrdU/DCX-ir cells in the DG two weeks, compared to vehicle-treated females [p =0.067, Cohen's d = 4.139; interaction effect of hormone by week: $F(4, 25) = 2.96$, $p = 0.040$; Fig. 4.4 A and 4.4 B], but not after one week or three weeks of hormone treatment/after BrdU injection (p's > 0.188). Furthermore, the percentage of BrdU/DCX-ir cells was greater in the dorsal DG compared to the ventral DG after two weeks hormone exposure/ BrdU injection [interaction effect of week by region: $F(2, 25) = 5.04$, $p = 0.015$, partial $\eta^2 = 0.287$; post-hoc: $p = 0.011$, Cohen's d = 1.249]. There were main effects of week [$F(2, 25) = 2467.46$, $p < 0.001$, partial $\eta^2 = 0.995$] and region [$F(1, 25) = 4.62$, $p = 0.042$, partial $\eta^2 = 0.156$]. There were no other significant main or interaction effects on the percentage of BrdU/DCX-ir cells (p > 0.306).

As expected, the percentage of BrdU/NeuN-ir neurons increased with time such that a greater percentage of BrdU/NeuN-ir cells at three weeks compared to two weeks ($p < 0.001$, Cohen's d = 2.159), and a greater percentage of BrdU/NeuN-ir cells at two weeks compared to one week of estrogen exposure/BrdU injection ($p < 0.001$, Cohen's d = 2.932) [main effect of week: $F(2, 24) = 104.616$, $p < 0.001$, partial $\eta^2 = 0.897$: Fig. 4.4 C and 4.4 D]. Estradiol-treated females, compared to vehicle-treated females, showed a lower percentage of BrdU/NeuN-ir cells in the dorsal DG after three weeks of treatment [a priori: $p = 0.0017$, Cohen's d = 1.630; interaction of region by hormone by week $F(2, 24) = 2.230$, $p =0.096$, partial $\eta^2 = 0.271$]. However, estradiol-treated females also had a higher percentage of BrdU/NeuN-ir at one week after hormone exposure/BrdU injection although this just failed to reach significance with

Bonferroni corrections (p=0.0045). There was a trend for a significant main effect of region [F(1, 24) = 3.136, p = 0.089, partial η^2 = 0.116], but no other significant main or interaction effects on the percentage of BrdU/NeuN-ir cells in the DG (p > 0.202).

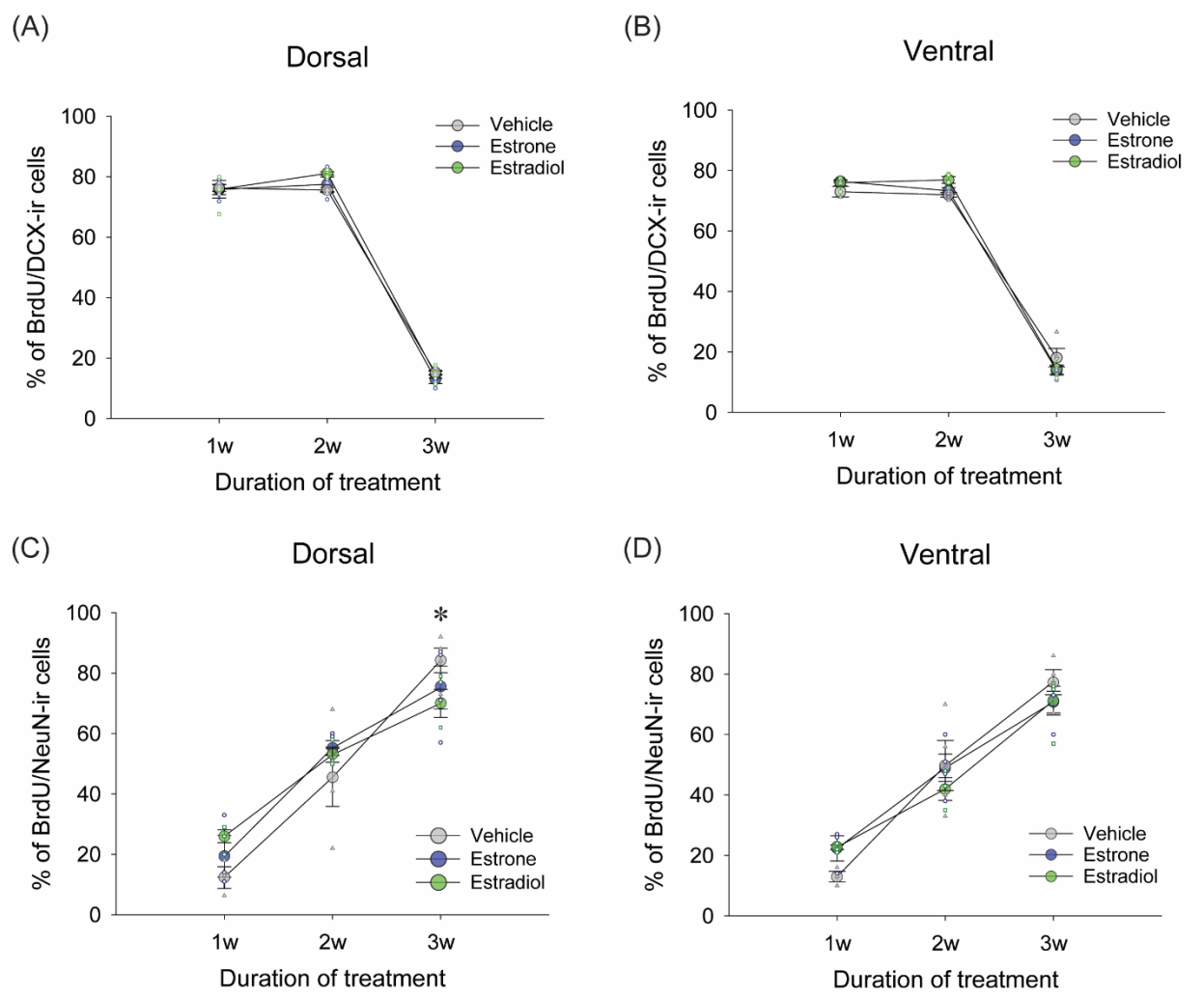

Fig. 4.4. Estrogen treatment and the maturation rate of new neurons. (A-B) Mean (±SEM) percentage of BrdU/DCX-ir cells in the dorsal dentate gyrus (A) and the ventral dentate gyrus (B). Estradiol-treated female rats showed a trend of greater percentage of BrdU/DCX-ir cells compared to vehicle-treated female rats two weeks after BrdU injection. (C-D) Mean (±SEM) percentage of BrdU/NeuN-ir cells in the dorsal dentate gyrus (C) and the ventral dentate gyrus (D). Estradiol-treated females, compared to vehicle-treated females, showed a greater percentage of BrdU/NeuN-ir cells in the dorsal DG three weeks after BrdU injection/hormone exposure. * indicates p < 0.05.

4.4 Discussion

Both estrogens had a dynamic effect on different characteristics of neurogenesis that depended on duration of exposure to estrogens. Shorter exposure to estrogens (one week) increased the density of new neurons and enhanced early maturation of new neurons compared to vehicle exposure. However, longer duration of exposure to estrogens (2-3 weeks) resulted in greater attrition of immature neurons such that there was no longer a significant difference in the number of new neurons after three weeks of exposure to estrogens. These findings suggest that the pathways to neurogenesis differ with estrogens which may reflect early but not sustained neurogenic properties of estrogens. Perhaps surprisingly we found that estradiol decreased the density of neural stem/progenitor cells in the dorsal DG, while estrone decreased the density of neural stem/progenitor cells in the ventral DG compared to vehicle-treated ovariectomized rats. These findings highlight the importance of studying estrogen type and duration in females, which have important implications for treatments that promote hippocampal plasticity.

4.4.1 Estrone and estradiol reduce expression of neural stem/progenitor cells in the DG

The present study found that chronic administration of estradiol and estrone to ovariectomized rats reduced the density of neural stem/progenitor cells in the DG. To our knowledge, this is the first study to report the effect of different estrogens on the density of neural stem/progenitor cells. Interestingly, the effect of estrogens on neural stem/progenitor cells varied along the dorsoventral axis, where estradiol reduced neural stem/progenitor cells in the dorsal region and estrone in the ventral region. The dorsoventral axis of the hippocampus has differing functions where the dorsal region plays an important role in reference memory, and the ventral region plays an important role in stress, anxiety and working memory (reviewed in Leary

and Cryan, 2014). Previously, we found that intact females had a greater density of Sox2-ir cells in the ventral DG compared to dorsal DG (Chapter 2: Yagi et al., 2020) and this same dorsoventral axis difference was seen in vehicle-treated and estradiol-treated females, but not in estrone-treated females. Underlying mechanisms or functional consequences for the differential regulation of estrogens on neural stem/progenitor cells along the dorsoventral axis have yet to be determined.

4.4.2 Estradiol enhances, whereas estrone reduces, cell proliferation in the DG one week after treatment

Estradiol enhanced cell proliferation in the DG one week, but not after two or three weeks of hormone exposure. Interestingly, estrone had the opposite effect as it reduced cell proliferation after one week of exposure. These results suggest that estradiol, but not estrone, enhances cell proliferation in a limited time window (up to one week) in naïve rats. These results are consistent with previous work demonstrating that a single dose of estradiol enhances cell proliferation in the DG, whereas three weeks of repeated administration of estradiol had no significant effects on cell proliferation (Chan et al., 2014; Ormerod et al., 2003b; Tanapat et al., 2005). In contrast, the present study found that estrone decreased cell proliferation in the DG after one week of treatment. Previous work shows that a single dose of estrone treatment enhanced cell proliferation 30 minutes after administration (Barha et al., 2009), whereas three weeks of repeated administration of estrone had no significant effects on cell proliferation (McClure et al., 2013). These findings indicate that chronic estrone treatment has detrimental effects on cell proliferation depending on the duration of treatment. Given the changes in cell proliferation with the different estrogens, but that the estrogens did not differ in neurogenesis levels after three weeks, this suggest that estradiol and estrone modulate neurogenesis via

different pathways. Taken together, estrogens modulate proliferation in a type and duration-dependent manner.

4.4.3. Estrone and estradiol increase the density of one-week old neurons after one week of exposure and increase the attrition of new neurons between one and two weeks.

