**Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions**

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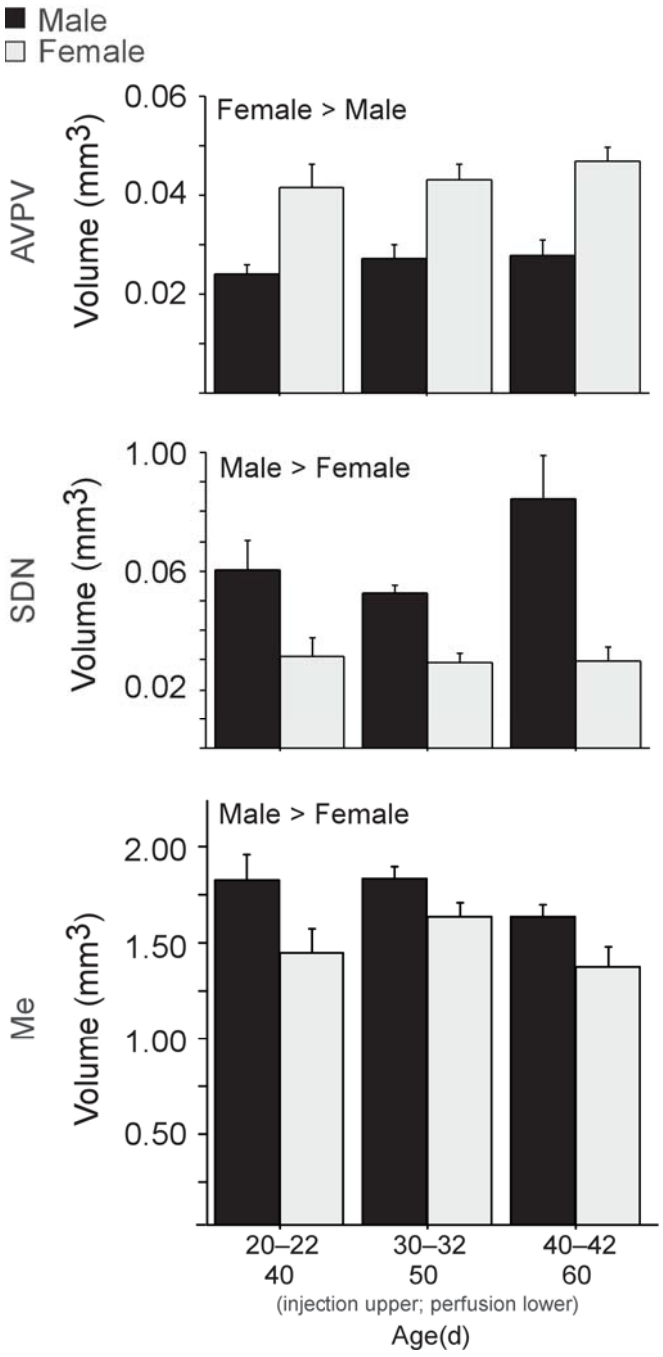
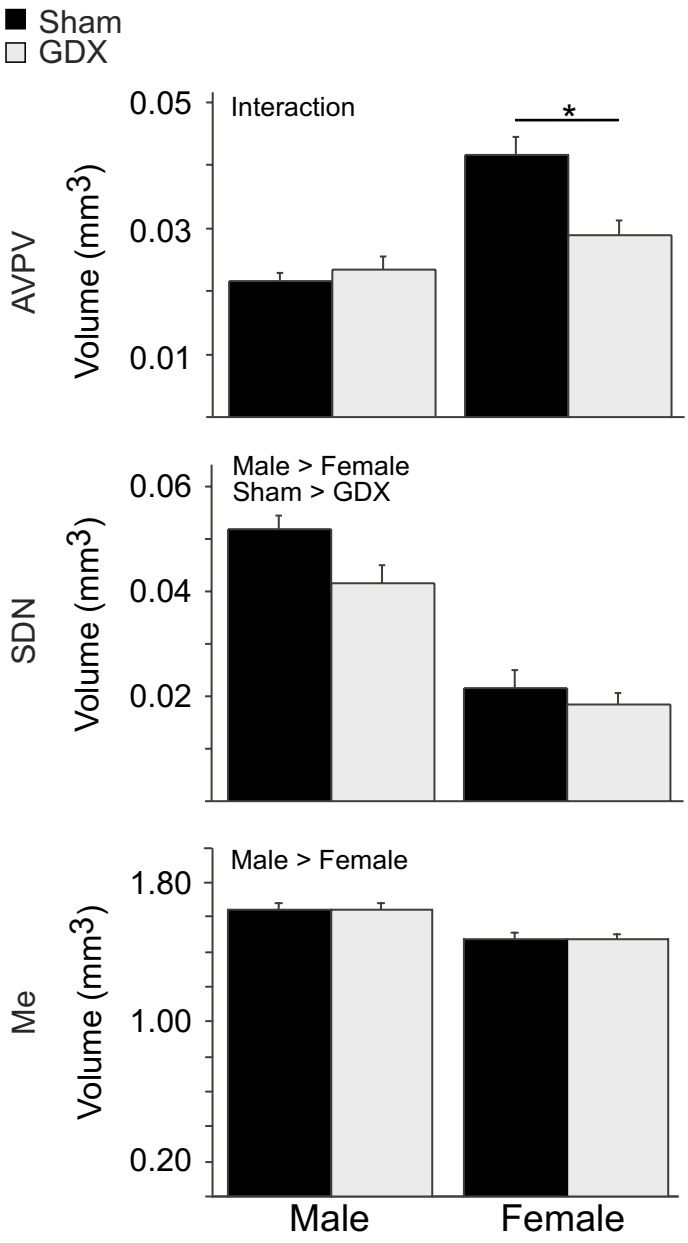
Supplementary Information

