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A developmental sex difference in hippocampal neurogenesis is mediated by endogenous oestradiol

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Abstract

Background: Oestradiol is a steroid hormone that exerts extensive influence on brain development and is a powerful modulator of hippocampal structure and function. The hippocampus is a critical brain region regulating complex cognitive and emotional responses and is implicated in the aetiology of several mental health disorders, many of which exhibit some degree of sex difference. Many sex differences in the adult rat brain are determined by oestradiol action during a sensitive period of development. We had previously reported a sex difference in rates of cell genesis in the developing hippocampus of the laboratory rat. Males generate more new cells on average than females. The current study explored the effects of both exogenous and endogenous oestradiol on this sex difference.

Methods: New born male and female rat pups were injected with the mitotic marker 5-bromo-2-deoxyuridine (BrdU) and oestradiol or agents that antagonize oestradiol action. The effects on cell number, proliferation, differentiation and survival were assessed at several time points. Significant differences between groups were determined by two- or three-Way ANOVA.

Results: Newborn males had higher rates of cell proliferation than females. Oestradiol treatment increased cell proliferation in neonatal females, but not males, and in the CA1 region many of these cells differentiated into neurons. The increased rate of proliferation induced by neonatal oestradiol persisted until at least 3 weeks of age, suggesting an organizational effect. Administering the aromatase inhibitor, formestane, or the oestrogen receptor antagonist, tamoxifen, significantly decreased the number of new cells in males but not females.

Conclusion: Endogenous oestradiol increased the rate of cell proliferation observed in newborn males compared to females. This sex difference in neonatal neurogenesis may have implications for adult differences in learning strategy, stress responsivity or vulnerability to damage or disease.

Introduction

Sex differences in the brain are widespread but of variable magnitude. Differences in the size of specific structures or subnuclei are well characterized, as are sex differences in the density and number of excitatory and inhibitory synapses within particular brain regions. Many sex differences in the brain are induced during a perinatal sensitive period by oestradiol following its central aromatization from testicular derived androgen precursors (for review see [1]). The most robust

neuroanatomical sex differences are found in the brain areas directly involved in reproduction, such as the pre-optic area, hypothalamus and spinal cord [2-6]. The impact of steroids on these brain regions across the lifespan is codified in the Organizational/Activational Hypothesis of sexual differentiation first postulated over 50 years ago [7]. Gonadal steroids, including oestradiol, also potently regulate synaptic profiles in adult brain regions not directly associated with reproduction, such as the hippocampus [8-10] and the amygdala [11].

The hippocampus subserves important behavioural and physiological functions that are influenced by sex. There are subtle, identifiable, sex differences in hippocampal volume [12] and the morphology of hippocampal

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cells [13-15]. There are also subtle sex differences in hippocampal associated behaviours such as spatial learning strategies, stress responsivity, and the long-term impact of negative early life events [16-20]. There are also subtle but complex sex differences in the developing hippocampus that are associated with parameters responsive to oestradiol, including calcium entry in response to depolarizing GABA [21-24] and cell genesis [25].

The hippocampus is comprised of subregions (for example, dentate gyrus (DG), CA1 and CA3) and each subregion contains distinctive cell types characterized by distinctive rates of maturation. The pyramidal cells, the principle cells of Ammon's horn, are largely formed before birth [26-29]. In contrast, granule cells, which comprise the major cell type found in the DG, are predominantly born during the first 2 weeks of postnatal life [28,30]. The development of granule cells in the molecular layer of the DG is faster in males than females [30], suggesting a sex difference in cell proliferation.

Hormonally-mediated sex differences in cell death are central to the sexual differentiation of many brain areas (for review see [31]), but less is known about sex differences in cell genesis, particularly during development. However, we recently reported that neonatal males have more new cells as indicated by 5-bromo-2-deoxyuridine (BrdU) in the DG and CA1, relative to females, with no corresponding sex difference in the number of pyknotic cells [25]. Both oestradiol and testosterone treatment increase the number of BrdU+ cells in females to the level found in males. However, whether endogenous steroids mediate the sex difference in cell proliferation is unknown. It is also unknown whether developmental steroid effects on cell proliferation are transient or organized and, as a result, endure across the lifespan. Here we report that endogenous oestradiol can generate higher rates of cell proliferation in males but plays no role in females. Consistent with previous results, treatment of females with exogenous oestradiol increases cell proliferation both acutely and 3 weeks later, suggesting that oestradiol might have an organizational role in neurogenesis. The sex difference in neurogenesis reported here may reflect a critical time period in hippocampal development that contributes to sexual dimorphisms in the adult hippocampus.

Methods

Animals

Newborn male and female Sprague-Dawley rats were obtained from breeder females at the University of Maryland School of Medicine. The day of birth was defined as postnatal day 0 (PN0). The animals were housed under a 12:12 h light: dark cycle, with food and water freely available. All procedures were approved by the University of Maryland School of Medicine

Institutional Animal Care and Use Committee and followed National Institutes of Health Guidelines.

Hormonal treatment of animals

On PN0, male and female rat pups were randomly distributed into different experimental groups and marked for identification by a subcutaneous (sc) ink injection in either the front or hind paws and injected sc with either oestradiol benzoate (100 µg / 0.1 mL in sesame oil), formestane (100 µg / 0.1 mL in sesame oil), tamoxifen (100 µg / 0.1 mL in sesame oil) or vehicle (sesame oil; see Figure 1 for the experimental timeline). The high dose of oestradiol is required in order to overcome the sequestering capacity of alpha-fetoprotein in the neonatal bloodstream [2] and is a dose routinely used by this laboratory to induce sexual differentiation of reproductive parameters [32]. Moreover, benzoate moiety does not alter the oestradiol's biological activity but does prolong its bioavailability. The dose of formestane has been previously used in this laboratory to decrease endogenous oestradiol in the neonatal brain to near undetectable levels [2]. The dose of tamoxifen was based on previously published literature using this drug *in vivo* [33]. Both formestane and tamoxifen readily cross the blood-brain-barrier [34,35]. Two hours after the steroid injection, all pups were injected intraperitoneally (ip) with BrdU on PN0 and PN1 (0.1 mL 0.9% sterile saline containing 100 mg/kg of BrdU), except for the experiments in which animals were injected with BrdU only on PN0. The rationale for administering BrdU 2 hours post hormone injection was based on previously published work in our laboratory in which a hormonal effect on BrdU+ cells was observed [25]. The injection sites were sealed with cyanoacrylate Vetbond Surgical Adhesive (3 M Animal Care Product, MN, USA).