Both estrogens given for one week increased the density of one-week old new dentate granule cells (DGCs; BrdU-ir cells) compared to vehicle treatment. However, by three weeks, there was no longer a significant effect of estrogens on the survival of new DGCs after three weeks of hormone treatment. Thus, the attrition of new DGCs was quite different between treatments with estrogens or vehicle as both estrogens, but not vehicle treatment, significantly reduced the density of new DGCs between one and two weeks. As majority of BrdU-ir cells (80%) at one and two weeks expressed an immature neuronal marker (DCX), the attrition of new DGCs were most likely due to the reduction of immature neurons. Indeed, the attrition of immature neurons (the density of BrdU/DCX-ir cells) between one and two weeks was also observed after treatment with both estrogens, but not after treatment with vehicle. It is possible that had we looked at an earlier time point than one week we would have seen a greater attrition of BrdU/DCX-ir cells as we saw the largest density of BrdU/DCX-ir cells 24 hours after BrdU injection in intact females (Chapter 2: Yagi et al., 2020). In terms of mature new neurons (BrdU/NeuN-ir cells), only vehicle treatment increased the density across the weeks. These results indicate that although estrone and estradiol initially enhance adult neurogenesis (BrdU/DCX-ir and a trend for BrdU/NeuN-ir) at one week compared to vehicle treatment, due to significant attrition in these new neurons with exposure to estrogens across weeks, there is no pro-neurogenic effect of estrogens in a long term.

4.4.4 Estrogens' ability to increase maturation of new neurons is diminished after sustained exposure

In estradiol-treated rats, a slightly higher percentage of new DGCs expressed a mature neuronal marker (NeuN) at one week, whereas a lower percentage of DGCs expressed NeuN three weeks after BrdU injection and hormone treatment compared to vehicle-treated rats. In terms of an immature neuronal marker (DCX), estradiol slightly increased the percentage of DGCs expressing DCX at two weeks after BrdU injection. These results suggest a possibility that estradiol initially enhances maturation of new neurons whereas prolonged exposure to estradiol delays maturation of new neurons in the adult DG, or that estrogens with time lose their effectiveness to enhance maturation of new neurons over time. Our previous work demonstrates that males show faster maturation of new neurons compared to females (Chapter 2: Yagi et al., 2020), and the present interpretation partially supports the hypothesis that estradiol contributes to the slower maturation time course of new neurons in females. Therefore, further research examining functional characteristics of these immature neurons is required to make a solid conclusion on the effects of estrogens on the maturation of new neurons.

4.4.5 Implications

Our results suggest that both the type of estrogens and duration of exposure to estrogens can significantly influence neurogenesis in the hippocampus. These findings are interesting as both animal and human studies suggest duration of exposure to estrogens influences a variety of factors. Estrogen exposure has differential effects on cell proliferation depending on time since ovariectomy surgery as estrogens enhance cell proliferation after short term (one week) ovarian hormone depletion (Barha et al., 2009; Mazzucco et al., 2006; Ormerod et al., 2003b; Tanapat et

al., 2005, 1999), whereas estrogens do not significantly influence cell proliferation after long term (four weeks) depletion (Tanapat et al., 2005). Here we found that one week of exposure to estradiol, but not longer exposure, enhanced both cell proliferation and maturation of new neurons after one week of ovarian hormone depletion. Our findings are reminiscent of findings from hormone therapy studies, as early initiation relative to menopause of hormone therapy increases hippocampal volume, whereas late treatment initiation relative to menopause has no such beneficial effects on hippocampal volume (Erickson et al., 2010). In addition, we and others have found that the type of estrogens used can have opposing effects in humans and animal studies on neuroplasticity (Boccardi et al., 2006; Joffe et al., 2006; Linzmayer et al., 2001; Maki et al., 2007; McClure et al., 2013; Phillips and Sherwin, 1992; Resnick et al., 2009; Shaywitz et al., 2003). Here we found that one week of exposure to estradiol enhanced cell proliferation but one week of exposure to estrone decreased cell proliferation. This is consistent with findings that estradiol enhanced survival of new neurons after three weeks of exposure, whereas estrone decreased the survival, in rodents that underwent cognitive training in the Morris water maze (McClure et al., 2013). Indeed, estradiol-based hormone therapy improves verbal memory and increase hippocampal volume (Boccardi et al., 2006; Joffe et al., 2006; Phillips and Sherwin, 1992), whereas conjugated estrone-based hormone therapy can have a detrimental effect in post-menopausal women (Maki et al., 2007; Resnick et al., 2009; Shaywitz et al., 2003). Thus, in humans and in rodents, different estrogens modulate neuroplasticity and cognition depending not only on initiation of treatment relative to menopause/ovariectomy, but also on the type of hormone therapy (Barha et al., 2009; Barker and Galea, 2008; Boccardi et al., 2006; Chan et al., 2014; Maki et al., 2007; McClure et al., 2013; Ormerod et al., 2003b; Phillips and Sherwin, 1992; Resnick et al., 2009; Shaywitz et al., 2003; Tanapat et al., 2005, 1999).

4.5 Conclusion

Here we report that estrogens influence different facets of neurogenesis dependent on the type and duration of exposure to estrogens. Our findings add to the growing literature that estrone and estradiol have similar but not equivalent effects on neurogenesis. We also show that the duration of exposure to estrogens have dynamic effects on neurogenic parameters with proneurogenic effects within one week of exposure that are no longer evident with prolonged exposure to estrogens. Our findings shed a light on importance of studying short and long-term consequences of exogenous estrogens on adult neurogenesis and may lead to a greater understanding of how hormone therapy modulates neuroplasticity based on duration and type of estrogen treatment.

Chapter 5: High Estradiol reduces adult neurogenesis but strengthens functional connectivity within the hippocampus during spatial pattern separation in adult female rats

5.1. Introduction

New neurons are continuously generated in the subgranular zone of the dentate gyrus (DG) in the hippocampus in adulthood. These new neurons play a critical role in pattern separation (Clelland et al., 2009), a process that enables discrimination and episodic memory by separating similar memory patterns to make distinct neural representations (Marr, 1971). Estradiol modulates both neurogenesis and spatial ability in a dose-dependent manner (reviewed in Duarte-Guterman et al., 2015) but to our knowledge, no studies have examined whether estradiol can modulate pattern separation. Therefore, the present study aimed to elucidate the effects of two different doses of estradiol on pattern separation, adult neurogenesis and neural activation in the hippocampus. We hypothesized that estradiol would modulate the ability for pattern separation, adult neurogenesis and zif268 activation in the hippocampus.

5.2 Materials and methods

5.2.1 Subjects

Twenty-five two-month-old female Sprague Dawley rats were purchased from Charles River Canada (St-Constant, Quebec, Canada). Rats were initially pair-housed for two weeks after arrival and single-housed afterward throughout entire experiment. 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 (A20-0147). All efforts were made to reduce the number of animals used and their suffering during all procedures.

5.2.2 Animal husbandry

Rats were housed in opaque polyurethane bins (48 × 27 × 20 cm) with paper towels, polyvinylchloride tube, and cedar bedding. Rats have free access to water and normal lab chow, and maintained under a 12 : 12 hour light/ dark cycle (7A.M. light-on).

5.2.3 Apparatus

The radial arm maze (RAM) had an octagonal center platform (36 cm in diameter) and 8 arms (53 cm long × 10 cm wide) that were set 80 cm above the floor in the center of a dimly lit room. Large extramaze cues were placed on all four walls of the room and were not moved throughout the study. Metal gates were used to block entries to arms.

5.2.4 Experimental design

All animals were handled every day for 2 minutes beginning one week after arrival. All rats were bilaterally ovariectomized two weeks after arrival (Fig. 5.1A). Ovariectomized rats received daily subcutaneous injections of 0.32 µg (Low) or 5 µg (High) estradiol benzoate, or vehicle in 0.1ml of sesame oil beginning one week after surgery (Day 0) until the end of experiment. One day after the initiation of hormone or vehicle treatment, one intraperitoneal injection of bromodeoxyuridine (BrdU; 200mg/kg; Sigma-Aldrich, Oakville, ON, Canada) was administered to all animals two hours after the second hormone/vehicle treatment (Day1). Four days after BrdU injection, all animals were food restricted and maintained their weight at 87-92% of their original weight throughout entire behavioral testing. On Day 8 to Day 10, rats were habituated to the RAM and on Days 11-13, rats were shaped (food reward placed on arms of radial arm maze) for 5 minutes each day. Following shaping, all rats were tested in the delayed nonmatching to position (DNMP) RAM task for 14 days (Day14-27; Fig. 5.1B) one hour after

hormone/vehicle treatment, which was followed by an activation and probe trial (Day28; Fig. 5.1C). On the last day (Fig. 5.1D), rats received one activation trial (same procedure as the spatial pattern separation task). Rats then received a probe trial to determine whether they were idiothetic or place strategy users 80 minutes after the activation trial. Ninety minutes after the activation trial, all rats were perfused and underwent tissue collection. Brain sections were immunohistochemically stained for Ki67, zif268 and BrdU/NeuN (all methods are described in detail in the Extended Experimental Procedures). The present study analyzed the dorsal and ventral hippocampus separately as the dorsal hippocampus is important for spatial learning and memory, whereas the ventral hippocampus is important for regulation of anxiety and stress response (Kjelstrup et al., 2002; Moser et al., 1993).

Ovariectomy and hormone replacement

Rats were anesthetized using an initial flow rate of 5% isofluorane (Boxter Corp., Mississauga, ON, Canada) and 2-3% during surgery. 35 mg/kg Ketamine (Merial Canada Inc, Baie-d'Urfe, QC, Canada) and 1.5 mg/kg Xylazine were administered intraperitoneal, and 4mg/kg Marcaine HCL and 5mg/kg Anafen were administered subcutaneously (s.c.) before surgery. To prevent dehydration, 5 ml Lactated Ringer Solution (Braun Medical Inc, Scarborough, ON, Canada) was injected s.c. After a recovery phase of six days, the rats were divided into three groups, eight rats for high dose estradiol group, eight rats for low dose estradiol group and nine rats for vehicle treated group. High dose estradiol group received a s.c. injection of 5 µg estradiol benzoate (E2B: Sigma) in 0.1 ml sesame oil, low dose estradiol group received 0.32 µg E2B in 0.1ml sesame oil and vehicle treated group received 0.1 ml sesame oil for 29 consecutive days including the probe trial.

