Sex differences in proliferation and attrition of pubertally born cells in the rat posterior dorsal medial amygdala☆

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ABSTRACT

The rodent posterodorsal medial amygdala (MePD) evaluates and assigns valence to social sensory stimuli. The perception of social stimuli evolves during puberty, when the focus of social interactions shifts from kin to peers. Using the cell birthdate marker bromo-deoxyuridine (BrdU), we previously discovered that more pubertally born cells are added to the rat MePD in males than females. Here we addressed several questions that remained unanswered by our previous work. First, to determine whether there are sex differences in cell proliferation within the MePD, we examined BrdU-immunoreactive (-ir) cells at 2 and 4 h following BrdU administration on postnatal day 30 (P30). The density of BrdU-ir cells was greater in males than in females, indicating greater proliferation in males. Proliferation was substantiated by double-label immunohistochemistry showing that MePD BrdU-ir cells colocalize proliferating cell nuclear antigen, but not the cell death marker Caspase3. We next studied longer time points (2–21 days) following BrdU administration on P30 and found that the rate of cell attrition is higher in males. Finally, triple-label immunohistochemistry of P30-born MePD cells revealed that some of these cells differentiate into neurons or astrocytes within three weeks of cell birth, with no discernable sex differences. The demonstration of pubertal neuro- and gliogenesis in the MePD of male and female rats adds a new dimension to developmental plasticity of the MePD that may contribute to pubertal changes in the perception of social stimuli in both sexes.

1. Introduction

The medial amygdala is a key node within a limbic circuit that mediates male and female social behaviors (Newman, 1999). The sexually dimorphic posterodorsal quadrant of the rodent medial amygdala (MePD) evaluates and assigns valence to sensory stimuli that trigger social interactions. The perception of social stimuli evolves during puberty and adolescence, as the focus of social interactions shifts from kin to peers and social behaviors such as sex and aggression progress into their adult forms (De Lorme et al., 2013). To meet the emerging social demands of adulthood, the MePD is structurally and functionally remodeled during puberty and adolescence. Sex differences in the mechanisms underlying pubertal reorganization of the MePD are likely because the sensory stimuli and hormones that activate social behavior and the behaviors themselves are distinct in males and females. Yet, the full scope of the structural remodeling that underlies ongoing functional sexual differentiation of the MePD during puberty remains to be fully elucidated.

Some sex differences in structural and functional features of the MePD exist prior to the onset of puberty. Prepubertally, MePD volume in rats is larger in males than in females (Cooke and Woolley, 2005; Johnson et al., 2013). This prepubertal sex difference is correlated with greater dendritic elaboration in males (Cooke et al., 2007), as well as a larger number of astrocytes and excitatory synapses in males (Cooke and Woolley, 2005; Cooke et al., 2007; Johnson et al., 2013). The sex differences in MePD volume and number of astrocytes become even more pronounced during pubertal maturation, likely due to androgen-dependent mechanisms in males (Johnson et al., 2013). Other features of the MePD are not as prominently sexually dimorphic in prepubertal rats, notably neuron density (Cooke and Woolley, 2005; Cooke et al., 2007) and astrocyte complexity (Johnson et al., 2008, 2013). In adulthood, however, robust sex differences exist in both these

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measures, with more neurons in males and greater astrocyte complexity in males in the left hemisphere (Johnson et al., 2012, 2013; Morris et al., 2008). Thus, during puberty, not only are preexisting sex differences in MePD morphology amplified, but also new sex differences emerge.