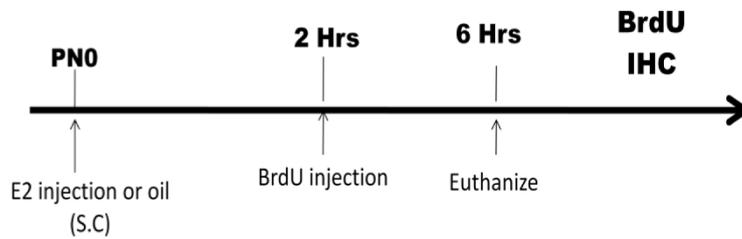
Tissue collection

Pups were deeply anaesthetized with sodium pentobarbital Fatal Plus (250 mg/kg) and transcardially perfused with 0.9% saline until there was no blood trace and then fixed with 4% paraformaldehyde (PFA). Brains were removed and fixed for 24 h in 4% PFA, followed by 48 h in 30% sucrose in PFA before being sectioned coronally on a cryostat, with each slice being 45 µm thick. Slices used for quantification were separated from each other along the rostral/caudal axis so that two contiguous sections were not analysed in the same animal. Sections were collected in series such that each animal generated 4-5 series of sections with 6-8 sections per series obtained from each brain.

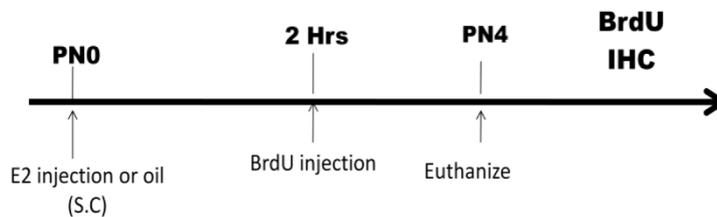
Immunohistochemistry

Free-floating tissue sections were rinsed with 0.1 M phosphate buffered saline (PBS) and then incubated

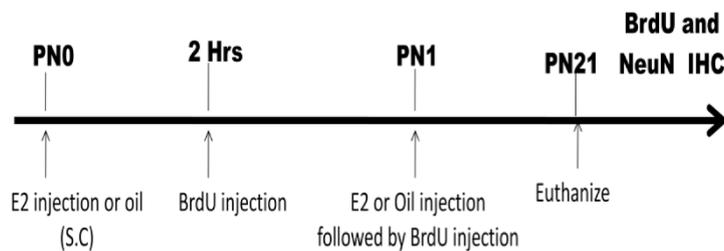
A. Sex differences and the effect of estradiol on cell proliferation.



B. Sex differences and effect of estradiol on short-term new cell survival.



C. Sex differences and effect of estradiol on long-term cell survival and cell fate.



D. Sex differences and effect of antagonizing endogenous estradiol on cell proliferation.

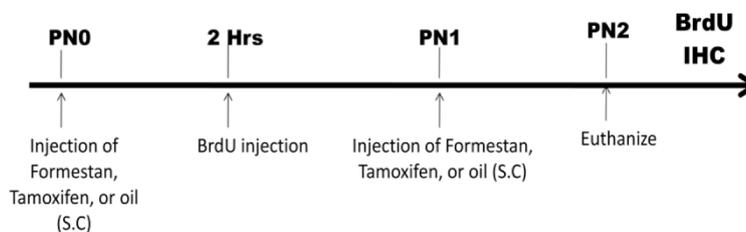


Figure 1 Timeline for 5-bromo-2-deoxyuridine (BrdU) injections and hormone treatments for each experiment. (A) Sex differences and the effects of oestradiol on cell proliferation were tested by postnatal day (PN) 0 hormone treatment with euthanasia 6 h after injection of BrdU. (B) Sex differences and the effects of oestradiol on short-term cell survival were tested by PNO hormone treatment followed by euthanasia at PN4. (C) Sex differences and the effects of oestradiol on long-term survival and cell fate were tested by PNO and PN1 hormone and BrdU treatments followed by euthanasia at PN21 and IHC processing for BrdU+ cells. (D) Sex differences and the effects of antagonizing endogenous oestradiol were tested by treatment on PNO and PN1 with formestane, tamoxifen or vehicle and BrdU followed by euthanasia at PN2 and IHC processing for BrdU+ cells.

with 3% hydrogen peroxide in PBS for 30 min. For BrdU immunohistochemistry, tissue sections were further incubated with 2N hydrogen chloride (HCl) for 60 min at 37°C in order to denature the DNA. After HCl incubation, the sections were rinsed with Borate buffer solution followed by PBS rinses. Finally, sections were incubated with 5% goat serum in PBS with 0.4% Triton X-100 (PBS-T) for 60 min followed by incubation in PBS-T with a monoclonal antibody against BrdU (1: 10,000, Caltag Laboratories, CA, USA) at room temperature (RT) for 60 min, then for 24 h at 4°C. Sections were rinsed in PBS and incubated with biotinylated anti-mouse secondary in PBS-T (1: 1000, Vector, Auckland, New Zealand) rinsed with PBS and incubated in Vectastain Elite ABC reagents (1: 1000, Vector). BrdU-positive cells were detected with diaminobenzidine (DAB) as chromogen, creating a dark brown colour in BrdU-positive nuclei.

For Ki-67 immunohistochemistry, tissue sections were treated as stated above with the omission of the HCl incubation step. Tissue sections were incubated with a polyclonal antibody against Ki-67 in PBS-T (1: 5000, Millipore, Darmstadt, Germany) for 60 min at RT and then 4°C for 48 h followed by biotinylated anti-rabbit secondary antibody (1: 1000). The Ki-67 positive cells were detected with DAB.

Double-label fluorescence immunohistochemistry

Fluorescent immunohistochemistry was used to quantify the extent of colocalization of BrdU and the neuronal specific marker, NeuN. Free-floating tissue sections were rinsed with 0.1 M PBS, incubated with 3% hydrogen peroxide in PBS for 30 min, rinsed and incubated with 2 N HCl for 60 min at 37°C. After HCl incubation, sections were rinsed with Borate buffer solution followed by PBS rinses, incubated with 0.3 M glycine in 0.4% Triton X-100 (PBS-T) for 30 min, and rinsed. Sections were co-incubated with primary antibodies against anti-rat BrdU (1:500, Abcam, Cambridge, UK) and anti-mouse NeuN (1: 500, Millipore) in PBS-T, which contained 10% bovine serum albumin (BSA), for 60 min at RT and overnight at 4°C then rinsed in PBS and incubated with biotinylated anti-rat secondary (1:300, Vector) in PBS-T for 90 min followed by co-incubation with streptavidin Alexa 488 (1:1000; Invitrogen, CA, USA) and anti-mouse Alexa 568 (1:500, Invitrogen) in PBS-T for 60 min in the dark. Rinsed tissue sections were mounted onto gelatin-subbed slides and coverslipped in the dark using Vector Vectashield.

