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Serotonin-specific lesions of the dorsal raphe disrupt maternal aggression and caregiving in postpartum rats

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Abstract

The behavioral modifications associated with early motherhood, including high aggression, caring for young, and low anxiety are all affected by acute pharmacological manipulation of serotonin signaling. However, the effects on these behaviors of permanently disrupting serotonin signaling at one of its primary sources, the dorsal raphe (DR), have not been examined in detail. To address this, serotonin-specific lesions centered on the dorsomedial DR (DRdm; subregion most implicated in emotional behaviors) were induced at mid-pregnancy (day 15) or early postpartum (day 2) in rats using a saporin-conjugated neurotoxin targeting the serotonin transporter (Anti-SERT-SAP). Prepartum or postpartum Anti-SERT-SAP reduced DRdm serotonin immunoreactivity by ~40–65%, and postpartum Anti-SERT-SAP also reduced it in the ventromedial and lateral wings of the DR, as well as the median raphe. Serotonin-immunoreactive fibers were significantly reduced in the anterior hypothalamus, but not medial preoptic area, of lesioned dams. Pre- and postpartum lesions both greatly reduced maternal aggression, but while prepartum lesions did not affect later undisturbed maternal caregiving, the larger postpartum lesions prevented the postpartum decline in kyphotic nursing and reduced pup licking. Serotonin lesions did not affect pup retrieval, but the prepartum lesions temporarily increased maternal hovering over and licking the pups observed immediately after the disruptive retrieval tests. Dams' anxiety-like behaviors and litter weight gains were unaffected. These findings suggest that DRdm serotonin projecting to the AH is particularly critical for maternal aggression, but that more widespread disruption of midbrain raphe serotonin is necessary to greatly impair maternal caregiving. Postpartum anxiety may rely more on other neurochemical systems or different midbrain serotonergic cell populations.

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Keywords

aggression; anxiety; lactation; maternal behavior; pregnancy; serotonin

1. Introduction

The postpartum period is characterized by a number of behavioral and physiological modifications that include maternal protection and care of offspring, blunted anxiety, and the production of milk. The brain systems underlying these and other aspects of postpartum behavior and physiology are affected by many neurochemicals, including serotonin (5-HT). For example, maternal aggression is reduced by suppressing serotonin output from the midbrain raphe nuclei via infusion of an autoreceptor agonist, or by infusing serotonin receptor agonists into the dorsal periaqueductal gray (PAG) or corticomedial amygdala [1–4]. Furthermore, postpartum caregiving in mice is greatly disrupted by null mutation of *Pet-1* (the transcription factor necessary for differentiation of serotonergic neurons) or *TPH2* (the enzyme necessary for neuronal synthesis of serotonin) genes [5, 6], and caregiving by postpartum rats is impaired by acute administration of serotonin receptor agonists and antagonists [7–10]. Serotonin also influences postpartum affective behaviors, as indicated by lower anxiety after systemic injection of the 5-HT_{1A} receptor agonist, 8-OH-DPAT [11]. Finally, serotonin released in the ventromedial hypothalamus is essential for the elevated circulating prolactin necessary for milk production [12, 13].

The largest single collection of serotonergic cells in the brain is found in the midbrain dorsal raphe nucleus (DR) [14]. The DR projects to many forebrain regions [14–18], including sites involved in maternal behaviors and physiology such as the medial preoptic area, anterior hypothalamus (AH), paraventricular hypothalamus, nucleus accumbens, lateral septum, bed nucleus of the stria terminalis, and amygdala [19, 20]. Serotonergic neurons in the DR are affected by female reproduction and have a higher spontaneous firing rate during late pregnancy, parturition, and early lactation compared to during the estrous cycle [21]. Serotonin metabolism is elevated in the laboratory rat DR and some of its forebrain targets (*e.g.*, preoptic area, bed nucleus of the stria terminalis) during pregnancy and/or early lactation [22–24], and the maternal DR shows neuroplastic changes involving altered survival and death of newborn cells [22]. Such effects of female reproduction are presumably due to the DR expressing the receptors for numerous steroids and peptides - including estrogens, progestins, glucocorticoids, and oxytocin (OT) [25–30] - that are known to influence serotonin synthesis and release [31–35] as well as regulate the peripartum onset and later maintenance of motherhood [19, 20].

While serotonin influences postpartum maternal aggression, caregiving, and anxiety, there is not much known about serotonin specifically originating from the DR in the control of these postpartum behaviors. Early studies by Barofsky et al. [36, 37] explored the role of the DR in maternal behavior and lactation by infusing the serotonin-selective neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) and found high litter mortality in the serotonin-lesioned group. They attributed this to lower suckling-induced prolactin release, given that maternal caregiving appeared to be normal after lesioning. This result probably led to the three

decades of little interest in the DR as a possible regulator of postpartum behaviors. However, Borofsky et al.'s observations of maternal behavior were cursory, and maternal aggression and anxiety were not examined. In addition, 5,7-DHT is taken up not only by somata, but also the terminals, of serotonergic cells [38]. Thus, serotonin cells projecting to the DR from other midbrain serotonin-rich nuclei would have been inadvertently affected.

