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Motherhood and infant contact regulate neuroplasticity in the serotonergic midbrain dorsal raphe

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Abstract

The adult brain shows remarkable neuroplasticity in response to hormones and the socioemotional modifications that they influence. In females with reproductive and maternal experience, this neuroplasticity includes the birth and death of new and existing cells in several forebrain regions involved in maternal caregiving and postpartum affective state. Such plasticity in midbrain sites critical for these processes has never been examined, though. Visualizing bromodeoxyuridine (BrdU) to label mitotic cells, I NeuroD for neuronal precursors, and TUNEL to identify dying cells, we found that the midbrain dorsal raphe nucleus (DR, the source of most ascending serotonergic projections) exhibited significant neuroplasticity in response to motherhood. Specifically, BrdU analyses revealed that DR newborn cell survival (but not proliferation) was regulated by reproductive state, such that cells born early postpartum were less likely to survive compared to cells born during late pregnancy. Many of the surviving cells in the DR were NeuN immunoreactive, suggesting a neuronal phenotype. Similarly, late postpartum rats had fewer NeuroD-immunoreactive DR cells than early postpartum rats. Maternal experience contributed to the late postpartum reduction in DR newborn cell survival because removing the litter at parturition increased cell survival and reduced cell death. Unlike cytogenesis in the maternal hippocampus, which is reduced by circulating glucocorticoids, DR newborn cell survival was unaffected by postpartum adrenalectomy. These effects of reproductive state and motherhood on DR plasticity were associated with concurrent changes in DR levels of serotonin's precursor, 5-HTP, and its metabolite, 5-HIAA. Our results demonstrate for the first time that cytogenesis occurs in the midbrain DR of any adult mammal, that DR plasticity is influenced by female reproductive state and maternal experience, and that this plasticity is accompanied by changes in DR serotonergic function. Because serotonin is critical for postpartum caregiving behaviors and maternal affective state, neuroplastic changes in the DR may contribute to the neurochemical changes necessary for successful motherhood.

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Contributors

M.A. Holschbach and J.S. Lonstein participated in the research and article preparation. M.A. Holschbach conceived of and designed the study with guidance from J.S. Lonstein. M.A. Holschbach acquired and analyzed the data. M.A. Holschbach interpreted the data with invaluable help from J.S. Lonstein. M.A. Holschbach and J.S. Lonstein worked together to draft and revise the article and both approve of the submitted version.

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Graphical abstract

Keywords

plasticity; maternal; serotonin; dorsal raphe; cytogenesis; cell death

1. Introduction

Circulating hormones act during numerous periods of the lifespan to help drive neurobehavioral change and do so, in part, by modifying central nervous system structure and function (for reviews see Sisk, Lonstein, Gore, 2013, Juraska & Willing, 2016). In adult females, the transition to motherhood involves dramatic fluctuations in steroid and other hormones, resulting in a suite of behavioral, emotional, and physiological adaptations that are required for successful rearing of offspring (Lonstein et al., 2014; Numan et al., 2006). These modifications include sensitive caregiving of the young, maternal aggression toward threats, reduced anxiety, milk production and letdown, and blunted endocrine responses to many stressors. All of these changes revert back to a prepartum state around the time of weaning the offspring (Lonstein et al., 2014; Numan et al., 2006).

To support such changes in behavior and physiology, mothers undergo equally remarkable neuroplasticity. The birth and death of new cells are two of the mechanisms through which hormones modify the structure, connectivity, and chemical function of the developing and adult nervous systems (Pawluski et al., 2009; Del Pino Sans et al., 2015; Juraska & Willing, 2016), and numerous studies have found that motherhood alters forebrain cytogenesis as well as cell death (Bridges & Grattan, 2003; Erbil et al., 2014).

For example, pregnant and early postpartum laboratory rodents have a prolactin-mediated increase in cytogenesis in the subventricular zone (SVZ) that gives rise to new olfactory neurons (Furuta & Bridges, 2005; Bridges & Grattan, 2003; Shingo et al., 2003), and early postpartum maternal experience increases newborn cell survival in other sites involved in maternal behavior including the nucleus accumbens and bed nucleus of the stria terminalis (Akbari et al., 2007). On the other hand, early postpartum rats also experience a

corticosterone-mediated decrease in cytogenesis in the hippocampal dentate gyrus (DG) (Pawluski et al., 2009; Pawluski & Galea, 2007; Leuner et al., 2007) and pregnant mice have more cell death in the DG and prefrontal cortex compared to nulliparae (Erbil et al., 2014). Motherhood-associated neuroplasticity has never been reported in the midbrain, though, perhaps because studies of adult cytogenesis almost invariably focus on forebrain sites apposing or close to the forebrain ventricular system - where cytogenesis is traditionally thought to occur (Kokoeva et al., 2007). However, the lining of the midbrain cerebral aqueduct is known to be an important proliferative niche from early development through adulthood and aging (Zhao & Lang, 2009; Zhao et al., 2003). One brain site that is both adjacent to the proliferative cerebral aqueduct and involved in postpartum behavior and physiology is the dorsal raphe nucleus (DR). The DR contains 80% of forebrain-projecting serotonergic neurons (Lowry et al., 2008) and selectively lesioning DR serotonergic neurons in postpartum rats increases pup mortality (Barofsky et al., 1983). Furthermore, maternal behaviors are severely impaired by knocking out *Pet-1* (transcription factor for serotonin neuron differentiation) or *TPH2* (enzyme necessary for neuronal serotonin synthesis) (Angoa-Perez et al., 2014; Lerch et al., 2008), or after acute pharmacological manipulation of serotonin signaling (Veiga et al., 2010; Zhao & Li, 2009). Postpartum physiological processes such as the suckling-induced prolactin and oxytocin surges necessary for milk production and release, also rely on DR serotonin (Barofsky et al., 1983). Given these roles for serotonin in postpartum behavior and physiology, one might predict that reproduction and motherhood are associated with neuroplastic changes in the DR.