Data analysis

Each subregion of the hippocampus (CA1, CA3 and DG) was analysed using a Nikon Eclipse E600 microscope and the NeuroLucida Software System (MicroBrightfield,

VT, USA). Cell density estimates for each subregion were determined by counting the immunopositive BrdU+, and Ki-67+, cells from the pyramidal layer of CA1 and CA3 as well as granule cell layer of the hippocampus. Cell quantification consisted of using six gridded counting frames that were within the specific subregion of the hippocampus. Each individual counting frame measured 100 × 100 µm. For all three subregions, we calculated the number of immunopositive cells that were inside the counting frames, in both the left and right hemisphere of each section. This procedure was done in four to five sections throughout the rostral hippocampus of each animal. The total number of immunopositive cells across all counting frames was averaged to give one mean value per animal per subregion. All quantifications were performed using a 40× objective. Furthermore, our cell quantification was performed with adherence to appropriate stereological principles, which included but was not limited to: (1) the presence of a nucleus; (2) immunopositive cells were of homogeneously shape; and (3) labelling intensities of immunopositive cells were distinguishable from background staining. Figure 2A-D is a photomicrograph that includes cells considered BrdU + and Ki67+ by both investigators conducting the analyses. Lastly, the sex and hormonal condition of the animals was unknown to the investigators conducting the analyses.

For immunofluorescence, each subregion of the hippocampus was analysed using a Nikon Eclipse 80i grid confocal microscope equipped with an OptiGrid structured light source. Volocity Grid Confocal software (Improvision, Warwickshire, UK) was used to perform the analysis of Z-stacks measuring 10-12 µm collected in two channels at 0.5 µm intervals using a 100× oil objective. BrdU+ cells were selected in each subregion and the number of cells co-expressing the neuronal marker NeuN+ and BrdU+ were quantified. The immunopositive cells were counted bilaterally within each subregion, in four sections (45 µm thick), throughout the hippocampus from each animal using an 80 × 100 µm counting frame.

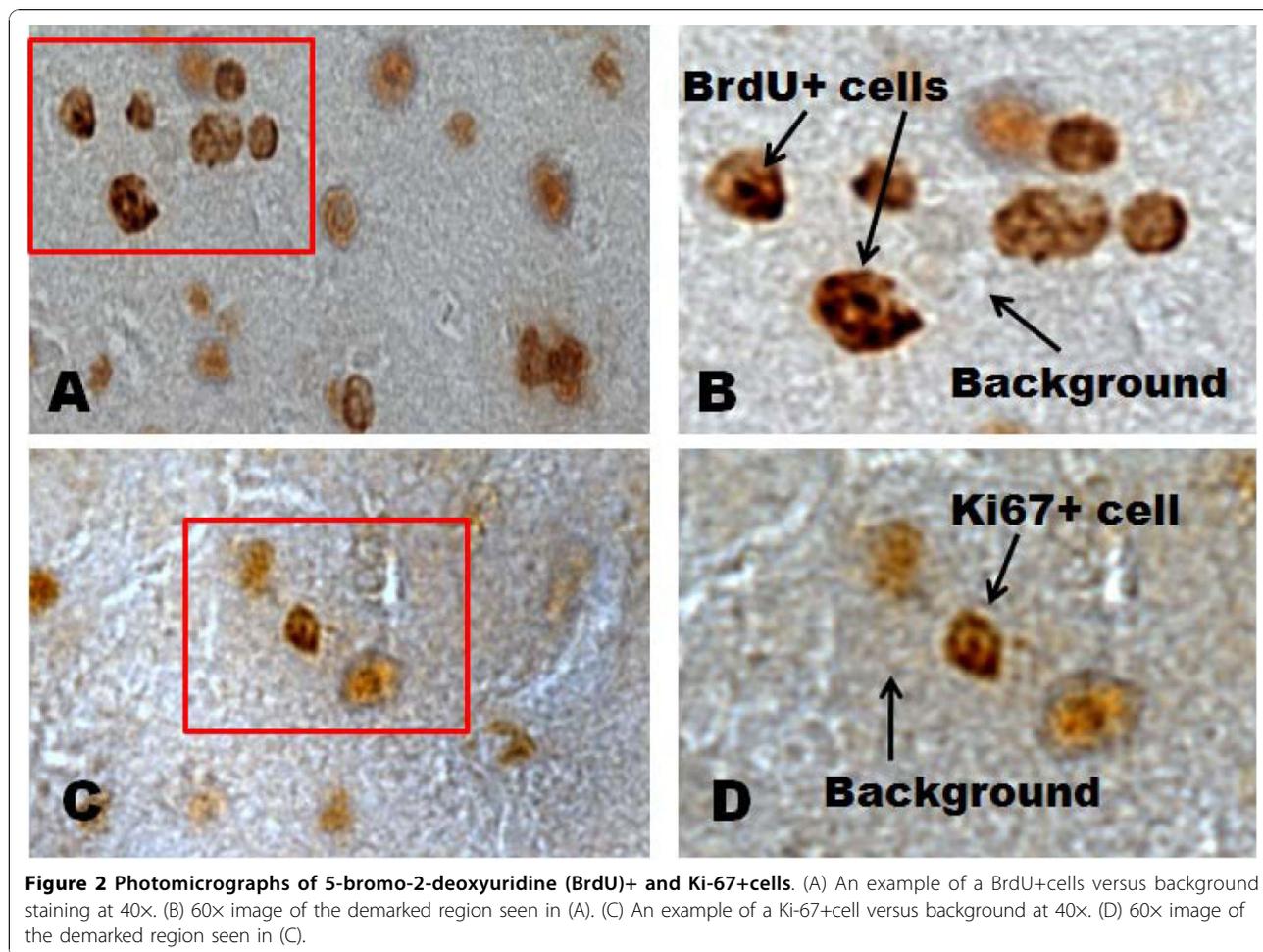
Statistics

The detection of differences in the mean number of cells per region per animal between groups was determined using a three-factor ANOVA with sex, treatment and brain region as fixed factors followed by *post hoc* pairwise comparisons using $P < 0.05$ as the criterion for significance. All *post hoc* comparisons were performed using a Bonferroni correction to control for familywise error.