The goal of the current study was to test the hypothesis that serotonin originating from the DR influences a constellation of postpartum socioemotional behaviors in female rats. To accomplish this, we infused a saporin-conjugated antiserum targeting the serotonin transporter (SERT) to selectively destroy DR serotonergic neurons [39–42]. These infusions were performed either during pregnancy (prepartum experiment) or soon after parturition (postpartum experiment). We then observed the dams' maternal aggression, caregiving behaviors under undisturbed conditions, retrieval of scattered pups to the nest, and anxiety-related behaviors in an elevated plus maze and light-dark box. The results revealed that a prepartum or postpartum reduction in serotonin particularly in the dorsomedial DR impaired maternal aggression, that maternal caregiving behaviors were more greatly affected by larger postpartum DR serotonin lesions, and that dams' anxiety-like behavior and lactation were unaffected.

2. Methods

2.1 Subjects

Female Long-Evans rats descended from rats purchased from Harlan Laboratories (Indianapolis, IN) were born and raised in our colony, as described in detail previously [22, 24]. They were housed with 1 or 2 same-sex littermates in polypropylene cages (48 cm x 28 cm x 16 cm) with wood chip bedding. Food (Tekland rat chow, Indianapolis, IN) and water were freely available, and the room was maintained on a 12:12 light/dark cycle (lights on at 0700 h) at 22 ± 1 °C and relative humidity of 40–50%. Subjects were vaginally smeared daily and impregnated between 70–100 days old by being paired overnight on proestrus with male breeders from our colony. After mating, subjects were housed with one or two other pregnant females, then singly housed starting on pregnancy day 15 and given cotton nestlets that were replenished weekly until the start of behavior testing. Within 12 hr of parturition on postpartum day (PPD) 0, litters were culled to contain 4 males and 4 females. 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.

2.2 Stereotaxic Surgery

Stereotaxic surgery was performed on pregnancy day 15 (prepartum experiment; Figure 1a) or on postpartum day 2 (postpartum experiment; Figure 1b). Subjects were anesthetized with ketamine (90 mg/kg IP; Butler, Dublin, OH) and xylazine (8 mg/kg IP; Butler, Dublin, OH), given buprenorphine (0.015 mg/kg IP) as an analgesic, and placed in a Kopf stereotaxic apparatus. A small hole was drilled in the skull above the DR (A/P = 8.2 mm, M/L = 0.0 mm from bregma) and a saporin-conjugated antiserum targeting the serotonin transporter (SERT) was infused (Anti-SERT-SAP; Advanced Targeting Systems, San Diego, CA). For infusion,

a Neuros^{TM} Hamilton syringe was slowly lowered 6.7 mm ventral from the dorsal skull surface and 1 μ L of 0.1M anti-SERT-SAP or a control conjugate provided by the manufacturer slowly infused into the DR (prepartum experiment- 13 lesion, 9 control; postpartum experiment - 13 lesion, 11 control). The needle remained for 10 min and then slowly retracted. Scalps were closed with surgical staples and subjects received twice-daily subcutaneous injections of buprenorphine (0.015 mg/kg) for two days. The dorsomedial subregion (DRdm) was targeted as the center of the infusion because it sends serotonergic projections to a number of areas involved in maternal aggression, caregiving, and emotional behaviors including the medial prefrontal cortex, nucleus accumbens, medial preoptic area, lateral septum, bed nucleus of the stria terminalis, and anterior hypothalamus [44–48]. Moreover, the DRdm is unique in that some of its serotonergic neurons also produce corticotropin releasing hormone (CRH) [46], which could contribute to its role in postpartum behavior and physiology [49]. Most, if not all, DR serotonin somata express SERT while other cells there do not [43]. While terminals of serotonin cells originating elsewhere (*e.g.*, MR) project to the DR and express SERT, Anti-SERT-SAP has no mechanism allowing it to be retrogradely transported to destroy those projecting cells. Consistent with this, we have found that cortical infusion of Anti-SERT-SAP does not produce a long-term reduction in local TPH2 or SERT content (Holschbach and Lonstein, unpublished data). Anti-SERT-SAP has previously been infused into other raphe nuclei to examine serotonin's role in urethro-genital reflexes, baroreflexes, and ventilatory responses [40–42].

Prepartum subjects were left undisturbed after surgery until the day of parturition. During postpartum surgery, litters were fostered to surrogate dams and given back to their own mothers following their dams' recovery from anesthesia. One postpartum-lesioned subject attacked her pups after their return, so for all subsequent subjects in that experiment the pups were placed in their dams' cages two at a time after surgery and dams given more pups only after accepting the previous two. In two cases, the full litter was not returned until the following morning (one postpartum control and one postpartum lesion), but the full litter was in place at least two hr before undisturbed observations began that day. In both experiments, litters were weighed and fostered daily among subjects and surrogate dams from our colony to ensure that all dams were interacting with healthy, vigorous pups. Dams' body weight and core temperature (Dorel TH060 high-speed rectal thermometer held in place for 5 sec) were measured every other day, starting on postpartum day 3, as indicators of their general health.

2.3 Behavior Testing

2.3.1 Observations of undisturbed maternal behavior—Undisturbed observations of maternal behavior occurred for 30 min every morning between 9:00–10:00 hr and again in the afternoon between 14:00–15:00 hr on postpartum days (PPD) 1–7 (prepartum experiment; Figure 1 top) or on PPD 1 and then PPD 3–8 (postpartum experiment) (Figure 1 bottom). Dam behaviors were recorded every 30 sec and included both maternal (hovering over pups in the nest, nursing in upright kyphosis or while supine, licking pups, retrieving and short-distanced mouthing of pups, sniffing pups, nest-building) and non-maternal (self-grooming, eating/drinking, resting alone, exploring the home cage) activities [50]. Quality of

nests was scored during each observation on a scale of 0–3 (0 = no nest, 1 = poor nest, 2 = partial nest, 3 = complete nest with high walls) [51].