We here tested the hypothesis that motherhood involves DR neuroplasticity by conducting a series of experiments in which we identified mitotic cells using 5-bromo-2'-deoxyuridine (BrdU), young neurons using immunohistochemical detection of NeuroD, and dying cells using Terminal dUTP-Nick End Labeling (TUNEL). We measured cytogenesis in female rats across different reproductive states, in postpartum rats with or without maternal experience, and in adrenalectomized mothers to examine if corticosterone affects DR cytogenesis as it does in the hippocampus (Pawluski et al., 2009; Pawluski & Galea, 2007; Leuner et al., 2007). We also colocalized BrdU and Neuronal Nuclei antigen (NeuN), a common neuronal marker. We also examined if reproductive state alters DR expression of NeuroD, a neurogenic transcription factor expressed by neuroblasts and terminally differentiating neurons (Gao et al., 2009). Lastly, we measured numerous aspects of the serotonin system across reproductive states to evaluate whether changes in DR plasticity occur contemporaneously with neurochemical modifications. We found that reproduction and motherhood alter DR newborn cell survival and cell death, as well as the serotonin synthetic and metabolic pathway.

2. Methods

Subjects

Subjects were female Long-Evans rats born and raised in our breeding colony and maintained as previously described (Smith et al., 2013). Pregnant females were singly housed approximately five days before parturition, and litters culled to contain four males and four females within 24 hours after birth. The day of parturition was considered PPD 0.

All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Michigan State University.

2.1. Experiment 1: Effects of reproductive state on DR cytogenesis and cell death in adult female rats

2.1.1. BrdU injections—To measure cytogenesis in the DR and elsewhere in the brain, female rats were injected with BrdU, a thymidine analogue permanently incorporated into the DNA of dividing cells (Taupin, 2007). A dose of 150 mg/kg bodyweight was chosen to approach a saturating dose that does not have deleterious effects on fetuses in the pregnant dams (Taupin, 2007; Cameron & McKay, 2001). Rats were injected once with BrdU dissolved in 0.9% sterile saline (IP; 10 mg/mL; Sigma-Aldrich, St. Louis, MO) either on a day of diestrus (determined by cytology in vaginal smears), on pregnancy day (PD) 19, or on postpartum day (PPD) 7 (Figure 1). Subjects were sacrificed one day after BrdU injection to examine cell proliferation ($n = 8$ per group), or 12 days after injection to examine short-term cell survival ($n = 10$ per group). The 12-day survival period was chosen for several reasons. First, this survival period allowed us to measure cell survival during the onset and early maintenance of postpartum behaviors and physiology (from PD 19 to PPD 8) and then have another discrete measure as these events wane (from PPD 7 to PPD 19), thus allowing us to evaluate cell survival during the two most salient periods of behavioral change in mothers. In addition to these behavioral changes, the physiological events of parturition could affect survival of cells in the maternal brain, which could further contribute to differences between these two groups. Second, because newborn cells migrate from the cerebral aqueduct to the substantia nigra (SN) within 10 days (Zhao et al., 2003), newborn cells still residing within the even closer DR 12 days after BrdU injection were unlikely to be migrating through the structure but instead have probably reached their final destination there. Third, 12 days after mitosis is sufficient time for expression of the neuronal marker, NeuN (Kuhn et al, 1996), which we used to determine the phenotype of the newborn neurons.

2.1.2 BrdU immunohistochemistry (IHC)—Subjects were overdosed with sodium pentobarbital and perfused with 150 mL of 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were postfixed overnight in 4% PFA, and stored in 20% sucrose until they were sectioned into 40- μ m-thick sections on a microtome, collected into four alternate series, and stored in a sucrose-based cryoprotectant at -20° C. BrdU IHC was performed on free-floating sections. Sections were rinsed three times $\times$ 5 min in Tris buffered saline (TBS) before each step except between applications of the blocking solution and the primary antiserum. All treatments occurred at room temperature unless otherwise specified. Sections were incubated in 50% formamide in 1.0 M standard saline citrate buffer at 60° C for two hours, 2.0 M hydrochloric acid for 30 min, borate buffer for 10 min, and 1% H_2O_2 in 0.3% Triton-X in TBS for 10 minutes. After blocking with 3% horse serum (HS; Vector Laboratories, Burlingame, CA) in 0.1% Triton-X in TBS for 25 minutes, sections were incubated overnight at 4° C in the primary antisera (0.10 μ g/mL; monoclonal mouse anti-BrdU; Roche Diagnostics, Germany) in 0.3% Triton-X in TBS. Sections were incubated in biotinylated horse-anti-mouse secondary (6 μ g/mL; Vector Laboratories, Burlingame, CA) in 0.3% Triton-X TBS for two hours, then the signal was amplified with avidin-biotin

complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA) for one hour, and staining was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO). Sections were then rinsed and stored in TBS until they were mounted onto microscope slides.

