Sex differences in maturation and attrition rate of adult born neurons in the hippocampus of rats

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ABSTRACT

Sex differences exist in the regulation of adult neurogenesis in the hippocampus in response to hormones and cognitive training. Here we investigated the trajectory and maturation rate of adult-born neurons in the dentate gyrus (DG) of male and female rats. Sprague-Dawley rats were perfused one, two or three weeks after BrdU injection, marking newly dividing cells. Adult-born neurons (BrdU/NeuN-ir) matured faster in males compared to females. Males had a greater density of neural stem cells (Sox2-ir) in the dorsal, but not in the ventral, DG and had higher levels of cell proliferation (Ki67-ir) than females. Males had a greater reduction in neurogenesis between one and two weeks after mitosis, while females showed similar levels of neurogenesis throughout. The faster maturation and attrition of new neurons suggests greater potential for neurogenesis to respond to external stimuli in males compared to females and emphasizes the importance of studying sex on adult hippocampal neurogenesis.

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; Gould et al., 1999a; Knoth et al., 2010; Kuhn et al., 1996; Kornack et al., 1999; 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 does exist in humans and is modulated by disease, age, and perhaps sex (Cipriani et al., 2018; Epp et al., 2013; 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) 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 Figure 1A 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). The IPCs can undergo multiple symmetrical or asymmetrical cell divisions but generally differentiate into neurons (Bonaguidi et al., 2011; Cameron et al., 1993; Encinas et al., 2011; Kempermann et al., 2004; 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 the second day or 1 week after the initial mitosis in mice of undisclosed sex (Encinas et al., 2011) or in male rats (Cameron et al., 1993), respectively. Adult-born cells in the DG start to die off and show a rapid decrease in the number of new cells between one and three weeks after the initial cell division in male rodents (Cameron et al., 1993; Encinas et al., 2011; Snyder et al., 2009). The subset of IPCs (type2b), neuroblasts 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, 1 week after mitosis in rats (Brown et al., 2003; Snyder et al., 2009). New neurons integrate into the existing neural circuitry, and play an important role in pattern separation and stress resilience (Hill et al., 2015; Clelland et al., 2009; Snyder et al., 2011; França et al., 2017).

However, there are species differences in the maturation rate of adult born neurons, where male mice show slower maturation of new neurons than male rats (Snyder et al., 2009) but 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 2011), and despite one study in females (Brown et al., 2003) no study has directly compared female to male rats. 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 males 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). These data collectively suggest that either neurogenesis in the hippocampus is not stimulated during cognitive training in females or that the CA3 region (the projection area of new neurons) may not be as active in females, leading to the inability of cognitive training to boost survival of new neurons. However, evidence suggests neither scenario is correct, as females show increased zif268 expression in the CA3 compared to males after training (Yagi et al., 2016; 2017) and activation of new neurons or the number of new neurons is associated with better performance in females compared to males (Yagi et al.,2016; Chow et al., 2013). Furthermore, work from the Shors laboratory has shown that females do show greater neurogenesis in the ventral DG following eyeblink conditioning (Dalla et al., 2009), showing that neurogenesis can be stimulated following some tasks. These findings, collectively, 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 aims to elucidate sex differences in the maturation and trajectory of the amount of new neurons with time as well as the number of neural stem cells. 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, and neural stem cells, favouring males.

2. Methods

2. 1. Animals

Twenty-four age-matched (two-month old) *Sprague-Dawley* rats were bred at the University of British Columbia and used in this study (n=12 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.

2. 2. Procedure

One intraperitoneal (i.p.) injection of BrdU (200mg/kg) was given to all rats between 11am-12 pm. Rats were perfused one, two or three weeks after the BrdU injection, but otherwise were left undisturbed after BrdU injection except for weekly cage changes (see Figure 1B). 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.

2.3. Radioimmunoassay for 17β-estradiol and testosterone

Previous studies reported that 17β-estradiol increases cell proliferation in females but not males (Tanapat et al., 1999; Barker and Galea, 2008) and androgens can influence survival of new neurons in males but not females (Spritzer and Galea, 2007; Duarte-Guterman et al., 2019).Thus, we examined serum levels of 17β-estradiol and testosterone in females and males, 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. None of the females were in proestrus at the time of sacrifice.

2. 4. Immunohistochemistry

2. 4. 1. 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). Brain tissue was prewashed three times with 0.1 M TBS and left overnight at 4 °C after the third

wash. The tissue was 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 Biotechnnology; 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. Tissue was 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, tissue was 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. Tissue was rinsed three times in TBS for 10 minutes each. 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 TBS for 24 hours at 4 °C. Tissue was then incubated 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 rinses with TBS, tissue was mounted onto microscope slides and cover-slipped with PVA DABCO.

2. 4. 2.Ki67 or Sox2 immunofluorescent staining

Actively dividing cells express Ki67 which is expressed during all stages of the cell cycle except G_0 and thus is expressed at higher levels than BrdU 24 h after injection (Kee et al., 2002). Randomly selected brains were also immunohistochemically stained with anti-Ki67 or anti-Sox2 (n=8 per sex). The tissue was prewashed with 0.1 M PBS and left to sit overnight at 4 °C. The next day, tissue was incubated in 10mM sodium citrate buffer for 45 minutes at 90 °C to retrieve antigens of Ki67. Then tissue was 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 Biotechnnology; 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 (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, tissue was incubated in 1:5000 DAPI in PBS for 3 mins, and slices were mounted onto slides and cover-slipped with PVA DABCO.

2. 5. 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 6.20-3.70mm from the interaural line defined as dorsal and sections 3.70-2.28mm from the interaural line as ventral (see Figure 1C). 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 time lines (Snyder et al., 2012). For example, the dorsal hippocampus is associated with spatial learning and memory, while the ventral hippocampus is associated with stress and anxiety (Moser et al., 1993; Kjelstrup et al., 2002).