2.3.2 Retrieval of scattered pups—Because retrieval of young pups rarely occurs during undisturbed observations [52], litters were experimentally displaced from the nest to induce this behavior as an indicator of maternal motivation. Retrieval testing occurred immediately after the afternoon undisturbed observations on PPD3, PPD5, and PPD7. Litters were removed from the home cage for 15 min and placed in an incubator set at nest temperature (34 °C). The pups were then scattered in the home cage opposite the nest and latencies for the dams to retrieve each pup and then hover over them in the nest were recorded. If a subject failed to retrieve all 8 pups within 5 min, experimenters moved any remaining pups into the nest and a latency of 600 sec was given for each pup that was not retrieved. After all pups were grouped in the nest site (by the dam or by the experimenter) maternal behaviors were observed and recorded as described above every 30 sec for an additional 10 min.

2.3.4 Elevated plus maze—Between 15:00–16:00 hr on PPD8, dams with their litters were carried in their home cage to a nearby room for a 10-min test in an elevated plus maze [53]. The maze was 50 cm above the floor, made of black plastic with four arms emerging from a 10 × 10 cm center square. The arms were 50 × 10 cm, two open and two with 40-cm high walls. The open arms were illuminated by dim overhead light at 28 lx and the ends of the closed arms were at 2 lx. Subjects were quickly removed from their home cage and placed in the central square of the maze facing an open arm. The home cage containing the litter was brought to in an adjacent room. A Panasonic low-light-sensitive camera and Magnavox DVD recorded the maze from above. An experimenter in an adjacent room simultaneously scored subjects' behavior from a monitor and computerized data acquisition system. The percentage of time spent and percentage of entries made in the open arms were used as indicators of anxiety-like behavior, and the number of entries into closed arms as an indicator of general locomotion. Entries and exits from arms were scored when the subject's head and two front paws crossed into or out of an arm as described previously [54]. After testing, dams were removed from the maze and returned to their home cage containing their pups, then returned to the colony room. The elevated plus maze was cleaned with 70% ethanol and dried between subjects.

2.3.5 Light-dark box—Between 9:00–10:00 hr on PPD9, subjects with their litters were again carried in their home cage to the testing room for light-dark box testing as described previously [55]. The light chamber of the light-dark box was illuminated at 624 lx and the dark chamber at 3 lx. Subjects were placed in the light chamber of the box for 10 min and behavior was video recorded and scored from an adjacent room. Behaviors in the light-dark box scored included the duration of time spent in the light chamber, the number of full-body transitions between chambers, relative frequency of rears in the light chamber (# of rears × 100/time spent in the light chamber), number of stretches from the dark chamber into the light chamber, latency to enter the dark chamber, and latency to reenter the light chamber [56]. After testing, dams were removed from the light-dark box and returned to their home

cages with their litters, but remained in the behavior testing room until assessment of maternal aggression.

2.3.6 Maternal aggression—Four hr after light-dark box testing on the afternoon of PPD9, subjects received a 10-min test for maternal aggression toward a male intruder placed into the home cage with the litter present [57]. Intruders were experimentally naïve, post-pubertal (45–55 days old) male rats from our colony. Dams' aggressive behaviors toward the intruder (lateral threats, aggressive grooming, kicking, boxing, biting, and attacking [lunging, pinning, or biting]) and sniffing the male were videotaped and later scored [57]. Intruders were removed from the dams' cages immediately after the 10 min test and placed back in their home cages.

2.4 Sacrifice and Serotonin Immunohistochemistry

To determine the location and extent of the SERT-SAP lesions, 60 min after maternal aggression testing the subjects were overdosed with sodium pentobarbital and perfused with 200 mL saline followed by 250 mL 4% paraformaldehyde in 0.1 M sodium phosphate buffered saline (PBS, pH = 7.4). Brains were removed and post-fixed overnight in 4% paraformaldehyde in PBS. The following day, brains were placed in a 20% sucrose/PBS solution for 2–4 days. The midbrain and forebrains were separated, and the midbrains cut into 40- μ m thick sections. Sections were stored in a sucrose-based cryoprotectant at -20°C until immunohistochemical processing. Every fourth section of the midbrain was immunohistochemically processed to visualize serotonin [22]. Rinses were in 0.1 M Tris-buffered saline (TBS; pH = 7.4). Sections were incubated in 0.1% sodium borohydride in PBS for 10 min, 1% H_2O_2 /TBS for 10 min, blocked with normal goat serum with 0.3% Triton-X in TBS for 30 min, then incubated in 2% goat serum with 0.3% Triton-X/TBS solution containing a rabbit polyclonal anti-serotonin primary antiserum (1:7,000–10,000; Protos Biotech Corporation, New York, NY) at 4°C for 48 hr. Tissue was then rinsed in TBS and incubated for one hr in biotinylated anti-rabbit secondary antiserum (1:500; Vector Laboratories, Burlingame, CA). Following rinses in TBS, sections were incubated in avidin-biotin-horse radish peroxidase complex (Vector Labs, Burlingame, CA) solution for 90 min, then rinsed again in TBS and incubated in a solution of 3,3'-diaminobenzadine (DAB) and H_2O_2 in TBS for 10 min. Sections were rinsed, mounted onto microscope slides, and coverslipped.