BrdU-immunoreactive (BrdU-ir) DR cells were counted in 5 sections in one of the tissue series under 200 x magnification using a Nikon E400 light microscope. Sections were matched to levels 43 – 47 of Swanson's atlas of the rat brain (Swanson, 1998). For here and the subsequent experiments below, all five major subregions of the DR (Kelly et al., 2011) were analyzed on these sections using standardized traces covering the entire extent of each subregion and the total numbers of cells quantified were combined for final data analyses because preliminary analyses indicated no unique subregional differences. We also quantified the number of BrdU-ir cells in the median raphe (MR), as well as in several forebrain sites previously examined in this literature (i.e., nucleus accumbens core and shell, dorsal and ventral bed nucleus of the stria terminalis, and medial preoptic area). The results from only the DR are presented and discussed in detail because BrdU-ir cells in the MR were in very low abundance (all groups had an average of < 10 cells) and there were no significant differences among groups in cell proliferation or survival in any of the forebrain sites examined (Supplementary Tables 1 and 2).

One of the four series of sections from subjects injected with BrdU on PD 19 and those injected on PPD 7 were used to determine if surviving cells in reproductive females had a neuronal phenotype ($n = 4$ per group). Dual-label immunofluorescence was performed on free-floating sections as described previously (Mohr & Sisk, 2013; Wainwright et al., 2011) using a cocktail of a high-sensitivity monoclonal rat anti-BrdU primary antiserum (1 $\mu\text{g/mL}$; Serotec) and a monoclonal mouse anti-NeuN primary antiserum (clone A60, 1:1000), followed by a cocktail of biotin-SP-conjugated AffiniPure goat anti-rat secondary antiserum (1.3 $\mu\text{g/mL}$; Jackson ImmunoResearch, West Grove, PA) with Alexa Fluor 405 goat-anti-mouse secondary antiserum (4 $\mu\text{g/mL}$; Invitrogen, Grand Island, NY), followed by Cy-2 conjugated streptavidin (1.8 $\mu\text{g/mL}$; JacksonImmunoResearch, West Grove, PA). BrdU- and NeuN-ir nuclei were visualized using an Olympus FluoView 1000 Filter-based Laser Scanning Confocal Microscope configured on an Olympus IX81 automated inverted microscope platform using a blue diode laser (405 nm) and band pass emission filters for wavelength selection. High-resolution confocal fluorescence was collected through a single, variable pinhole aperture and recorded using three high-sensitivity photomultiplier tube detectors. Z-stack images were collected at 2.65 μm intervals through five sections containing the DR per animal. Thirty BrdU-ir nuclei in the DR were randomly identified for each animal and colocalization with NeuN was determined via 3D reconstruction using a Z-stack orthogonal viewer.

2.1.3 NeuroD immunohistochemistry (IHC)—To further confirm the existence of newborn neurons in the adult female DR, we immunohistochemically labeled cells containing Neuro D, which is a DNA-binding neurogenic transcription factor that is highly expressed by maturing neurons (Gao et al., 2009), including in the adult brain. Adult female rats were sacrificed and perfused as diestrus virgins, on PPD 8, or on PPD 19 (5 females/group), the same time points used above to examine the effects of reproductive state on DR

short-term cell survival. IHC was performed on a one-in-four series of free-floating sections using a monoclonal rabbit anti-NeuroD (A-10) primary antiserum (sc-46684; 1:1,000; Protos BiotechCorp, NY) and Alexafluor 568 goat anti-rabbit secondary antiserum (1:500; Invitrogen, Grand Island, NY). NeuroD-ir nuclei were visualized using a Zeiss Zen 2.1 Laser Scanning Confocal Microscope configured on a Zeiss Axio Examiner Z1 upright 880 microscope platform using a HeNe 543nm laser. Z-stack images were collected at 1.66 μ m intervals through five sections containing the DR per animal.

2.1.4. TUNEL—The number of dying or apoptotic cells was determined using an *In Situ* Cell Death Detection Kit (TMR Red; Roche Applied Sciences, Indianapolis, IN) that allows visualization of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Following the manufacturer's protocol, we included negative control tissue that was treated with Label Solution (without terminal transferase) instead of TUNEL reaction mixture and these sections showed no fluorescence. TUNEL+ nuclei were visualized on the same confocal microscope described in 2.1.2 using a green Helium Neon gas laser (543 nm) to visualize TUNEL. Z-stack images were collected at 2.65 μ m intervals through five sections containing the DR per animal and TUNEL+ nuclei were quantified using Image-J.

To examine the phenotype of apoptotic DR cells in virgin, PPD8, and PPD19 females, an alternate set of tissue was double-labeled to identify dying neurons. Immediately after TUNEL processing described immediately above, immunohistochemical labeling was processed using a NeuN antiserum (monoclonal mouse anti-NeuN; clone A60, 1:1000), as described for the BrdU/NeuN double-labeling above. Colocalization of TUNEL and NeuN was confirmed via 3D reconstruction using a Z-stack orthogonal viewer.

2.2. Experiment 2: Effects of maternal experience on DR cytogenesis and cell death in postpartum rats

2.2.1. Litter removal and BrdU injections—Subjects were monitored frequently beginning on day 20 of pregnancy for parturition. Within an hour after completion of parturition, litters were culled to contain 4 males and 4 females and either remained with the dams, or were permanently removed from the home cage to generate a maternally inexperienced group ($n = 9$ per group). To assess DR cytogenesis in these groups, the rats were injected twice daily with BrdU (IP; 150 mg/ kg bodyweight; 10 mg/mL; Sigma-Aldrich, St. Louis, MO) on PPD 7 and 8. These postpartum days were chosen in order to match the timing of BrdU injection of the late-postpartum rats in Experiment 1 above. The multiple injections of BrdU were delivered in an effort to label multiple pools of mitotic cells, but it is important to mention that this methodological change was not found to notably increase the number of BrdU-containing cells within the DR compared to what was found in Experiment 1 above, possibly because the full mitotic cycle takes approximately 25 hours to complete in the adult hippocampus (Cameron & McKay, 2001), and potentially longer in the midbrain. This finding is in line with previous reports that infusing BrdU intracerebroventricularly labels 5-fold more mitotic midbrain cells compared to larger, repeated IP injections (Zhao et al., 2003).