2. 5.1. BrdU and Ki67

Ki67-ir and BrdU-ir cells were counted under a 100x oil immersion objective lens (Figure 2B, 3A). 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 20 (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 estimates of the DG were calculated using Cavalieri's principle (Gundersen and Jensen, 1987) by multiplying the summed areas of the DG by thickness of the section (30µm). Area measurements for the DG were obtained using digitized images on the software ImageJ (NIH).

2. 5. 2. 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 (Figure 3A, 3B and 6A; method used by Banasr et al., 2006). The percentage of

BrdU/DCX-ir cells were also categorized into the three morphology types using the criteria used by Plümpe et al. (2006). Briefly, type-1 proliferative stage was defined as neurons with no or short plump processes, type-2 intermediate stage neurons possess medium-length processes or apical dendrites that reach the molecular layer, and type-3 postmitotic stage neurons possessing apical dendrites with at least one branching in the molecular layer (see Figure1D). The density of BrdU-ir cells was multiplied by the percentage of BrdU-ir cells that expressed DCX or Sox2 to calculate the density of BrdU/DCX-ir cells or BrdU/Sox2-ir cells, respectively.

2.5.3. 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; Figure 1C and 2A). 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. 6. Data analyses

All analyses were conducted using STATISTICA (Statsoft Tulsa, OK). The density of BrdU-ir cells, the percentage of BrdU-ir cells that express NeuN or DCX were each analyzed using repeated-measures analysis of variance (ANOVA), with maturation time (1w, 2w, 3w) and sex (male, female) as between-subject variables and with hippocampal region (dorsal, ventral) as within-subject variables. 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-3 morphology was analyzed using repeated-measures ANOVA with sex as between-subject variables and with 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 α=0.05 and effect sizes are given with Cohen's d or partial η^2.

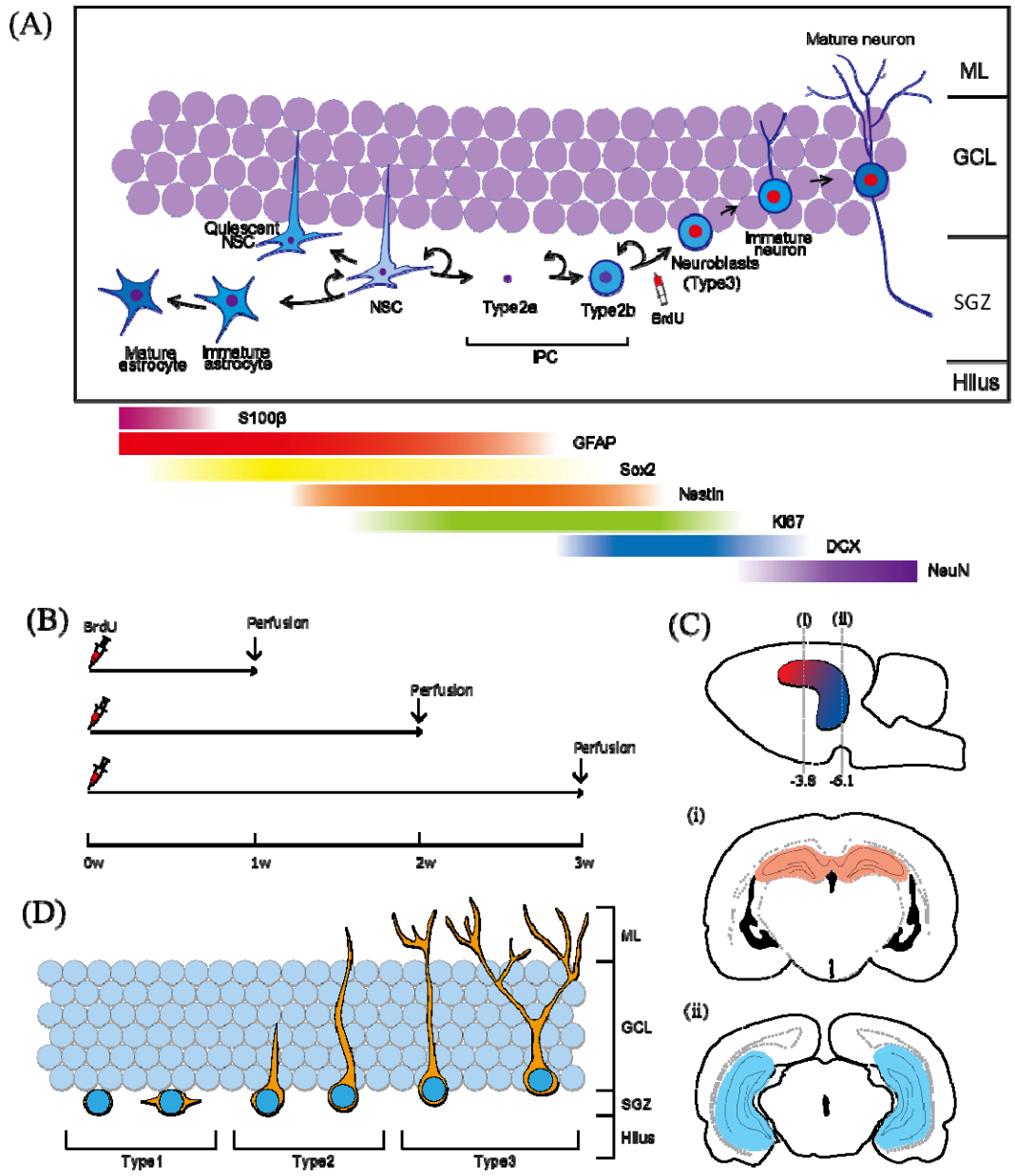

Figure 1. Schematic illustrations for the experimental design. (A) Schematic illustrations for the timeline of neural stem cell lineage with expression of stage-specific proteins (Amador-Arjona et al., 2015; Bonaguidi et al., 2011; Encinas et al., 2011; Micheli et al., 2018; Steiner et al., 2006). (B-D) Schematic illustrations for the experimental design: (B) The experimental timeline, (C) the definition of the dorsal (section (i): red), and ventral (section (ii): blue) hippocampus and (D) morphological phenotypes of DCX-ir cells.