The pubertal increase in the number of MePD astrocytes (Johnson et al., 2013), and the pubertal emergence of a sex difference in MePD neuron number suggests that during puberty, more cells are added to the male than to the female MePD. However, it is not clear whether MePD cells that are added during puberty already exist elsewhere and migrate in from other brain regions, or whether they are newly generated during puberty. Using bromo-deoxyuridine (BrdU) as a cell birthdate marker, we previously showed that after BrdU administration either before puberty (postnatal day 20, P20), near the onset of puberty (P30) or during early puberty (P40), BrdU-immunoreactive (-ir) cells are observed in the MePD three weeks later in both male and female rats, indicating that at least some pubertally added MePD cells are newly generated during puberty (Ahmed et al., 2008). In addition, we found that some of these three-week-old cells colocalize BrdU and markers for either mature neurons or astrocytes, indicating that pubertal addition of MePD cells involves both neurogenesis and gliogenesis. Finally, there are more pubertally born cells in the male than in the female MePD (Ahmed et al., 2008). Here we addressed several important questions that remained unanswered by our previous work. First, to determine whether progenitor cells exist within MePD itself and whether cells that incorporate BrdU are proliferating or dying, we examined MePD cells at very short time points (~24 h) following BrdU administration to male and female rats on P30. Next, to determine whether the male bias in pubertal addition of MePD cells is due to greater cell proliferation or cell survival in males, we studied a range of short and long time points (2–21 days) following BrdU administration on P30. Finally, we asked whether there are sex differences in the proportion of P30-born MePD cells that differentiate into mature neurons or astrocytes within three weeks of proliferation.

2. Methods

2.1. General methods

2.1.1. Animals

Weanling (P23 in Experiment 1; P21 in Experiments 2 and 3) Sprague-Dawley rats were ordered from Harlan Sprague-Dawley (Madison, WI) and single-housed (Experiment 1) or double-housed (Experiments 2–3) in 37.5 × 33 × 17 cm clear polycarbonate cages with ad libitum access to food (Teklad Rodent diet no. 8640; Harlan, Madison, WI) and water. They were maintained on a 14:10 light:dark cycle, with lights off at 1300 h (2 h after lights off). Rats were perfused as described above at 2 h, 4 h, 1 day, 2 days, 4 days, or 7 days after BrdU injection. Except for the 2 h and 4 h post-BrdU perfusion groups, all perfusions (n = 4/sex/time point) took place at approximately 1500 h (2 h after lights off).

2.2. Experiment 1: determine whether there is a sex difference in cell proliferation

2.2.1. Animals and brain processing

On P30, rats were given a single intraperitoneal (ip) injection of 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) at a dose of 200 mg/kg, dissolved in sterile saline (10 mg/mL solution) between 1200 and 1400 h. Rats were perfused as described above at 2 h, 4 h, 1 day, 2 days, 4 days, or 7 days after BrdU injection. Except for the 2 h and 4 h post-BrdU perfusion groups, all perfusions (n = 4/sex/time point) took place at approximately 1500 h (2 h after lights off).

2.2.2. Experiment 1a: single-label BrdU immunohistochemistry

To determine whether there is a sex difference in cell proliferation, we quantified the number of BrdU-ir cells in the MePD of rats perfused at the 2 h and 4 h time points. Five anatomically matched sections through the MePD corresponding to plates 54, 56, 58, 60, and 62 in the Paxinos and Watson rat brain atlas were processed for single-label BrdU immunohistochemistry. Immunohistochemistry for BrdU was performed exactly as previously described in (Mohr and Sisk, 2013) except with the addition of AffiniPure Fab Fragment donkey anti-rat IgG (Jackson ImmunoResearch, cat no. 712–007–003, 12 μg/mL) in the TBS blocking solution, as in (Mohr et al., 2016). If poor tissue quality prohibited bilateral quantification of BrdU-ir cells in all five sections for a given rat, then that rat was excluded from microscopic analysis. Final sample sizes were: 2 hr time point, n = 4/sex; 4 hr time point, n = 4 males, n = 3 females.

2.2.3. Expt 1b: double-label immunofluorescence for BrdU and PCNA

To determine if BrdU was incorporated into proliferating cells, labeling with BrdU and proliferating cell nuclear antigen (PCNA), a protein involved in DNA replication, was performed. Tissue containing the MePD from animals sacrificed 2 h, 4 h, 1d, 2d, 4d, or 7d after BrdU injection (n = 2–3/sex/time point) was processed for immunofluorescence to detect both BrdU and PCNA. Three sections containing the medial amygdala were selected for each animal at the level of the rostral, middle or caudal MePD. Double-label immunofluorescent labeling for BrdU and PCNA was performed exactly as described in (Mohr et al., 2016).