Habituation, shaping and behavioral testing for spatial pattern separation

During habituation, rats were placed on the center platform and allow them to freely explore all the arms for 10 minutes. During the first day of shaping, three quarters of Froot Loops (Kellog's) were located along each arm at equidistant intervals and a quarter was placed in a cup (3 cm in diameter) at the end of arms. During the second and the third day of shaping, each arm was baited only with a quarter of Froot Loops placed in each cup.

Rats received four trials of delayed non-match place (DNMP) version of pattern separation task in the radial arm maze (RAM) each day (two trials of each separation pattern) for 14 consecutive days (56 trials total with 28 trials of each separation). The first trial of each day began between 10 a.m. and 11 a.m two hours after hormone or vehicle treatment. Every rat received one trial before the first rat began their next trial to maximize the intervals between each trial. One trial consisted of two phases, a sample phase and a choice phase (40 seconds interval between the two phases). Rats were tested in their ability to discriminate the newly-opened arm during choice phases. During the sample phase, a start arm and a sample arm were open and all the 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 returned to their cage from the maze after spending ten seconds in the sample arm after eating the reward or exiting the sample arm. During the choice phase, all arms were closed 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 (entry to the sample arm or start arm) were permitted to self-correct and retrieve the reward from the correct arm. Rats were retrieved from the maze after rewarded or after one minute had passed and returned to the colony room. During the 40-second interval between a sample phase and a choice phase, the maze was rotated

to minimize the ability of rats to reach the correct arm by utilizing intra-maze cues such as odor. After the rotation, the location of the start and sample arms relative to extra-maze cues, but not the arms themselves, were held constant during each trial. The RAM was wiped with distilled water after each rat.

Two sets of sample-correct arm pairs were used in this study, ADJACENT and DISTINCT. Correct arms during ADJACENT trials were 45° away from the sample arm and correct arms during DISTINCT trials were 135° away from the sample arm. Start arms were located perpendicular to either the correct or sample arms (Figure. 1B). 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. The order of rats to be tested each day was randomized every day throughout this experiment.

Activation trial and probe trial

On Day 28, rats received hormone or vehicle injection in the morning between 9 a.m. and 10 a.m. One hour after hormone or vehicle treatment, rats received one set of activation trial (same procedure as DNMP version of spatial pattern separation task described previously). Following 80 minutes after the activation trial, rats received a probe trial to determine whether they were response strategy users (relying more on idiothetic response cues) or place strategy users (relying more on spatial extra-maze cues). A probe trial consisted of a sample phase and a choice phase with 40 seconds of interval between the two phases. A sample phase of the probe trial has the same rules as a sample phase of testing trials. 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. The start arm was moved to a new position and two choice arms (135° away from each other) were opened after the rotation. One choice arm (Idiothetic arm) was held the same position as the sample arm relative to the extra-maze cues and the other choice arm (Place arm) was perpendicularly right from the new start arm. A place strategy user chose the newly opened arm by using extra-maze cues, while an idiothetic strategy user chose the sample arm with ignoring extra-maze cues as the orientation of start-Place arm pair (90°) during the choice phase was the same as that of start-sample arm pair (90°) during the sample phase (Fig. 1C). Immediately after the probe trial, rats were perfused (about 90 minutes after the activation trial).

Perfusion and tissue processing

Rats were administered an overdose of sodium pentobarbitol (500 mg/kg, i.p.) and perfused transcardially with 60 ml of 0.9% saline followed by 120 ml of 4% formaldehyde (Sigma-Aldrich). Brains were extracted and post-fixed in 4% formaldehyde overnight, then transferred to 30% sucrose (Fisher Scientific) solution for cryoprotection and remained in the solution until sectioning. Brains were sliced into 30 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.

5.2.5 Immunohistochemistry

Ki-67 DAB staining

Brain sections were rinsed overnight with 0.1 MPBS 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- Ki67 antibody (Vector Laboratories), 0.3% Triton-X, and 3% normal goat serum (NGS; Vector Laboratories) in 0.1 M PBS for 24 hours at 4 °C. Following rinsing the sections four times, the sections were 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 sections were then incubated in AB solution (Vector Laboratories) for 1 hour at room temperature. The sections were then visualized with diaminobenzidine (DAB; Sigma) solution and mounted onto microscope slides, followed by dehydrated, cleared with xylene and cover-slipped with Permount (Fisher Scientific; Ottawa, ON, Canada).

Zif268 DAB staining

Brain tissue was rinsed overnight with 0.1 MPBS 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 (Milli- pore; MA, USA) or anti-cFos (Milli- pore; MA, USA), 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 AB solution (Vector Laboratories) for 1 hour at room temperature. Tissue slices were then visualized with diaminobenzidine (DAB; Sigma) solution and mounted onto microscope slides, followed by dehydrated, cleared with xylene and cover-slipped with Permount (Fisher Scientific; Ottawa, ON, Canada).

BrdU/NeuN double fluorescent 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 rinsed three times with PBS, tissue was washed with 4% formaldehyde, 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 rinsed with PBS, tissue was mounted onto microscope slides and cover-slipped with PVA DABCO.

5.2.6 Cell counting

All counting was conducted by an experimenter blind to the group assignment of each animal using a Nikon E600 microscope. Locations of immunoreactive cells were examined whether in the dorsal or ventral dentate gyrus using the criterion defined by Banasr and others (2006), with sections 6.20-3.70mm from the interaural line defined as dorsal and sections 3.70-2.28mm from the interaural line as ventral. 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 emotional responses (Moser et al., 1993; Kjelstrup et al., 2002).

BrdU-immunoreactive (ir) cells were counted under a 100x oil immersion objective lens. For BrdU-ir cells, every tenth section of the hilus or the granule cell layer (GCL) that includes the subgranular zone and total immunoreactive cells per region was estimated by multiplying the aggregate number of cells per region by ten. Density of BrdU-ir cells was calculated by dividing the sum of immunoreactive cells in the hilus or GCL by volume of the corresponding region. Volume estimates of the dentate gyrus were calculated using Cavalieri's principle by multiplying the summed areas of the dentate gyrus by distance between sections (300μm). Area measurements for the dentate gyrus were obtained using digitized images on the software ImageJ (NIH).

The percentages of BrdU/NeuN-ir cells were obtained by randomly selecting 50 BrdU-ir cells and calculating the percentage of cells that co-ir with NeuN under 40x objective lens using a Nikon E600 epifluorescent microscope (Figure. XX).

Optical density of zif268 expression in the dentate gyrus, CA3 and CA1 subregions was analyzed as an estimate of the proportion of immunoreactive cells in the subregions. Images of the hippocampus were acquired at 100× magnification from three sections from the dorsal hippocampus and three sections from the ventral hippocampus on a Nikon E600 light microscope (Figure. 2D-I). The proportion of area that exhibited above-threshold zif268 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. The background gray levels were the mean gray values that were obtained from five randomly selected areas without immunoactivity. 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.

5.2.7 Statistical analyses

All analyses were conducted using Statistica (Statsoft Tulsa, OK, USA) and significance level was set at $\alpha = 0.05$. The percentage of correct choices during ADJACENT or DISTINCT trials were each analyzed using two-way analysis of variance (ANOVA), with strategy choice (spatial, idiothetic) and treatment (Vehicle, Low dose, High dose) as between-subject variables. Chi-square analyses were used for strategy choice across the treatment. Two-way ANOVAs were used for each dorsal (d) and ventral (v) DG to analyze the density of Ki67-ir cells and BrdU/NeuN-ir cells, and for each subregion of the hippocampus (dDG, vDG, dCA3, vCA3, dCA1, vCA1) to analyze the density of zif268-ir cells with strategy choice and treatment as between-subject variables. Post-hoc tests utilized the Neuman-Keuls procedure. As a measure of functional connectivity, Pearson product-moment correlations were also calculated between the density of zif268-ir cells among the hippocampus subregions and a brain network map for each treatment was calculated with coefficient of correlations (r) for each inter-regional correlation. The coefficient of variation (r^2) for each inter-regional correlation for each treatment group was calculated to measure the proportion of variance accounted for between the two subregions of the hippocampus, which was used as a measure of the strength of connectivity (Liu et al., 2022). The r^2 values were analyzed using one-way ANOVA with treatment as the between-subject variables. In addition, Pearson product-moment correlations were calculated between the density of BrdU/NeuN-ir cells in the dDG or vDG and the density of zif268-ir cells in each subregion of the hippocampus. A priori comparisons and Pearson product-moment correlations were subjected to Bonferroni corrections.