3. Results

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 comparisons [$p = 0.012$; region by sex interaction: $F(1,22) = 4.61$, $p = 0.043$, Cohen's $d = 1.26$; see Table1]. 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 1

Mean (±SEM) volume of the dorsal and ventral dentate gyrus (DG) and hormone concentrations in male and female rats (mm^3). Females had a smaller dorsal dentate gyrus volumes

	Dorsal DG	Ventral DG	Hormone concentration
Male	0.905±0.056	1.334±0.083	Testosterone 1.067±0.43 (ng/ml)
Female	0.688±0.043*	1.593±0.195	17β-Estradiol 14.41±1.30 (pg/ml)

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.

Sox 2 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 ($p = 0.023$, Cohen's $d = 1.39$; sex by region [$F(1,16) = 6.34$, see Figure 2C). Females had a greater density of Sox2-ir cells in the ventral DG compared to the dorsal DG ($p = 0.0054$, 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 [$F(1,16) = 4.20$, $p = 0.057$].

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

We also examined the cell proliferation marker Ki67 and found that 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 Figure 2D]. 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 ($p=0.11$). There were no significant correlations between the density of Ki67-ir cells and the levels of 17β-estradiol or testosterone (all ps' > 0.268).

3.4. Males, but not females, show greater attrition of BrdU-ir cells from one to two 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 1,2 and 3 weeks after injection. Males had a greater density of one week old BrdU-ir cells compared to all other time points ($p < 0.001$; interaction effect of sex by weeks [$F(2,18) = 4.41$, $p = 0.028$, partial $\eta^2 = 0.329$; see Figure 3C]). Males had a greater density of BrdU-ir cells than females only at the one week time point ($p = 0.0005$, Cohen's $d = 2.26$) but not at the two or three week timepoints (p's = 0.603, 0.759, respectively). Furthermore, there were no significant differences in the density of BrdU-ir cells across the time points in females ($p > 0.777$). There were main effects of sex [$F(1,18) = 11.56$, $p = 0.003$, Cohen's $d = 0.953$], weeks [$F(2, 18) = 10.86$, $p = 0.0008$, partial $\eta^2 = 0.547$] and regions [$F(1, 18) = 5.82$, $p = 0.027$, Cohen's $d = 0.351$] but no other significant interactions (p's > 0.226).