To determine the effects of midbrain SERT-SAP lesions on serotonin-immunoreactive fibers in the anterior hypothalamus (AH) and medial preoptic area (mPOA), brain sites involved in aggression and caregiving [19, 58, 59], the forebrains from a randomly selected subset of the prepartum-lesioned and control dams ($n = 8/\text{group}$) were also sectioned and underwent serotonin immunohistochemistry as described above using a primary antiserum incubation of 72 hr. Due to a cryoprotection failure, the AH and mPOA from the postpartum-lesioned and control subjects could not be analyzed for serotonin fibers.

2.5 Microscopic Analysis

Slides were randomized and coded. Serotonin immunoreactivity in the DR was visualized with a Nikon E400 camera and photomicrographs were taken on every fourth section

through the rostrocaudal extent of the DR (~7.06 mm through -8.52 mm, 5–7 sections per subject). Photomicrographs of the MR were also taken in the same sections. Immunoreactivity was analyzed using NIS-Elements software. Each subregion of the DR was analyzed separately, with the dorsomedial and ventromedial DR corresponding to atlas plates 96–101, the DR lateral wings to atlas plates 95–100, and the caudal DR to atlas plates 102–104 of Paxinos and Watson [60]. Images were captured at 100x magnification and the average light level kept constant across sections. Boxes of a standardized size for each DR subregion and the MR were superimposed upon the images and the area covered by pixels above a standardized light threshold optimized to capture dark immunoreactivity was calculated using NIS Elements [61]. Degree of the lesion was determined by comparing the percent area of the selected region that was covered by serotonin immunoreactivity. In addition to its optical density, the relatively fewer and mostly non-overlapping serotonin-ir cells of the MR made them readily quantifiable, which was done to further determine the extent of the lesions. Serotonin-ir fibers were analyzed bilaterally in the AH (2 sections from atlas plates 34–36) and mPOA (3 sections from atlas plates 43–46 of Paxinos and Watson) [60] with NIS Elements by placing a standardized box ($435 \times 326 \mu\text{m}$) in the middle of the sites on each section. Fibers in both sites were relatively sparse, so instead of obtaining total optical density, the fibers were analyzed using a digital tablet to trace the length of each serotonin-immunoreactive fiber visible. Optical density of DR and MR serotonin immunoreactivity, number of serotonin-ir MR cells, or total length of all serotonin-ir fibers in the AH and mPOA, was summed between hemispheres and sections for each animal for each brain site, and the sums used for data analyses.

2.6 Data Analyses

Analysis of the brains indicated no loss of serotonin immunoreactivity anywhere in the DR or MR of 2 prepartum experiment and 4 postpartum experiment subjects from the intended lesion group, which may have been due to clogged Hamilton syringes. These animals were removed from the lesion groups and placed into the appropriate control groups, resulting in final *ns* of 11 prepartum lesions, 11 prepartum controls, 9 postpartum lesions, and 15 postpartum controls. Repeated measures ANOVAs involving test day and group as factors were used to analyze the data from the undisturbed observations (maternal and non-maternal behaviors), retrieval tests (latencies to retrieve each pup), number of pups retrieved, and the general health measurements (dam and litter weights, dam core temperatures). Independent samples two-tailed t-tests were used to analyze serotonin immunoreactivity in the DR, MR (% area covered above threshold, number of cells) and the two forebrain sites (fiber length), anxiety-related behaviors in the EPM and light-dark box, and maternal aggression. In a few cases where groups had unequal variances, Welch's t-tests were used. Statistical significance was indicated by $p < 0.05$.

3. Results

3.1 Serotonin immunoreactivity

As expected, prepartum or postpartum infusion of Anti-SERT-SAP reduced DR serotonin immunoreactivity (Figure 2A–D). The prepartum infusions produced a ~40% decrease in the optical density of serotonin immunoreactivity in the DR dorsomedial subregion ($t_{20} = 2.87$,

$p < 0.05$; Figure 2E) and a non-significant ~30% decrease in the ventromedial subregion ($t_{20} = 1.37$, $p = 0.18$). The lateral wings of the DR ($t_{20} = 0.546$, $p = 0.591$), caudal DR ($t_{20} = 0.51$, $p = 0.62$), and MR ($t_{20} = 0.53$, $p = 0.60$) were spared. In addition, the numbers of serotonin-ir cells in the MR were similar between the lesioned and control groups ($t_{20} = 0.74$, $p = 0.47$).

The postpartum DR infusion of Anti-SERT-SAP reduced the optical density of serotonin immunoreactivity by 64% the DRdm ($t_{19} = 2.94$; $p = 0.008$), and unexpectedly by 90% in the ventromedial DR ($t_{19} = 4.40$; $p < 0.001$) and 80% in the lateral wings ($t_{19} = 3.20$, $p = 0.005$; Figure 2F) compared to control dams. It did not affect the caudal DR ($t_{19} = 1.19$, $p = 0.25$). Postpartum Anti-SERT-SAP also reduced the optical density of serotonin immunoreactivity in the MR by almost 50% ($t_{19} = 2.35$; $p = .03$), but this appears to be due to a loss of serotonin-ir fibers rather than neurons, because the numbers of serotonin-ir cells in the MR were similar between the groups ($t_{19} = 0.50$; $p = 0.62$).

Prepartum infusion of Anti-SERT-SAP into the DR significantly reduced the total length of serotonin-ir fibers in the AH by 50% ($t_{14} = 2.54$; $p = 0.02$), but it did not significantly affect fiber length in the mPOA ($t_{14} = 0.61$; $p = 0.55$; Figure 3).