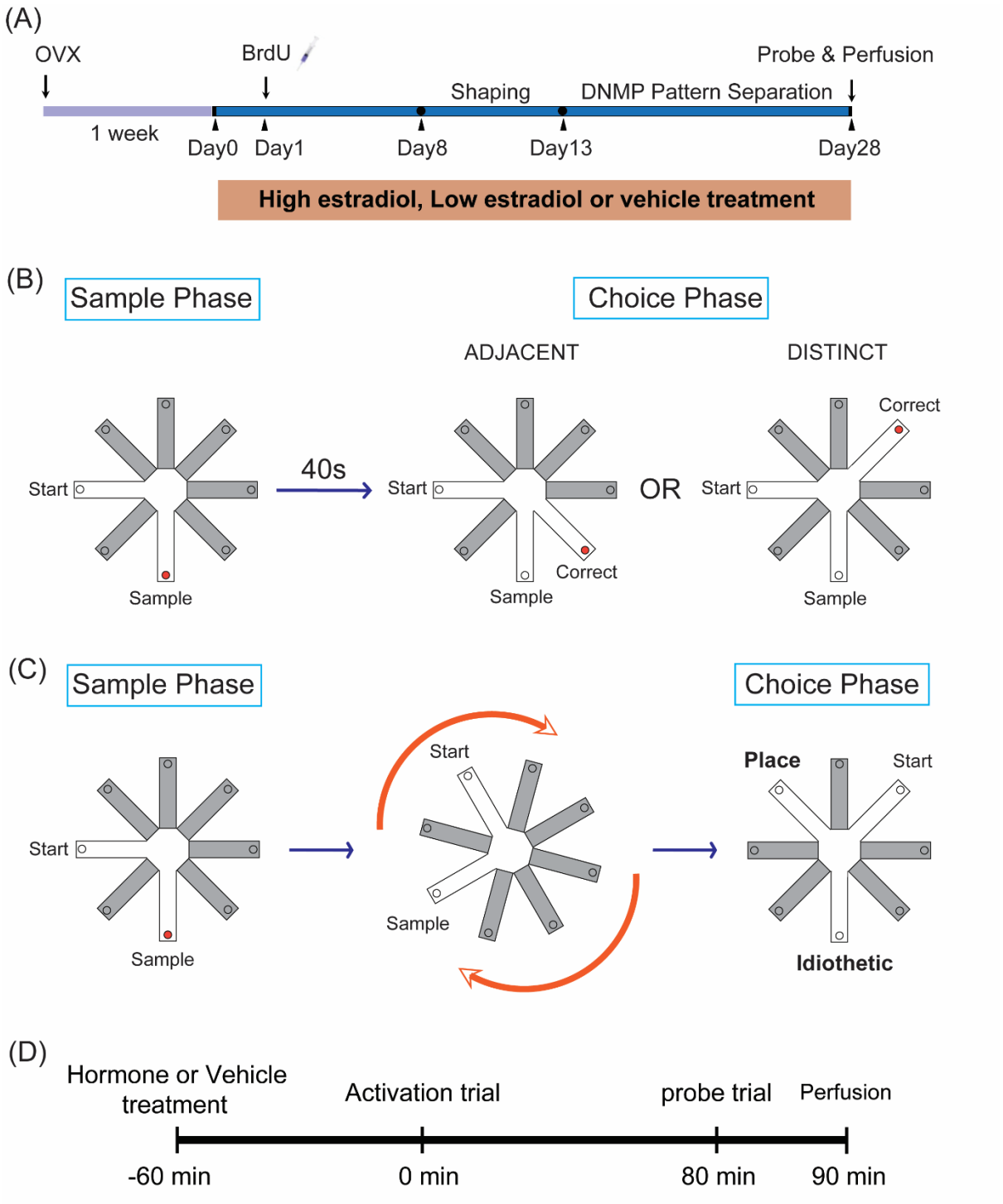
(A)
OVX
BrdU
Probe & Perfusion
Shaping
DNMP Pattern Separation
1 week
Day0
Day1
Day8
Day13
Day28
High estradiol, Low estradiol or vehicle treatment
(B)
Sample Phase
Choice Phase
ADJACENT
DISTINCT
Start
40s
Start
OR
Start
Correct
Correct
Sample
Sample
Sample
(C)
Sample Phase
Choice Phase
Start
Start
Sample
Sample
Place
Start
Idiothetic
(D)
Hormone or Vehicle treatment
Activation trial
probe trial
Perfusion
-60 min
0 min
80 min
90 min

Fig. 5.1. Experimental design of the spatial pattern separation task in ovariectomized rats. (A) Timeline of the experiment. (B) The spatial pattern separation task utilized the delayed non-match to position task in the radial arm maze, and rats received four trials each day (two trials of each separation arm pattern – distinct and adjacent). Each trial consists of sample phase and choice phase where rats were tested in their ability to discriminate the newly-opened arm during choice phases. During the sample phase, a rat was placed on the start arm and allowed to retrieve a food reward (indicated with orange circle in Fig. 1B) at the end of Sample arm. During the choice phase, the additional/correct arm, but not the sample arm, was baited (Correct arm). (C) Rats were classified as a place strategy user or an idiothetic strategy user based on their arm choice during the choice phase of probe trial. After the sample phase of the probe trial, the maze was rotated and the start arm was moved to a new location and two choice arms were presented. One choice arm (Idiothetic arm) was held the same position as the sample arm relative to the extra-maze cues and the other choice arm (Place arm) was perpendicularly right from the new start arm. A place strategy user will choose the newly opened arm by using extra-maze cues, whereas an idiothetic strategy user will choose the sample arm with ignoring extra-maze cues as the orientation of start-Place arm pair (90°) during the choice phase was the same as that of start-sample arm pair (90°) during the sample phase. (D) Timeline on the last day. All rats were perfused 90 minutes after the activation trial.

5.3 Results

5.3.1 There was no significant effect of estradiol on strategy use or on the ability for separating either adjacent or distinct patterns

Neither chronic administration of High nor Low estradiol affected strategy use nor the percentage of correct arms chosen regardless of the pattern (adjacent or distinct) compared to vehicle treatment (p's > 0.345; Supplemental Fig. 5.1A-B).

5.3.2 High estradiol reduced the density of BrdU/NeuN-ir cells in the dDG

High estradiol-treated rats had significantly less density of BrdU/NeuN double-ir cells in the dDG compared to vehicle-treated rats ($p = 0.011$, Cohen's $d = 1.846$) and a trend compared to Low estradiol-treated rats ($p = 0.065$, Cohen's $d = 1.045$) [main effect of treatment: $F(2, 17) = 4.621$, $p = 0.025$, partial $\eta^2 = 0.352$; Fig. 5.2A]. There were no other significant main or interaction effects on the density of BrdU/NeuN-ir cells (p > 0.299) or Ki67-ir cells (p's > 0.116; Supplemental Fig. 5.1C-D) in the DG.

5.3.3. zif268 expression in the vCA1 was greater in High estradiol-treated rats than the other groups

High estradiol-treated rats tended to have a greater density of zif268-ir cells in the vCA1 compared to vehicle-treated rats and Low estradiol-treated rats [vehicle: $p = 0.058$, Cohen's $d = 1.320$; Low: $p = 0.070$, Cohen's $d = 1.158$; main effect of treatment: $F(2, 18) = 3.167$, $p = 0.066$, partial $\eta^2 = 0.352$; see Fig. 5.2B]. There were no main or interaction effects on the density of zif268-ir cells in the hippocampus (p's > 0.222).

5.3.4. High estradiol-treated rats had distinct inter-regional correlations compared to the other groups

For both estradiol-treated groups there were significant intra-hippocampal correlations of zif268 activity between the six regions, but there were no significant correlations in the vehicle group (3 in High, 1 in Low, 0 in Vehicle). Intriguingly, all the three correlations in the High estradiol-treated group involved the DG (2 involved dDG and 1 involved vDG) but not in the Low estradiol-treated group. However, after applying Bonferroni corrections, the High estradiol-treated rats had two significant correlations in the density of zif268-ir cells between the dDG and the dCA1 [$r(7) = -0.963$, $p = 0.002$; Fig. 5.2C] and the vDG and the dCA3 [$r(6) = 0.940$, $p = 0.002$; Fig. 5.2D]. There were no significant inter-regional correlations in the hippocampus in the other two groups after Bonferroni corrections (p's > 0.030). Furthermore, High estradiol-treated rats had greater coefficients of variation (mean $r^2 = 0.445 \pm 0.073$) in the hippocampus compared to Low estradiol-treated rats (mean $r^2 = 0.240 \pm 0.054$) and vehicle-treated rats (mean $r^2 = 0.153 \pm 0.043$) [Low estradiol: $p = 0.016$, Cohen's $d = 0.827$, Cohen's $d = 1.265$; vehicle: $p = 0.003$; main effect of treatment: $F(2, 42) = 6.746$, $p = 0.003$, partial $\eta^2 = 0.243$]. There was no significant difference between Low estradiol-treated rats and vehicle-treated rats ($p = 0.292$).

In addition, there were no significant correlations between the density of BrdU/NeuN-ir cells and the density of zif268-ir cells in any hippocampal subregions after Bonferroni correction (p's > 0.035).

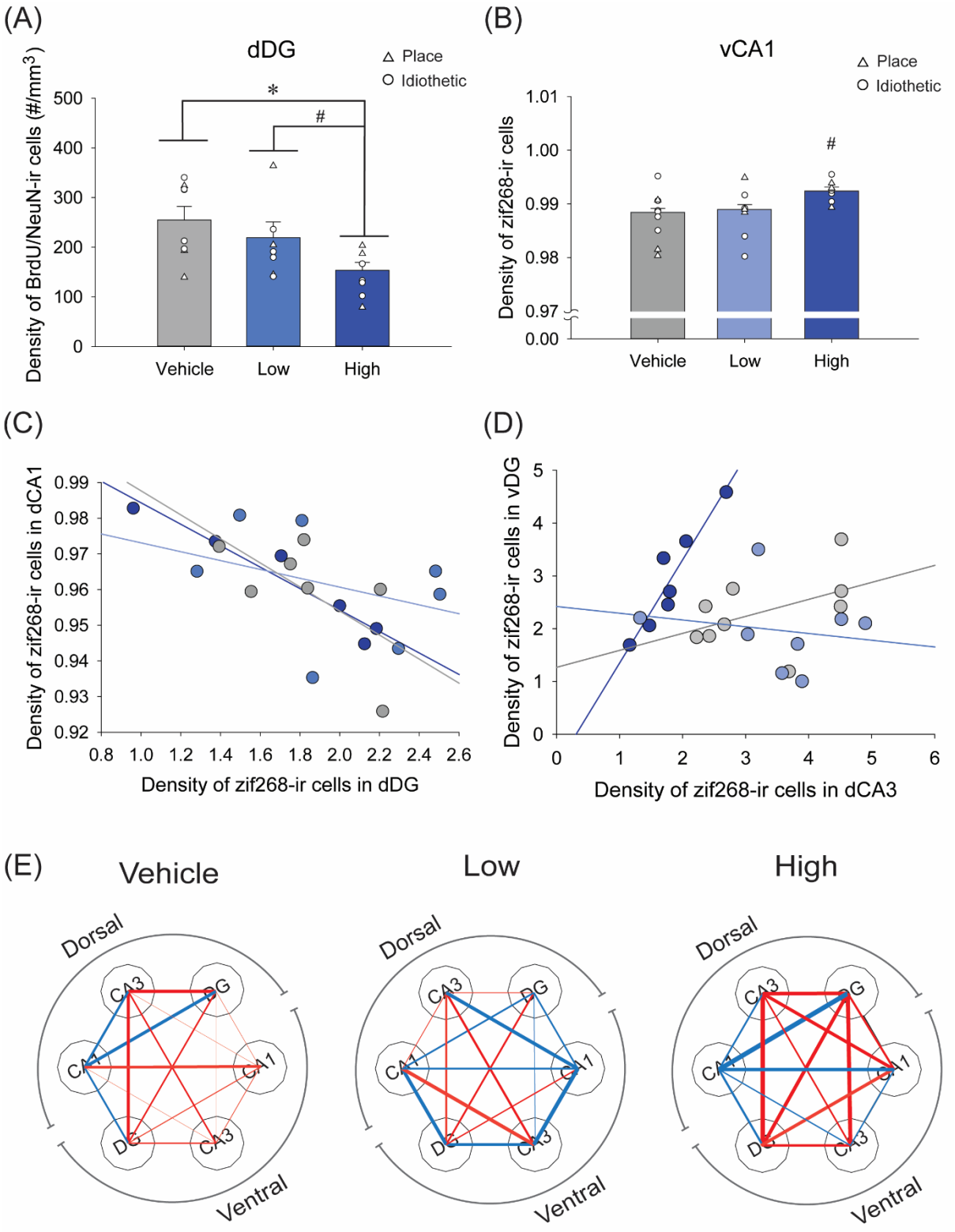
(A)
dDG
Place
Idiothetic
Density of BrdU/NeuN-ir cells (#/mm3)
500
400
300
200
100
0
*
#
Vehicle
Low
High
(B)
vCA1
Place
Idiothetic
Density of zif268-ir cells
1.01
1.00
0.99
0.98
0.97
0.00
#
Vehicle
Low
High
(C)
Density of zif268-ir cells in dCA1
0.99
0.98
0.97
0.96
0.95
0.94
0.93
0.92
0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6
Density of zif268-ir cells in dDG
(D)
Density of zif268-ir cells in vDG
5
4
3
2
1
0
0 1 2 3 4 5 6
Density of zif268-ir cells in dCA3
(E)
Vehicle
Low
High
Dorsal
Ventral
CA3
DG
CA1
DG
CA3
CA1