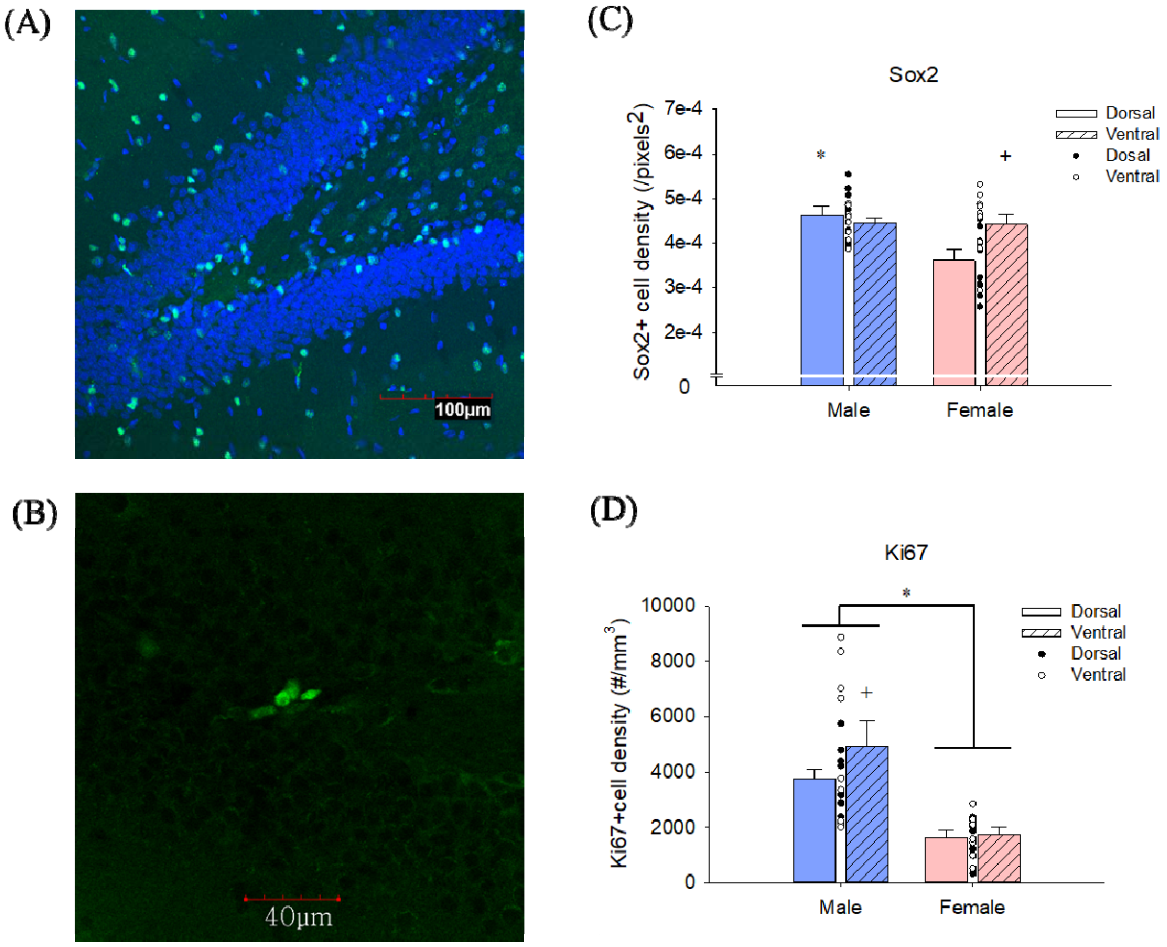

Figure 2. Sex differences in neural stem cell population and proliferating cell population in the dentate gyrus. (A-B) Photomicrographs of Sox2 (green) with DAPI (blue) (A) and Ki67 (B). (C) Mean (+SEM) density of Sox2-ir cells: Males, compared to females, had a greater density of Sox2-ir cells in the dorsal dentate gyrus. The dorsal dentate gyrus of females, but not males, had a greater density of Sox2-ir cells compared to the ventral dentate gyrus. (D) Mean (+SEM) density of Ki67-ir cells: Males had a greater density of Ki67-ir cells compared to females. A priori showed that the ventral dentate gyrus had a greater density of Ki67-ir cells compared to the dorsal dentate gyrus in males. + indicates significant regional differences ($p<0.05$) and * indicates significant sex differences ($p<0.05$).

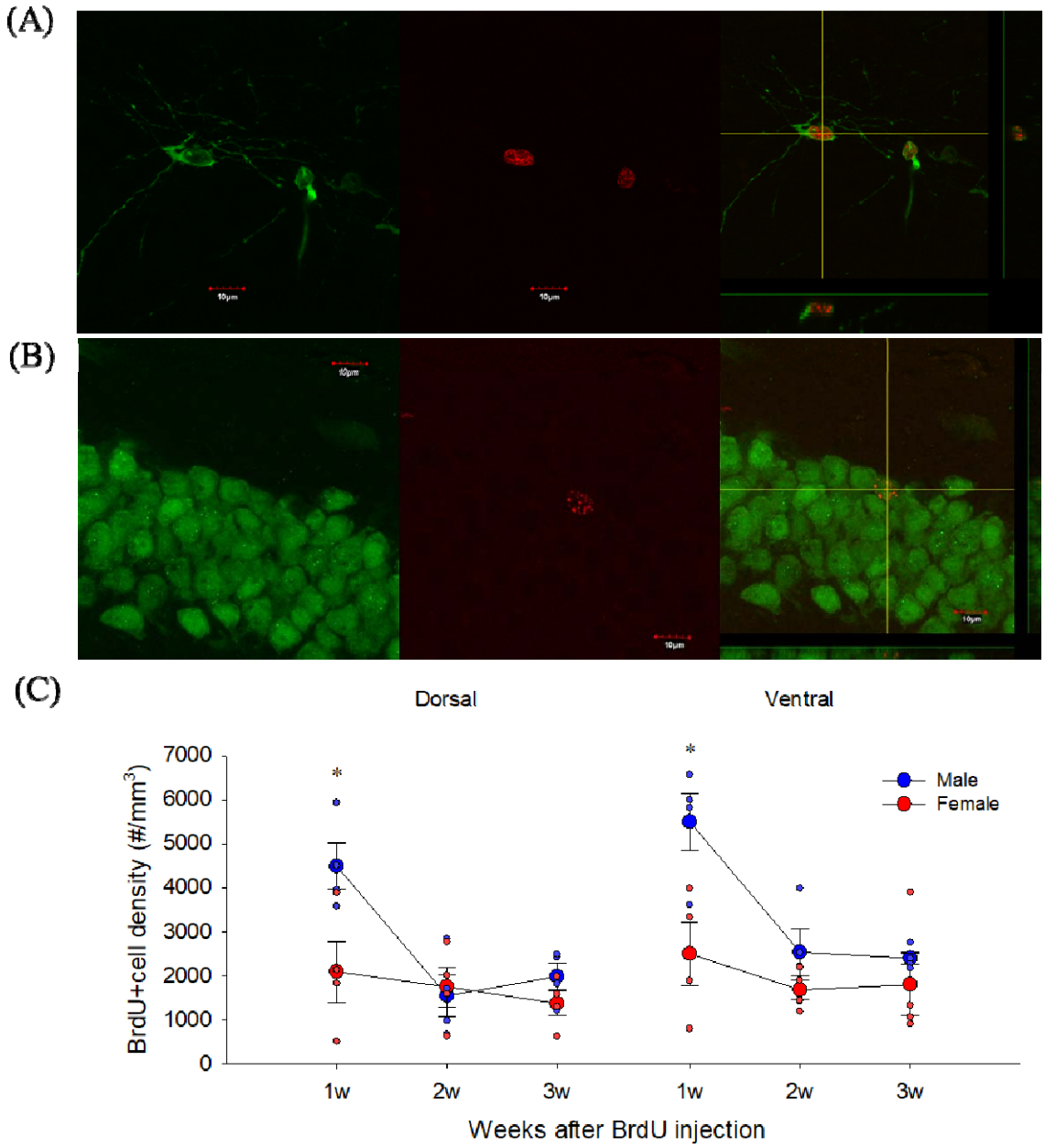

Figure 3. Sex differences in the trajectory of adult-born BrdU-ir cells. (A-B) Photomicrographs of BrdU (red)/DCX (green)-ir cells (A) and BrdU (red)/NeuN (green)-ir cells (B). (C) Mean (±SEM) density of BrdU-ir cells. Males had a greater density of BrdU-ir cells at one week compared to females, but males showed greater reduction between one and two weeks after BrdU injection. * indicates $p<0.05$.

