Sex Differences and Similarities in Hippocampal Cellular Proliferation and the Number of Immature Neurons during Adolescence in Rats

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Abstract

Adolescence is associated with significant reductions in hippocampal cellular proliferation and neurogenesis, the physiological and behavioral implications of which are unclear. Though sex differences exist in these proliferative processes in adulthood, relatively little is known about the role sex plays in these adolescent-related changes. To address this gap, we examined cross-sectional area of the dentate gyrus and cellular proliferation, as measured by Ki-67 immunohistochemistry, in pre- (30 days), mid- (45 days), and post-adolescent (70 days) male and female rats. We also investigated the number of immature neurons using doublecortin (DCX) immunohistochemistry in pre- and post-adolescent males and females. Despite increases in the size of the dentate gyrus during adolescence, we found significant adolescent-related decreases in hippocampal proliferation in both males and females, with a more dramatic decrease in males, indicating both age- and sex-dependent changes in the dentate gyrus. We also found an adolescent-related decline in the number of immature neurons in the dentate gyrus of male rats and a female-biased sex difference in the number of immature neurons in adults. Given these significant changes in the dentate gyrus, these data suggest that this period in de-

velopment might be particularly sensitive to internal and external factors known to modulate neurogenesis, with potential sex-specific neurobehavioral ramifications.

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Introduction

Adolescent maturation is associated with many structural and functional changes in the brain, particularly within cortical and limbic regions [1–3]. One such change is in the number of newly born cells in the dentate gyrus of the hippocampal formation. For instance, studies in rats and mice have indicated a substantial decline in cellular proliferation in this structure that occurs at pubertal onset and is maintained into adulthood [4–11]. The implications of these changes during this developmental stage are unclear but might contribute to the significant shifts in neurobehavioral functions noted during adolescence [12].

In addition to development, sex of the individual affects cellular proliferation and neurogenesis in the hippocampal formation [13]. Adult female rats have higher levels of cellular proliferation in the dentate than males [14], and gonadal hormones can further modulate the difference in the birth and survival of newly born cells [15]. Specifically, ovarian hormones in females, such as estradiol and progesterone, promote both cellular prolif-

eration and survival [14, 16], while testicular hormones in males, such as testosterone, support cell survival [17]. Though levels of gonadal hormones substantially change during pubertal maturation [18], the adolescent-related decrease in hippocampal cell proliferation occurs independent of these hormonal changes, at least in males [10]. That is, males castrated prior to puberty continue to show the decline in both cellular proliferation and neurogenesis into adulthood [10].

It is currently unknown whether there are sex differences in cellular proliferation or the number of immature neurons in the peripubertal dentate gyrus. It has been reported that neonatal males have greater cellular proliferation in the dentate gyrus than females [19, 20], so it is possible that these sex differences persist into the post-weanling, pubertal stages of development. Furthermore, as changes in cortical and limbic gray matter often display curvilinear patterns [1], with differences between males and females [21–23], the decline in cellular proliferation from pubertal onset to adulthood might also display similar nonlinear patterns with unique differences between the sexes.

To bridge this gap in our understanding of adolescent- and sex-dependent changes in cellular proliferation and the number of immature neurons in the hippocampal formation, we examined these parameters in the dentate gyrus of both male and female rats before, during, and after adolescent development. We hypothesized that there would be adolescent-related decreases in the number of proliferating cells and immature neurons, with significant differences between males and females.

Materials and Methods

Animals

Male and female Sprague-Dawley rats were obtained from our breeding colony at Barnard College. All animals were weaned at 21 days of age and housed 2 per cage in clear polycarbonate cages (45 × 25 × 20 cm) with wood chip bedding. All animals were maintained on a 12-h light-dark schedule (lights on at 09.00 h). Animals had ad libitum access to food and water, and the animal room was maintained at 21 ± 2 °C. All procedures were carried out in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Columbia University.