2.2.4. Expt 1c: double-label BrdU/Caspase-3 immunofluorescence

To determine whether BrdU was incorporated into dying, apoptotic cells at the time of BrdU injection, as well as to determine if attrition of pubertally born cells occurs with cell age via apoptosis, labeling with BrdU and the apoptosis marker cleaved caspase-3 was performed.
## Table 1

| Primary Antibody | Secondary Antibody | Manufacturer, catalog # | Concentration | Amplification or conjugate (if applicable) |
|------------------|--------------------|--------------------------|---------------|------------------------------------------|
| **Expt 1a**       |                    |                          |               |                                          |
| monoclonal rat anti-BrdU | AbD Serotec, MCA 2060 | 1 µg/mL                  | Biotin-SP-conjugated | ABC Elite Kit Vector DAB (3,3'-diaminobenzidine) 0.25 mg/mL w/ 0.012% H₂O₂ |
|                   |                    |                          |               |                                          |
| **Expt 1b**       |                    |                          |               |                                          |
| monoclonal rat anti-BrdU | AbD Serotec, MCA 2060 | 1 µg/mL                  | AlexaFluor 488 goat anti-rat, 1:250 dilution | monoclonal mouse anti-PCNA; 4 µg/mL µg/mL mouse, 4 µg/mL µg/mL |
|                   |                    |                          |               |                                          |
| **Expt 1c**       |                    |                          |               |                                          |
| monoclonal rat anti-BrdU | AbD Serotec, MCA 2060 | 1 µg/mL                  | AlexaFluor 488 goat anti-rat, 1:250 dilution | polyclonal rabbit anti-cleaved caspase-3; 3.33 µg/mL µg/mL |
|                   |                    |                          |               |                                          |
| **Expt 2**        |                    |                          |               |                                          |
| monoclonal rat anti-BrdU | AbD Serotec, MCA 2060 | 1 µg/mL                  | ABC Elite Kit Vector DAB; 0.25 mg/mL w/ 0.012% H₂O₂ | monoclonal rat anti-BrdU | AbD Serotec, MCA 2060 | 1 µg/mL                  | Biotin-SP-conjugated | ABC Elite Kit Vector DAB (3,3'-diaminobenzidine) 0.25 mg/mL w/ 0.012% H₂O₂ |
|                   |                    |                          |               |                                          |
| **Expt 3**        |                    |                          |               |                                          |
| BrdU/NeuN         |                    |                          |               |                                          |
| monoclonal mouse anti-NeuN | Dako, Z0334 | 1 µg/mL                  | Cy3-conjugated AffiniPure goat anti-rat, 1:250 dilution | monoclonal mouse anti-NeuN | Dako, Z0334 | 1 µg/mL                  | Cy3-conjugated AffiniPure goat anti-rat, 1:250 dilution |

Procedures for immunofluorescence were the same as above for PCNA except that a polyclonal rabbit anti-cleaved caspase-3 antisera was used (Cell Signaling, cat no. 9661, 3.33 µg/mL) with Alexa Fluor 635 goat anti-rabbit as the secondary antisera (Invitrogen, A-31577, working dilution 1:250).

### 2.2.5. Microscopic analyses

For Experiment 1a, cells were identified as BrdU-ir if microscopic examination revealed dense nuclear dark brown-black reaction products. Analyses were performed on an Olympus BX51 microscope with brightfield illumination, as well as epi-illumination (mercury arc lamp with DAPI filter) using Neurolucida software (MBF Bioscience; Williston, Vermont). The MePD was located and traced bilaterally at 100x magnification using the DAPI stain as a guide. BrdU-ir cells were counted at 400x magnification using an UPlanSapo 40x (0.9 NA) objective.

For Experiment 1b, only undamaged sections with an intact MePD on at least one side of the brain were analyzed by confocal microscopy, therefore the number of analyzed sections per animal varied from 1 to 3 sections/side. A Zeiss 510 LSM laser scanning confocal microscope with 405 diode laser, an Argon 458,477,488,514, and a red helium neon (HeNe) 633 laser equipped with version 5 of the LSM browser software was used for analysis. Settings were optimized on a standardized section and then used for every animal in the analysis. For colocalization of BrdU with PCNA, a 5 × 5 set of tiles taken at 250x magnification was stitched together to create tiled montages of the MePD. When the individual tiles were stitched together, the montage was used to trace the contour of the MePD. Position markers were set within the contour so that the entire MePD could be imaged in Z-stacks without the stacks overlapping. At each marker, a Z-stack was imaged at 250x magnification. Each stack was 21 µm (2.6 µm step), beginning at the point when the DAPI came into maximal focus. The number of Z-stacks collected per animal per hemisphere ranged from 3 to 15 with a mean of 8. A modified stereological approach was taken to counting cells. The Z-stacks were flattened into a single plane and cells were counted using an optical dissector that ensured that cells were not counted twice. The stacks were also viewed in the orthogonal plane to ensure there were no overlapping labeled cells in the depth of the stack. The number of BrdU-ir and BrdU-ir/PCNA-ir cells were recorded. Data are expressed as percent of BrdU-ir cells colocalized with PCNA-ir.

