Pubertally born neurons and glia are functionally integrated into limbic and hypothalamic circuits of the male Syrian hamster

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During puberty, the brain goes through extensive remodeling, involving the addition of new neurons and glia to brain regions beyond the canonical neurogenic regions (i.e., dentate gyrus and olfactory bulb), including limbic and hypothalamic cell groups associated with sex-typical behavior. Whether these pubertally born cells become functionally integrated into neural circuits remains unknown. To address this question, we gave male Syrian hamsters daily injections of the cell birthdate marker bromodeoxyuridine throughout puberty (postnatal day 28–49). Half of the animals were housed in enriched environments with access to a running wheel to determine whether enrichment increased the survival of pubertally born cells compared with the control environment. At 4 wk after the last BrdU injection, animals were allowed to interact with a receptive female and were then killed 1 h later. Triple-label immunofluorescence for BrdU, the mature neuron marker neuronal nuclear antigen, and the astrocytic marker glial fibrillary acidic protein revealed that a proportion of pubertally born cells in the medial preoptic area, arcuate nucleus, and medial amygdala differentiate into either mature neurons or astrocytes. Double-label immunofluorescence for BrdU and the protein Fos revealed that a subset of pubertally born cells in these regions is activated during sociosexual behavior, indicative of their functional incorporation into neural circuits. Enrichment affected the survival and activation of pubertally born cells in a brain region-specific manner. These results demonstrate that pubertally born cells located outside of the traditional neurogenic regions differentiate into neurons and glia and become functionally incorporated into neural circuits that subserve sex-typical behaviors.

adolescence | gliogenesis | neurogenesis

Puberty and adolescence represent the pivotal stage of development during which a child transforms into an adult. This transformation includes the hormonal changes that underlie sexual maturation, as well as the neural changes that underlie cognitive, emotional, and social maturation (1). The extensive remodeling of the adolescent brain recapitulates many of the neurodevelopmental processes used during initial construction of the nervous system, including neurogenesis and gliogenesis, cell death, synapse proliferation and pruning, and myelination.

Previous work in our laboratory has shown that cells are added during puberty to sexually dimorphic limbic and hypothalamic regions in a sex- and brain region-specific manner, and that sex differences in pubertal cell addition are driven by gonadal hormones (2). The addition of new cells to the adolescent brain may be a unique mechanism facilitating the transformation from childhood to adulthood, particularly for the maturation of sex-typical behaviors. Whether pubertally born cells become incorporated into neural circuits that subserve sex-typical behaviors remains to be elucidated, however. In this work, we sought empirical evidence for the functional incorporation of pubertally added cells into the neural circuit that underlies social-sexual behavior in male Syrian hamsters.

Our approach used sexual experience-induced expression of Fos (protein product of the immediate early gene *c-fos*, a marker of neuronal activation) immunoreactivity within pubertally born cells as an assay for cell activation and functionality (3). Specifically, male hamsters received injections of the cell birthdate marker bromodeoxyuridine (BrdU) during puberty. In adulthood, they were allowed to interact and mate with sexually receptive female hamsters, an experience known to induce a robust Fos response within limbic and hypothalamic regions (4, 5). Environmental enrichment (6, 7) and opportunities for physical activity (8, 9) are well-documented ways to increase the addition of adult neurons to the dentate gyrus (DG) of the hippocampus. In an attempt to boost the number of BrdU-immunoreactive (BrdU-ir) cells that survived into adulthood, we housed some hamsters in enriched environments with running wheels. Thus, we were also able to determine whether enrichment increases the likelihood of long-term survival of pubertally born cells in limbic and hypothalamic regions. We found that some proportion of cells added to limbic and hypothalamic regions during puberty differentiate into either mature neurons or mature astrocytes and become functionally incorporated into neural circuits. In addition, enrichment increases the likelihood of long-term survival of pubertally born cells in some, but not all, of the hypothalamic and limbic regions studied.

Results

Some Pubertally Born Cells Become Either Mature Neurons or Mature Astrocytes. BrdU-ir cells were analyzed within regions of interest (ROIs), including the DG of the hippocampus, medial preoptic area (MPOA), arcuate nucleus, and medial amygdala (Me), which was subdivided into the anterior (MeA), anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD), and posteroventral (MePV) areas (Fig. 1). Within each ROI, 80 BrdU-ir cells were examined for colocalization with mature neuron marker neuronal nuclear antigen (NeuN) or astrocytic marker glial fibrillary acidic protein (GFAP); in Fig. 2, circular histograms depict the proportion of BrdU-ir cells that colabeled with NeuN and/or GFAP, or neither marker.