Perfusion and Tissue Collection

Rats were perfused at 30 (pre-adolescent), 45 (mid-adolescent), or 70 (post-adolescent) days of age with heparinized saline rinse and 4% paraformaldehyde ($n = 6$ –8 per age and sex). Prior to perfusion, animals were weighed and deeply anesthetized with ket-

amine (80 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). Brains were post-fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) and then incubated in 20% sucrose in 0.1 M PB at 4 °C. Brains were sectioned coronally in 40- μ m-thick sections into 4 serial sets on a cryostat and stored in cryoprotectant (1:1 of 20% sucrose in 0.1 M PB and ethylene glycol) at –20 °C until processed for histology (see below).

Cross-Sectional Area Measurement of Dentate Gyrus

To assess possible effects of age and sex on the cross-sectional area of the dentate gyrus, the first set of sections for each animal were Nissl stained. These sections were adjacent to the sections used for Ki-67 immunohistochemistry (see below), separated by 120 μ m, and anatomically matched across subjects (corresponding to plates 56–62 in a standard rat atlas [24]). Measurements were made using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) from images captured under a 2.5 $\times$ objective. The cross-sectional area of each dentate gyrus was measured from each section, averaged for each animal, and presented as the average cross-sectional area (mm²).

Ki-67 and DCX Immunohistochemistry

For immunohistochemistry, the second (Ki-67) and third (DCX) set of sections were washed in 0.1 M PB and incubated for 10 min in 0.05% H₂O₂ in 0.1 M PB saline (PBS). Sections were then washed in 0.1 M PB with 0.1% Triton-X-100 (PBT), blocked for 1 h in 2% normal goat serum in PBT, and then incubated in either rabbit anti-Ki-67 (1:1,000; AB15580; Abcam, Cambridge, MA, USA) or guinea pig anti-DCX (1:10,000; AB2253; Millipore Sigma, Burlington, MA, USA) in normal goat serum block for 24 h at 4 °C. The tissue was washed in PBT and incubated for 1 h in either goat anti-rabbit or anti-guinea pig secondary antibodies (1:200; Vector Laboratories) in PBT at room temperature and then incubated in Avidin–Biotin Complex at room temperature (1:250; Vectastain ABC Kit, Vector Laboratories) for 1 h. The sections were then washed in PBS and incubated for 5 min in 3,3'-diaminobenzidine in a 3 M sodium acetate buffer containing 0.05% H₂O₂. For Ki-67 immunostaining, the 3,3'-diaminobenzidine was nickel enhanced, while no nickel enhancement was used for DCX immunostaining. Tissue was then washed in PBS, mounted on Fisher Brand Plus slides (Fischer Scientific, Pittsburgh, PA, USA), dried, dehydrated in 70, 95, and 100% ethanol, cleared in xylenes, and coverslipped with DPX Mountant (Sigma-Aldrich, St Louis, MO, USA).

Microscopy and Quantification

Six or three anatomically matched sections from the dentate gyrus, separated by 120 μ m, were used to measure Ki-67- and DCX-positive cells, respectively (corresponding to plates 56–62 or 58–60, respectively; [24]). For Ki-67 analysis in the dentate, cells were counted under a 40 $\times$ objective across the entire dentate gyrus of each section and averaged. As we also measured the cross-sectional area of each dentate gyrus, we present these data as average number of Ki-67-positive cells per unit area (mm²) of the dentate gyrus. For DCX analysis in the dentate, cell counts were made under a 40 $\times$ objective in the dentate gyrus by placing a grid of 10,000 μ m² superimposed on the images. Bilateral counts from the upper and lower blades of the dentate gyrus were made for each brain section and averaged. Data are presented as the estimated average number of DCX-positive cells per mm². It is important to

Fig. 1. Mean ($\pm$ SEM) cross-sectional area (mm²; **a**) and number of Ki-67-positive cells per mm² (**b**) in the dentate gyrus of pre-adolescent (30 days of age), mid-adolescent (45 days of age), and post-adolescent (70 days of age) male (white bars) and female (grey bars) rats. Bars that share a letter are not significantly different from one another.

note that only the animals in the 30 and 70 days of age groups were processed for DCX immunohistochemistry, as the greatest change in cellular proliferation was observed between these 2 ages.