For analysis of cleaved caspase-3 and BrdU colocalization in Experiment 1c, one section at the level of the rostral, middle and caudal MePD was selected for each animal (3 sections/animal). The number of stacks per animal per hemisphere ranged from 3 to 16 with a mean of 9. BrdU-ir and BrdU/caspase-3-ir labeled cells were counted as for BrdU/PCNA. Data are expressed as percent BrdU cells colocalized with caspase-3. In addition to BrdU-ir and BrdU/caspase-3-ir labeled cells, sections were reanalyzed to calculate all caspase-3 labeled cells in the MePD; these data are expressed as total number of caspase-3-ir cells/mm² x 10⁶.

### 2.2.6. Statistical analyses

For Experiment 1a, a 2 × 2 ANOVA was performed with sex (male, female) and post-BrdU survival time (2 h, 4 h) as independent variables to determine if the density of BrdU-ir cells (number of cells/mm² x 10⁶) differed between sexes or as a function of cell age (hours post-BrdU injection).

For experiments 1b and 1c, separate 2 × 6 ANOVAs were performed with sex (male, female) and post-BrdU survival time (2 h, 4 h, 1 d, 2 d, 4 d, 7 d) as independent variables to determine if the percentage of BrdU-ir cells that colocalize with PCNA-ir (1b) or Caspase-3 (1c) differed between sexes or as a function of cell age (hours or days post-BrdU injection). Significant main effects were followed-up with Tukey’s multiple comparisons tests.
2.3. Experiment 2: determine whether there is a sex difference in cell attrition

2.3.1. Animals and brain processing

On P30, rats received 3 ip injections of BrdU (200 mg/kg, dissolved in warm sterile saline; 10 mg/ml) at ~ 0900 h, 1700 h, and 0100 h. Three BrdU injections were given in this experiment in order to increase the number of P30-born cells that would be both visualized in this experiment and phenotyped in Experiment 3. Rats were perfused 2d, 4d, 7d, 14d or 21d after BrdU injection (n = 5/sex/time point). All perfusions took place at approximately 1400 h.

2.3.2. Single-label BrdU immunohistochemistry and microscopic analyses

A 1-in-6 series of free-floating sections through the MePD was processed for single-label BrdU immunohistochemistry and analyzed as described in Expt 1a, with the exception that analyses were performed on a Leica DMR microscope equipped with a Retiga CCD camera (Surrey, BC, Canada) for epifluorescence and a MicroPublisher 5.0 RTV camera (Q-Imaging; Surrey, BC, Canada) for brightfield imaging. DAPI was used to identify the boundaries of MePD within each section and 3–5 sections/animal were analyzed.

2.3.3. Statistical analysis of single-label BrdU

A 2 × 5 ANOVA was performed with sex (male, female) and post-BrdU survival time (2d, 4d, 7d, 14d, and 21d) as independent variables to determine if the density of BrdU-ir cells (number of cells/mm² × 10⁶) differed between sexes or as a function of cell age (days post-BrdU injection). To evaluate the rate of attrition of P30-born MePD cells in males and females, linear regression analyses were performed on the time course data after first determining that the goodness of fit was best for linear (r² ~ 0.96 for males) vs. quadratic or exponential curves (r² values below 0.3).

2.4. Experiment 3: phenotype of P30-born cells three weeks after birth

To determine whether P30-born cells differentiate into mature neurons or astrocytes, we performed triple-label immunofluorescence for BrdU, the astrocyte marker, glial fibrillary acidic protein (GFAP), and the mature neuronal marker, Neuronal Nuclei (NeuN) on sections from a subset of animals perfused 21 days post-BrdU injection (n = 3/sex). The methods were performed as previously described (Mohr et al., 2016).