# Supplemental Figure 1.
(Sisk)



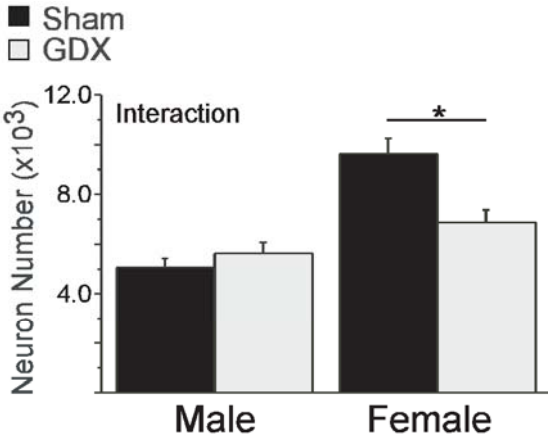

Supplemental Figure 1. Sex differences in volume of AVPV, SDN, and Me in male and female rats positively correlate with sex differences in the number of BrdU-ir cells shown in manuscript Fig 1. Data are presented as means ± SEM.The x-axis shows the age at which brain tissue was collected, which was 20 days after BrdU injections were administered. Top panel: AVPV volume was significantly larger in females than in males (ANOVA $F_{1,26}=41.83$, $p<0.0001$). AVPV volume did not vary with age and no interaction between age and sex was observed. Middle panel: SDN volume was significantly larger in males than in females (ANOVA $F_{1, 31}=34.76$, $p<0.0001$). No effect of age and no interaction between age and sex were found. Bottom panel: Me volume was significantly larger in males than in females (ANOVA $F_{1,30}=11.31$, $p=0.0021$). No effect of age and no interaction between age and sex were found.

Supplemental Figure 2.
(Sisk)




Supplemental Figure 2. The effects of prepubertal gonadectomy on volume of AVPV and SDN, but not Me, parallel effects on number of BrdU-ir cells shown in manuscript Fig 3. Male and female rats were either gonadectomized (GDX) or sham GDX at P20, injected with BrdU on P30-32, and perfused on P50. Data are presented as means ± SEM. Top panel: Prepubertal GDX significantly decreased AVPV volume in females but not in males (ANOVA, interaction F1, 27=10.34, p=0.0034). Asterisk indicates $p<0.05$, Fisher's post hoc test. A similar interaction was seen on number of BrdU-ir cells (manuscript Fig. 3). Middle panel: Male SDN volume was significantly greater than that of female SDN (ANOVA F1, 27= 79.00, $p<0.001$). There was a main effect of GDX on SDN volume (ANOVA F1, 27=5.07, p=0.0327), and no interaction with sex. This main effect appears to be driven primarily by a decrease in SDN volume in GDX males, similar to the interaction between sex and GDX seen on BrdU-ir cell number (manuscript Figure 3). Bottom panel: Me volume was significantly greater in males than in females (ANOVA F 1,27 = 29.7, $p<0.001$). There was neither an effect of GDX nor an interaction with sex on Me volume. Thus, the interaction between sex and GDX in which GDX decreased Me BrdU-ir cells in males but not females, was not paralleled by similar changes in volume, perhaps in part because of the larger overall volume of Me compared with AVPV and SDN, and in part because volumes of anterior regions of Me are not sensitive to fluctuations in circulating gonadal steroids.