Fig. 5.2. Estrogenic regulations for adult neurogenesis and inter-regional correlations in the hippocampus. (A) Mean density of bromodeoxyuridine (BrdU)/neuronal nuclei (NeuN)-immunoreactive (ir) cells in the dorsal (d) dentate gyrus (DG). High estradiol-treated rats had a significantly less density of BrdU/NeuN-ir cells and Low estradiol-treated rats had a trend of less density of BrdU/NeuN-ir cells in the dDG compared to vehicle-treated rats. (B) Mean density of zif268-ir cells in the ventral (v) Cornu Ammonis (CA) 1. High estradiol-treated rats had a trend of greater density of zif268-ir cells in the vCA1 compared to Low estradiol-treated rats and compared to vehicle-treated rats. (C-D) Inter-regional correlations of the density of zif268-ir cells in vehicle-treated (grey), Low estradiol-treated (light blue) and High estradiol-treated rats (dark blue). High estradiol-treated rats had a negative correlation between the dCA1 and the dDG (C) and a positive correlation between the vDG and the dCA3 (D). (E) Brain network maps were generated with correlations with red lines indicating positive correlations and blue lines indicating negative correlations with wider lines indicating larger coefficients in vehicle-treated (Vehicle), Low estradiol-treated (Low) and High estradiol-treated (High) rats. High estradiol treated rats had greater overall r^2 values compared to other groups. Error bars represent ± SEM. * indicates $p < 0.05$ and # indicates $0.05 < p < 0.10$.

5.4 Discussion

5.4.1 High levels of estradiol decreased adult-born neurons in the dDG

The present study demonstrates that high levels of estradiol decreased the density of new neurons (BrdU/NeuN-ir) in the dDG compared to vehicle-treated rats, consistent with past studies in female rats that did not undergo spatial testing (Chan et al., 2014). Estradiol can upregulate or downregulate neurogenesis dependent on the timing of estradiol and BrdU injection (Ormerod et al., 2003b) and to cognitive training (McClure et al., 2013). Because in the present study we injected BrdU 26 h after the first injection of high estradiol benzoate, this likely lead to the reduction in survival of new neurons, due to lower cell proliferation at that time (Ormerod et al., 2003b). The ability of estradiol to reduce cell proliferation after 24-48 h in

females is due to estradiol's effects to increase adrenal steroids, but not on estradiol's effects on NMDA receptor activation (Ormerod et al., 2003a). Further research is warranted to elucidate molecular, cellular and systemic mechanisms underlying the effect of timing of estradiol on adult neurogenesis in females and how cognitive training may influence this.

5.4.2 Estradiol altered patterns of neural activation and functional connectivity in the hippocampus

Previous studies demonstrate that estradiol increases CA1 dendritic spine density through activation NMDA receptors and increases synaptic excitability through activation of AMPA receptors (Wong and Moss, 1992; Woolley and McEwen, 1994). Thus, it is possible that greater neural activation in the vCA1 in the present study is due to the long-term effect (increasing dendritic spines) or the short-term effect (increasing excitability) of estradiol. However, in this study we used 29 days of treatment with estradiol, while others have used acute treatment of estradiol on the electrophysiological properties of CA1 neurons, thus the influence of chronic estradiol treatment on electrophysiological properties are still unknown. The present study also demonstrates that high estradiol rats have an inverse association of the neural activation between the dDG and dCA1, and neural coactivation between vDG and dCA3. Furthermore, high estradiol rats had greater overall amount of variance accounted for across inter-regional correlations in the hippocampus compared to the other groups. These findings are important as it suggests that chronic high levels of estradiol enhance functional connectivity among hippocampal subregions during spatial learning, although this did not affect learning outcomes. Previous work in humans and rodents demonstrate that functional connectivity in the hippocampus decreases with age, which is associated with spatial learning and memory (Dalton et al., 2019; Liang et al., 2020). Taken together, it is possible that high estradiol treatment can

restore age-related decline in functional connectivity in the hippocampus, and potentially improve hippocampus-dependent cognition in aged subjects.

5.4.3 Strategy use was not altered by estradiol dose

Estradiol dose did not affect strategy use in the present study and this is inconsistent with findings from other studies examining strategy use across the estrous cycle (Korol et al., 2004). The present study also failed to demonstrate significant strategy differences in zif268 activation in the dorsal hippocampus or significant correlations between the density of zif268-ir cells and the density of new neurons in the dorsal hippocampus. These results indicate that the hippocampus was equally recruited during the activation trial between the two strategy users. The present study used daily injections of 5µg or 0.32µg of estradiol benzoate into young ovariectomized rats, which gives serum concentrations of estradiol equivalent to proestrous or diestrous females, respectively (Becker and Rudick, 1999). However, repeated exogenous estrogens may influence adult neurogenesis and cognition differently than naturally fluctuating estrogens. Proestrus is associated with a hippocampus-dependent place strategy, while diestrus is associated with striatum-dependent response strategy (reviewed in Yagi and Galea, 2019). Furthermore, low estradiol phase females perform better at spatial learning tasks in the Morris water maze compared to high estradiol phase females (Galea et al., 1995; Warren and Juraska, 1997) and thus perhaps other ovarian hormones work to influence strategy use.

In the present study we also saw no significant effects of chronic estradiol benzoate treatment on spatial pattern separation performance, inconsistent with past work that indicated that low and high chronic estradiol benzoate modulated working memory in the spatial radial arm maze (Holmes et al., 2002). Consistent with other studies, the present study shows that long-

term exposure to exogenous estradiol loses the enhancing effect of estradiol on cell proliferation(reviewed in Duarte-Guterman et al., 2015). Therefore, further research is needed to elucidate complex effects of estradiol on cognition and underlying mechanisms.

5.5 Conclusion

The present study demonstrates that estradiol benzoate modulates neurogenesis and activity within the hippocampus of young adult female rats in a dose dependent manner, with no significant effect of estradiol on pattern separation performance. As female estradiol levels vary across the estrous cycle, females have different hippocampal neural network activation depending on the estrous cycle stage. These findings highlight the importance of considering the hormonal status when studying brain connectome and hippocampal neural plasticity.

Chapter 6: General Discussion

6.1 Summary of experimental findings

Here in this dissertation, I examined sex differences in basic characteristics of adult neurogenesis such as the maturation rate and trajectory of new neurons (Chapter 2), and in the ability for contextual pattern separation and functional connectivity of new neurons (Chapter 3). Furthermore, I examined the estrogenic regulation of the basic characteristics of neurogenesis (Chapter 4) and the ability for spatial pattern separation in females (Chapter 5).

I found that adult-born neurons (BrdU/NeuN-ir) matured faster in males compared to females (Yagi et al., 2020: Chapter 2). Males had a greater density of neural stem cells (Sox2-ir) in the dorsal, but not in the ventral, dentate gyrus and had higher levels of cell proliferation (Ki67-ir) than non-proestrous females. However, males showed a greater reduction in neurogenesis between one week and two weeks after mitosis, whereas females showed similar levels of neurogenesis throughout the weeks. The faster maturation and greater attrition of new neurons in males compared to females suggests greater potential for neurogenesis to respond to external stimuli in males (Chapter 2).

In Chapter 3, I found that females, but not males, showed contextual discrimination during the last days of training. However, both sexes displayed similar levels of freezing on the last day, indicating equivalent fear memory for fear associated context. Despite similar fear memory, males showed more positive correlations of zif268 activation between the limbic regions and the striatum, whereas females showed more negative correlations among these regions. Females showed greater activation of the frontal cortex, dorsal CA1, and 3-week-old adult-born dentate granular cells compared to males (Chapter 3).

In Chapter 4, I found that estradiol reduced the density of neural stem cells in the dorsal DG, whereas estrone reduced the density of neural stem cells in the ventral DG. Furthermore, estradiol enhanced, whereas estrone reduced, cell proliferation after one week but not after longer exposure to hormones. Both estrogens increased the density of immature new neurons (BrdU/DCX-ir cells) after one week of exposure but showed greater attrition of new neurons between one and two weeks after exposure. Lastly, estradiol decreased the percentage of mature new neurons (BrdU/NeuN-ir cells) in the dorsal dentate gyrus after three weeks of treatment. These results demonstrate that estrogens have differential effects to modulate several aspects of adult hippocampal neurogenesis in the short term, but fewer effects after long-term exposure (Chapter 4).

Overall, these results highlight the importance of studying sex differences and hormonal regulation in hippocampal neuroplasticity and the contribution of adult neurogenesis to hippocampus dependent cognition. Studying sex and hormone differences will lead to a better understanding how the sexes show different susceptibility to hippocampus-related diseases such as post-traumatic stress disorder and Alzheimer's disease.