Results

Neonatal oestradiol treatment increased BrdU+ cells in the newborn female but not the male hippocampus

In order to assess the effects of oestradiol on cell proliferation in developing hippocampus, pups were treated

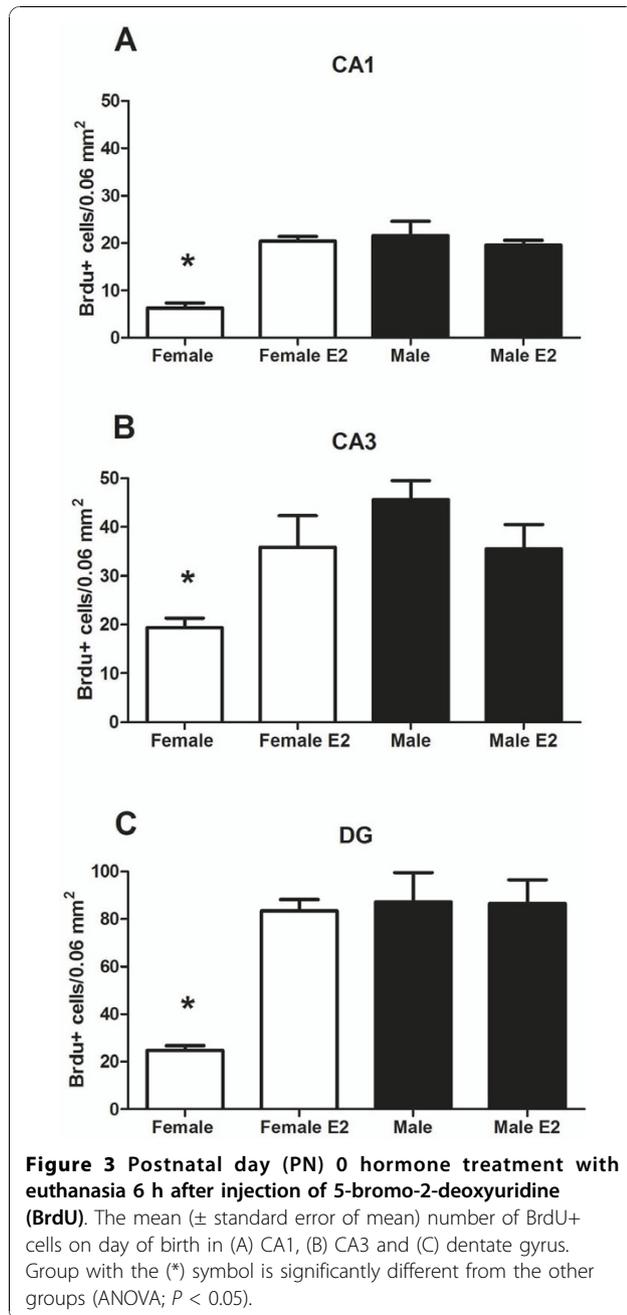


on PN0 with oestradiol followed 2 h later with a single injection of BrdU and euthanized 6 h post-BrdU injection (for procedural timeline see Figure 1-A). A $2 \times 2 \times 3$ ANOVA indicated a significant main effect of sex [$F(1,54) = 28.43, P < 0.001$] confirming that males have a higher mean number of BrdU+ cells than females. There was also a main effect of treatment [$F(1,54) = 14.68, P < 0.001$] with oestradiol increasing the mean number of BrdU+ cells over vehicle. The three-way interaction involving sex \times treatment \times brain region was also significant [$F(2,54) = 3.85, P < 0.03$], due to the larger number of new cells being born in the DG compared to CA1 and CA3. Regardless of the total number of new cells, *post hoc* pairwise comparisons indicated vehicle treated females had significantly fewer BrdU+ cells in all three subregions of the hippocampus than either oestradiol treated females or males treated with vehicle or oestradiol (Figure 3A-C; $P < 0.01$). In females, oestradiol treatment increased the number of BrdU+ cells to the equivalent level seen in males, whereas there was no effect of oestradiol on the number of BrdU+ cells in males ($P > 0.05$).

Cells generated in the presence of oestradiol survive for both short- and long-term periods in the female hippocampus

In order to assess the short- and long-term survival of newborn cells in the neonatal hippocampus, pups were treated with oestradiol followed 2 h later with a single injection of BrdU on PN0 (for procedural timeline see Figure 1-B). At PN4 (short-term period), males again had more BrdU+ cell than females [$F(1, 57) = 7.96, P < 0.007$] and oestradiol treatment increased the number of BrdU+ cells in DG and CA1 [$F(1, 57) = 5.55, P < 0.03$]. A significant three-way interaction [$F(2,57) = 4.76, P < 0.03$] followed by *post hoc* comparisons showed control females had fewer BrdU+ cells in both the DG and CA1 than either oestradiol treated females or males (Figure 4A-C; *Post hoc*, $P < 0.01$).

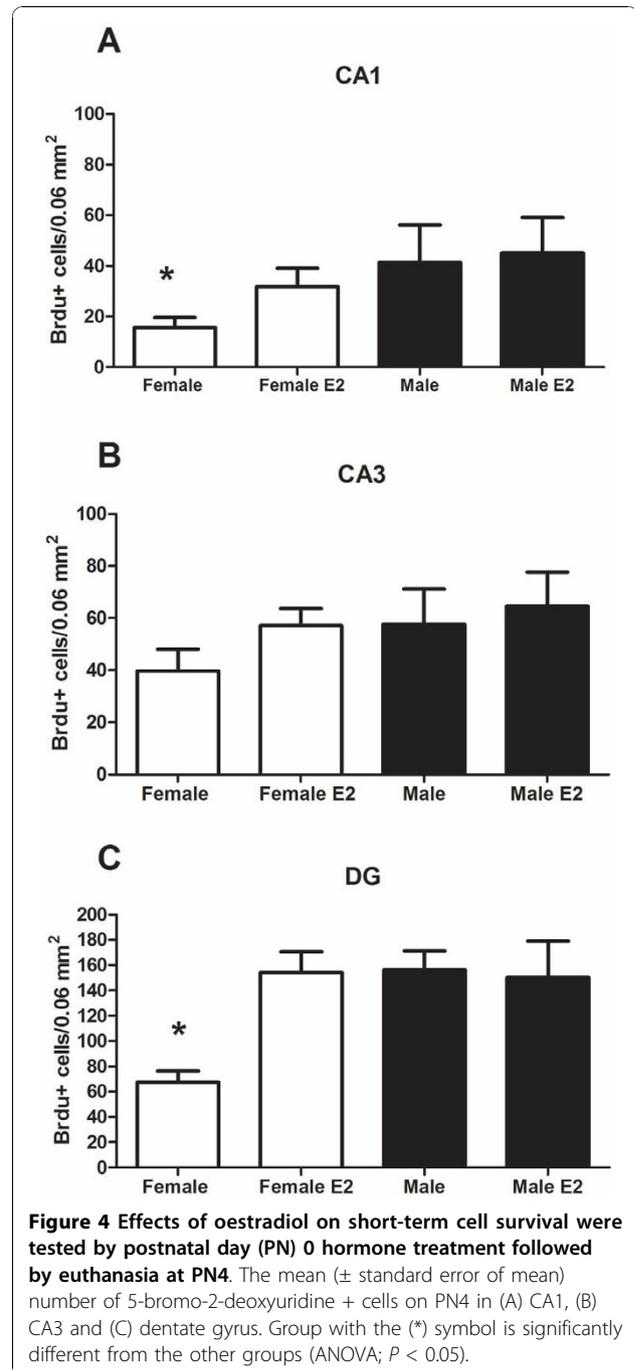
In order to assess whether cells generated under the influence of oestradiol survive over a long period of time, pups were treated with oestradiol and BrdU on PN0 and PN1, then sacrificed on PN21 (for procedural timeline see Figure 1C). The same general pattern of effects observed on PN4 was again apparent on PN21,