2.4.1. Confocal microscopic analyses of triple-label BrdU//NeuN/GFAP

Confocal microscopy was performed using an Olympus Fluoview 1000 confocal laser-scanning microscope equipped with FV1000 ASW software. The multiline argon laser (488 nm), green HeNe laser (543 nm), and red HeNe laser (633 nm) were used to image BrdU-, GFAP- and NeuN-positive cells, respectively. Confocal stacks were taken at 0.5-micron steps spanning the Z-axis of the cell. All BrdU positive cells were imaged in 3–5 sections/animal, yielding 28–55 images of BrdU-ir cells/animal for phenotypic analysis. Hemisphere was noted during image acquisition. Data are expressed as the percentage of BrdU-ir cells that were either NeuN-ir or GFAP-ir.

2.4.2. Statistical analyses of triple-label BrdU//NeuN/GFAP

To determine if there were sex differences in the phenotype of P30-born MePD cells, multiple t-tests were performed with sex (male, female) as the independent variable and cell phenotype (% BrdU-ir colocalize with NeuN-ir, GFAP-ir, or neither) as the dependent variable.

3. Results

3.1. Qualitative results

In single label studies using DAB as the chromogen, BrdU-ir appeared as a dense nuclear dark brown reaction product (Fig. 1). Within a given section, BrdU-labeled cells were evenly distributed throughout the MePD, even at the earliest time points analyzed (i.e., 2 h and 4 h post-BrdU). The density of BrdU-labeled cells appeared slightly higher in the caudal sections than in the rostral sections. The intensity of immunolabel within BrdU-labeled cells did not appear to decrease with increasing cell age (i.e., time post-BrdU).

3.2. Proliferation of MePD cells on P30 was higher in males than in females

When we examined proliferation by quantifying the density of BrdU-ir MePD cells at two time points within 4 h of BrdU administration on P30, higher numbers of BrdU-ir cells/mm² x 10⁶ were found in males compared with females (Fig. 2). Two-way ANOVA with cell age (i.e., time post-BrdU injection) and sex as independent variables and the number of BrdU-ir cells/mm² x 10⁶ as the dependent variable revealed a significant main effect of sex (F(1,11) = 5.72, p = 0.0357), such that overall males had 1.5 times more BrdU-ir cells compared with females. There was no main effect of cell age or interaction between sex and cell age.

3.3. BrdU was incorporated into proliferating cells in the MePD

Confocal z-stacks demonstrated colocalization of BrdU with PCNA, a marker of active proliferation (Fig. 3a). Over 90% of cells born on P30 were colocalized with PCNA at 2 h and 4 h after BrdU administration (Fig. 3b). By 1d after BrdU administration, the percentage of BrdU-ir cells that were immunoreactive for PCNA declined to roughly 60%, and further declined to below 50% by 2 days, and by 7 days of cell age very few colocalized cells were observed. Statistical analysis revealed a
main effect of cell age (F(5,19) = 20.9, p < 0.001). Posthoc analyses showed that the %PCNA/BrdU-ir co-localized cells at 2-hr, 4-hr, and 1 day cell ages was significantly greater than that observed at the 2-, 4-, and 7-day cell ages (Tukey’s multiple comparisons, p’s < 0.0034). There was no main effect of sex (F(1,19), p = 0.4839) or an interaction (F(5,19) = 0.222, p = 0.9487).

3.4. BrdU-ir cells were not dying at the time of BrdU uptake on P30

Less than 5% of BrdU-ir cells co-localized the apoptotic marker activated caspase-3 at the early survival points post-BrdU, suggesting that the vast majority of cells that incorporated BrdU on P30 were not dying at the time of injection (Table 2). The absolute number of cells that colocalized caspase-3 and BrdU in each animal examined was very small, ranging from 0 to 8; the average number of BrdU-ir/caspase-3-ir cells was 0.67. Although most BrdU-ir cells were not also caspase-3-ir cells, the two cell types were often in close proximity (Fig. 4). Additionally, the presence of many caspase-3-ir cells that were BrdU-negative prompted investigation of caspase-3-only densities in the MePD. The density of caspase-3-labeled cells was relatively high (roughly 350–630 per mm²), but also quite variable, and no sex or age differences were apparent with these small sample sizes (Table 3).