Supplemental figure 3.
(Sisk)




Supplemental Figure 3. The effect of prepubertal gonadectomy on number of AVPV neurons parallels effects on number of BrdU-ir cells shown in manuscript Fig 3 and on AVPV volume shown in Supplemental Figure 2. Male and female rats were either gonadectomized (GDX) or sham GDX at P20, injected with BrdU on P30-32, and perfused on P50. Stereological estimates of the total number of neurons are presented as means ± SEM. Prepubertal GDX significantly decreased the number of neurons in females but not in males (ANOVA, interaction $F_{1, 28}=10.91$, $p<0.003$). Asterisk indicates $p<0.05$, Fisher's post hoc test.

## Supplemental Methods

### Animal Housing and Care

Litters of 18 day old male and female Sprague Dawley rats were purchased from Harlan Sprague-Dawley (Madison, WI). Animals were randomly assigned to treatment groups at arrival and were housed with dams and littermates until weaning on postnatal day 21. After weaning, animals were housed 2-3/cage (37.5 x 33 x 17 cm clear polycarbonate cages) in same sex groups with free access to food and water. The room was environmentally controlled with temperature set at 22 ±2 °C and a light/dark schedule of 14:10 (lights off at 1700 h EST). All rats were held in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Approval for experimental procedures was obtained from the Michigan State University Institutional Animal Care and Use Committee before initiation of experiments.

### Experimental Design

***Experiment 1:*** To test for the effects of sex and age on the pubertal addition of new cells to sexually dimorphic brain regions, male and female rats were randomly assigned to receive injections of the cell birth marker 5'-bromo-2'-deoxyuridine (BrdU) beginning on either P-20, -30 or -40 (n=6-8/age and sex group; see below for details of BrdU injections). These ages correspond to prepubertal, early pubertal and mid-pubertal stages of development in rats[1]. Brains were collected on P-40, -50 or -60, respectively.

***Experiment 2:*** To test whether gonadal hormones influence the number of cells added during puberty, males and females were gonadectomized or sham gonadectomized (n=8/group) before puberty on P20 under isoflurane anesthesia. All animals were injected with BrdU on P30-32 (detailed below), and then perfused on P50.

**BrdU injections**

Animals received a daily injection of the cell birth marker BrdU (cat. no. B-9285, Sigma, St. Louis, MO; 300 mg/kg bw, 10 mg/ml BrdU in 0.9% saline, ip) for three consecutive days at 0900 EST, for a total of three BrdU injections per animal. For all experimental groups, brains were collected 20 days after the first BrdU injection. This time interval is typically sufficient to allow newly generated cells to undergo neuronal or glial differentiation[2].

**Brain processing and histological procedures**

Twenty days after the first BrdU injection (at P40, 50 or 60), animals were anesthetized with sodium pentobarbital (90 mg/kg, ip, Nembutal, Abbott Laboratories, North Chicago, IL), and perfused intracardially with heparinized phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in cold 0.1M phosphate buffer. The brains were removed, post-fixed in 4% paraformaldehyde overnight, and stored in a 30% sucrose solution at 4°C. Brains were cryostat-sectioned into three (AVPV and SDN) or six (Me and DG) sets of 40 µm coronal sections, which were stored in ethylene glycol-based cryoprotectant at –20°C until further processed. The first set was Nissl-stained with thionin to identify the AVPV and SDN. A second adjacent set of sections

was processed for single-label BrdU immunocytochemistry. Double- or triple-label immunofluorescence and confocal microscopy were performed on a third set of sections.

***BrdU immunocytochemisty:*** Free-floating brain sections were processed for BrdU immunoreactivity according to Kempermann and Gage[3]. In brief, sections were transferred to 50% formamide in standard saline citrate (SSC) buffer (0.3 M Nacl, 0.03 M sodium citrate) at 65°C for 2 h, rinsed in 2X SSC-buffer, placed in 2N HCl for 30 min at 37°C, and rinsed in 0.1M borate buffer pH 8.5 for 10 min. After three rinses in Tris-buffered saline (TBS), sections were blocked in TBS containing 0.1% Triton X-100 and 3% donkey serum (Jackson ImmunoResearch Lab.,Inc, West Grove, PA) for 30 min. Sections were then incubated overnight at 4°C in monoclonal primary antibody rat anti-BrdU (cat. no. MCA2060, Serotec, Ltd, Oxford, UK) at a working dilution of 1:1000. Sections were incubated with biotinylated donkey anti-rat secondary antibody at a dilution of 1:250 (cat. no. 712-065-150, Jackson ImmunoResearch Lab.,Inc, West Grove, PA) for 2 hours at room temperature, rinsed, then incubated with ABC reagent (ABC Elite kit; Vector Laboratories, Burlingame, CA) for 60 min at room temperature, and reacted for 6 min in diaminobenzidine (DAB; Sigma; 0.67 mg/ml in TBS with 0.01% $H_2O_2$). Sections were thoroughly rinsed in TBS before and between all incubations. Finally, sections were mounted, dehydrated, cleared with xylene and cover slipped.