Statistical analyses revealed significant differences among brain regions in the proportion of all three cell phenotypes: BrdU/NeuN cells ($F_{1,3} = 219.007$, $P = 0.001$), BrdU/GFAP cells ($F_{1,3} = 34.218$, $P = 0.010$), and BrdU-only cells ($F_{1,3} = 656.877$, $P < 0.001$). Post hoc analyses revealed significantly more BrdU/NeuN cells in the DG than in the arcuate ($P = 0.007$), MeA ($P = 0.028$), MeAV ($P = 0.024$), MePD ($P = 0.007$), MePV ($P = 0.008$), and MPOA ($P = 0.011$). In contrast, there were significantly more BrdU/GFAP cells in the arcuate nucleus than in the DG ($P = 0.002$), MeA ($P = 0.036$), MeAD ($P = 0.034$), MePV ($P = 0.027$), and MPOA ($P = 0.042$). There were also significantly more BrdU/GFAP cells in the MePD compared with the MeA ($P = 0.003$) and MPOA ($P = 0.022$). Finally, there were

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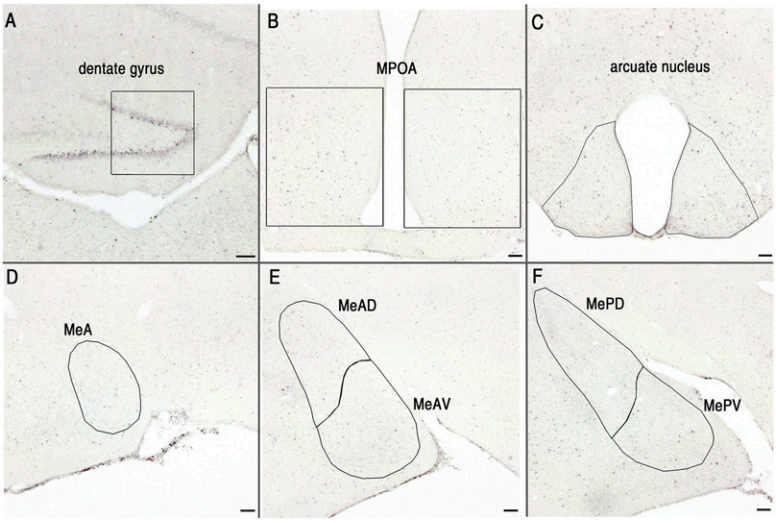

Fig. 1. Representative photomicrographs of BrdU-ir tissue sections showing placement of boxes/contours in ROIs. BrdU-ir cells were counted in anatomically matched sections within two 0.25-mm^2 boxes placed bilaterally in two sections of DG (*A*), two 1.5-mm^2 rectangular boxes in one section of MPOA (*B*), contours in 15 sections of arcuate nucleus (*C*), and contours traced in 13 sections of Me, including MeA (*D*), MeAD and MeAV (*E*), and MePD and MePV (*F*). (Scale bars: 100 μm.)

significantly fewer BrdU only cells in the DG compared with the arcuate (P = 0.038), MeA (P = 0.022), MeAD (P = 0.025), MeAV (P = 0.026), MePD (P = 0.022), MePV (P = 0.004), and MPOA (P = 0.004), and significantly more BrdU-only cells in the MPOA compared with the MeAV (P = 0.049) and MePD (P = 0.014). Confocal Z-stacks with orthogonal views confirmed colocalization of pubertally born cells with the astrocytic marker GFAP (Fig. 3*A*), or the mature neuronal marker NeuN (Fig. 3*B*).

Certain Pubertally Born Cells Are Activated During Sociosexual Behavior. BrdU-ir cells that also expressed Fos-ir (Fig. 4) were present in all ROIs, signifying that some pubertally born cells are activated during sociosexual behavior in adulthood. The proportion of BrdU-ir cells that also expressed Fos-ir ranged from ∼1% to 8%, depending on the ROI involved (Fig. 5). Enrichment had no effect on the percentage of BrdU-ir cells expressing Fos-ir in the DG, MPOA, arcuate nucleus, or MeAD (P = 0.095, 0.407, 0.527, and 0.566, respectively; Fig. 5). In contrast, enrichment roughly doubled the percentage of BrdU-ir cells expressing Fos-ir in the MePD ($F_{1,22}$ = 7.925, P = 0.01; Fig. 5*G*) and MePV ($F_{1,22}$ = 9.828, P = 0.005; Fig. 5*H*), from ∼2% in the control environment to 4% in the enriched environment. Group-by-hemisphere interactions were found in the MeA ($F_{1,22}$= 4.448, P = 0.047; Fig. 5*D*) and MeAV ($F_{1,22}$= 4.974, P = 0.036; Fig. 5*F*), in which animals housed in enriched environments had significantly more double-labeled BrdU-ir/Fos-ir cells than control animals only within the right MeA, and animals housed in enriched environments had significantly fewer double-labeled BrdU-ir/Fos-ir cells than control animals only within the right MeAV.