Table 2
Percent of BrdU-ir cells that were also caspase-3-ir in the MePD.

| Survival time post-BrdU injection | Male | Female |
|----------------------------------|------|--------|
| 2 h                              | 3.6  | 1.9    |
| 4 h                              | 1.6  | 4.8    |
| 1d                               | 2.4  | 5.3    |
| 2d                               | 0.8  | 0.0    |
| 4d                               | 5.5  | 2.9    |
| 7d                               | 3.4  | 0.8    |

Values are expressed as the mean percent of BrdU-ir cells that colocalized with caspase-3-ir. n = 2–3/sex/time point.

3.5. Attrition of P30-born cells is greater in males than in females

To determine if there is a sex difference in survival of P30-born cells in the MePD, the density of BrdU-ir cells was examined from 2 days to 21 days after BrdU injections. Two-way ANOVA with sex and cell age as independent variables and the density of BrdU-ir cells as the dependent variable indicated a significant main effect of sex (F(1,40) = 4.19, p = 0.0472), such that there were approximately 1.2 times more BrdU-ir cells/mm² x 10⁶ in the male MePD compared with the female MePD. To probe whether the rate of attrition of P30-born MePD cells is different between the sexes, regression analyses were performed. The slope of the line of best fit was significantly different in males and females (−2.97 and −0.85, respectively, F(1,6) = 10.79, p = 0.0167; Fig. 5), and only in males did the line of best fit significantly differ from zero (F(1,3) = 67.68, p = 0.0038), indicating that there was a sex difference in the rate of attrition of P30-born cells.

3.6. Some P30-born BrdU-ir cells differentiate into neurons or astrocytes within three weeks of proliferation

Confocal Z-stacks with orthogonal views demonstrate colocalization of BrdU with the mature neuronal marker NeuN (Fig. 6a), whereas other
BrdU-labeled cells were immunoreactive for the astrocyte marker GFAP (Fig. 6b). Overall, 3–4% of the P30-born BrdU-ir cells colocalized NeuN, and 9–11% colocalized GFAP when examined 21 days after BrdU administration. The relative proportion of these two cellular phenotypes were not significantly different between sexes (multiple t-tests, p values > 0.05).

4. Discussion

Here we extend our previous finding that cells born during early puberty are added to the rat MePD, with more cells added in males than in females (Ahmed et al., 2008). Time course studies using the cell birthdate marker BrdU revealed that on P30, more cells proliferate in males than in females. Attrition of P30-born cells was also greater in males over three weeks following BrdU administration. In both sexes, approximately 15% of MePD cells born on P30 differentiate into either mature neurons or astrocytes within three weeks of cell birth. Our findings establish that pubertal remodeling of the MePD involves neuro- and gliogenesis, demonstrating that the generation of new cells is potentially a facet of brain plasticity during puberty and adolescence, when MePD-mediated evaluation of social stimuli is transformed to the adult state.

4.1. MePD cell proliferation on P30 is greater in males than in females

Within 2–4 h after a single injection of BrdU on P30, BrdU-ir cells were detectable in the MePD, with a greater density of BrdU-ir cells in males than in females. This male bias in P30-born MePD cells contrasts with the female bias in P30-born AVPV cells observed in these same animals (Mohr et al., 2016). These brain region-specific sex differences correlate with previously reported sex differences in volume and neuron number of the MePD (M > F; (Morris et al., 2008)) and AVPV (F > M; (Sumida et al., 1993)). However, during early postnatal development (P4) of the rat MePD, cell proliferation is greater in females (Krebs-Kraft et al., 2010), indicating that sex differences in cell proliferation do not necessarily foretell sex differences in volume or cell number in adulthood, and that different developmental processes are at play during early postnatal and pubertal development of the MePD.

At the 2- and 4-hr post-BrdU time points studied here, approximately 95% of P30-born MePD cells expressed the cell proliferation marker PCNA. During the cell cycle, PCNA expression rises during the growth (G1) stage, reaches maximum expression during the synthesis (S) phase, and starts to decline during the second growth (G2) and mitotic (M) phases (Kurki et al., 1988). Thus, the BrdU-ir/PCNA-ir colocalization observed within hours of BrdU administration reflects cells that were actively undergoing cell division at the time of tissue collection. The sharp decline in the number of BrdU-ir/PCNA-ir cells after the one-day time point indicates that the vast majority of four- and seven-day-old cells were no longer in a mitotic condition.