2.2.2. Immunohistochemistry—Subjects were overdosed with sodium pentobarbital and perfused with 150 mL of 0.9% saline followed by 4% paraformaldehyde (PFA) 12 days after BrdU injection (PPD 19). Brain harvesting, BrdU IHC, and TUNEL were performed as described in Experiment 1.

2.3. Experiment 3: Effects of adrenalectomy on DR cyto genesis in postpartum rats

Lactation is characterized by high circulating corticosterone, which suppresses cyto genesis in the postpartum hippocampus (Leuner et al., 2007). We examined if the same is true for cyto genesis in the maternal DR by comparing sham-operated dams (Sham) and two groups of dams that were adrenalectomized on PPD 1 (Figure 2). Subjects were anesthetized with ketamine (90 mg/kg, ip) and xylazine (8 mg/kg, ip) and adrenalectomized or sham operated as described elsewhere (Leuner et al., 2007). Litters were fostered to surrogate dams during surgery and given back to their dams after recovery from anesthesia. One group of adrenalectomized dams received a very low dose of corticosterone in their drinking water to maintain general neuronal health (ADX; 15 µg/mL in 0.5% saline, 0.4% ethanol) and the other received full corticosterone replacement (ADX+Cort; 100 µg/mL) (Rees et al., 2004). Because adrenalectomy can reduce milk production and output (Leuner et al., 2007), litters were weighed and cross-fostered daily among the three groups of dams in the experiment and an additional group of unmanipulated, lactating surrogates from our colony. Sham-operated and adrenalectomized female rats were injected with BrdU twice daily on PPD 7 and 8. Twelve days later, subjects were overdosed with pentobarbital and ~3 mL of blood was collected from a cardiac puncture, then animals were perfused, and brains were harvested for BrdU IHC as described in Experiment 1. Blood was centrifuged to collect plasma, which was used to measure circulating corticosterone in a Mouse and Rat Corticosterone ELISA according to the manufacturer's protocol (cat no: 55-CORMS-E01, ALPCO, Salem, NH).

2.4. Experiment 4: Effects of reproductive state on the serotonin neurochemical pathway

2.4.1. Immunohistochemistry—Adult female rats were sacrificed and perfused as diestrus virgins, on PPD 8, or on PPD 19 (13–20 females/group), the same time points used to examine the effects of reproductive state on DR cyto genesis and cell death in Experiment 1. IHC was performed on a one-in-four series of free-floating sections using a monoclonal rabbit anti-serotonin primary antiserum (NT-102 5HTrab; 1:10,000; Protos BiotechCorp, NY) and a biotinylated goat anti-rabbit secondary antiserum (1:1000; Vector Laboratories, Burlingame, CA). Images of the DR were captured at 100x magnification and the average light level measured by NES Elements was adjusted in order to keep it constant for every image. Immunoreactivity was differentiated from background by using an optimized light threshold that was also kept constant for every image. Standardized traces were superimposed upon the images and the area covered by pixels above the standardized light threshold was calculated using NIS-Elements, as described elsewhere (Smith et al., 2012).

2.4.2. Western Blotting—Diestrus virgin, PPD 8, and PPD 19 rats were narcotized by brief (< 90 sec) exposure to carbon dioxide ($n = 8$ per group). Brains were harvested, frozen with cold isopentane, and stored at -80°C until being sectioned into 500-µm-thick sections on a cryostat. Sections were mounted onto microscope slides and stored at -80°C until the

DR was microdissected using a Harris Micropunch (Sigma-Aldrich, St. Louis, MO) and homogenized in 50 μ L RIPA buffer (Santa Cruz Biotechnology SC-24948, Santa Cruz, CA). Samples (10 μ g protein / subject) were denatured at 100 °C for five minutes, electrophoresed on 10% Bis-Tris Plus precast gels (BG00100; Thermo Scientific; Rockford, IL) for 50 min at 165 V using a Powerpac Basic (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes using an iBlot system (Invitrogen #iB4010, Grand Island, NY). Membranes were stored in ultrapure water overnight at 4 °C, saturated in iBind Solution, then loaded into an iBind Western Blotting system that drew through the membranes 2 mL each of the following solutions: 1) primary antiserum diluted 1:500 in iBind solution (rabbit anti-TPH2, Thermo Scientific PA1-778, Rockford, IL), 2) iBind solution, and 3) secondary antiserum diluted 1:1000 in iBind Solution (goat anti-rabbit Cell Signaling #70745, St. Louis, MO). Finally, 6 mL of iBind solution was drawn through the membranes. Membranes were then temporarily placed in ultrapure water and immunoreactive bands were detected using a chemiluminescence kit (Bio-Rad #170-5060, Hercules, CA) and placed on films (Optimum blue Sensitive X-ray film, XR-0810-100; Life Sciences Products; Frederick, CO) that were developed and fixed with a Kodak X-OMAT 100A Processor (Kodak Co., Rochester, NY). After developing, films were scanned (PSC 1410, Hewlett Packard) and images of TPH2-ir bands were analyzed using Image J (National Institutes of Health, Bethesda, MD, USA) to determine their integrated density. Membranes were rinsed 3 times in ultrapure water, then processed similarly to detect the loading control protein GAPDH using appropriate primary (1:500; mouse anti-GAPDH; Millipore #MAB374, Temecula, CA) and secondary (1:4000; rabbit anti-mouse, Sigma-Aldrich #A9044, St. Louis, MO) antisera. GAPDH integrated density in the DR was found to not differ among groups ($F_{2,20} = 2.03$, $p = .16$), so TPH2 integrated density was standardized to corresponding GAPDH integrated density for each subject.