Statistical Analysis

Two-way ANOVAs (sex $\times$ age) were used for statistical analyses, and significant effects were further analyzed with Tukey's honestly significant difference post hoc tests. Data are reported as the mean $\pm$ SEM, and differences were considered significant at $p < 0.05$. All statistical analyses were performed using GraphPad PRISM, version 5.04 (GraphPad Software Inc., San Diego, CA, USA).

Results

For the cross-sectional area of the dentate gyrus, 2-way ANOVAs revealed a significant interaction between age and sex, such that there is an increase in the

area of the dentate in both males and females as they progress through adolescence, but this increase occurs earlier in females ($F [2, 40] = 6.14, p < 0.05$; Fig. 1a). Specifically, the cross-sectional area of the female dentate gyrus increases between 30 and 45 days of age, while the cross-sectional area in males increases only after 45 days of age.

A 2-way ANOVA also revealed a significant interaction between age and sex in the context of Ki-67-positive cell number in the dentate gyrus ($F [2, 40] = 16.62, p < 0.05$; Fig. 1b). On this measure, pre-adolescent males had significantly more Ki-67-positive cells than pre-adolescent females, and both males and females demonstrated a decline in Ki-67-positive cell number from 30 to 70 days of age. No sex differences were observed in overall cell proliferation at 45 or 70 days of age, indicating the pre-adolescent to post-adolescent decrease in cellular proliferation in the dentate is greater in males compared to females. In fact, the decrease in males reaches approximately 75%, while in females the decrease is about 55%. Figure 2 shows representative photomicrographs of these differences in the number of Ki-67 cells in dentate gyrus of pre- and post-adolescent males and females (Fig. 2).

To determine if changes in proliferation are paralleled by changes in the number of immature neurons, we conducted a 2-way ANOVA on the number DCX-positive cells in the dentate between pre-adolescent and post-adolescent males and females and found a significant interaction between age and sex ($F [1, 20] = 5.61, p < 0.05$; Fig. 3). Specifically, there was a significant decline in DCX-positive cell number in males from 30 to 70 days of age, while 70-day-old females had significantly more DCX-positive cells than 70-day-old males.

Discussion

The present study demonstrates that there are significant age- and sex-related changes in cellular proliferation and the number of immature neurons in the dentate gyrus of the hippocampal formation throughout adolescence. Specifically, pre-adolescent males show significantly more cellular proliferation than pre-adolescent females, but these levels of proliferation are equal by the time both males and females reach young adulthood. Males also had a pubertal decline in the number of immature neurons, paralleling the observed reduction in proliferation. In comparison, females show no pubertal decline in the number of young neurons and adult females had more immature neurons than adult males.

Fig. 2. Representative photomicrographs of Ki-67-positive cells in the dentate gyrus of a pre-adolescent (30 days of age) male (**a**) and female (**c**) and post-adolescent (70 days of age) male (**b**) and female

(**d**) rats. Scale bar = 50 μ m. The inset in panel A is a higher magnification picture of 6 Ki-67-positive cells (scale bar = 25 μ m) from the area in the box with hashed lines.

Though the implications of these neurobiological changes are currently unknown, they might contribute to the significant physiological and behavioral alterations reported to occur during adolescence, which often show sex differences, such as regulation of hormonal stress reactivity and associative memory functions [25, 26].

Previous studies have reported sex differences in hippocampal cell proliferation shortly after birth, such that neonatal male rats have more proliferation, as measured by either Ki-67 or BrdU immunohistochemistry, in the dentate gyrus than neonatal females [19, 20]. The present study suggests that this sex difference is maintained, at least until the onset of puberty, as the pre-adolescent males have about 40% more proliferating cells than the

pre-adolescent females. Though it is unclear what purpose this difference in proliferation serves the developing animal, these data indicate that this considerable sex difference in proliferation is eliminated by time adolescent development is complete, with no differences in proliferation between our 70-day-old, post-adolescent males and females. It should be noted that an earlier study did find a female-biased sex difference in cellular proliferation in the adult dentate gyrus [14], which is different from the current results. However, this earlier study used short-term exposures to the thymidine analog, BrdU, to label proliferating cells [14], while we used Ki-67 as a marker of proliferation. Thus, methodological differences between these studies may account for these discrepant