The origin(s) of P30-born MePD cells is not known, but our results suggest that at least some of them proliferate in situ. We observed BrdU-ir cells within the MePD as early as 2 h following BrdU injection, and similarly, newborn neurons and glia (evidenced by Tuj1 and NG2 staining, respectively) have been observed in the MePD of the adult male meadow vole in as little as 30 min following BrdU injection (Fowler et al., 2003). If any pubertally born MePD cells do originate in the SVZ and have a migration rate similar to the rates estimated for other cells migrating from the SVZ (ranging from ~23-100 μm/h; (Luskin and

![Fig. 6.](image-url) Some P30-born MePD cells differentiate into neurons or astrocytes. Photomicrographs with orthogonal views of BrdU-ir cells in the MePD that colocalize with NeuN-ir (A) or GFAP-ir (B). Scale bars = 10 µm. Percentage of BrdU-ir cells in the MePD that colocalize with NeuN (C), GFAP (D), or do not colocalize with either phenotypic marker (E).
programmed cell death, suggesting that these cells were undergoing apoptosis at the time of BrdU administration. We also unexpectedly found that the density of P30-born cells was indistinguishable between males and females. While females did not demonstrate the same levels of cell proliferation as males, there was virtually no attrition in females, resulting in similar stable numbers of P30-born cells three weeks post-injection. This pattern of results is different from those we observed in the AVPV of these same animals, in which the rate of attrition of P30-born cells was similar in females and males (Mohr et al., 2016). Because proliferation of AVPV cells was also greater in females, the number of P30-born AVPV cells was larger in females than in males when examined 21 days post-BrdU. The gradual reduction in density of P30-born MePD in males over the study period could be the result of either cell death or migration of P30-born cells out of the MePD.

A small percentage (<5%) of BrdU-ir cells across the time points studied were also immunoreactive for activated caspase-3, a marker of programmed cell death, suggesting that these cells were undergoing apoptosis at the time of BrdU administration. We also unexpectedly observed many caspase-3-ir cells in the MePD on P30 that were not also BrdU-ir. Cell death maps of the mouse medial amygdala show a steady decline in activated caspase-3 throughout postnatal development, with hardly any caspase-3 expression by P11 (Ahern et al., 2013). Another study reports caspase-3 expression in the rat medial amygdala during early development, at P0 and P7 (Zuloaga et al., 2011). If the postnatal decline in caspase-3 expression is the same in rats as it is in mice, then our results suggest that puberty may evoke an additional wave of caspase-3 expression and programmed cell death.

4.3. Some pubertally born MePD cells become neurons or astrocytes

Within 21 days of proliferating, approximately 15% of cells born on P30 in both males and females had differentiated into either mature neurons or mature astrocytes, as evidenced by colocalization of BrdU with either NeuN or GFAP, respectively. We saw no trend for any sex differences, although sample sizes were small, and phenotyping was done for cells born on a single day during early puberty. However, the proportion of P30-born cells that become astrocytes (~12%) is 4 times more than the proportion that become mature neurons (~3%) in both sexes. In the AVPV of these same animals, 19% of P30-born cells were astrocytes and 15% were neurons (Mohr et al., 2016). This pattern of differentiation (relatively more astrogens than neurons) is similar to a previous study in male hamsters in which ~23% of pubertally born MePD cells were astrocytes and ~14% were mature neurons (Mohr and Sisk, 2013). In that study, BrdU was given every day for 3 weeks during the time of puberty, and tissue was collected 4 weeks after the last BrdU injection, therefore phenotyping was done on cells that ranged from four to seven weeks of age. The P30-born cells phenotyped in the current study were just 21 days old, and their younger age could account for the relatively smaller proportion of mature phenotypes seen here than in the previous study in hamsters.