3.2 Maternal and litter health

There were no effects of prepartum or postpartum Anti-SERT-SAP infusion on daily litter weight gains, the dams' body weight, or the dams' core body temperature (Table 1).

3.3 Behavior

3.3.1 Undisturbed maternal behaviors—There was no significant main effect of prepartum DR infusion group (lesion or control), or interaction between group and postpartum time, on the frequency of any undisturbed maternal or non-maternal behavior measured. There were the expected significant main effects of postpartum time on some behaviors, though (Table 2).

In contrast, postpartum DR infusion of Anti-SERT-SAP affected numerous maternal behaviors during the undisturbed observations. Whereas the total frequency of nursing in any posture did not differ between groups ($F_{(1,19)} = 0.80$; $p = 0.38$), the lesioned group showed a significantly higher frequency of kyphosis ($F_{(1,19)} = 4.54$; $p = 0.05$; Figure 4A) and tended to nurse less often in a supine posture ($F_{(1,19)} = 3.39$; $p = 0.08$; Figure 4B) compared to controls. There was also a significant interaction between group and postpartum time on kyphosis, such that the control animals showed less kyphosis over time, but the lesioned females did not ($F_{(6,114)} = 2.58$; $p = 0.02$). Significant interactions between group and postpartum time on the frequency of hovering over the litter ($F_{(6,114)} = 2.03$; $p = 0.05$) and licking pups ($F_{(6,114)} = 2.59$; $p = 0.02$) were found, with the lesioned group showing these behaviors less often than controls on some days (Table 2). The postpartum lesions also reduced the frequency of self-grooming ($F_{(1,19)} = 5.69$; $p = 0.03$). Similar to the prepartum experiment, the frequency of most maternal behaviors changed across postpartum time (Table 2).

3.3.2 Retrieval of scattered pups—Prepartum DR infusion of Anti-SERT-SAP did not affect the dams' later latency to retrieve pups ($F_{(1, 21)} = 1.13$, $p = 0.30$), the total number of pups retrieved ($F_{(1, 21)} = 0.86$, $p = 0.36$), or the latency to begin hovering over the pups after retrieving them ($F_{(1, 21)} = 0.14$, $p = 0.71$; Table 2). However, during the 10-minute observations immediately following the retrieval tests, prepartum-lesioned dams had a higher frequency of pup-directed behaviors, including hovering over the litter ($F_{(1, 21)} = 5.50$, $p < 0.05$; Figure 4C) and licking them ($F_{(1, 21)} = 6.88$, $p < 0.05$; Figure 4D), compared to controls. Prepartum-lesioned dams also tended to explore their home cage less often during the 10-min observations following retrieval ($F_{(1, 21)} = 4.22$, $p = 0.053$) and there was a significant interaction between group and postpartum time on cage exploration following retrieval ($F_{(2, 38)} = 3.73$, $p < 0.05$).

Similar to the prepartum lesions, postpartum-lesioned dams retrieved their first pup ($F_{(1, 18)} = 3.23$; $p = 0.09$) and gathered the entire litter into the nest site ($F_{(1, 18)} = 0.35$; $p = 0.85$), as quickly as controls (Table 2). Unlike the prepartum-lesioned dams, postpartum-lesioned dams did not differ from controls in their frequency of any behavior scored during the 10-min observations following retrieval (data not shown).

3.3.3 Anxiety-like behavior—Prepartum DR infusion of Anti-SERT-SAP did not affect anxiety-like behaviors in either the EPM or the light-dark box, as indicated by the similar percentage of time that the two groups of dams spent in the open arms of the EPM ($t_{20} = 1.37$, $p = 0.19$), percentage of entries made into the open arms of the EPM ($t_{20} = 1.24$, $p = 0.23$), and time spent in the light chamber of the light-dark box ($t_{20} = 0.89$, $p = 0.38$).

Similarly, postpartum DR infusion of Anti-SERT-SAP did not affect the percentage of time dams spent in the open arms of the EPM ($t_{19} = 0.70$; $p = 0.50$), the percentage of entries made into the open arms of the EPM ($t_{19} = 0.70$; $p = 0.50$), or time spent in the light chamber of the light-dark box ($t_{19} = 0.29$; $p = 0.28$). Other behaviors in the EPM and light-dark box were also similar between the two groups in both experiments (Table 3).

3.3.4 Maternal aggression—Both the prepartum and postpartum serotonin lesions greatly reduced maternal aggressive behaviors toward the male intruders. Compared to controls, prepartum-lesioned dams showed an ~85% reduction in total attack time ($t_{20} = 2.46$, $p = 0.03$; Figure 5A), ~75% fewer attacks ($t_{20} = 2.93$, $p = 0.01$) and ~85% fewer pins ($t_{20} = 2.39$, $p = 0.03$). Prepartum-lesioned dams also showed a non-significant ~60 % reduction in the average duration of individual attacks ($t_{20} = 1.07$, $p = 0.30$; Figure 5C). Postpartum serotonin lesions also reduced maternal aggression, although to a smaller degree than the prepartum lesions, with lesioned dams showing a ~70% reduction in total attack time ($t_{19} = 1.98$; $p = 0.07$; Figure 5B) and a ~60% reduction in the average length of each attack ($t_{19} = 2.51$; $p = 0.03$; Figure 5D; Table 4).