Enrichment Affects the Number of BrdU-ir Cells in a Brain Region-Specific Manner. As expected, animals housed in enriched environments (i.e., larger cage with additional nesting materials and a running wheel) had more BrdU-ir cells in the DG of the

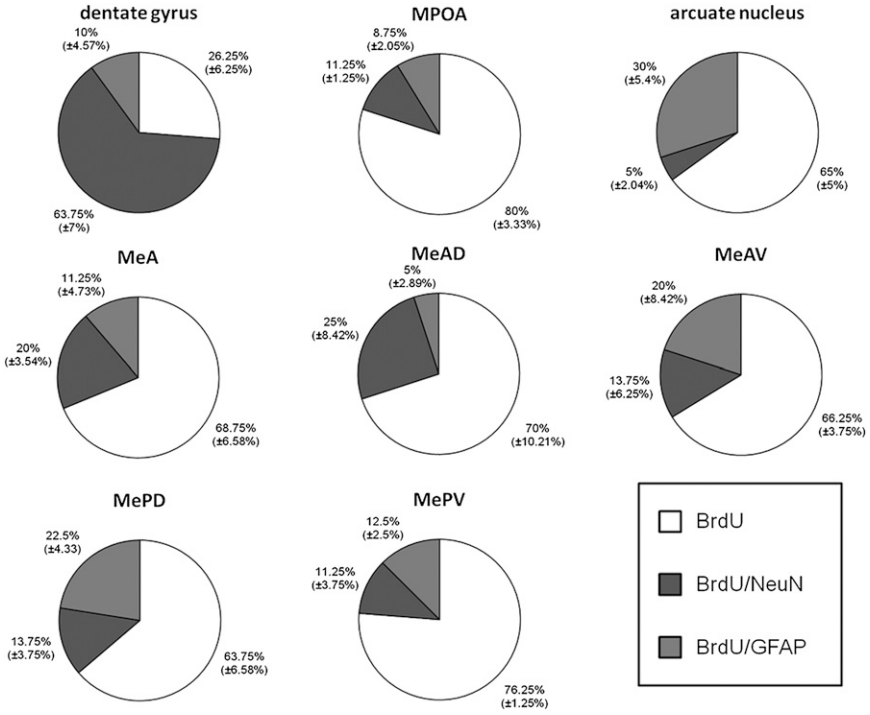

Fig. 2. Some pubertally born cells acquire mature neuronal and glial phenotypes. Pie charts represent the percentage of BrdU-ir cells (± SEM) double-labeled with mature neuronal marker NeuN, astrocytic marker GFAP, or neither.

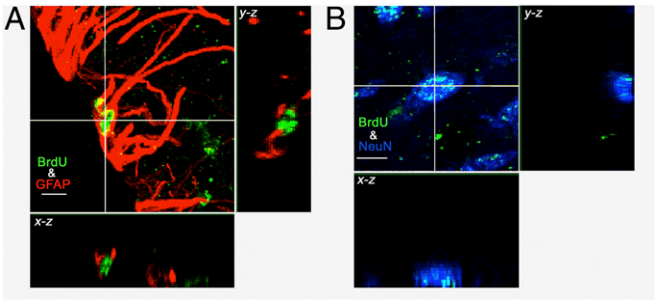

Fig. 3. Representative confocal images with orthogonal views of pubertally born cells in the arcuate nucleus with a glial phentotype (*A*) and in the MeAD with a neuronal phenotype (*B*). (Scale bars: 10 μm.)

hippocampus compared with animals housed in control environments ($F_{1,10}$ =7.443, P = 0.023; Fig. 6*A*). Similarly, the enriched environment resulted in more BrdU-ir cells in the MPOA ($F_{1,10}$ = 7.902, P = 0.020; Fig. 6*B*), the MeAD ($F_{1,10}$ = 5.278, P = 0.047), and the MePD ($F_{1,10}$ = 5.648, P = 0.041; Fig. 6*D*) compared with the control environment. There was no effect of enrichment on the number of BrdU-ir cells in the arcuate nucleus of the hypothalamus (P = 0.179), MeA (P = 0.176), MeAV (P = 0.071), or MePV (P = 0.519).

Discussion

This study phenotyped pubertally born cells in limbic and hypothalamic regions in adulthood, and demonstrates that these cells become incorporated into neural circuits. In each brain region analyzed, some pubertally born cells were found to express either mature neuronal or astrocytic markers, with the proportion of cells expressing these markers differing by region. Approximately three-fourths of pubertally born cells in the DG differentiated into mature neurons (64%) or astrocytes (10%), whereas only one-third of the pubertally born cells in the amygdala and hypothalamic regions expressed markers for either mature neurons or astrocytes (with relative proportions varying by region).

Importantly, on average, ∼3% of pubertally born cells expressed Fos after a sexual experience, indicating that these cells were activated as a result of the social interaction, and providing compelling evidence of functional connectivity of these cells with other components of the circuit underlying male sexual behavior. This relatively small percentage of BrdU-ir cells expressing Fos is similar to that reported in previous studies using BrdU and immediate early genes to show functional incorporation of adult-born neurons (10, 11), which is not surprising given that most pubertally born cells were not identified as either mature neurons or astrocytes (except for the DG), and that not all pubertally born neurons would be expected to have acquired similar function.