6.2 The maturation of new neurons in neurodegenerative diseases

Previous studies demonstrate that the maturation of new neurons is compromised in Huntington's disease, Alzheimer's disease and other types of dementia, which is associated with impairment in hippocampus dependent cognition (Moreno-jiménez et al., 2019; Terreros-Roncal et al., 2021). Previous studies demonstrate that the maturation and survival of adult-born new neurons in the dentate gyrus are regulated by various transcriptional factors such as CREB, Kruppel-like factor 9 (Klf9) and NeuroD1 (Gao et al., 2009; Jagasia et al., 2009; Kuwabara et al.,

2009; Scobie et al., 2009). Overexpression of NeuroD1 in the dentate gyrus accelerates neuronal maturation of adult-born neurons, and enhanced maturation of new neurons, via NeuroD1, restores cognitive impairment in a mouse model of Alzheimer's disease (Richetin et al., 2015). These studies indicate that delayed maturation of new neurons plays an important role in cognitive impairment in neurodegenerative diseases, and interventions modulating the maturation time course of new neurons could be a potential therapeutic for such diseases.

Here I demonstrate that a higher percentage of new neurons express mature neuronal phenotypes in males compared to females suggesting faster maturation of new neurons in healthy young male rats compared to females. However, no study has investigated how sex differences in the maturation time course of new neurons affects the function of the dentate gyrus. As there are sex differences in hippocampus dependent cognition (as discussed in Chapter 1), it is possible that these sex differences in basal characteristics of neurogenesis in the dentate gyrus may result in the sex differences in hippocampus dependent cognition, neural networks engaging with the hippocampus, and strategy use during learning and memory. Although no study has investigated how the maturation time course may be altered with normal aging or in pathological conditions of neurodegenerative diseases between males and females, slower maturation may be associated with more severe symptoms in females in certain disorders. Therefore, future research should examine how the maturation time course changes within healthy aging and in neurodegenerative diseases in males and females.

6.3 How can the overall neurogenesis be increased in males and females?

Previous work demonstrates that various interventions such as environmental enrichment and cognitive training can enhance adult neurogenesis via increasing the survival of new neurons, and enhanced neurogenesis can improve certain forms of cognition and rescue some

depressive-like symptoms in rodents (Choi et al., 2018; Epp et al., 2011; Fabel et al., 2009; Micheli et al., 2018; Nilsson et al., 1999; Schloesser et al., 2010; van Praag et al., 1999; Veena et al., 2009). However, some of the pro-neurogenic effects have a limited time window as cognitive training enhances the survival of new neurons only when the interventions occur between one and two weeks after mitosis in male rats (Epp et al., 2011; Elizabeth Gould et al., 1999). It is important to note that males are more likely to respond to interventions targeting the survival of new neurons between one and two weeks, compared to females, as I found in Chapter 2 that males have a greater density of new neurons one week after mitosis. In contrast, the characteristics of new neurons development in females suggests that females require not only those interventions that enhance the cell survival between one and two weeks, but also may require enhancement of cell proliferation or early survival of new neurons (earlier than one week). This is intriguing as these findings suggest that males and females may show differential efficacy to potential treatments for Alzheimer's disease and depression with targeting neurogenesis in the hippocampus.

Interestingly, male mice with reduced proliferation in the dentate gyrus fail to show the beneficial effects of environmental enrichment on cognition (Schloesser et al., 2010). Furthermore, female rats that receive combined interventions of aerobic exercise (enhancing proliferation and survival) and environment enrichment (enhancing survival) show greater enhancement in neurogenesis and greater improvement in depressive-like behaviour compared to each intervention alone (Fabel et al., 2009). In addition, estradiol treatment (enhancing proliferation) coupled with spatial learning in the Morris water maze enhances overall neurogenesis via enhancing the survival of new neurons born under the influence of estradiol (McClure et al., 2013). It is important to note that the enhancing effect of estradiol on cell

proliferation is eliminated with long-term estradiol treatment, although this may depend on the timing of estradiol to BrdU injection (Chapter 4). Thus, the timing of each intervention must be carefully considered to optimize the efficacy of treatment. These findings support the idea that males and females may need different therapeutic strategies to prevent or delay the progression of hippocampus-related diseases.

6.4 Estrogenic regulation of neurogenesis as a potential mechanism of sex differences in the maturation and trajectory of new neurons

Any time sex differences are seen in a phenotype, this suggests that sex hormones or sex chromosomes or some combinations are involved. Given the sex differences in the maturation and trajectory of adult-born neurons (Yagi et al., 2020: Chapter 2), I also examined the effect of estrogens on the maturation time course and trajectory of new neurons in young adult female rats. In Chapter 4, I found that estrogens modulated the basic characteristics of neurogenesis in young adult females dependent on type of estrogen and duration of treatment (Chapter 4). However, the magnitude of effects in Chapter 4 do not account for all of the sex differences seen in Chapter 2. Thus, sex differences in the maturation rate and trajectory of new neurons in Chapter 2 are likely driven in concert by other factors such as progesterone or testosterone, and genetic differences between males and females.

Ovaries and testicles also produce progesterone, which plays important roles in hippocampal plasticity and cognition (reviewed in Mahmoud et al., 2016). Although testicles produce progesterone, the hormone is found in much lower levels in males compared to females (Jeyaraj et al., 2001). Similar to estrogens, acute administration of progesterone enhances cell proliferation in ovariectomized young rats, whereas chronic administration of progesterone has no significant effect on neurogenesis (Chan et al., 2014), suggesting desensitization of chronic

progesterone treatment on neurogenesis similar to my findings in Chapter 4. Although there is no study examining the effects of progesterone on the maturation time course of new neurons in the dentate gyrus, it is less likely that progesterone has an impact on the attrition of new neurons in females according to the findings in Chan et al. (2014).

Testosterone is found in higher levels in males, which modulates adult neurogenesis (reviewed in Mahmoud et al., 2016). Briefly, testosterone enhances the survival of adult-born new neurons in castrated young rats, whereas there is no significant effect of testosterone on cell proliferation (Spritzer and Galea, 2007). This enhancing effect of testosterone on the survival of new neurons can be blocked by administration of androgen receptor antagonist or in rats with testicular feminization mutation, which renders the ARs non-functional (Hamson et al., 2013). In Chapter 2, I found that males had greater cell proliferation compared to females. As testosterone does not influence cell proliferation, the sex differences may be driven by different factors such as estradiol levels in females. Previous studies demonstrate that female rats during proestrus (high estradiol) have significantly greater cell proliferation compared to non-proestrous females (Rummel et al., 2010; Tanapat et al., 1999). Indeed, all females in Chapter 2 had low levels of estradiol (equivalent to diestrus). Furthermore, previous studies used BrdU to assess cell proliferation whereas I used an endogenous marker, Ki67, which may lead inconsistent results. In terms of maturation time course, as no study has examined the effect of testosterone on the maturation rate of new neurons throughout 1w-3w, future studies should explore the effects of testosterone on the maturation rate of new neurons. Overall, further research is warranted to elucidate underlying mechanisms of sex differences in the maturation of adult-born neurons, and its behavioural outcomes in males and females.

6.5 Sex differences in the functional connectivity and cognition

In Chapter 3, I demonstrated that males and females have distinct patterns of inter-regional functional connectivity in response to fear memory retrieval whereas there is no significant sex difference in behaviour (Chapter 3). In particular, males have more functional connectivity within the hippocampus, whereas females have more functional connectivity between the hippocampus and other brain regions. This result suggests that males and females recruit different neuronal networks along with different strategies during fear memory retrieval even though they show similar performance in the task. Previous studies have reported that males and females show different behaviours in a fear conditioning task, where both male and female rats exhibit freezing behaviour, whereas only females show darting behaviour in response to conditioned fear stimulus during the cued fear conditioning paradigm (Gruene et al., 2015). This finding suggests that females choose a more active strategy to avoid potential threats compared to males. Although I failed to find darting behaviour in both male and female rats in the contextual fear discrimination task (Chapter 3), it is plausible that males and females differently processed fear memory as the two sexes showed distinct patterns of neural activation. Future research should explore whether neural activation in particular brain regions contribute to fear responses in males and females as this may give us important clues how sex differences develop in susceptibility to anxiety disorders, and brain regions that could be targeted for treatment. Furthermore, as functional connectivity is rather dynamic than static (Hutchison et al., 2013), it is important to study the contribution of hippocampal plasticity to the dynamics of brain neural network for learning and memory.

6.6 Effects of stress on hippocampal cognition and adult neurogenesis in males and females

Previous studies examining learning after stressful experiences show sex differences in hippocampal plasticity and in performance during hippocampus dependent tasks (Bowman et al.,

2001; Galea et al., 1997; Wood and Shors, 1998). For instance, repeated restraint stress (21 days) reduces dendritic complexity in the apical or basal dendrites of CA3 pyramidal neurons of males and females, respectively (Galea et al., 1997). However, this same stress regime results in opposing effects on spatial acquisition with impairments in males but improvements in females although the sexes were not directly compared in one study for learning outcomes (reviewed in Luine, 2002). Thus, seemingly small differences (apical vs basal) to the architecture of dendrites can lead to opposing effects on spatial memory between the sexes. This association of a reduction in branching leading to an improvement in learning outcomes, or an increase in spines related to a time of impairments in learning outcomes, is a pattern that is often seen in females. The idea that female performance may be more sensitive to stress, may explain some sex differences seen in the Morris water maze. When rats were pre-exposed to the environment these studies failed to demonstrate the sex difference in spatial performance (Hvoslef-Eide and Oomen, 2016; Jonasson, 2005). Pre-exposure to the water maze reduces the stress levels and circulating levels of stress hormones during the testing sessions and it is possible that females perform better under a less stressful environment (Beiko et al., 2004; Engelmann et al., 2006). As stress modulates learning and memory, and neural plasticity in the hippocampus differently between sexes, it is possible that the sex differences in contextual pattern separation in Chapter 3 is due to sex differences in stress response or in the ability for stress adaptation.