4. Discussion

4.1 Serotonin lesions impair maternal aggression

Motherhood involves a number of salient behavioral characteristics that often include maternal aggression toward threats to the dams' litter and territory, interest in neonates and

maternal caregiving activities, and a modified affective state. Serotonin has been implicated in all of these, and while previous studies found that serotonergic lesions of the midbrain raphe nuclei increased pup mortality [36, 37, 62], the behavioral consequences of the lesions that contributed to this mortality remained largely unknown. We hypothesized that serotonin from the DR is an important modulator of postpartum behaviors because of the links between serotonin and socioemotional behaviors in non-parous rodents, and studies specifically showing that genetic or pharmacological manipulations of serotonin affect maternal aggression [1–4, 63], caregiving [5–10], and anxiety [11]. We predicted that serotonin-specific lesions in the DR would reduce maternal aggression, impair caregiving, and increase postpartum anxiety. We centered our SERT-SAP infusion at the DRdm because serotonin cells in this subregion influence emotional behaviors [46, 64, 65] and project to forebrain sites involved in maternal aggression and caregiving [17, 44, 45, 47, 48].

Our most significant finding was that depleting DRdm serotonin either prepartum or postpartum (the latter also extending outside the DRdm) produced similar impairments in maternal aggression. In both experiments, attack duration was reduced by over 60% and the results collectively suggest that 1) the onset of the lesion does not need to be temporally close to the time of maternal aggression testing (*i.e.*, our prepartum-lesioned dams did not recover by postpartum testing two weeks after surgery) and 2) the lesion does not need to extend beyond the DRdm to affect maternal aggression. This decrease in maternal aggression is consistent with a studies in male rats reporting that pharmacologically inhibiting DR neurons reduce inter-male aggression, and that aggressive males have more Fos in DR serotonin cells after an encounter compared to unaggressive males [66]. Similarly, dominant female hamsters have elevated Fos in DR serotonin neurons after an aggressive encounter with a subordinate female [67].

This literature is quite equivocal, though, and other research has shown that acutely stimulating serotonin 1A autoreceptors in the DR (which reduces DR activity) increases maternal aggression [1] and that serotonin receptor agonism in DR projections such as the prefrontal cortex, amygdala or periaqueductal gray can increase or decrease maternal attacks [1, 2, 4]. Meta-analysis reveals that most experiments increasing serotonin signaling find blunted aggression [68], but while this meta-analysis accounted for sex, only <10% of subjects were female. The analysis also did not take into account the subjects' hormonal status. This is critical even in males because serotonin depletion increases territorial aggression in gonadally intact or androgen-replaced castrated male rats and mice, but not in castrated males not given hormone replacement [69, 70]. Perhaps serotonin from the DR has a facilitatory effect on maternal aggression because dams are in a unique state of lactational diestrus, which is characterized by chronically low estrogens and low-to-rising progesterone [71, 72]. Furthermore, a sex difference in how serotonin affects aggression was recently found, with serotonin 1A receptor agonism in the anterior hypothalamus increasing attacks by female hamsters but decreasing them in males; systemic fluoxetine mimicked this sexually different effect [67]. Clearly, more research on how sex and gonadal hormone status affect serotonin's control of aggression would be valuable.

Our analysis of forebrain serotonin-ir fibers after prepartum SERT-SAP infusion into the DR found that lesioned dams had shorter total fiber length in the AH compared to controls,

suggesting that the AH is a particularly relevant target of DR serotonin for maternal aggression. The AH receives almost half of its serotonin innervation from the DR and the remainder from the MR [73–75]. Disrupting only the projection from the DR is apparently sufficient to reduce maternal aggression and studies selectively targeting the DRdm-to-AH serotonin pathway, perhaps using optogenetic stimulation or inhibition, would help confirm this. Most research on hypothalamic control of maternal aggression has focused on the ventromedial and paraventricular nuclei [76–82], but a bout of maternal aggression does increase immediate-early gene expression in dams' AH [83] and a male placed in the home cage increases Fos in aggressive postpartum mice but not in non-aggressive virgins [84]. On the other hand, there is a large literature on the AH, including some studies on its serotonin innervation, for aggression in male rodents [85, 86]. Serotonin produces some of its effects on male aggression by interacting with AH vasopressin [86, 87] and this may eventually be found for maternal aggression [88].

4.2 Serotonin lesions affect maternal caregiving

In addition to reducing maternal aggression, DR serotonin lesions caused abnormalities in some maternal caregiving behaviors. Unlike maternal aggression, the timing and/or extent of the lesions mattered for their effects on caregiving. Prepartum-lesioned dams temporarily showed more hovering over and licking the pups after retrieval testing compared to controls, but the groups were behaviorally similar during the undisturbed observations. In contrast, the postpartum lesions affected caregiving only during the undisturbed observations, such that lesioned dams nursed more often in kyphosis and licked their pups less on some test days compared to controls.

Two previous studies have examined the effects of DR lesions on postpartum maternal behavior and physiology [36, 89], but both studies left unanswered questions that we aimed to address. Barofsky and colleagues [36] injected 5,7-DHT into the DR after parturition and destroyed many, but not all, of its serotonergic cells. These lesions produced a ~60% decrease in serotonin content in the hypothalamus. Nursing appeared to be unaffected, but this was evaluated only on two postpartum days by determining whether three or more pups were attached to teats when dams were lifted by their tails. Barofsky and colleagues also reported that lesioning the DR did not affect pup retrieval. This is not surprising, given that dams were given 45 minutes to do so, and retrieval of 8 scattered pups usually takes control rats less than 5 minutes [90, 91]. Their DR lesions did reduce litter growth and led to offspring mortality, which in the absence of gross changes in maternal behavior, they concluded was due to impaired suckling-induced prolactin [36]. Localizing the effects they found to the DR is difficult because 5,7-DHT is retrogradely transported [92], so their lesions would have also destroyed serotonergic neurons projecting to the DR, which include MR cells that also provide serotonin to the forebrain [93–95]. In fact, 5,7-DHT lesions of the MR do produce cannibalism and temporarily impair retrieval [37].