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, NeuN and DCX across the three weeks. Males, compared to females, had a greater percentage of BrdU-ir cells that expressed NeuN two weeks after BrdU injection ($p = 0.003$, Cohen's $d = 2.14$) but not one week ($p=0.99$) or 3 weeks ($p=0.54$) after mitosis (interaction effect of sex by week [$F(2, 17) = 3.52$, $p = 0.05$, partial $\eta^2 = 0.293$; see Figure 4A]). There were also main effects of sex: [$F(1, 17) = 7.14$, $p = 0.016$, partial $\eta^2 = 0.296$] and week [$F(2, 16) = 41.92$, $p < 0.00001$, 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 increased by three weeks after BrdU injection in both males and females and the percentage of BrdU-ir cells that express NeuN of females reached the same level as male BrdU-ir cells (90%; Figure 4A) and did not significantly differ at 3 weeks ($p = 0.583$).

As expected, in both sexes across both regions, the percentage of BrdU-ir cells that also express DCX decreased significantly as weeks progressed with greater expression in week one compared to week two ($p=0.043$), which had greater expression compared to week three ($p<0.00016$) [main effect of weeks: $F(2, 16) = 110.12$, $p < 0.0001$; Cohen's $d = 0.93$; see Figure 4B]. There were no other significant main or interaction effects on the percentage of BrdU-ir cells that express DCX (p's > 0.43).

3.6. Males, compared females, have a greater density of BrdU/DCX-ir cells at one week, and a greater reduction between one to three weeks after BrdU injection.

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 one week time point ($p=0.00043$, Cohen's $d = 2.61$) but not at the two ($p = 0.616$) or three ($p = 0.951$) week time point [interaction effect of sex by weeks: $F(2,16) = 6.27$, $p = 0.001$, partial $\eta^2 = 0.439$; see Figure 4C]. There were also main effects of sex [$F(1,16) = 8.51$, $p = 0.010$, Cohen's $d = 0.560$], weeks [$F(2, 16) = 24.2$, $p < 0.001$, partial $\eta^2 = 0.828$] and a trend for a main effect of region [$p = 0.053$] but no other significant interactions (p's > 0.176).

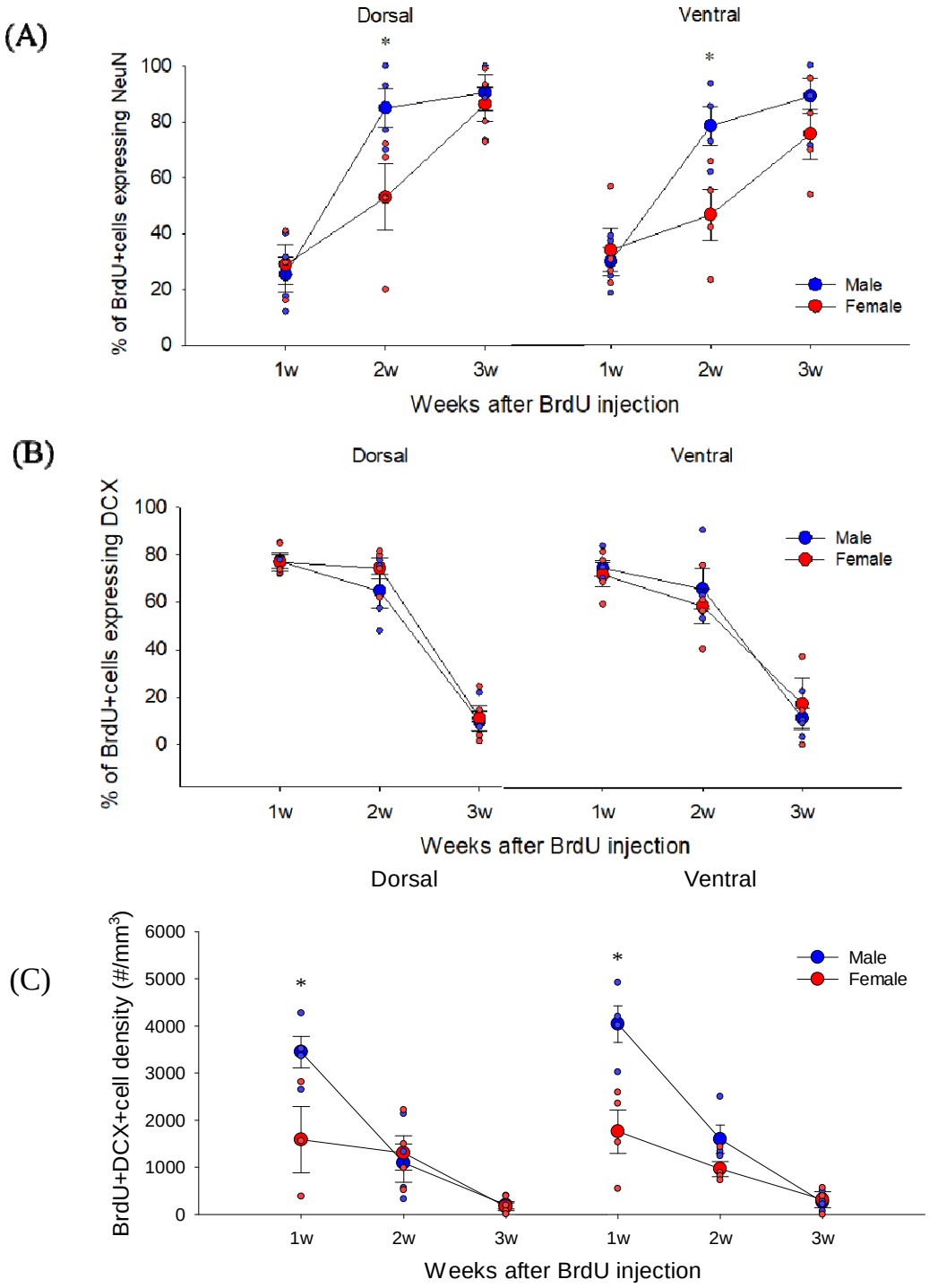

Figure 4. Sex differences in the maturation rate of adult-born neurons in the dentate gyrus. (A) Mean (±SEM) percentages of BrdU-ir cells that express NeuN. Males had a greater percentage of BrdU-ir cells that express NeuN at two weeks in the dorsal and ventral dentate gyrus. (B) Mean (±SEM) percentages of BrdU-ir cells that express DCX. There was no significant sex difference in the percentage of BrdU-ir cells that express DCX (C) Mean (±SEM) density of BrdU/DCX-ir cells. Males had a greater density of BrdU-ir cells that express DCX cells at one week. * indicates $p<0.05$.