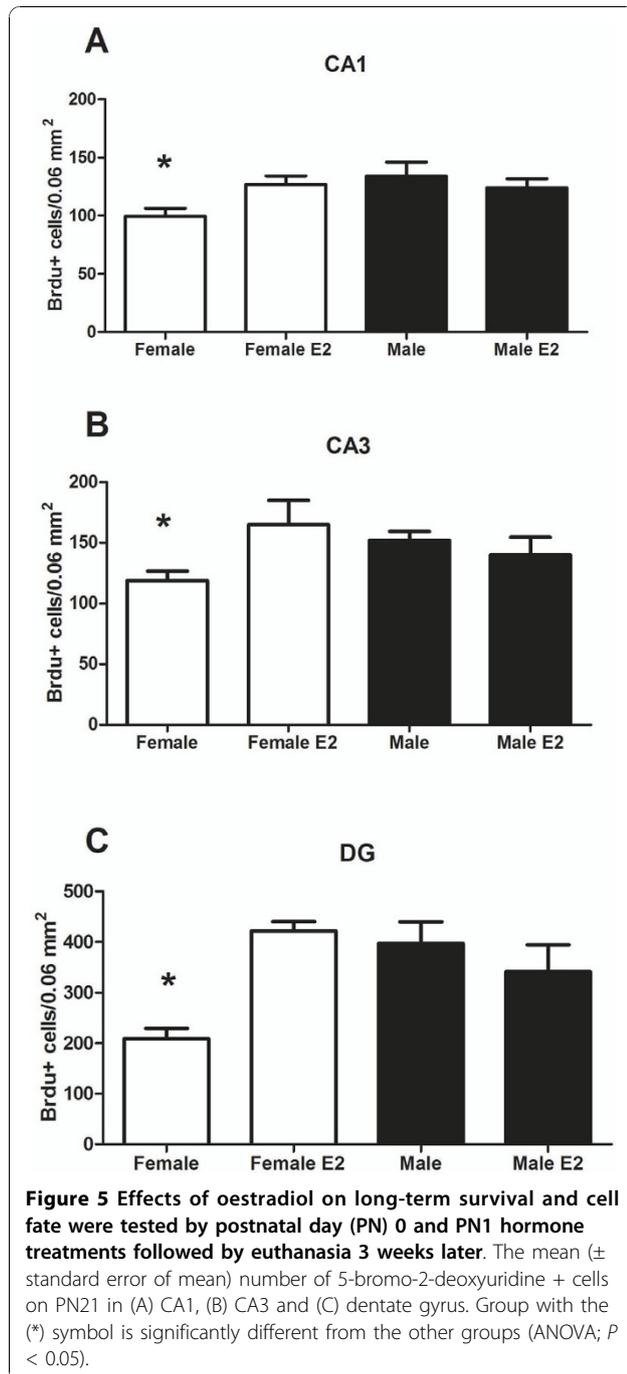
with males having more BrdU+ cells than females [$F(1, 66) = 6.99, P < 0.01$]; oestradiol treatment increased the number of BrdU+ cells in all three subdivisions of the hippocampus of females but not in males [$F(2, 66) = 7.20, P < 0.002$; Figure 5A-C, *Post hoc*, $P < 0.01$].

The majority of cells born in the presence of oestradiol become neurons

In order to determine the fate of cells born on the first few days of postnatal life, we performed fluorescent immunohistochemistry and quantified the proportion of



BrdU+ cells that are also positive for NeuN, a marker of mature neurons. The quantification of BrdU+/NeuN+ cells at PN21 indicated that both male and female groups had proportionally similar numbers of BrdU+ cells that were co-labelled with NeuN in the pyramidal cell layer of CA3 and the granular cell layer of DG (73%; \pm 2% in CA3 and 81%; \pm 4% in the DG). However, males had, overall, ~10% more BrdU+/NeuN+ cells than females [$F(1, 33) = 8.73, P < 0.01$] with the DG having



the highest number of BrdU+ cells that co-labelled with NeuN in both sexes [$F(2, 33) = 5.28, P < 0.05$]. *Post hoc* comparison following a significant three-way interaction [$F(2, 33) = 4.04, P < 0.03$] revealed significantly fewer co-labelled BrdU+/NeuN+ cells in area CA1 for control females compared to either oestradiol treated females and oestradiol treated males or control males ($P < 0.05$; Figure 6). In control females, 41% of cells in CA1 born on PN0-1 were identified as neurons on PN21.

In contrast, for females treated neonatally with oestradiol, 77% of the surviving cells were NeuN+, which is comparable to the 80% and 78% observed in control and oestradiol treated males, respectively.

The sex difference in cell proliferation is still apparent at PN21 and increased in females by neonatal oestradiol treatment

In order to assess whether the neonatal oestradiol-induced increases in cell proliferation endure beyond the period of steroid exposure, we quantified cells expressing Ki-67, an endogenous protein that labels actively proliferating cells, on PN21. *Post hoc* comparison following a significant three-way interaction [$F(2, 36) = 4.49, P < 0.02$] revealed that control females had significantly fewer Ki-67+ cells in the DG, as compared to control males and both neonatal oestradiol treated females and males ($P < 0.01$; Figure 7). There were no significant main effects for sex or treatment (P 's > 0.05).

Inhibiting aromatase activity or blocking oestrogen receptor binding reduces cell proliferation in the developing male but not in the female hippocampus

In order to assess the impact of endogenous oestradiol on cell proliferation, we quantified the number of BrdU+ cells in pups that were treated on PN0 and PN1 with formestane (an aromatase inhibitor), tamoxifen (an oestrogen receptor (ER) antagonist) or vehicle, followed 2 h later with an injection of BrdU (for procedural timeline see Figure 1D). Treatment with formestane or tamoxifen significantly reduced the number of BrdU+ cell compared to controls [$F(2, 152) = 30.63, P < 0.001$]; this effect was detected in all three subdivisions of the hippocampal complex [$F(2, 152) = 42.54, P < 0.001$]. *Post hoc* comparison following a significant two-way interaction of sex \times treatment [$F(2, 152) = 30.97, P < 0.001$] indicated that the effect of formestane and tamoxifen treatment was restricted to males ($P < 0.01$; Figure 8).