Finally, we found that environmental enrichment increased the number of pubertally born cells surviving into adulthood in certain brain regions. Thus, we propose that pubertal neurogenesis and gliogenesis are mechanisms by which the adolescent brain is remodeled, and that a gain of function of pubertally born cells contributes to the adolescent maturation of sex-typical social behaviors.

Here we report the functional incorporation of pubertally born cells into brain regions outside the canonical adult neurogenic regions, that is, the DG and olfactory bulbs. Pubertally born cells in the hypothalamus and amygdala expressed Fos after a sociosexual experience, and it seems unlikely that this could have occurred without some sort of connection or communication between these cells and cells normally involved in social interactions. It is possible that the Fos expression is a response to aspects of the social experience other than mating per se, such as the novel environment or handling, or even that some BrdU-labeled cells constitutively express Fos; however, we believe it more likely that Fos expression in the Me and MPOA in particular is specific to sexual interaction with the female, given the robust Fos response in both regions after mating or exposure to vaginal secretions, compared with the near absence of Fos-ir in these regions in hamsters just placed in a clean cage (12).

Using an experimental approach similar to ours, Huang and Bittman (13) investigated whether adult-generated cells in male Syrian hamsters are incorporated into circuits supporting sociosexual behaviors. They detected BrdU-ir/Fos-ir cells in the male hamster olfactory bulb, but not in the Me or MPOA, after exposure to a receptive female. The discrepancy between that study and ours most likely reflects differences in the BrdU injection regimen, with Huang and Bittman labeling adult-born cells (three injections at age 2.5 mo) compared with our labeling of pubertally born cells [21 injections on postnatal day (P) 28–P49]. Other studies have reported a higher rate of neurogenesis in the DG in adolescence than in adulthood (14); thus, both the younger age of hamsters and the larger number of BrdU injections likely contributed to the detection of BrdU-ir/Fos-ir in the Me and MPOA in the present study, but not the study of Huang and Bittman. However, in addition, the functional incorporation of newly born cells into the amygdala and hypothalamus may be more likely during adolescence than in adulthood, because of the greater degree of plasticity of the adolescent brain. The results of the present study support the idea that ongoing neurogenesis and gliogenesis during puberty may contribute to the regulation of social behaviors that emerge during adolescence and are essential for reproductive success in adulthood.

Accumulating evidence supports a contribution of adult cell proliferation to structural and functional plasticity in hypothalamic (15) and limbic regions (16) of the mammalian brain. Recent reports implicate cell proliferation in the adult olfactory bulb and DG as important for the expression of species-typical sociosexual behaviors (17–19). For example, experimental blockade of cell proliferation by intracerebroventricular delivery of the antimitotic agent cytosine arabinoside in male rats inhibits sexual behavior (20), and treatment of adult female mice with arabinofuranosyl cytidine abolishes the otherwise characteristic preference for odors from dominant male mice (19). Our results extend this emerging literature by showing that pubertally born cells in hypothalamic and limbic regions are

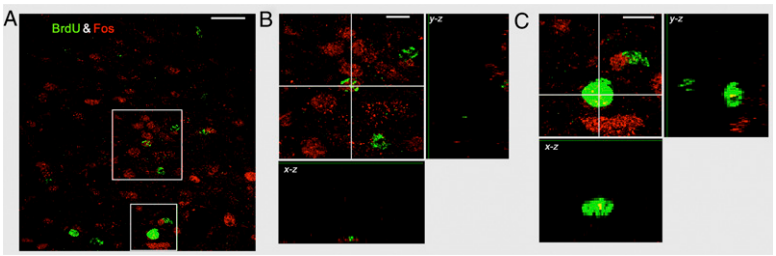

Fig. 4. Representative photomicrograph showing two pubertally born cells in the MePD that are active during sociosexual behavior. The upper box in *A* is enlarged in *B*, and the lower box in *A* is enlarged in *C*. Orthogonal views in *B* and *C* confirm colocalization of BrdU and Fos. (Scale bars: 30 μm in *A*; 10 μm in *B* and *C*.)