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-3 morphology compared to females at two weeks in the dorsal DG [a priori: $p = 0.017$, Cohen's $d = 1.84$; effect of week: $F(2, 18) = 5.39$, $p = 0.015$, partial $\eta^2 = 0.37$; see Figure 5) but not at one week ($p = 0.95$) or three weeks ($p = 0.84$) after BrdU injection.

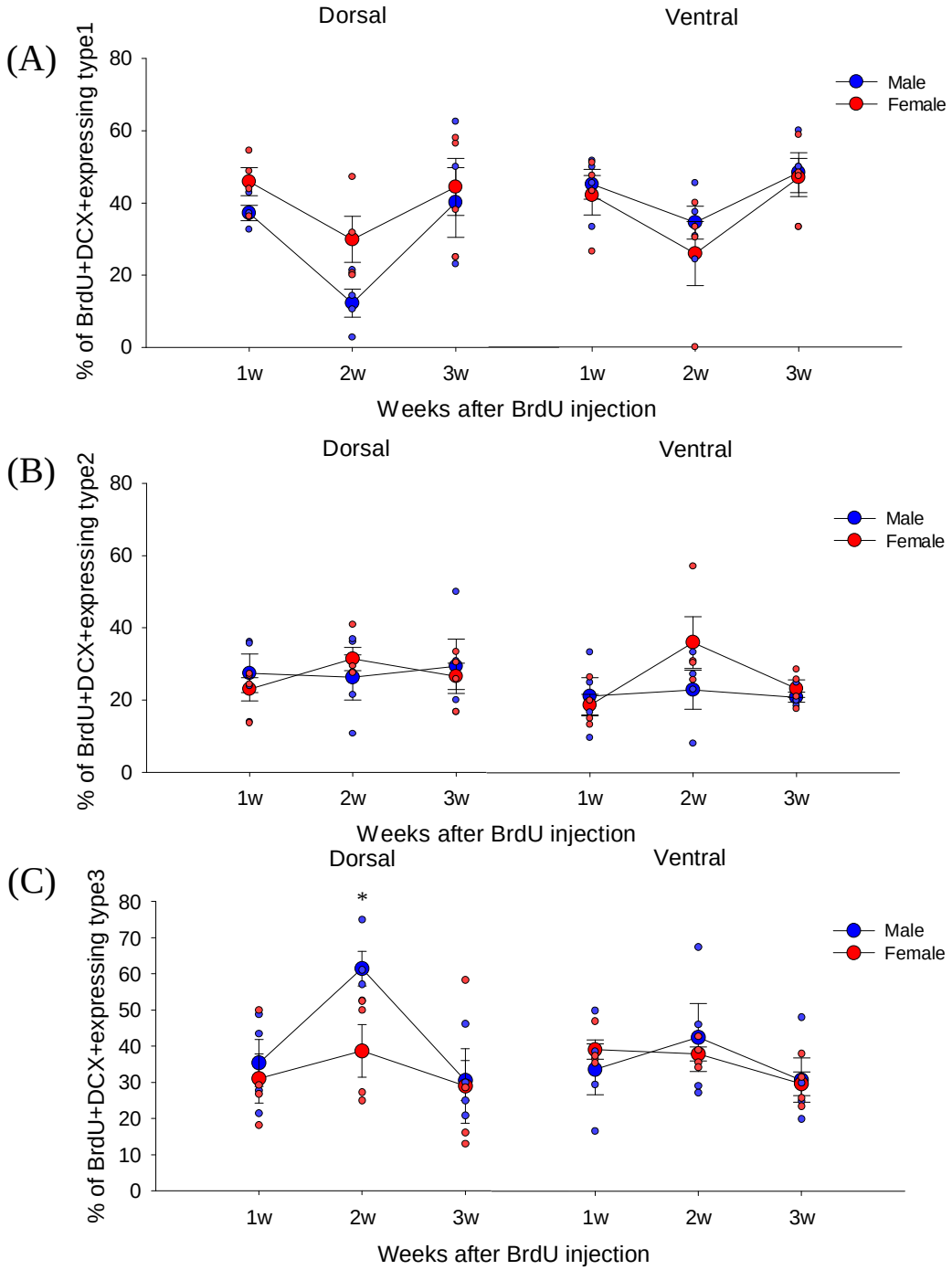

Figure 5. Sex differences in the morphological phenotypes of BrdU/DCX-ir cells. (A-C) Mean (±SEM) percentages of BrdU/DCX-ir cells that showed type1 (A), type2 (B) or type3 (C) morphological phenotypes. A priori showed that males had a greater percentage of BrdU/DCX-ir cells that showed the type3 morphological phenotype at two weeks compared to females in the dorsal dentate gyrus. * indicates $p<0.05$

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3.7. Males have a greater density of BrdU/Sox2-ir cells in the ventral DG at one week compared to females.

To understand if there may be differences in the time course of actively dividing neural stem cells between sexes, we examined the density of BrdU/Sox2-ir cells at one, two and three weeks after BrdU injection as well as the percentage of BrdU-ir cells that also expressed Sox2-ir compared to those that did not.