2.4.3. HPLC—Another set of diestrus virgin, PPD 8, and PPD 19 rats were exposed to carbon dioxide ($n = 8$ per group) and the brains removed and sectioned on a cryostat. DR tissue punches were homogenized in 200 nL cold 0.1 M perchloric acid, centrifuged at 10,000 rpm for 10 min, then the supernatant collected and stored at -80°C until analysis was conducted using HPLC at a core facility at Michigan State University (Smith et al., 2013). The limits of detection for serotonin, its precursor (5-HTP), and its metabolite (5-HIAA) were all 0.5 ng/mL. Norepinephrine and dopamine in the DR were also measured but did not differ among the groups studied (data not shown).

2.5. Data analyses

All histological and neurochemical analyses were performed by experimenters who were naïve to subjects' experimental condition. To estimate the total numbers of BrdU-ir and TUNEL+ nuclei in the DR for Experiments 1–3, an Abercrombie correction factor of 0.93 was applied based on our section thickness and an average measured DR nuclear diameter of 3.1 μ m. The number of BrdU-ir cells was not normally distributed among the groups of females compared across reproductive states or adrenal status (Experiments 1 and 3), so these data were analyzed using Kruskal-Wallis tests followed by Mann Whitney U tests for the post-hoc analyses. NeuroD-ir and TUNEL+ cells in virgin, PPD8, and PPD19 females in Experiment 1 were analyzed using one-way ANOVAs, followed by Tukey HSD tests for

post-hoc analyses. Independent samples t-tests were used to analyze the number of BrdU-ir and TUNEL+ cells in maternally experienced and inexperienced postpartum rats (Experiment 2). Serotonin pathway measures from IHC, Western Blot, and HPLC were analyzed in Experiment 4 using one-way ANOVAs, followed by Tukey HSD tests for post-hoc analyses. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Experiment 1: Effects of reproductive state on DR cytogenesis and cell death in adult female rats

As has been reported previously in adult male mice (Zhao & Lang, 2009; Zhao et al., 2003), the dorsal and lateral aspects of the SVZ surrounding the cerebral aqueduct were densely populated by BrdU-ir cells in most of our female rats (Figure 3A, 3B). There were also numerous BrdU-ir cells within the DR of all three groups of females studied (Figure 3B, 3C). In addition to newborn DR cells that likely migrated from the nearby SVZ, pairs of BrdU-ir nuclei were also frequently observed (Figure 3C), suggesting that these cells may have divided within the DR itself (Kokoeva et al., 2007).

Reproductive state did not significantly affect the number of proliferating (1-day old) BrdU-ir cells in the DR (χ^2 (2, $N = 24$) = 1.54, $p = .46$; Figure 3G), but it did significantly affect the number of BrdU-ir cells surviving until 12 days after injection (χ^2 (2, $N = 32$) = 8.66, $p = .034$; Figure 3H). PPD 19 females (i.e., those injected with BrdU early postpartum) had fewer BrdU-ir cells in the DR compared to PPD 8 females (i.e., those injected with BrdU during late pregnancy) ($p = .005$). A majority of the 12-day old BrdU-ir cells were also NeuN-ir, suggesting a neuronal phenotype (Figure 3D–F). The percentage of BrdU-ir cells that were NeuN-ir was similar between the late-postpartum females (range = 43–57%; mean = 51%) and early-postpartum females (range = 30–83%; mean = 62%). By contrast, none of the BrdU-ir nuclei lining the cerebral aqueduct were NeuN-ir (Figure 3B), which was expected because newborn cells in this location would be proliferating rather than differentiating. The overall levels of BrdU-ir cells in DR was site-specific because very few BrdU-ir labeled cells were detected in the periaqueductal grey adjacent to the DR (Figure 3B) or in the MR.

Analysis of NeuroD-ir cells in the DR revealed hundreds of cells in our female rats, further confirming the presence of neuronal precursors in this site during adulthood (Figure 3I). Reproductive state affected the number of NeuroD-ir cells in the DR ($F_{2,14} = 23.32$, $p < .0001$) in a similar pattern to that of BrdU - PPD 8 females had the highest number of NeuroD-ir cells (744 ± 60), which was significantly higher than both diestrus virgins (259 ± 74 ; ($p = .001$) and PPD 19 females (287 ± 22) ($p = .001$). Diestrus virgins and PPD 19 females did not significantly differ from each other in NeuroD-ir cells ($p = .90$).

Using TUNEL to measure DR cell death (Figure 4A–D), we found no significant effect of reproductive state on the number of dying cells ($F_{2,24} = 1.03$, $p = .37$), despite 34% more TUNEL+ nuclei in the DR of the PPD 19 females compared to PPD 8 females (Figure 4E). The percentage of TUNEL+ cells that were neurons (i.e., NeuN-ir) was stable at 30–40% for all three reproductive states ($F_{2,22} = 0.37$, $p = .70$, Figure 4D).