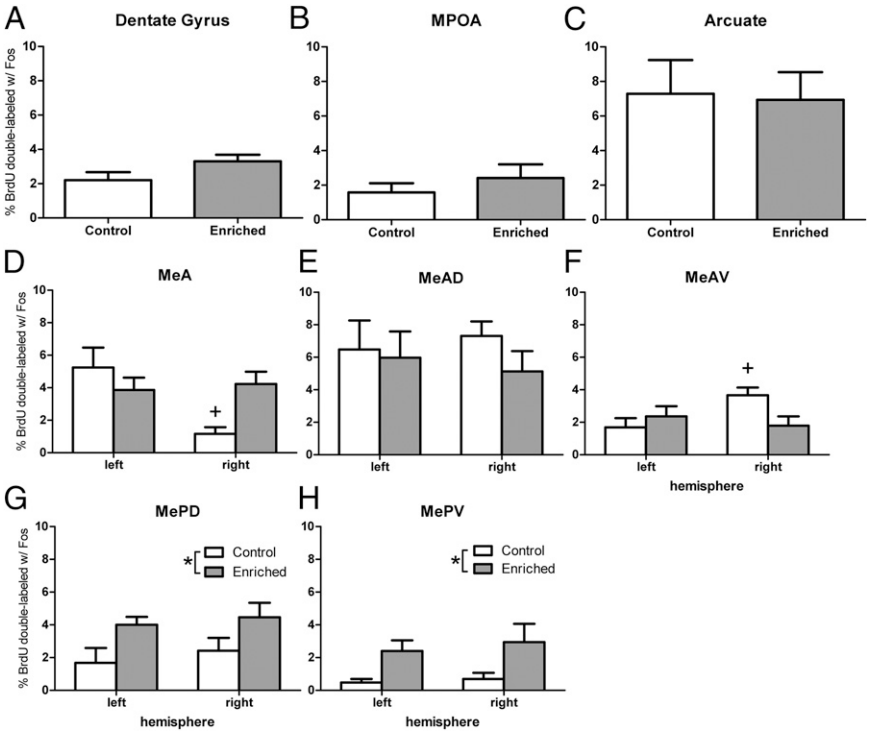

Fig. 5. Pubertally born cells are active during sociosexual behavior in adulthood, with no effect of enrichment in the DG (*A*), MPOA (*B*), or arcuate nucleus (*C*), but with subregion- and hemisphere-specific effects of enrichment in the Me. Control animals showed a lower percentage of BrdU-ir cells double-labeled with Fos-ir in the right MeA (*D*) and a greater percentage of BrdU-ir cells colabeled with Fos-ir in the right MeAV (*F*). There was no effect of enrichment in either hemisphere of the MeAD (*E*). Enriched animals showed a greater percentage of BrdU-ir cells colabeled with Fos-ir in the MePD (*G*) and MePV (*H*) compared with control animals, with differences between hemispheres. Data are mean ± SEM percentage of BrdU-ir cells double-labeled with Fos-ir cells. $n = 6$ for control; $n = 7$ for enriched. *Main effect of group, $P \leq 0.01$; +Group-by-hemisphere interaction, $P \leq 0.05$.

activated by sociosexual experience, pointing to a role for pubertal cell proliferation in the maturation of adult social behavior.

The majority of pubertally born cells in the hypothalamic and limbic regions studied here expressed neither the mature neuronal marker NeuN nor the astrocyte marker GFAP. At the time of tissue collection, the age of the BrdU-labeled cells would have ranged from 4 wk to 7 wk, sufficient time for differentiation, migration, targeting, and synaptic integration to have occurred (21). Although GFAP is also expressed in neural progenitor cells, we are confident that the BrdU-ir/GFAP-ir cells detected in the present study are indeed astrocytes and not neural progenitor cells, based on the highly branched morphology of the GFAP staining. Thus, the cells that were not phenotyped by NeuN or GFAP expression could be microglia, oligodendroglia, tanycytes, immature neurons at various stages of differentiation, or undifferentiated precursor cells.

The percentage of BrdU-ir cells colabeled with NeuN in this study is low compared with that reported in adult mouse (8, 22) and rat (23) DG. This suggests that there is a species difference in the addition and survival of neurons to the DG, that the proportion of newly proliferated cells differentiating into mature neurons depends on whether proliferation occurs during puberty or in adulthood, or, less likely, that there is a species difference in the ability of NeuN to label hamster neurons compared with rat or mouse neurons. Future work will examine the phenotype of pubertally born cells that are not mature neurons or astrocytes, providing insight into whether these cells have specialized functions, such as sources of growth factors or other support of neighboring cells, or whether they may be destined for differentiation in the face of new challenges, for example, parenthood or seasonal changes in resources.

The arcuate nucleus is a hub for the convergence of neuroendocrine and homeostatic signals, and arcuate glial cells play an important role in regulating the secretion of hypothalamic-releasing hormones into the pituitary portal system (reviewed in ref. 24). In the present study, the arcuate nucleus contained the highest proportion (30%) of pubertally born cells that differentiated into mature astrocytes. Lorenz et al. (25) found that astrocytes express androgen receptor in adult, but not juvenile, male rats, indicating a pubertal increase in the number of androgen-sensitive glial cells. Thus, the BrdU-ir/GFAP-ir arcuate cells seen in this study may reflect newly added androgen-sensitive arcuate astrocytes that potentially participate in pubertal activation of reproductive hormone secretion and behavior.