Fig. 3. Mean ($\pm$ SEM) estimated number of DCX-positive cells per mm² in the dentate gyrus of pre-adolescent (30 days of age) and post-adolescent (70 days of age) male (white bars) and female (grey bars) rats (**a**). In (**a**), bars that share a letter are not significantly different from one another. **b** Is a representative photomicrograph of DCX-positive cells (black arrows) on a portion of the lower blade in the dentate gyrus of an adult female rat (**b**). Bar = 50 μ m.

results. Regardless, this body of work suggests that sex differences in the rate of hippocampal cellular proliferation during earlier neonatal and peri-adolescent stages of development are significantly attenuated by the time reproductive maturity is attained.

The findings that the decline in proliferation is paralleled by a decrease in immature neurons in male rats from pre-adolescence into adulthood are consistent with previous studies [10, 27]. Interestingly, however, a similar decline in immature neurons was not found in the females across the adolescent period, despite the noted reduction in cellular proliferation. These data suggest that proliferating cells in males are following a different maturational path than those in females during adolescence. While we quantified prolifer-

ation levels and the number of immature neurons, we did not measure survival of newly born cells nor did we examine the possibility that proliferating cells are differentiating into nonneuronal cell types, such as glia. No differences in cell survival have been found between adult males and females [14], but it is possible that more newly born cells in adult females differentiate into neurons, while more proliferating cells in adult males differentiate into glial cells. Along these lines, pubertal development has been shown to affect the number and morphology of microglia [28] and gonadal hormones are known to modulate the morphology of astrocytes in males and females [29, 30], suggesting a potential role for glial elements contributing to these observed changes. Another possibility that could account for these different results is that these maturational events might occur earlier in females than males. In fact, our data on the area of the dentate gyrus indicate that some of these adolescent-related changes arise sooner in females. Therefore, significant decreases in both cellular proliferation and the number of newly born neurons may begin earlier than 30 days of age in females. Though these possibilities are not mutually exclusive, future studies quantifying both neurogenesis and gliogenesis across the peri-adolescent period and into adulthood will be needed to determine potential maturational differences and the ultimate fate of these proliferating cells in the adolescent dentate gyrus of both males and females.

Contrary to the results of the current study, a previous experiment comparing the density of DCX-positive cells in the dentate gyrus of adult rats did not find a significant difference between males and females [31]. This inconsistency might be due to the differences in the age of the experimental subjects used, as the adult rats in the present study were significantly younger (i.e., 70 days of age) than the 4-month-old animals that were used in this previous study where no sex differences were observed [31]. If this were the case, these data would suggest that there are further sex-dependent changes in the number of proliferating cells and immature neurons in the dentate gyrus after young adulthood.

Cross-sectional area of dentate was found to increase significantly in both males and females, with the increase in females occurring earlier than males. These data support previous studies in male rats that found increases in the area of the dentate gyrus from the adolescent stage of development to adulthood [10, 32]. This increase in the area of dentate gyrus in both males and females is paradoxical given the significant decrease in cellular proliferation happening in both sexes during this time. However, several factors might mediate this increase in area of the dentate gyrus that are independent of changes in the number of cells, such as changes in dendritic morphology and somal size of gran-

ule neurons in the dentate gyrus, factors known to increase during neonatal and pubertal development [33].

In summary, these data indicate significant changes in the number of proliferating cells and immature neurons occur during adolescence within the dentate gyrus of rats, with unique developmental trajectories in males and females. While sex differences in hippocampal structure, function, and development are well-established [34], few studies have examined the impact of pubertal and adolescent development on hippocampal proliferation and neurogenesis. As newly born cells in the hippocampal formation are vital to cognitive functions, stress resilience, and memory formation [35–37], it will be important for future investigation to assess whether disrupting proliferation and/or neurogenesis during this dynamic stage of dentate gyrus development contribute to the neurobehavioral vulnerabilities that often emerge during adolescence.

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Statement of Ethics

All procedures were carried out in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Columbia University.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.S. and R.D.R.: designed and conducted these experiments and both authors contributed to the writing and editing of the manuscript.

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