3.2. Experiment 2: Effects of maternal experience on DR cyto genesis and cell death in postpartum rats

Removing the litter at parturition significantly increased the number of 12-day old BrdU-ir cells in the DR of PPD 19 rats ($t_{(17)} = 2.44$, $p = .026$, Figure 5A). Conversely, litter removal at parturition significantly decreased the number of TUNEL+ cells in the dams' DR ($t_{(17)} = 2.90$, $p = .01$, Figure 5B).

3.3. Experiment 3: Effects of adrenalectomy on DR cyto genesis in postpartum rats

As expected, adrenalectomy reduced circulating corticosterone such that most ADX dams had non-detectible plasma levels of the hormone while all Sham and ADX+Cort dams had measureable corticosterone ($\chi^2 (2, N = 32) = 9.50$, $p = .009$). Correspondingly, ADX dams had significantly lower plasma corticosterone levels compared to the control groups (0.56 ± 3.82 vs. 37.96 ± 14.13 ng/ml; $t_{(30)} = 2.56$, $p = .017$). Consistent with the literature (Leuner et al., 2007), daily relative weight gains were lower in litters fed by ADX females than by control dams, and corticosterone replacement partially alleviated that deficit ($F_{(2,28)} = 10.49$, $p = .0005$, Figure 5C). Adrenalectomy did not, however, affect newborn cell survival in the DR ($F_{(2,25)} = 1.63$, $p = .22$, Figure 5D).

3.4. Experiment 4: Effects of reproductive state on the serotonin neurochemical pathway

Neither the immunohistochemical ($F_{(2,38)} = 0.31$, $p = .74$, Figure 6A–B) nor HPLC ($F_{(2,26)} = 1.74$, $p = .20$, Figure 6F) analyses of DR serotonin revealed significant differences across reproductive states. Western blotting for TPH2 also revealed no differences among groups ($F_{(2,20)} = 1.10$, $p = .36$, Figure 6C–D). However, the HPLC analysis revealed that early-postpartum rats had higher DR levels (130–150%) of serotonin's precursor, 5-HTP ($F_{(2,26)} = 6.79$, $p = .005$, Figure 6E), and serotonin's metabolite, 5-HIAA ($F_{(2,26)} = 8.53$, $p = .002$, Figure 6G), compared to the late-postpartum rats or diestrus virgins. Serotonin turnover (5-HIAA/serotonin) did not significantly differ among groups, but followed a similar pattern ($F_{(2,26)} = 3.27$, $p = .055$).

4. Conclusion & Discussion

These experiments demonstrate the plastic nature of the adult midbrain serotonergic DR. We show for the first time that cyto genesis and early neuronal differentiation occur in the DR of an adult mammal, that this plasticity is influenced by female reproductive state and motherhood, and that changes in newborn cell survival are associated with concurrent changes in the pathway for serotonin synthesis and metabolism. This DR plasticity and the synchronous neurochemical changes could be essential for the onset, maintenance, and termination of the behavioral and physiological hallmarks of motherhood. Importantly, similar plasticity may occur in the female DR during other reproductive milestones including puberty and reproductive senescence.

There have been numerous studies of motherhood-induced cyto genesis, and many links between serotonin and postpartum behavior and physiology, but no previous examinations of motherhood-induced neuroplasticity in the midbrain DR. We hypothesized that motherhood modifies the DR by changing cyto genesis and cell death, which could be associated with

changes in the DR's neurochemical function. We found that fewer DR cells born (i.e., containing BrdU) during the early postpartum period survived 12 days (until soon before weaning - PPD 19) when compared to the number of cells born during late pregnancy that survived for 12 days (until PPD 8). Similarly, late postpartum (PPD 19) rats had fewer DR neuronal precursors (i.e., NeuroD-ir cells) when compared to early postpartum (PPD 8) rats. Adult, virgin female rats had intermediate levels of cell survival in the DR that did not significantly differ from either postpartum group, but both virgin and late-postpartum rats had fewer young DR neurons than early postpartum rats. These results collectively indicate that young DR neurons are less likely to survive when maternal interactions with offspring are low or declining. At the same time that late postpartum dams showed reduced DR cell survival and fewer neuronal precursors, there was a concomitant (but non-significant) 34% increase in DR cell death, and significantly decreased levels of both serotonin's precursor, 5-HTP, and its metabolite, 5-HIAA. Moreover, preventing maternal experience by removing pups precluded the low cell survival and high cell death of late-postpartum females, suggesting that DR plasticity requires mothering experience rather than simply reproduction.

The concurrent changes in DR newborn cell survival and serotonin synthesis/metabolism suggest that these events are causally related, but the directionality of any relationship cannot be determined by our studies. This could be determined by depleting or infusing serotonin into the DR and measuring newborn cell survival, or conversely, reducing newborn cell survival by infusing an antimetabolic agent into the DR and measuring the serotonin biosynthetic pathway. Serotonin promotes survival of cells born in the forebrain SVZ by stimulating 5-HT_{1A} receptors (Soumier et al., 2010) and may act similarly in the midbrain. If so, early postpartum enhancement of local serotonin could support the survival of newborn DR cells, whereas newborn cells fail to survive in the late postpartum DR when serotonergic activity wanes. Of course, the reciprocal relationship is possible - changes in the survival of newborn DR cells may affect serotonin. This could occur because newborn cells can increase levels of local neurotrophic factors, such as brain derived neurotrophic factor (BDNF). Transplanting mitotic cells into the adult brain increases BDNF (Mendonca et al., 2015) and administering BDNF elevates serotonin content and reuptake in DR neurons (Martinowich & Lu, 2008). Thus, a drop in newborn cell survival in late-postpartum mothers may have reduced BDNF in their DR, thereby reducing serotonergic activity back to a prepartum state. To be clear, we do not suggest that the newborn neurons observed in our studies are (or eventually become) serotonergic neurons themselves, but rather that they may influence or respond to changes in DR serotonergic function. Chemical phenotype of these cells was not explored because previous work indicates that cells born in the lining of the cerebral aqueduct that migrate to the substantia nigra do not express tyrosine hydroxylase until three weeks after BrdU incorporation (Zhao et al., 2003). Future experiments involving longer post-BrdU survival times would be necessary to explore the chemical phenotype of any persisting newborn DR cells.