The influence of enrichment on the survival and activation of pubertally born cells was confined to particular brain regions. Our results are in agreement with many previous studies reporting that environmental enrichment increases the survival of cells in the DG (6–9, 26), and demonstrate that enrichment also increases the survival of pubertally born cells in particular cell groups within the hypothalamus and amygdala. This region-specific effect of enrichment could be related to regional

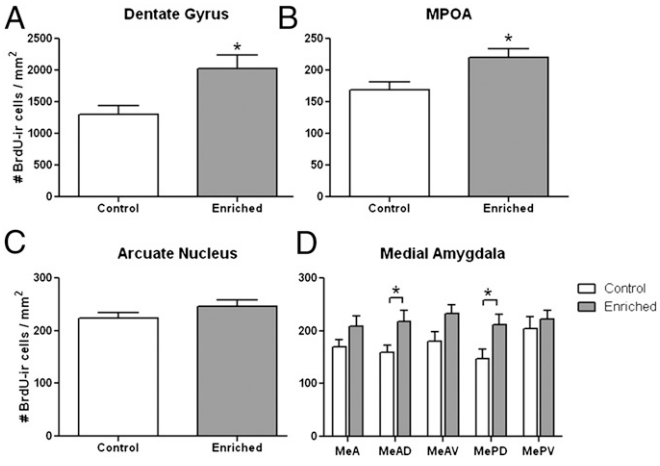

Fig. 6. Enrichment significantly increased the density (number of BrdU-ir cells/mm^2, mean ± SEM) of pubertally born cells in specific brain regions. Enrichment significantly increased the number of BrdU-ir cells in the DG (*A*), MPOA (*B*), and MeAD and MePD (*D*), but had no effect on the number of BrdU-ir cells in the arcuate nucleus (*C*). $n = 5$ for control; n = 6 for enriched. *$P \leq 0.01$.

differences in the local expression of neurotrophic factors (27) or neurohormones (28) that are known regulators of cell proliferation or survival (29).

The current experimental design allowed identification only of pubertally born cells that survived 4–7 wk, and thus the increases in BrdU-ir in the DG, MPOA, MeAD, and MePD in enriched animals compared with control animals could be related to a higher rate of cell proliferation during puberty and/or a lower rate of cell death after puberty. Snyder et al. (30) used BrdU injection regimens that distinguished between the effects of wheel running on cell survival and on proliferation, and found that running increased both cell proliferation (indicated by proliferating cell nuclear antigen-ir) and survival of new hippocampal granule cells. They also found that voluntary running increased the proportion of young granule cells expressing the immediate early gene *Arc* after a kainate-induced seizure, consistent with our finding that enrichment increased the proportion of BrdU-ir cells expressing Fos in the MePD, MePV, and MeA.

Conclusion

This study documents that pubertally born cells located outside of the canonical neurogenic regions survive into adulthood and become functionally integrated into neural circuits, and that environmental enrichment can bolster the survival and activation of these cells in certain brain regions. These results highlight the malleability and sensitivity to experience of the adolescent brain, and identify neurogenesis and gliogenesis as mechanisms that contribute to brain remodeling during this critical period of postnatal development.

Materials and Methods

Animals and Experimental Design. Fourteen P21 sexually naïve male Syrian hamsters were purchased from Harlan Sprague-Dawley and individually housed on arrival with ad libitum access to food and water on a 14:10 light: dark cycle (lights out at 1400 h). Animals in the enriched group ($n = 7$) were housed in large clear polycarbonate cages (37.5 × 17 × 33 cm) with cotton bedding and cardboard igloos on arrival and given access to running wheels (Super Pet, purchased from PetSmart; epoxy-coated metal, 5.75″ diameter) at P23. In this study, enrichment is defined as both environmental enrichment and voluntary wheel running. Rosenzweig (31) defined environmental enrichment as the "combination of complex inanimate and social stimulation," but because male Syrian hamsters are solitary creatures, the present study did not include social stimulation as a part of enrichment. It should be noted that although environmental enrichment and voluntary exercise have been shown to influence adult hippocampal neurogenesis via dissociable pathways (22), no distinction was made between environmental enrichment and voluntary exercise in the present study.

Animals in the control group ($n = 7$) were housed in clear polycarbonate cages (30.5 × 10.2 × 20.3 cm) with no enrichment. All animals remained in enriched or control environments until the end of the experiment, and were treated in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Michigan State University's Institutional Animal Care and Use Committee approved all protocols.

Between P28 and P49, animals received daily i.p. injections of BrdU at a dose of 150 mg/kg body weight (Sigma-Aldrich), dissolved in 0.9% sterile saline (10 mg/mL), occurring at ~1230 h. On P77, in dim red light, each animal was allowed to mate with a sexually receptive stimulus female for 15 min. Sexual receptivity was induced in ovariectomized female hamsters by injection of estradiol benzoate (10 μg s.c.) and progesterone (500 μg s.c.) at 52 h and 4–5 h, respectively, before mating. The interactions were videotaped, and sexual behaviors were scored as described previously (32); mounts, ectopic mounts, intromissions, rhythmic thrusts, anogenital investigations, and genital grooms were quantified by an observer blind to the experimental groups. There were no statistically significant differences between enriched and control males in any of these sexual behaviors, and every male was observed to achieve ejaculation.