A later study by Yurino and colleagues [89] induced radiofrequency lesions restricted to the rostral DR. They reported no effect on pup retrieval or the frequency of pup licking during 30-minute retrieval tests. These results are limited because their subjects were non-lactating, postpartum females that did not cohabitate with pups, but instead only interacted with them

during retrieval testing, so there were no measures of undisturbed mother-litter interaction or litter growth. Also, separation from pups causes bursts of caregiving in the first few hours after reunion [96–100], so Yurino et al.'s observations were in the context of very high maternal motivation that probably obscured any lesion effects that might have been found under undisturbed conditions [101, 102].

Consistent with these two previous studies, our experiments using relatively more extensive observations of mother-litter interactions and more restricted disruption of raphe serotonin found no lesion effects on retrieval even within a 5-minute test. Also consistent with Barofsky et al. [36], we found that both groups of dams spent a similar amount of time nursing. However, our behavioral analyses revealed that prepartum serotonin-lesioned dams spent slightly but significantly more time hovering over and licking the litter immediately after retrieval testing, which involved briefly removing the pups from the home cage and returning them. These behaviors were unaffected during undisturbed observations. As noted above, even a brief separation from pups elicits higher maternal caregiving upon reunion, so perhaps the prepartum-lesioned mothers spent more time hovering over and licking pups after retrieval testing because they were more aroused than controls by the separation from and reunion with the litter.

In contrast to the prepartum-induced lesions, the postpartum-induced lesions decreased licking during daily undisturbed observations. Yurino et al. [89] previously found that DR radiofrequency lesions did not affect post-retrieval licking, but they did not assess it while undisturbed. The lower licking by our postpartum-lesioned dams is consistent with previous work showing that genetic and pharmacological manipulations reducing serotonin signaling decrease licking and other maternal behaviors [6–9]. The differences between the prepartum and postpartum lesions in their effects on maternal licking (and nursing - see discussion below) may have been due to the timing of the lesion, such that inducing the lesion closer to behavior testing caused more impairment in caregiving because of insufficient time for the system to adapt or even regenerate [103–105]. It is also very possible that differences between the prepartum and postpartum studies on maternal behavior are due to the extent of the lesions. Despite using identical procedures (including the stereotaxic surgery, aliquot of Anti-SERT-SAP and its volume/dose), our postpartum lesions were more extensive and reduced serotonin immunoreactivity in the DRvm and DRlw, in addition to the targeted DRdm. We have no obvious explanation for this difference, but pre- and postpartum rats may differ in the abundance, affinity, or efficiency of DR SERT; the degree to which SERT is transported to the plasma membrane; or levels of extracellular serotonin that would affect competitive binding for reuptake. In fact, a study run in our laboratory contemporaneous to the present studies found that postpartum rats had ~30% more SERT in the DR compared to nulliparous virgin females [106].

Dams with postpartum, but not prepartum, DR serotonin lesions also spent more time nursing in the upright kyphosis posture and tended to spend less time nursing in a supine position. Kyphosis is the predominant nursing posture during early lactation and facilitates pups' ability to attach to teats, breathe, and suckle [107]. As pups age, they more independently achieve these tasks, allowing their dams' to nurse them more often while supine [107]. Our lesions prevented this decline in kyphosis and increase in supine nursing,

and this effect may have been more dramatic if the observations continued into late lactation when the pups were even older. DR serotonin could affect nursing posture by modulating both motor and sensory pathways. Lesioning serotonergic input to the motor cortex increases the neural activity required to initiate changes in movement, whereas stimulating the DR reduces this threshold [108]. The more extensive DR lesions in our postpartum study may have disrupted serotonin in the motor cortex enough to cause sustained bouts of immobile kyphosis for longer than normal. These lesions also may have prevented the postpartum plasticity in the somatosensory cortex [109–111], making dams less sensitive to changes in tactile input from their maturing offspring, thus preventing the progression from kyphotic nursing to a supine posture. A lack of shifting from kyphotic to supine nursing, plus a possible decrement in prolactin release after lesioning [36, 62, 112], may have been expected to reduce litter weights but our daily fostering of litters among lesioned, control, and surrogate dams to ensure litter health may have prevented such effects.

Brain sites that receive a DR serotonin projection and influence maternal caregiving behavior include the mPOA. Approximately 12–15% of all DR serotonergic cells project to the mPOA [113], and serotonin turnover [23] and serotonin 2A receptor expression [114] are higher in the mPOA of postpartum rats compared to nulliparae. We found that mPOA serotonin fiber length did not significantly differ between prepartum DR serotonin lesioned and unlesioned dams, despite some differences in their maternal behavior after retrieval testing. The mPOA receives about 20% of its serotonergic input from the DR, 50% from the MR [73, 115], and the remainder from other hindbrain sources [116–118]. Raphe lesions that damage the MR-to-mPOA serotonin projection may be necessary to greatly impair caregiving, and postpartum MR 5,7-DHT lesions do briefly impair dams' initiation of contact with and retrieval of pups [37].