Discussion

In the present study, we found that exogenous oestradiol treatment promoted cell proliferation and survival in the neonatal female but not the male hippocampus, whereas antagonizing endogenous oestradiol synthesis or action reduced cell proliferation in the male but not in the female hippocampus. These results confirm and extend our previous report describing a sex difference and oestradiol-induced increase in the number of new cells in the neonatal hippocampus [28] by confirming a sex difference in cell genesis that is regulated by oestradiol. Our previous report included evidence of a male biased sex difference and oestradiol-induced increase in BrdU+/glial fibrillary acid (GFAP)+ expressing cells.

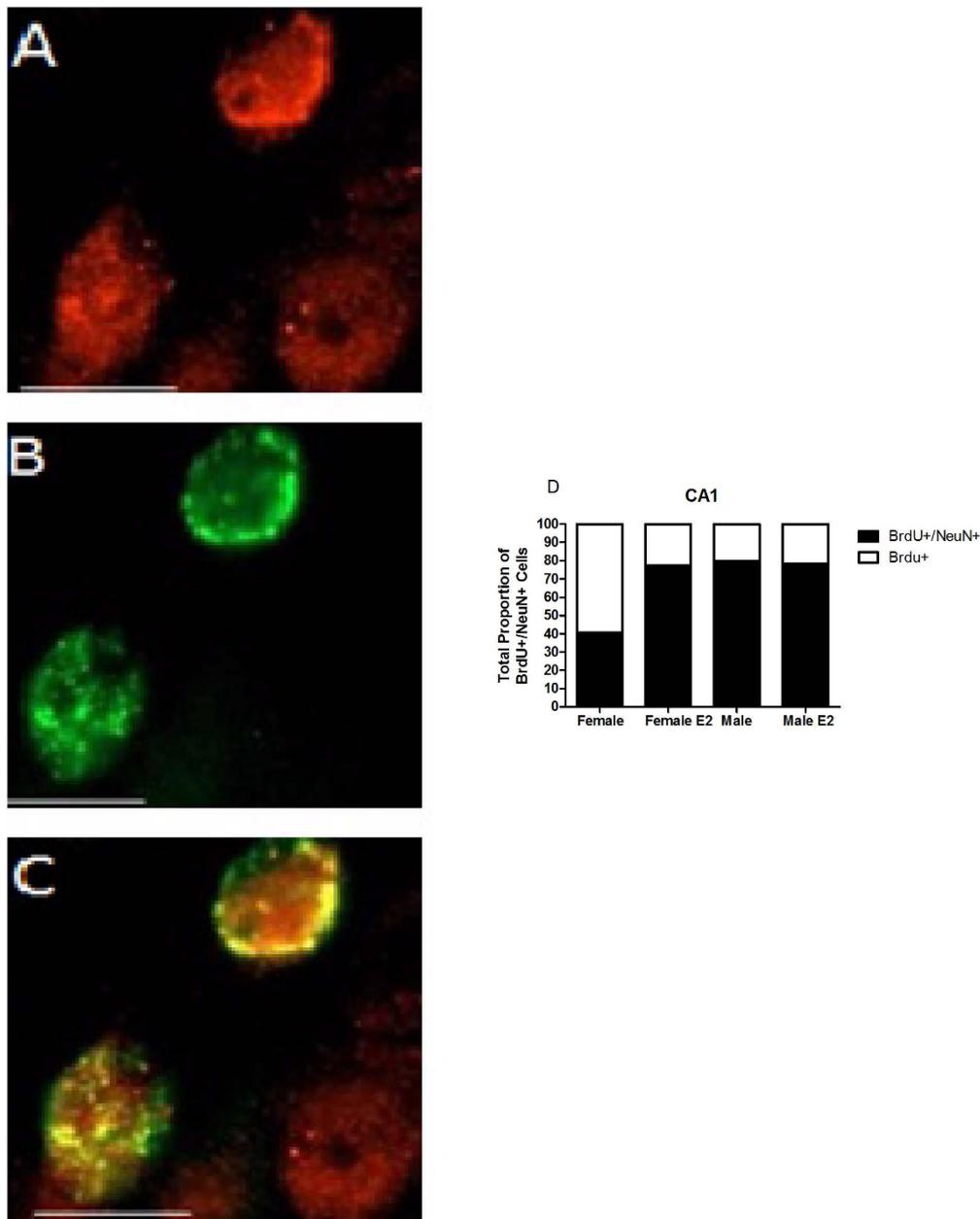
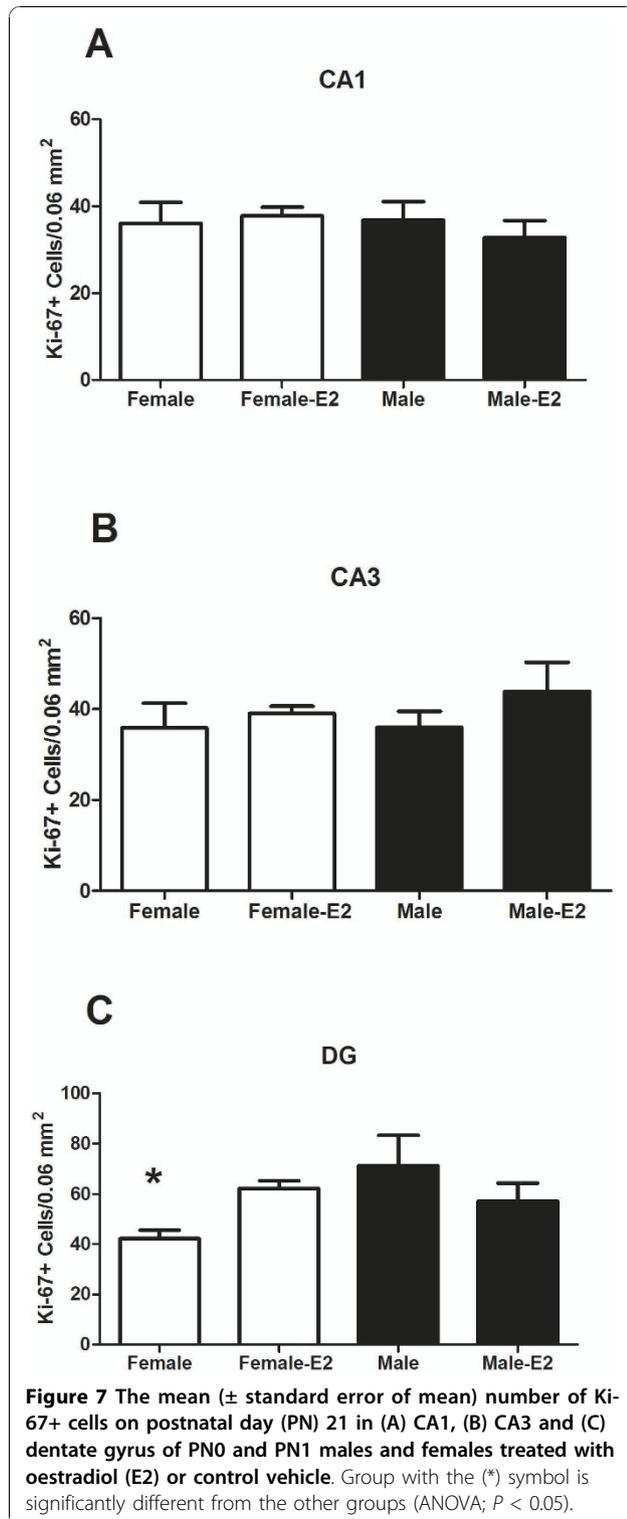