Tissue and Blood Collection. At 1 h after sexual behavior, male hamsters were given an overdose of sodium pentobarbital (130 mg/kg i.p.), had a terminal blood sample obtained via cardiac puncture, and were intracardially perfused with a PBS rinse, followed by 4% paraformaldehyde in cold 0.1 M PBS. Brains were removed and postfixed overnight in 4% paraformaldehyde, then stored in 30% sucrose solution at 4 °C. Plasma testosterone concentrations were measured in triplicate 50-μL samples using the Coat-A-Count Total Testosterone Kit (Diagnostic Products). The intra-assay coefficient of variation was 4.7%, and the minimum limit of detectability was 0.1 ng/mL.

Brain Processing and Histological Procedures. Brains were sectioned at 40 μm with a cryostat into four series, placed in cryoprotectant solution, and stored at −4 °C. The first series was thionin Nissl-stained and coverslipped. Series were processed as described below.

BrdU immunohistochemistry. The second series of sections was subjected to single-label immunohistochemistry for BrdU, as described previously (2), with a few minor changes. In brief, after rinses in Tris-buffered saline (TBS; 0.05 M), free-floating sections were incubated for 30 min in 0.6% H_2O_2, then for 2 h at 65 °C in 50% formamide in SSC buffer, rinsed in 2× SSC, placed in 1.86 N HCl for 30 min at 37 °C, and then rinsed for 10 min in borate buffer (0.1 M; pH 8.5). Sections were blocked for 30 min in TBS containing 0.01% Triton X-100 and 3% donkey serum (Jackson ImmunoResearch), then incubated overnight at 4 °C in monoclonal primary antibody rat anti-BrdU (catalog no. MCA2060; Serotec) at a working concentration of 1 μg/mL. Sections were then incubated for 2 h in biotinylated donkey anti-rat secondary antibody (cat no. 712–065-150; Jackson ImmunoResearch) at a working concentration of 2 μg/mL, then for 60 min with avidin/biotinylated enzyme complex reagent (ABC Elite Kit; Vector Laboratories), and then reacted with nickel-enhanced 3,3′-diaminobenzidine tetrahydrochloride (catalog no. D5905; Sigma-Aldrich) for ~4 min. Sections were thoroughly rinsed in TBS before and between all incubations, and all incubations were performed at room temperature unless noted otherwise. Finally, sections were mounted, dehydrated, and cleared with xylene before being coverslipped. Controls excluding primary and secondary antibodies were run using experimental tissue that contained no ROIs, with minimal nonspecific background staining.

Double-label immunofluorescence. Double-label immunofluorescence for BrdU and Fos was performed to examine whether pubertally born cells that survive into adulthood are active during a sexual encounter in adulthood. For this, sections were rinsed in TBS and incubated for 30 min in 0.6% H_2O_2, placed in 0.93 N HCl for 30 min at 37 °C, then rinsed for 10 min in borate buffer (0.1 M; pH 8.5). Blocking was done in TBS plus 0.1% Triton X-100 and 6% normal goat serum (Pel-Freez) for 45 min, followed by incubation for 48 h at 4 °C in a primary mixture solution containing monoclonal rat anti-BrdU (Serotec) at a working concentration of 1 μg/mL and rabbit anti–c-Fos (4) (sc-52; Santa Cruz Biotechnology) at a working concentration of 0.02 μg/mL.

On removal from the primary solution, sections were incubated for 2 h with a secondary antibody mixture solution containing Biotin-SP–conjugated AffiniPure goat anti-rat (catalog no. 112–065-003; Jackson ImmunoResearch) at a working concentration of 1.3 μg/mL and Cy3-conjugated AffiniPure goat anti-rabbit (catalog no. 111–165-144; Jackson ImmunoResearch) at a working concentration of 1.5 μg/mL, followed by a 1-h incubation in Cy2-conjugated streptavidin (catalog no. 016–220-084; Jackson ImmunoResearch) at a working concentration of 1.8 μg/mL. Sections were thoroughly rinsed in TBS before and between all incubations, and all incubations were performed at room temperature unless noted otherwise. Finally, sections were mounted onto slides and coverslipped using SlowFade Gold antifade reagent (catalog no. S36936; Invitrogen). Controls containing BrdU primary antibody with Fos secondary antibody and Fos primary antibody with BrdU secondary antibodies were run separately and showed no cross-reactivity, as demonstrated by a lack of nonspecific background staining. Controls excluding primary and secondary antibodies were run using experimental tissue containing no ROIs, with minimal nonspecific background staining.