Males had a greater density of BrdU/Sox2-ir cells at one week compared to all other groups [all p's < 0.012; interaction effect of sex by weeks: $F(2, 13) = 5.84$, $p = 0.016$; partial $\eta^2 = 0.473$, Cohen's d = 2.27 for males to females at one week]. However, this is driven by the ventral region as males had a greater density of BrdU/Sox2-ir cells in the ventral DG at one week time point compared to all other groups (a priori: all p's < 0.0001, Cohen's d = 2.98 for males versus females at one week; three-way interaction effect p=0.12; see Figure 6). There were also significant main effect of weeks ($F(2, 13) = 6.06$, $p = 0.014$, partial $\eta^2 = 0.483$) and region ($F(1, 13) = 10.31$, $p < 0.007$, partial $\eta^2 = 0.442$) and interactions of region by weeks $F(2, 13) = 4.39$, $p = 0.035$, partial $\eta^2 = 0.403$) but no other main or interaction effects (both p's >0.119).

(A)

(B)

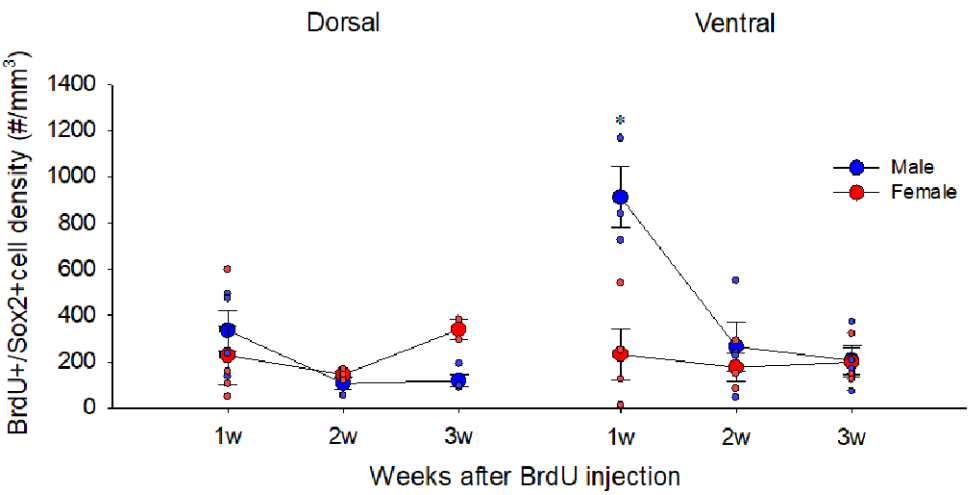

Figure 6. Sex differences in the density of BrdU-ir cells that express Sox2. (A) Photomicrographs of BrdU (red) /Sox2-ir (Green) cells. (B) Mean (±SEM) density of BrdU-ir cells that express Sox2. Males had a greater density of BrdU-ir cells that express Sox2 in the ventral dentate gyrus at one week. * indicates $p<0.05$.

4. Discussion

Our findings indicate that adult-born neurons mature faster in males one week earlier compared to females. We found notable sex differences in the attrition or survival rate of BrdU-ir cells across weeks, with males showing a reduction from one to two weeks, but females showing no appreciable reduction in BrdU-ir cells across 3 weeks. The sex differences in maturation and attrition of new neurons, to our knowledge, has not been reported before and has important implications for our understanding of adult neurogenesis. Furthermore, males had a higher density of neural stem cells (Sox2) in the dorsal DG and greater levels of cell proliferation (Ki67) compared to females. The greater levels of cell proliferation in males also point to a possible sex difference in attrition, as there was no sex difference in the density of new neurons after three weeks. Intriguingly, the density of BrdU-ir cells two weeks after production was comparable between males and females, indicating that potential sex differences in neurogenesis outcomes would be more likely using treatment or experimental perturbations 1 week after production in rats. Altogether, these sex differences in the trajectory and maturation rate of adult neurogenesis, may explain the sex differences seen in the amount of adult neurogenesis after stress and cognitive training (Chow et al., 2013; Hillerer et al., 2013; Yagi et al., 2016). These findings underscore that although a tremendous amount of research has unveiled the characteristics of neurogenesis in the adult hippocampus, we cannot assume that the same characteristics will be similar in females. This has profound implications for our understanding of neurogenesis and the use of the modulation of neurogenesis as therapeutics in the general population.

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. Male rats showed a rapid increase in the percentage of BrdU-ir cells that expressed NeuN across the weeks [27.6±4.3% (1 week), 81.7±4.0% (2 weeks) 90.5±6.3%(3 weeks)] but female rats showed a more gradual increase in the percentage of BrdU-ir cells that expressed NeuN across weeks [27.7±2.4% (1 week),

49.8±9.7% (2 weeks), 84.1±8.6% (3 weeks)]. These results suggest that adult-born neurons mature faster in male rats compared to female rats, as they co-express NeuN earlier (at two weeks) compared to females. Consistent with this finding we also found that there was a greater percentage of BrdU/ type3 DCX-ir cells (most mature morphology of DCX-ir cells) in male rats at two weeks. Thus, more new cells had mature morphological phenotype at two weeks after BrdU injection in males compared to female rats, consistent with our BrdU/NeuN results. These data demonstrate that male adult-born granule neurons mature faster compared to female adult-born granule neurons in the DG.