Stress also acts on adult neurogenesis in a sex-dependent manner (Falconer and Galea, 2003; Hillerer et al., 2013). For example, acute predator odor decreases cell proliferation in male, but not female rats (Falconer and Galea, 2003) and this was not dependent on levels of testicular hormones in adult males (Kambo and Galea, 2006) or ovarian hormone levels in adult females (Falconer and Galea, 2003). Similarly, repeated restraint stress reduced the number of

proliferating stem cells in adult males, but not females (Hillerer et al., 2013). However, 9 days after 12 days of restraint stress, neurogenesis was reduced in adult females, but not adult males (Hillerer et al., 2013), suggesting that the duration of time after stress exposure had more impact in females compared to males. Other studies find that both males and females show reduced neurogenesis to stress (footshock, water restraint) and these discrepancies are likely dependent on the nature of the stressor (Tzeng et al., 2014). Together, these studies indicate the nature, duration and timing of the stressor as well as what aspect of neurogenesis is measured (proliferation, immature neurons, mature neurons) will affect the expression of sex differences on neurogenesis in the dentate gyrus. Therefore, future studies should include detailed information about experimental procedures especially factors that potentially influence behavioral outcomes such as length of handling, housing conditions, and type of behavioural paradigm.

6.7 Species differences in adult neurogenesis such as maturation time course

It is important to note that the kinetics of the maturation of newly produced neurons vary between species (Snyder et al., 2009). For instance, newly produced neurons of male rats exhibit mature neuronal marker and activity dependent immediate early gene expression 1-2 weeks earlier than in male mice (Snyder et al., 2009). At 3 weeks of age after cell division, about 90% of adult born neurons in male mice but only 18% in male rats express immature neuronal marker, doublecortin (DCX) indicating faster maturation of new neurons in rats compared to mice (Snyder et al., 2009). The maturation of adult-born neurons also differs in primates compared to rodents, as approximately 84% of adult-born granule cells at 6 weeks after cell division, and more than 40% of new dentate granule cells at 13 weeks of age express DCX in macaque monkeys (Kohler et al., 2011: Fig. 6.1 A), whereas none of adult-born neurons expresses DCX at

10 weeks in either rats or mice (Snyder et al., 2009: see Fig. 6.1 B-C). Furthermore, the majority of adult born neurons at 5-6 weeks of age in the dentate gyrus of rhesus monkeys are morphologically immature in terms of branch the number and size of somata (Kohler et al., 2011; Ngwenya et al., 2006). Collectively these studies indicate that the timing of the maturation of a new neuron in the dentate gyrus is very different in rodents compared to primates. Therefore, it is possible that the findings in this dissertation may be very different in humans in terms of maturation time course and trajectory of adult-born neurons. Further research examining the maturation time course of adult-born neurons is waited as future treatment targeting adult neurogenesis may bear more fruitful outcomes depending on the timing of intervention.

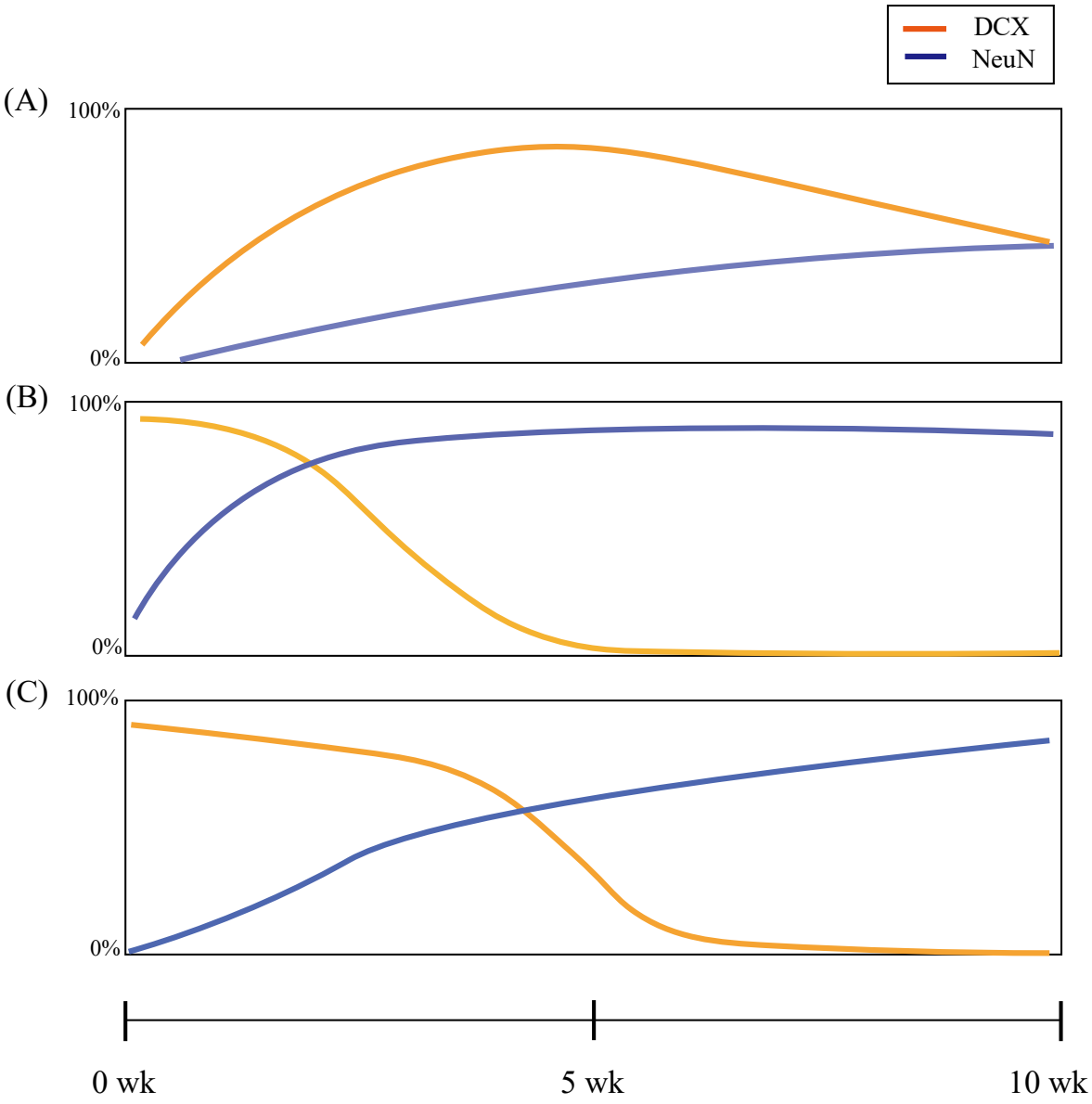

Fig. 6.1. Schematic images for species difference in expression of immature and mature neuronal markers (DCX: orange, NeuN; blue). (A-C) The percentage of adult-born granule cells expressing DCX or NeuN in primates (A), rats (B) and mice (C). X-axis indicates the time (week) after cell division. A greater proportion of adult-born neurons of rats lose DCX expression and started to exhibit NeuN in early time point compared to mice and primates.

6.8 Ovariectomized rats with exogenous estrogens and naturally cycling estrogens

In Chapter 4, young ovariectomized rats were used to examine the effects of estrogens on neurogenesis in the dentate gyrus. It is important to note that exogenous estrogens may act differently from naturally cycling estrogens (Discussed in Chapter 4).

Furthermore, ovariectomies in rodents are often used as a preclinical animal model of menopause, though surgical menopause and naturally occurring menopause show differences in the physiology (reviewed in Brinton, 2012). Circulating levels of estradiol decline during perimenopause and drop to steady low levels post menopause in humans, and during the transition, the levels of estradiol greatly fluctuate (reviewed in Gordon and Sander, 2021). In contrast, the fluctuation is not observed in ovariectomized rodents as it rapidly eliminates circulating estrogens post-surgery. These differences between surgical menopause in rodents and natural menopause in humans must be taken into consideration when translating preclinical findings into human clinical settings. The experimental results shown in Chapter 4 demonstrate the significant effects of estrogens on adult neurogenesis in young female rats. Previously, no study has examined the effects of estrogens on the basal characteristics of neurogenesis (maturation and trajectory) in different age groups or with previous reproductive experience, whereas these are important factors that modulate the efficacy of estrogens on hippocampal plasticity and cognition (discussed in Chapter 1). Therefore, further research examining effects of estrogen treatment in middle-aged and aged subjects, with or without reproductive experience, is needed to unravel the complexities of estrogenic regulation of adult neurogenesis in females throughout the lifetime.

6.9 Conclusion

The evidence presented in this thesis demonstrates significant sex differences in adult neurogenesis and in the ability for pattern separation. Brain connectome analysis using zif268 protein activation in this thesis also indicates that males and females demonstrate distinct neuronal networks of hippocampal neurons with other brain regions during fear memory retrieval. Lastly, this thesis demonstrates that estrogens are powerful modulators of adult neurogenesis in females. As adult neurogenesis in the hippocampus is related to some forms of cognition and stress regulation, these findings suggest that modulation of neurogenesis may be one pathway through which estrogens could modulate cognition and stress in females. Women are more vulnerable to Alzheimer's disease and stress-related disorders such as depression, and women exhibit more severe cognitive decline in these disorders compared to men. Although, to date, the underlying mechanisms of the sex differences in incidence and severity of these disorders remain unknown, sex differences in basal characteristics of adult neurogenesis and alterations in hippocampal plasticity in males and females may play important roles in such diseases. Therefore, future studies examining the role of adult neurogenesis in hippocampal function, such as cognition and the stress response, should include both male and female subjects with analyzing their data with sex as a discovery variable. Furthermore, studies should include careful investigation of menstrual/estrous cycle stages, age, and reproductive experience as these factors have an impact on adult neurogenesis and cognition in females. However, the vast majority of studies published in major journals in psychiatry and neuroscience lack such information in their studies as only 2% in 2009 and 5% in 2019 have conducted optimal analyses for discovery of potential sex differences (Rechlin et al., 2022). The academic and clinical communities should make more effort to encourage researchers to analyze potential sex differences given that sex can be a powerful modulator of neuroplasticity.

Overall, the work presented in this thesis emphasizes the importance of studying sex differences in hippocampal function. Future studies are encouraged to not only include males and females in their experiments, and to analyze their data stratified by sex, but also to consider age and hormone status in their studies to gain a clearer picture of how sex differences may be contributing to measures of interest. These efforts will provide the academic and clinical communities with invaluable information that we hope will lead to more efficient clinical treatment for both men and women.