***Double-label immunofluorescence:*** To determine the cellular phenotype of BrdU-labeled cells in AVPV and SDN, double-label immunofluorescence was performed for BrdU and either the neuron-specific nuclear protein marker NeuN or the astrocyte-specific marker glial fibrillary acidic protein (GFAP). Sections from 3 males and 3

females in each age group were pretreated as described above until the blocking step. A blocking step was applied with diluent containing 3% normal goat serum (Chemicon, Temecula, CA) and 0.1% Triton X-100, in 0.05M TBS. The sections were then incubated sequentially in the primary antibody solution directed against BrdU (1µg/ml, 48 hours, 4°C), biotinylated goat anti-rat secondary antiserum (Vector Laboratories, Burlingame, CA;1:250, 2 hours, room temperature), and Cy2-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:300, 1 hour, room temperature). Double labeling was then performed with a 72-hour incubation at 4˚C with either mouse monoclonal anti-NeuN (a marker for mature neurons; MAB377; Chemicon, Temecula, CA ;1µg/ml), or mouse monoclonal anti-GFAP, (a marker for mature astrocytes; clone G-A-5, cat. no. G3893, Sigma-Aldrich, St. Louis, MO; 4µg/ml). The NeuN and GFAP primary antibodies were used in conjunction with a Cy5 conjugated goat anti-mouse secondary antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:100, 2 hours, room temperature). Sections were thoroughly rinsed before and between all incubations in 25mM PBS, and all incubations and rinses were performed on a shaking platform. Lastly, sections were mounted on gelatin-coated slides. After drying for one hour, slides were cover slipped with aqueous Gel/mount containing anti-fading agents (Biomeda, Foster City, CA) and stored in the dark at 4ºC.

***Triple-label immunofluorescence:*** To determine the cellular phenotype of BrdU-labeled cells in Me, sections from the same animals used for double-label immunofluorescence were processed using a protocol adapted from several published studies[4-7]. As with single-label immunohistochemistry, sections were treated with

formamide, SSC, and HCl to expose BrdU-labeled DNA. After blocking for 30 min in 0.2% Triton X-100 and 3% goat serum (CAT#, Jackson ImmunoResearch Lab.,Inc, West Grove, PA) in TBS, sections were incubated over 2 nights (~40 hr) at 4ºC in a cocktail of three primary antibodies for monoclonal rat anti-BrdU (1:1000, MCA2060, AbD Serotec, Raleigh, NC), monoclonal mouse anti-NeuN (1:1000, MAB377, Chemicon International, Inc., Temecula, CA), and polyclonal rabbit anti-GFAP (1:400, CAT#, Dako USA, Carpinteria, CA). After rinsing and blocking a second time, sections were incubated for 3 hr in a cocktail of three secondary antibodies all at a working concentration of 1:200, including a Cy2 conjugated goat anti-rat (112-225-167, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), Cy3 conjugated goat anti-rabbit (111-165-144, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and Cy5 conjugated goat anti-mouse (115-175-166, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). These 3 secondary antibodies have been developed by Jackson Laboratories to have minimal cross-species reactivity. Finally, sections were rinsed in TBS, mounted onto gelatin-coated slides, and coverslipped using the anti-fade mounting medium Gel/Mount (Biomeda, Burlingame, CA). Slides were stored in the dark at 4ºC until microscopic analysis.

***Controls for immunohistochemistry and immunofluorescence.*** Single label fluorescence immunohistochemistry was used to verify that there was no overlap in the excitation and emission wavelengths of the chosen fluorophores. All of the following additional controls were included in every immunohistochemical run. To assess non-specific immunoreactivity, control sections were processed in the absence of either primary or secondary antibodies, with all other steps in parallel with the above protocols.

Omitting either the primary or secondary antibody resulted in no immunostaining. As a negative control, sections from non-BrdU treated animals were processed for BrdU immunohistochemistry; these did not exhibit any signal, demonstrating that the BrdU antibody did not bind endogenous rat IgG. As a positive control, we also processed tissue sections from a 21 day old rat born to a dam injected with BrdU on gestational days 14-16; extensive numbers of BrdU-immunoreactive (BrdU-ir) cells were observed in tissue from this animal in every run.