Mothers' behavior and physiology are not static across the postpartum period, but change synchronously with offspring development. These temporal changes rely on altered neurochemistry in many brain areas (Pereira & Ferreira, 2015) and our results suggest this could include DR serotonergic activity. Ovarian hormones increase DR serotonergic activity by increasing its synthesizing enzyme at the same time that they reduce both its

autoreceptor expression and its metabolism (Donner & Handa, 2009; Smith et al., 2004). Late-postpartum mothers had lower 5-HTP, the product of the rate-limiting step in serotonin synthesis. Serotonin content remained stable probably because its metabolism into 5-HIAA simultaneously decreased in this group of mothers. This decreased DR serotonin synthesis/metabolism as postpartum time progressed would presumably limit serotonergic activity at DR terminal sites, including the medial preoptic 2area and bed nucleus of the stria terminalis (Lowry et al., 2008). These regions have especially high serotonin turnover in new mothers and are essential for early postpartum caregiving and emotional behaviors (Lonstein et al., 2003; Smith et al., 2013). Decreased DR serotonergic activity in late-postpartum mothers could be partly responsible for the waning of maternal behaviors and return to a prepartum physiological state when lactation comes to an end.

Another possibly relevant effect of neuroplasticity-associated changes in DR serotonergic activity across reproduction involves serotonin's role in somatosensation (Arora et al., 2014). DR serotonin innervates the primary somatosensory cortex (SI) (Kirifides et al., 2001), which expands and contracts across lactation to heighten and then reduce female rats' ventrum sensitivity to tactile input from young (Xerri et al., 1994). Contact with offspring maintains most postpartum behavioral and physiological adaptations (Stern, 1996), and offspring removal prevents this SI plasticity (Xerri et al., 1994), and the low cell survival and high cell death we found in the DR. Thus, maternal experience induces plasticity in the DR in ways that may support maternal behavior and physiology directly by influencing serotonergic signaling in forebrain sites mediating these processes, or indirectly by affecting plasticity in SI and elsewhere in the brain.

As discussed above, the newborn neurons observed here were probably too immature to have developed a chemical phenotype (Zhao et al., 2003), but the dying cells we observed (i.e., TUNEL+) could have been neurochemically mature neurons. Because serotonergic DR neurons influence postpartum physiology and behavior (Barofsky et al., 1983), their loss would be particularly impactful for the mothers. Additionally, the rostral DR is densely populated by dopaminergic neurons that encode the negative valence of social isolation and then drive the motivation to socially interact (Matthews et al., 2016). Elevated loss of these dopaminergic neurons during late lactation may facilitate weaning of the pups, which could otherwise produce aversive social isolation for the dams. The timing of these postpartum changes in DR plasticity certainly supports such a role.

Many circulating steroids and peptides influence cell birth and death, and their levels dramatically differ among female reproductive states (Bridges & Grattan, 2003). Reducing corticosterone in mothers by adrenalectomy or removing the offspring increases DG cell proliferation (Leuner et al., 2007). Exogenous corticosterone prevents adrenalectomy's effect on cell proliferation (Leuner et al., 2007) and further decreases DG cell proliferation in intact dams (Brummelte & Galea, 2010). The DR densely expresses glucocorticoid receptors (Vincent & Jacobson, 2014), so we had hypothesized that high postpartum corticosterone was reducing DR newborn cell survival. Postpartum ADX did not prevent the drop in DR cytogenesis, however, so neurochemicals other than glucocorticoids must be responsible. The DR expresses receptors for numerous other hormones that influence cell survival (Alves et al., 2000). Prolactin is very high until PPD 15 in rats (Hansen et al., 1983),

but is neurogenic (Pawluski et al., 2009; Bridges & Grattan, 2003), so probably not responsible for reducing the survival of cells born on PPD 7. While it is possible that declining prolactin after PPD 15 promoted cell death by the time we counted cells on PPD 19, this also seems unlikely because removing the young increased DR cell survival but causes prolactin to rapidly fall due to the lack of suckling. Instead, ovarian hormones may have contributed to our results. Circulating estradiol and progesterone are high during pregnancy and temporarily decline after parturition (Lonstein et al., 2014), so the early postpartum absence of these neurogenic hormones may have contributed to low DR newborn cell survival. If so, removing the litter increased cell survival because the absence of suckling allowed neurogenic ovarian hormones to rise again in anticipation of dams' resumption of estrous cycling. In support, hormone replacement increases expression of proliferating cell nuclear antigen (PCNA) within the DR of ovariectomized adult and aged female monkeys (Bethea et al., 2016).