Figure 6 Confocal images of neurons in CA1 pyramidal layer cell layer of the hippocampus at 100 \times magnification. 5-bromo-2-deoxyuridine (BrdU) immunofluorescence was combined with immunostaining for the neuronal marker, NeuN, at 3 weeks (PN21). (A) Confocal image showing NeuN+ cells. (B) Confocal image showing BrdU+ cells. (C) Merged image of both BrdU+/NeuN+ cells. (D) Graph representing total proportion of BrdU+/NeuN+ cells in CA1. Scale bars = 10 μ m.

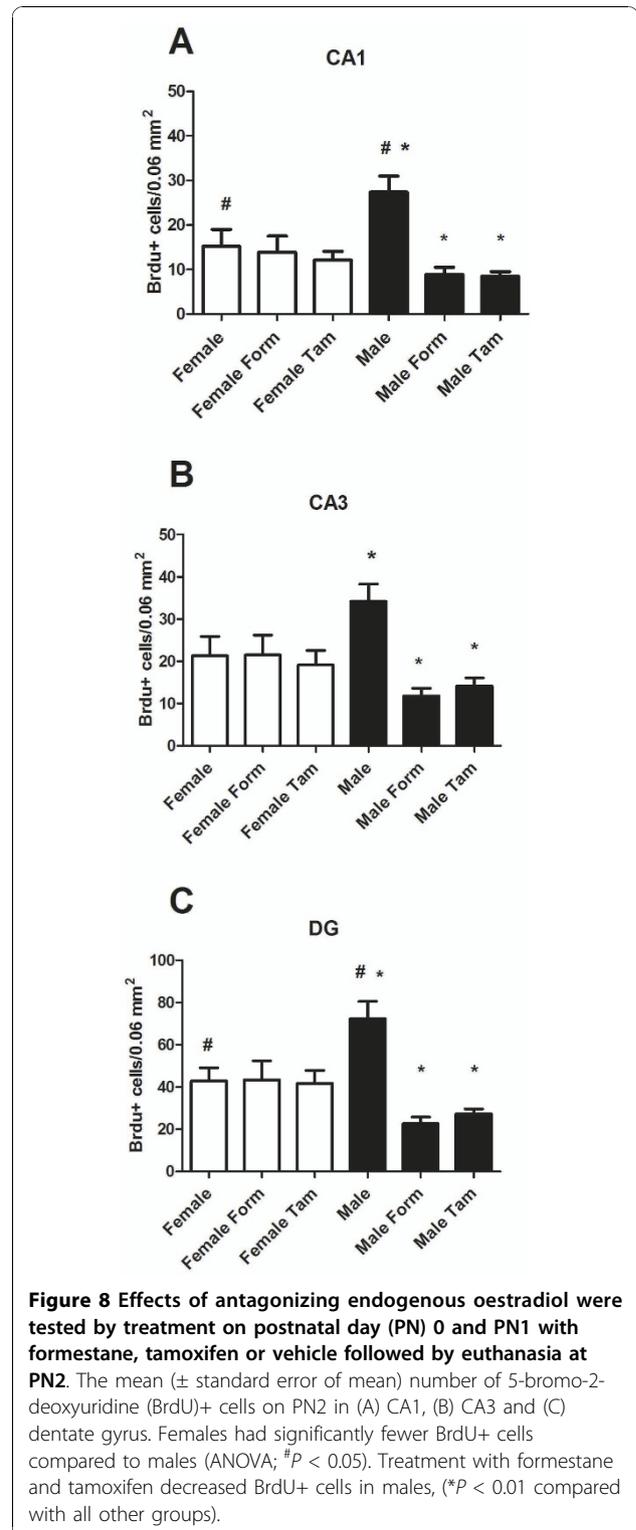
We also quantified the number of NeuN+ cells on PN4, but did not co-label them with BrdU as there was insufficient time for neurons born on PN0 to differentiate. We found more NeuN+ cells in males, but there was no significant increase following oestradiol treatment at PN0-1. This is, again, most probably due to insufficient time for neurons born on PN0 to differentiate into neurons. Therefore our previous study did

not assess neurogenesis *per se*, as opposed to the later developmental stage of PN21 used here. Moreover, many GFAP expressing cells ultimately become neurons [36] and it is possible the oestradiol-induced increase we observed in BrdU+/GFAP+ cells was a precursor to the later increase in BrdU+/NeuN+ cells seen here. The current analysis is a more accurate depiction of the fate of cells labelled at birth with



BrdU under the influence of oestradiol, whereas our previous results depict the fate of new cells within a few days of birth.

There are two mechanisms by which an increase in new cell number can occur; (1) an increased rate of



proliferation; and/or (2) a decreased rate of cell death. In our previous study we observed a significant increase in the number of new cells within 24 h of BrdU injection in males versus females and in females treated with

oestradiol versus vehicle treated females. In the current study the same pattern was observed at the even shorter post-BrdU injection time point of 8 h. The half-life of BrdU is approximately 2 h [37] whereas the cell cycle requires approximately 24 h to complete [38]. Therefore, differences in the number of BrdU+ cells within 24 h of injection are generally interpreted as differences in the rate of cell proliferation and not cell death [37]. Our observation of more BrdU+ cells in males and oestradiol-treated females at both 8 h and 24 h post-BrdU injection is, therefore, most consistent with a hormonally mediated sex difference in cell proliferation. However, given that many new cells die shortly after being born and would not be detected here, a potential contribution of cell death to the sex differences observed cannot be entirely ruled out.