**Microscopic Analysis and Quantification**

***Volume measurements and BrdU-ir cell counts***: Using Neurolucida (Version number 6 Microbrightfield, Williston, VT), the boundaries of the AVPV, SDN, Me, and DG were traced bilaterally at 40X magnification in each Nissl-stained section containing these nuclei. AVPV, SDN, and Me volumes were estimated by summing the traced cross-sectional areas and multiplying by the distance between sections (120 µm for AVPV and SDN, 240 µm for Me). Volume estimates were not obtained for DG. Tracings from Nissl sections through AVPV, SDN, Me, and DG were then superimposed onto adjacent BrdU-labeled sections, aligning landmarks. All BrdU-ir cells located within the tracing boundaries were counted at 400x magnification. BrdU-ir cells were identified by dark brown nuclear DAB reaction product. The total number of BrdU-ir cells was summed across sections for each animal, and the number of BrdU-ir cells/animal averaged for each age, sex, or treatment group.

***3D-confocal analysis***: Qualitative analysis for double-labeled immunofluorescent sections was performed with a Zeiss confocal LSM 510 scanning

laser microscope equipped with Argon (456 & 488 nm) and HeNe (543 nm) lasers, utilizing the Zeiss LSM Image Browser version 3.2. The AVPV and SDN were analyzed for BrdU co-localization with the neuronal and glial markers, and colocalization was confirmed via the z-stack orthogonal viewer in the confocal analysis software. All of the images presented in the figures represent a single z-level with a section thickness of 0.56 µm. Minimal contrast adjustments were made to the images, but images were otherwise unaltered.

For analysis of triple-labeled sections in the MA, laser-scanning confocal images were taken using an Olympus Fluoview microscope equipped with an Argon laser emitting 488 nm light, a green HeNe laser emitting 543 nm light, and a red HeNe laser emitting 633 nm light. Confocal Z-stacks were collected sequentially in the 3 channels at 1 µm intervals using a 40X oil objective (N.A. 1.40). BrDU-ir cells were examined in orthogonal views to determine whether cells were colocalized with NeuN or GFAP. Brdu-ir cells were categorized as being colocalized with NeuN, GFAP, or neither. Brdu, which is incorporated into DNA, has a different subcellular localization than NeuN, a protein contained in the nuclear envelope and some portions of the cytoplasm, and GFAP, a protein localized to the processes of astrocytes. Cells categorized as colocalized for Brdu and NeuN displayed both Cy2 and Cy5 fluorescence, but this fluorescence could be localized to different poles of the cell nucleus and soma. Cells categorized as colocalized for Brdu and GFAP displayed Cy2 and Cy3 fluorescence without adjacent Cy5 fluorescence. Cells not meeting these criteria for BrdU colocalization with NeuN or GFAP were categorized as unknown.

***Estimation of AVPV neuronal cell number:*** In Nissl-stained sections from Experiment 2, AVPV neurons were distinguished based on morphological characteristics under a high-power oil immersion (100X) lens. Neurons were identified by their large size and the presence of a large lightly stained nucleus that was surrounded by a rim of Nissl-stained cytoplasm and clearly defined nucleolus. Design-based stereological neuron counts were performed using the optical fractionator method (Stereoinvestigator, Version 7.0, MicroBrightfield, Williston, VT). For each animal, every third 40 µm section throughout the AVPV was analyzed. The software randomly laid a 100x50 µm grid over the brain region of interest. At each intersection of the grid, cells were counted using a 25x25 µm counting frame to allow for systematic random sampling throughout the region of interest. To avoid over sampling and cutting artifacts, upper and lower guard zones of 1µm along the Z dimension were excluded from counting. This method provides stereological estimates of cell number that are unbiased for cell size, orientation, shape, and packing density of cells. Sampling parameters were optimized for counting neurons such that 100 to 150 neurons per animal were counted across sections. The coefficient of error (CE) for neuron number was calculated by the software at each sampling site. On average, the estimated CE value was less than 10%.

**Statistical Analysis**

In experiment 1, two-way analysis of variance (ANOVA) was used to determine the effects of age and sex on the number of BrdU-ir cells and volume of AVPV, SDN, and Me. In experiment 2, two-way ANOVA was used to determine the effects of sex and

gonadectomy on the number of BrdU-ir cells in AVPV, SDN, Me, and DG, volume of AVPV, SDN, and Me, and the number of Nissl-stained AVPV neurons. For both experiments, significant main effects and interactions were probed using Fisher's PLSD post-hoc tests. Differences were considered significant when $p<0.05$.

## References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