Triple-label immunofluorescence. The cellular phenotype of pubertally born cells surviving into adulthood was evaluated using triple-label immunofluorescence for mature neuronal marker NeuN, astrocytic marker GFAP, and BrdU. All steps were the same as for double-label immunofluorescence described above, with the exception of the primary and secondary antibody mixture solutions. The primary antibody mixture solution contained monoclonal rat anti-BrdU (Serotec) at a working concentration of 1 μg/mL, monoclonal mouse anti-NeuN (MAB377; Chemicon International) at a working concentration of 1 μg/mL, and polyclonal anti-GFAP (catalog no. Z0334; Dako) at a working concentration of 0.58 μg/mL. The secondary antibody mixture solution contained Biotin-SP–conjugated AffiniPure goat anti-rat (Jackson ImmunoResearch) at a working concentration of 1.3 μg/mL, Alexa Fluor 405 goat anti-mouse (catalog no. A-31553; Invitrogen) at a working concentration of 4 μg/mL, and Cy3-conjugated AffiniPure goat anti-rabbit (Jackson ImmunoResearch) at a working concentration of 1.5 μg/mL Controls excluding primary and secondary antibodies (no primary with secondary, primary with no secondary, and no primary with no secondary) were run using experimental tissue containing no ROIs, with minimal nonspecific background staining.

Microscopic Analyses. ***BrdU-ir cell counts.*** All single-label analyses were performed with an Olympus BX51 microscope under bright-field illumination using Neurolucida version 7 (MBF Bioscience). Using the Morin and Wood hamster brain atlas as a reference (33), ROIs were located in Nissl-stained sections. The DG was chosen as a positive control because enrichment is known to increase adult neurogenesis in this area. The arcuate nucleus of the hypothalamus is a known site of adult neurogenesis (15). Boundaries of the Me and arcuate nucleus were traced bilaterally at 40× magnification in 13 and 15 anatomically matched sections, respectively, and boxes were placed bilaterally in 2 anatomically matched sections of the DG (0.5-mm^2 square area) and in 1 anatomically matched section of the MPOA (1.5-mm^2 rectangular area, as in ref. 34) in Nissl-stained sections. Tracings and boxes from Nissl sections were superimposed onto adjacent BrdU-labeled tissue. BrdU-ir cells were counted at 400× magnification using an UPlanSApo 40× (0.9 NA) objective.

BrdU-ir and Fos-ir analysis. Double-label fluorescent analyses of BrdU and Fos immunoreactivity were performed with an Olympus BX51 microscope under epi-illumination (mercury arc lamp with FITC and TRITC filters) using Neurolucida version 10. Tracings and boxes from Nissl-stained sections were superimposed onto adjacent BrdU- and Fos-labeled tissue. BrdU-ir cells were counted using an UPlanSApo 40× (0.9 NA) objective, and the percentage of BrdU-ir cells double-labeled with Fos-ir cells within each ROI was determined.

Three-dimensional confocal analysis. Triple-label analyses were performed with an Olympus FluoView FV1000 confocal laser-scanning microscope equipped with FV1000 ASW software, with Z-stacks visualized using an UPLFLN 40× oil objective (1.3 NA) with blue diode (405 nm), argon (488 nm), and helium neon (green HeNe; 543 nm) lasers. Four animals from the enriched group were analyzed. Twenty BrdU-ir cells were located within each ROI, for a total of 80 BrdU-ir cells per ROI, and were analyzed for BrdU colocalization with neuronal or astrocytic markers. Colocalization was confirmed via 3D reconstruction using a Z-stack orthogonal viewer.

Statistical Analysis. ***Single-label.*** To test whether the density of BrdU-ir cells differed between enriched and control groups, a separate between-subjects ANOVA was performed for the DG, the arcuate nucleus, and the MPOA, with experimental group as the independent variable and number of BrdU-ir cells per square millimeter as the dependent variable. Repeat-measures ANOVA was used to detect laterality between left and right Me hemispheres, but because no differences were found, the left and right hemisphere data are combined in the present analysis. To identify any variations in BrdU-ir cell density in the different subregions between the enriched and control groups, two-way ANOVA was performed for the Me, with subregion and group as independent variables and number of BrdU-ir cells per square millimeter as the dependent variable. Group-by-subregion interaction was followed up with univariate ANOVA within each subregion to identify any difference in BrdU-ir density between the enriched and control groups.

Double-label. A separate between-subjects ANOVA was done for each ROI to assess the effects of enrichment on the percentage of BrdU-ir cells double-labeled with Fos-ir cells. Experimental group, brain region, and hemisphere were independent variables, and percentage of double-labeled BrdU/Fos-ir was the dependent variable. Interactions were followed up by univariate ANOVA for the purposes outlined above.

Triple-label. A separate repeat-measures ANOVA was performed for each cell phenotype to determine whether the proportion of pubertally born (BrdU-ir) cells expressing NeuN, GFAP, or neither marker varied by region. Brain region was the independent variable, and phenotype was the dependent variable.

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