While BrdU labelling offers many advantages, such as long-term tracking of cell fate, there are also limitations associated with potential toxicity and non-specific labelling [37]. In contrast, Ki-67 is an endogenous protein that does not have any adverse effects on living cells and is expressed in all phases of the cell cycle except the resting phase and a short period at the beginning of the G1 phase [39-41]. Thus, Ki-67 is not present in quiescent and terminally differentiated cells and increased numbers of Ki-67 expressing cells is consistent with increased proliferation (but see [42]). In order to determine if sex differences or hormonal modulation of cell proliferation persisted outside of the early neonatal period we quantified the number of Ki-67 expressing cells in the hippocampus of 3-week-old animals and found males and neonatally oestradiol-treated females had more Ki-67+ cells than control females. This observation suggests a higher rate of cell proliferation was organized during the neonatal sensitive period. A precedent for the sexual differentiation of neurogenesis is evident in studies reporting differential sensitivity of adult males and females to exogenous oestradiol treatment or rates of cell birth and death in the DG. In the adult female hippocampus, oestradiol stimulates cell proliferation [43], enhances cell survival [44] and increases dendritic spine synapse density [45-48]. In contrast, the adult male hippocampus is insensitive to the spinogenesis or cell genesis inducing effects of oestradiol treatment [44,49,50]. Oestradiol effects are mediated via binding to the two oestrogen receptor isoforms, ER α and ER β [51,52]. Both isoforms are distributed throughout the brain, including the hippocampus, and both ERs colocalize with the proliferative marker Ki-67 in the adult hippocampus [50,53,54], although, there is a region specific variation in their distribution [54-57]. Moreover, in the adult hippocampus, there are relatively low levels ER α , which is in contrast to elevated levels of ER α that occur during early postnatal

development [58-60]. Nonetheless, it is unclear which receptor isoform is regulating the oestradiol's effects on cell proliferation in the developing hippocampus. Both the short-term increase in the number of BrdU cells and the higher density of Ki-67+ cells in oestradiol treated females indicate a change in proliferative rates of progenitor cells induced by oestradiol and this may be due to a change in the duration of the progenitor cell cycle. This was determined to be the case in neocortical neurogenesis [61]. Oestradiol recruits cells into the S-phase from either G1 or G0 phase, which effectively shortens the G-phase, and results in an increased rate of proliferation of dividing progenitor cells [61].

Both the amount of endogenous oestradiol and aromatase activity in the developing hippocampus are extremely low compared to the hypothalamus and do not appear to be sexually dimorphic [62], suggesting that hippocampal sensitivity to oestradiol is high and differs between the sexes, at least as indexed by hippocampal cell genesis. However, because oestradiol, the ER antagonist and the aromatase inhibitor were all administered systemically, it is possible the effects were not mediated directly at the hippocampus but, instead, were secondary to changes in other brain regions projecting to the hippocampus. Cholinergic neurons of the medial septum/diagonal band of Broca are essential for oestradiol-induced spinogenesis in adult CA1 hippocampus [63] and cholinergic input modulates maturation and integration of adult born DG granule cells [64]. Gonadally intact males release more acetylcholine into the hippocampus than females during locomotor tasks and this sex difference is organized by oestradiol during development [65,66]. The cholinergic system matures relatively early and more new septal cholinergic neurons are born in males during a brief period of gestation but the sex difference does not persist into adulthood [67]. Nonetheless, it is possible that the effects observed here are the results of oestradiol-induced acetylcholine release into the neonatal hippocampus during the early postnatal period. It is also possible that oestradiol is acting outside the central nervous system. In adults, systemic oestradiol alters the arterial cerebral blood flow in females, but not in males [68-71], via an interaction with nitric oxide [72,73]. Both ER α and ER β , as well as the transmembrane G protein-coupled receptor, GPR30, have been identified in blood vessels [74,75] and have been implicated in the rapid vasodilator effects of oestradiol [76]. One of the most powerful stimulators of adult neurogenesis is exercise [77,78]; an effect believed to be at least partly due to enhanced blood flow and increased delivery of growth factors. Lastly, activation of the stress axis has negative effects on adult neurogenesis and the injection of BrdU and steroidal agents to neonates is undoubtedly stressful. However, given that the

number of injections was carefully controlled for across groups, sex differences in stress responding can not entirely explain the current results.

Neurogenesis in the adult hippocampus is restricted to the proliferative zone of DG, with the majority of the new cells becoming granule neurons [79,80]. A notable difference in the profile of neurogenesis in the immature brain is the presence of ongoing proliferation in CA1 and CA3 of Ammon's horn. The colocalization of BrdU with NeuN in these areas indicates that the cells born early postnatally do become neurons but whether they become pyramidal neurons or interneurons is unknown. When, in development, this source of new cells is lost and neurogenesis becomes restricted to the DG is also unknown. An additional unknown is the ultimate role of these enduring neurons in the adult hippocampal function. We observed almost twice as many new cells being born in the neonatal male hippocampus compared to the female. However, when the overall size of the hippocampus is compared in males and females, either developmentally or in adulthood, the sex difference, while biased towards males, is of the order of 10%-12% [15,21]. The magnitude of the sex difference in new cells was just as strong at 3 weeks of age as when they were just a few days old. Therefore, it does not appear that these cells contribute significantly to the hippocampal volume. The early postnatal period is a time of olfactory imprinting and somatosensory stimulation from the dam. The intensity of maternal licking and grooming is greater toward male than female pups [81] and both the nature and function of olfactory learning at this time is likely to be different between the sexes. Whether the role of new neurons born during this period is related to olfactory or sensory learning remains to be determined.

Conclusion

The hippocampus is a critical brain region involved in a variety of cognitive functions (for example, learning and memory) and both the physiologic and emotional responses to stress. Abnormalities in the hippocampus are strongly associated with affective disorders such as major depressive disorder and schizophrenia [82-85], as well as neurologic diseases such as Alzheimers. The selective vulnerability of the hippocampus to hypoxia/ischaemia following stroke, both perinatally and in adulthood [86], further emphasizes the importance of this critical brain region and the need to understand the variables that impact upon it in both males and females. Many of the sex differences observed in the adult hippocampus appear to be the result of early life events, including the impact of gonadal steroid hormones on neurogenesis during the early postnatal period.

Abbreviations

BrdU: 5-bromo-2-deoxyuridine; DAB: diaminobenzidine; DG: dentate gyrus; ER: oestrogen receptor; GFAP: glial fibrillary acid protein; HCl: hydrogen chloride; ip: intraperitoneally; PBS: phosphate buffered saline; PFA: paraformaldehyde; PN: postnatal day; RT: room temperature; sc: subcutaneous.

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Authors' contributions

MB performed the immunohistochemistry, statistical analyses and drafted the manuscript. Both MB and JW treated the animals, processed the tissue samples and performed the cell counts. MM conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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