The remaining 85% of BrdU-ir cells that did not colocalize NeuN or GFAP could be a combination of multiple different cell types, including microglia, undifferentiated precursor cells, or immature neurons not yet expressing NeuN. It is also important to note that not all mature neurons express NeuN (Cannon and Greenamyre, 2009; Mullen et al., 1992; Sarnat et al., 1998; Saul et al., 2014) examined the phenotype of P30-born cells in the male rat amygdala 10 days after BrdU administration and found that 30% of BrdU-ir cells were also immunoreactive for doublecortin, a microtubule-associated protein expressed in immature neurons. In a separate study by the same group (Saul et al., 2013), about half of P30-born cells in the amygdala were immunoreactive for both BrdU and NG2 (nerve/glial antigen 2), a marker of oligodendrocyte precursor cells. Undifferentiated cells or immature neurons could be destined for eventual differentiation or cell death as internal signals or experience-dependent activity might dictate. For example, social experience during adolescence may promote differentiation and survival of pubertally born cells.

4.4. Methodological considerations

We previously found a significant sex difference (M>F) in BrdU-ir MePD cells 21 days post-BrdU (Ahmed et al., 2008), whereas in this study there was no sex difference at this time point. This disparity between the two studies is likely due to methodological differences. First, Ahmed et al. (2008) reported the total number of BrdU-ir cells counted in a one-in-six series of sections throughout the MePD; here we report the density (cells/mm² × 10⁶) of BrdU-ir cells to control for potential sex differences in MePD volume and because unequal numbers of sections were available for analysis across experiments. Because the density measure effectively normalizes BrdU-ir cell number to cross-sectional area, the density measure underestimates the sex difference in the absolute number of pubertally born cells. Second, in our earlier study, BrdU was administered on three consecutive days (P30-P32), which would yield a greater number of BrdU-ir cells and therefore more power to detect a sex difference.

The density of BrdU-ir MePD cells was an order of magnitude higher in Experiment 2 than in Experiment 1. Two methodological differences between the experiments likely account for the higher density in Experiment 2. First, a single injection of BrdU was given on P30 in Experiment 1, whereas three injections were given on P30 in Experiment 2. Second, rats in experiment 1 were singly housed, whereas they were doubly housed in experiment 2. Social interactions resulting from double housing may have resulted in greater survival of P30-born cells (Holmes, 2016).

4.5. Significance and functional implications

These studies add to a growing body of literature substantiating the pubertal addition of new cells to limbic and hypothalamic regions that control motivation behaviors and physiology (Ahmed et al., 2008; Curlik et al., 2014; Haan et al., 2013; Juraska et al., 2013; Lee et al., 2012, 2014; Matsuzaki et al., 2015; Mohr and Sisk, 2013; Mohr et al., 2016, 2017; Saul et al., 2014, 2015; Staffend et al., 2014). Pubertally born cells are functionally integrated into neural circuits (reviewed in (Yoo and Blackshaw, 2018)). For example, pubertally born MePD cells in male hamsters are activated (as evidenced by Fos expression) during mating (Mohr and Sisk, 2013). In female rats, pubertally born cells in the anteroventral periventricular nucleus (AVPV), a key node for generation of the ovarian hormone-dependent preovulatory LH surge, are activated by estradiol and progesterone treatment, and knockout of cell proliferation in the AVPV during puberty blunts the hormone-induced LH surge (Mohr et al., 2017).

Our finding that some P30-born MePD cells differentiate into mature neurons or astrocytes within three weeks of proliferation in both males and females provides evidence that pubertal neurogenesis and gliogenesis are part of the structural remodeling of the MePD during puberty and suggests that they contribute to its functional remodeling as well. A primary function of MePD in male rodents is to assign initial meaning
and valence to social chemosensory information relayed from the olfactory bulb, and the perception of social cues necessarily changes during puberty as the focus of social interactions shifts from kin to peers, a process known as social reorientation (De Lorme et al., 2013). For example, juvenile male hamsters are not attracted to female odors (Johnston and Coplin, 1979), preference for opposite-sex odors develops during puberty (Job and Cooke, 2015), and female odors acquire the properties of unconditioned reward during puberty (Bell et al., 2013; Bell and Sisk, 2013). We posit that the functional incorporation of puberty-born MePD neurons and astrocytes is one mechanism that contributes to this gain in function associated with the pubertal transition in the perception of social stimuli.

Author Contributions

MAM: conceived and designed analysis, collected data, performed analysis, wrote the paper. NSM: conceived and designed analysis, collected data, performed analysis, wrote the paper. CLS: conceived and designed analysis, wrote the paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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