Previous studies are consistent with our results (Brown et al., 2003; Snyder et al., 2009). These studies, while not comparing the sexes, 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). Our findings of a sex difference in the maturation time course may be due to a sex difference in the neural activity of the hippocampal network. Maturation of adult-born neurons in the DG is accelerated by enhanced electrophysiological activity of the hippocampus (Piatti et al., 2011). Indeed, 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., 2016; 2017). However, in the same studies, females show greater activation of zif268 in the dorsal CA3 compared to males, which is inconsistent with the interpretation. Another possible explanation for faster maturation of adult-born neurons in males is that faster maturing neurons have a greater chance to survive between one and two weeks after cell division. Adult-born new neurons start to become integrated into the existing neural network during this time period (4-10 days after mitosis in male rats (Hastings and Gould, 1999b), and greater spine formation of new neurons enhances integration of the neurons into the existing neural network (McAvoy et al., 2016). Indeed, the present study demonstrates that males have greater production of new neurons compared to females by two weeks, and therefore, those new neurons in males may be exposed to greater competition with existing and other new neurons for survival. Ultimately, however, under basal

conditions there is no sex difference in the density of new neurons by three weeks after mitosis. Further research is needed to examine the mechanisms of the sex differences in the maturation of new neurons.

4.2. Females had a greater density of neural stem cells in the ventral, compared to dorsal, dentate gyrus.

We found that males had a greater density of Sox2-ir cells in the dorsal, but not the ventral, DG compared to females. We also found that there was a regional difference in females, with more Sox2-ir cells in the ventral compared to the dorsal region while this same regional difference was not observed in males. To our knowledge, neither of these findings have been reported previously. These results suggest that males have more pluripotent neural precursor cells in the dorsal DG compared to females as Sox2 is a transcription factor for maintaining pluripotency of RGLs and type2a IPCs (Lugert et al., 2010; Steiner et al., 2006). However, 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 but there are some intriguing possibilities as males generally show better spatial learning (Jonasson, 2005; Voyer et al., 2017), whereas females show different stress reactions compared to males (Young and Korszun, 2010).

We also found that males produce more pluripotent cells that express BrdU-ir compared to females one week after initial cell division in the ventral DG. These results suggest that RGLs may differentiate into neuroblasts slower in the ventral DG compared to the dorsal DG in males. Previous studies show that adult-born neurons mature faster in the dorsal DG compared to the ventral DG in males (Piatti et al., 2011; Snyder et al., 2012), and it is also possible that the time course of neural differentiation is different between the dorsal and ventral DG. The present results emphasize the importance of further investigation in the associations between sex differences in the neural stem cell population and cognition, as the preservation of neural stem cells in the hippocampus is a potential treatment to delay the cognitive decline in Alzheimer's patients (Briley et

al., 2016) and given sex differences in Sox2 population as well as sex differences in the manifestation of AD, careful attention to biological sex is warranted.

4.3. Neurogenesis in males has a different trajectory/timeline compared to females

The present study found that males, but not females, showed a substantial reduction in the density of BrdU-ir cells between one and two weeks after BrdU labeling. The decrease was notable such that despite the fact that males showed greater density of 1 week old BrdU-ir cells there was no sex differences in density of 2-3 week old BrdU-ir cells. Our findings are consistent with previous studies that demonstrated that male Sprague Dawley rats rapidly reduce the amount of adult-born dentate granule cells between one and two weeks after [3H] thymidine or thymidine-analogue labelling (Cameron et al., 1993; Snyder et al., 2009; 2012). Furthermore, previous studies found that there was no significant sex difference in the amount of 2 or 3 week old BrdU-ir cells in cage controls (Barha et al., 2011; Chow et al., 2013 Tanapat et al., 1999), consistent with our findings. Although two studies have seen sex differences in 3 week old cells, (Hillerer et al., 2013; Lee et al., 2014), the inconsistencies are likely due to different conditions (i.e. handling, daily injections, single vs double housing). Collectively these results suggest that males and females regulate adult neurogenesis differently as males produce more new cells in the DG and decrease the number of new cells during the maturation, while females produce fewer new cells but these cells are preserved across the maturation cycle. These findings may explain why spatial learning and or estrogens given during immature neuronal stage increases the amount of survived new neurons at 21 days and 28 days after BrdU injection only in males, but not females (Chow et al., 2013; Epp et al., 2007; Ormerod et al., 2004; Yagi et al., 2016). It is important to note that spatial training occurring only during 6-10 days after BrdU injection can increase in the density of adult-born neurons in male rats (Epp et al., 2011; Gould et al., 1999). Interestingly, this time period for enhancing effects of spatial learning coincides with the greater reduction of new neurons observed in the present study. Taken together, these results suggest that spatial training between one and two weeks after production of new neurons can prevent the attrition of adult-born neurons in males.

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

Males had a greater density of proliferating cells (Ki67-ir) in the DG compared to females. Curiously, an older study had a similar finding that male meadow voles had greater cell proliferation than females during the breeding season only (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). Tanapat et al. (1999) found greater cell proliferation (2h BrdU-ir cell density) but only in proestrous female rats compared to male rats. None of the females in the present study were in proestrus and thus, we would expect lower levels of cell proliferation in our females. Furthermore, the inconsistency in the sex difference in cell proliferation may be due to differences between BrdU and Ki67. Although BrdU has been used to examine cell proliferation in many studies, BrdU is an exogenous marker that labels cells during the S-phase of cell cycle only for two hours after intraperitoneal injection of BrdU. In contrast, Ki67 is an endogenous protein produced during the S-phase, G_2 and mitosis, and the antigen is also detectable during the G_1 phase when the daughter cell continuously enters another cell cycle (Miller et al., 2018). Therefore, it is possible that injection-associated stress differently influences the density of BrdU-ir cells between males and females, as acute stress suppresses cell proliferation in male rats, but not female rats (Falconer and Galea, 2003). Furthermore, the inconsistency between the present study and those previous studies may be also due to the differences in experimental procedures, as the present study examining subjects with no intervention.

5. Conclusion

In the present study, we see clear sex differences in the neural stem cell population, cell proliferation, maturation rate and the attrition rate of adult-born neurons in the hippocampus. The trajectory of new neuron survival is quite 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. Further studies are needed to elucidate the physiological and environmental factors that modulate the maturation of adult-born neurons and maintenance of neural stem cells, which may give us important clues how we can prevent or delay the development of hippocampus related neurological disorders.

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