Very few experiments have examined plasticity in the midbrain of adults but the lining of the cerebral aqueduct expresses many markers (including nestin, FoxA2, Sox2, and vimentin) indicating that it is a neurogenic niche (Bonilla et al., 2008) and this neurogenic activity is known to continue through adulthood in laboratory rodents (Zhao et al., 2003; Zhao & Lang, 2009). The DR is adjacent to this well-characterized neurogenic niche, but cell genesis has never been reported within the DR of any mammal, and our results demonstrate that it occurs in abundance. BrdU is available for incorporation into DNA for approximately two hours (Cameron & McKay, 2001), so extrapolating from the numbers of newborn DR cells we found, there could be hundreds of new cells added daily to the DR of adult female rats. More than half of our 12-day old BrdU-ir DR cells did express NeuN, suggesting that a very large number of new DR neurons are born during adulthood. We also observed hundreds of NeuroD-ir cells within the DR of our adult female rats, further indicating the presence of many young neurons within this midbrain site. The BrdU-ir cells that did not express NeuN could be neuroblasts too immature to express NeuN, or cells that could eventually mature into glia. The latter could be relevant because astrocytes provide trophic factors necessary for survival of serotonergic raphe neurons (Eriksen et al., 2002), so reduced survival of newborn glia could also lead to high cell death for serotonergic DR neurons, as discussed above. It will be valuable in future studies to determine whether gliogenesis also occurs in the DR of adult mammals, and whether all the types of DR newborn cells are functionally integrated to support behavior and physiology during the postpartum period and other times of the reproductive lifespan (cf., Mohr & Sisk, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- New cells are added to the serotonergic dorsal raphe of adult females.
- Reproduction and maternal experience affect plasticity in the dorsal raphe (DR).
- There were contemporaneous changes in DR serotonin synthesis & metabolism.
- Dorsal raphe plasticity and changes in chemical output may promote postpartum behaviors.

Figure 1. Experimental time course to determine the effects of reproductive state on cell proliferation and survival

Adult female rats were injected with the mitotic marker BrdU as diestrus virgins, on day 19 of pregnancy (PD 19), or on day 7 postpartum (PPD 7). Females were sacrificed one day later to measure cell proliferation, or 12 days later to measure cell survival.

Figure 2. Experimental time course to determine the effects of corticosterone on cell survival
 Postpartum day 1 rats were sham operated (Sham) or adrenalectomized. Adrenalectomized dams received a very low dose of corticosterone in their drinking water to maintain neuronal health (ADX) or a high dose of corticosterone to reestablish normal postpartum levels (ADX + Cort). Rats were injected with the mitotic marker BrdU on days 7 and 8 postpartum and sacrificed 12 days later to measure cell survival.

Figure 3. Newborn cell proliferation and survival in the DR of virgin and reproductive female rats

A) Representative photomicrograph of BrdU-ir nuclei lining the lateral border of the cerebral aqueduct (aq). Scale bar = 50 μ m. B) Photomicrograph showing BrdU-ir nuclei lining the lateral border of the aq. Note that no NeuN immunoreactivity is seen in this proliferative niche, where mature neurons do not reside. Scale bar = 50 μ m. C) Representative photomicrograph of a pair of BrdU-ir nuclei in the DR. Scale bar = 20 μ m. D) Representative photomicrograph of BrdU immunofluorescence in the DR, bordered by the medial longitudinal fasciculi (mlf). E) Representative photomicrograph of NeuN immunofluorescence in the DR. F) Overlay of cells containing BrdU immunofluorescence (green), NeuN immunofluorescence (red), and colocalization (yellow) in the DR. Arrows indicate some colocalized nuclei. G) Effects of reproductive state on DR cell proliferation. H) Effects of reproductive state on newborn cells survival. Bars show means $\pm$ SEMs. Unique letters above bars indicate statistically significant differences between groups, $p < 0.05$. I) Representative photomicrograph of NeuroD immunofluorescence in the DR. Arrows indicate some NeuroD-immuno fluorescent cells.

Figure 4. Effects of reproductive state on DR cell death DR

A) Photomicrograph of TUNEL+ nuclei (red), NeuN immunofluorescence (blue), and NeuN/TUNEL colocalization (pink) in the DR. White frames indicate magnified insets. Scale bar = 100 μ m. B) Photomicrograph of TUNEL+ nucleus. C) Photomicrograph of a TUNEL+/NeuN-ir nucleus. D) Percentage of TUNEL+ cells that were NeuN-ir (colocalized nuclei/total TUNEL+ nuclei). E) Number of TUNEL+ nuclei in the DR of adult female rats. Bars show means + SEMs.

Figure 5. Effects of maternal experience and adrenalectomy on newborn cell survival and cell death in the postpartum DR

A) Effects of removing pups after parturition on 12-day survival of maternal DR cells born on PPD 7. B) Effects of removing the pups soon after parturition on cell death in the maternal DR. C) Effects of adrenalectomy and corticosterone replacement on litter weight gain. D) Effects of adrenalectomy and corticosterone replacement on the number of BrdU-ir nuclei in the postpartum DR. Bars show means + SEMs. Asterisks indicate significant differences between groups, $p < 0.05$

Figure 6. Effects of reproductive state on DR serotonin measures

A) Photomicrograph of serotonin immunoreactivity in the DR. Scale bar = 100 μ m. B) Effects of reproductive state on DR area covered by serotonin immunoreactivity. C) TPH2-ir Western blot bands from DR obtained from representative diestrus virgin, PPD 8 and PPD 19 rats. E–G) Effects of reproductive state on DR levels of serotonin's precursor (5-HTP), serotonin, and the serotonin metabolite (5-HIAA). Bars show means $\pm$ SEMs. Unique letters above bars indicate significant differences between groups, $p < 0.05$