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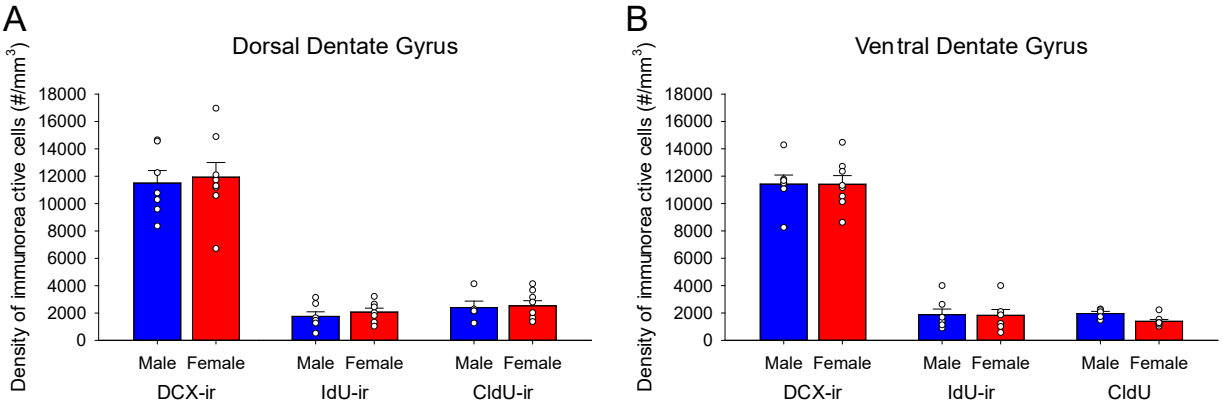
Appendices

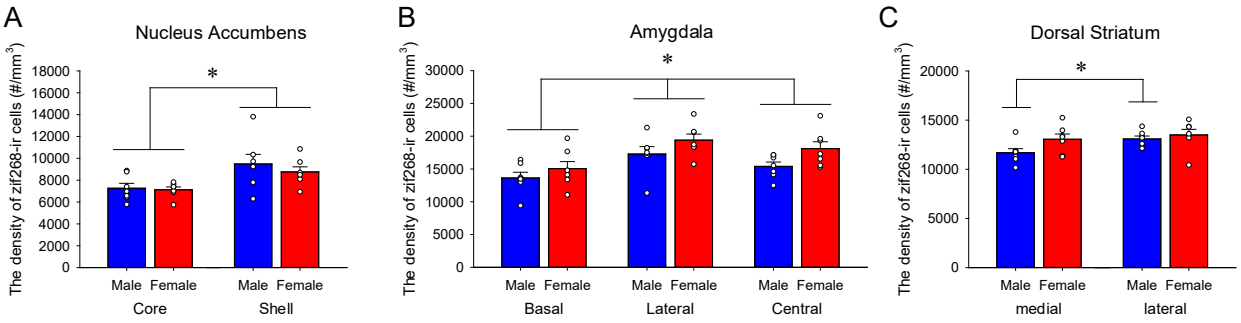
Appendix A Supplemental figures

A.1 Supplemental Figure 3.1



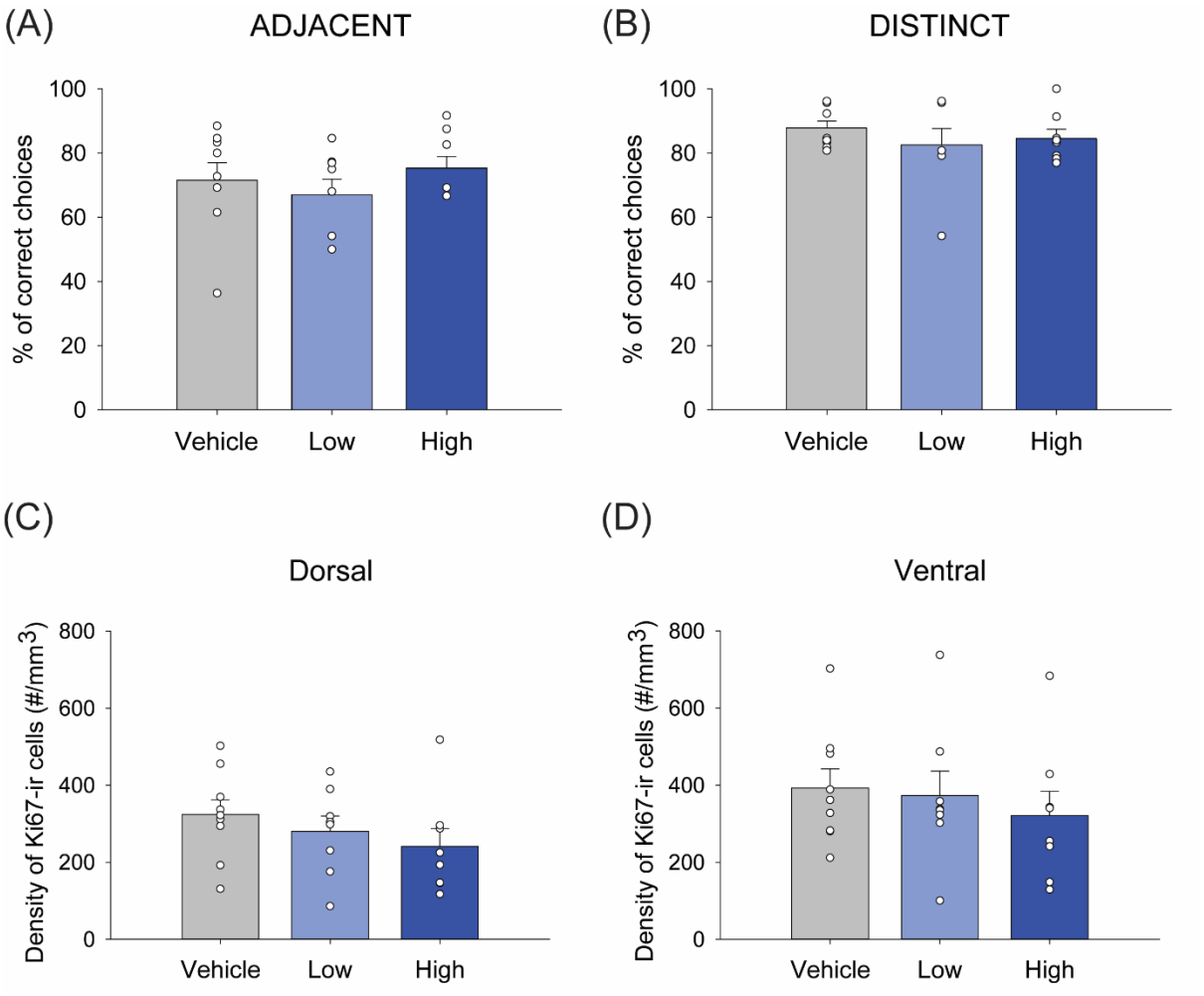
Supplemental Figure 3.1. A-B: Mean (±SEM) density of adult-born cells in the dorsal (A) and ventral (B) dentate gyrus. There were no significant sex differences in the density of DCX-ir cells, IdU-ir cells or CldU-ir cells in the dorsal or ventral dentate gyrus.

A.2 Supplemental Figure 3.2



Supplemental Figure 3.2. A-C: Mean (±SEM) density of zif268-ir cells in the nucleus accumbens (A), the amygdala (B) and the dorsal striatum (C). The density of zif268-ir cells in the nucleus accumbens shell is greater compared to the nucleus accumbens core (A). Females, compared to males, showed greater density of zif268-ir cells in the amygdala (B). A priori we found that the density of zif268-ir cells was significantly greater in the lDS compared to mDS in males, but not in females. * indicates $p < 0.05$.

A.3 Supplemental Figure 5.1



Supplemental Fig. 5.1. (A-B) Mean percentage of correct arm choices in ADJACENT trials (A) and DISTINCT trials (B). There were no significant main or interaction effect of treatment on the ability for spatial pattern separation. (C-D) Mean density of Ki67-immunoreactive (ir) cells in the dorsal (C) and ventral (D) dentate gyrus. There were no significant main or interaction effect of treatment on cell proliferation in the dentate gyrus. Error bars represent ± SEM. * indicates $p < 0.05$.

Appendix B Supplemental tables

B.1 Supplemental Table 3.1

A list of antibodies used in the present study

Product	Host Species	Manufacturer	Catalog #
Anti-BrdU antibody [BU1/75]	rat	Abcam	ab6326
Anti-BrdU antibody [B44]	mouse	BD Biosciences	347580
Anti-doublecortin	goat	Santa Cruz	sc-8066
Anti-EGR1/zif268	rabbit	Santa Cruz	sc-189
Anti-mouse Alexa Fluor 488	Donkey	Invitrogen	A21203
Anti-rat Alexa Fluor 594	Donkey	Invitrogen	A21209
Anti-goat Alexa Fluor 488	Donkey	Invitrogen	A11055
Anti-rabbit Alexa Fluor 594	Donkey	Invitrogen	A21207
Anti-rabbit Alexa Fluor 647	Donkey	Jackson ImmunoResearch	711-605-152

B.2 Supplemental Table 3.2

A list of reagents used in the present study

Product	Manufacturer	Catalog #
5-chloro-2'-deoxy-uridine	Cayman Chemical	50-90-8
5 Iodo 2' deoxyuridine	MP Biomedicals	210035705
Triton X-100	Sigma-Aldrich	T8787-250ML
Trizma hydrochloride	Sigma-Aldrich	T3253-1KG
Trizma base	Sigma-Aldrich	T6066-500G
Hydrochrolic acid	Fisher Scientific	SA431-500ML
Sodium Chrolide	Sigma-Aldrich	S9625-1KG
Tween 20	Sigma-Aldrich	P9416-50ML
Polyvinylalcohol	Sigma-Aldrich	P8136-250G
DABCO 33LV	Sigma-Aldrich	290734-100ML

B.3 Supplemental Table 3.3

Mean (±SEM) duration of rearing, grooming and non-specific behaviors in males and females during the activation trial. There were no significant sex differences in any of the behaviors ($p > 0.889$).

Behaviors (sec)		
Rearing	Grooming	Other
4.38±2.03	0	54.5±8.81
3.38±1.51	1.50±1.13	55.75±16.08