4.4 Serotonin-specific DR lesions did not affect postpartum anxiety

The extensive literature on the DR's role in anxiety-like behaviors in non-parous animals is often contradictory. Some studies have shown that serotonin-specific DR lesions have no effect on anxiety [119, 120], while others find that these lesions reduce it [121]. Depleting forebrain serotonin can also be anxiolytic or anxiogenic depending on the lesion extent and subject's sex [122–124]. Female rats show low anxiety-related behavior during the first week postpartum, which requires high central GABA and oxytocin in the face of low norepinephrine [24, 125]. The present study was the first analysis of how this postpartum anxiety may be affected by prepartum or postpartum DR serotonin lesions, and we found no significant effects in an EPM or light-dark box. These negative results suggest that: 1) serotonin from DR subregions other than those affected by our lesions, such as the caudal DR, are more relevant for postpartum anxiety, 2) serotonin from outside the DR completely, such as from the MR, is sufficient, or 3) serotonin from any source is irrelevant for dams' anxiety. The second possibility seems the most likely because serotonin-specific lesions of the MR can reduce anxiety in male rats [122, 126, 127], suppressing MR serotonergic output with a serotonin 1A receptor agonist is anxiolytic [128, 129], and serotonin 1A receptor antagonists in the MR are anxiogenic [128, 130].

4.5 Conclusions

The present experiments and other research manipulating the central serotonergic system demonstrate that this neurotransmitter regulates numerous postpartum behaviors. Serotonin emanating from the DRdm and likely projecting to the AH is particularly relevant for maternal aggression, while serotonin from the DRdm along with nearby DR subregions have an additional role in regulating postpartum nursing postures and pup licking. More complete serotonin lesions of the DR, possibly along with lesions that affect serotonin cells in the MR, may be necessary to broadly reduce serotonin input to multiple forebrain sites and severely impair the entire suite of postpartum socioemotional behaviors.

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Highlights

- Prepartum serotonin-specific lesions of the DRdm impaired maternal aggression
- Larger postpartum DR serotonin lesions affected both aggression and caregiving
- DR serotonin lesions did not affect postpartum anxiety
- Serotonin fibers in the anterior hypothalamus were reduced by DRdm lesions
- Serotonin from DRdm to AH may be especially involved in maternal aggression

Figure 1.

Schematic representation of the experimental time course used to determine the effects of prepartum (top) and postpartum (bottom) Anti-SERT-SAP lesions of the DR on postpartum behaviors. EPM = elevated plus maze, LD = light-dark box.

Figure 2.

Representative photomicrograph of the DR after prepartum infusion of the control conjugate (A,B) or Anti-SERT-SAP (C,D). Relative optical density (Mean + SEM) of serotonin immunoreactivity in the DR from the (E) prepartum and (F) postpartum experiments. DRdm = dorsomedial subregion of the DR, DRvm = ventromedial subregion of the DR, DRlw = lateral wings of the DR, DRc = caudal DR. Scale bar = 100 μ m. * $p < 0.05$.

Figure 3. Total length (Mean + SEM) of serotonin-ir fibers in the AH and mPOA of prepartum control and Anti-SERT-SAP lesioned rats. * $p < 0.05$.

Figure 4.

Frequency (Mean + SEM) of (A) kyphosis and (B) supine nursing during undisturbed observations of maternal behavior by postpartum-lesioned and control dams. Frequency of (C) hovering over pups and (D) licking pups by prepartum-lesioned and control dams during 10-minute observations after retrieval tests. * $p < .05$.

Figure 5. Total attack duration (Mean + SEM) by dams in the (A) prepartum experiment and (B) postpartum experiment. Average duration of attack bouts (Mean + SEM) in the (C) prepartum experiment and (D) postpartum experiment. * $p < 0.05$, # $p = 0.07$.

Table 1

Postpartum body weight and temperature, and daily weight gain of pups (Means $\pm$ SEM), of dams receiving prepartum or postpartum Anti-SERT-SAP or control infusion into the DR. PPD = significant effect of postpartum day.

	Prepartum Control	Prepartum Lesion	Significant Effects	Postpartum Control	Postpartum Lesion	Significant Effects
Dam weight (g)	305.9 $\pm$ 7.7	307.6 $\pm$ 7.7	PPD	298.4 $\pm$ 6.8	296.2 $\pm$ 8.2	PPD
Dam temperature ($^{\circ}$ C)	37.6 $\pm$ 0.1	37.5 $\pm$ 0.1	-	37.8 $\pm$ 0.2	37.4 $\pm$ 0.2	-
Daily litter weight gain (%)	16.3 $\pm$ 0.7	16.1 $\pm$ 0.7	PPD	13.4 $\pm$ 0.9	13.4 $\pm$ 1.2	PPD

Table 2

Frequencies of maternal and non-maternal behaviors (Mean $\pm$ SEM) during undisturbed observations and pup retrieval tests in dams receiving prepartum or postpartum Anti-SERT-SAP or control infusion into the DR. Asterisks indicate significant differences compared to the appropriate control group. PPD = significant effect of postpartum day.

	Prepartum Control	Prepartum Lesion	Significant Effects	Postpartum Control	Postpartum Lesion	Significant Effects
<i>Undisturbed Observations</i>						
Hover Over	23 $\pm$ 3	25 $\pm$ 3	PPD	23 $\pm$ 2	21 $\pm$ 3	PPD, PPD x Lesion
Kyphosis	62 $\pm$ 4	60 $\pm$ 4	PPD	65 $\pm$ 3	76 $\pm$ 4	PPD, PPD x Lesion
Prone Nursing	8 $\pm$ 2	11 $\pm$ 2	-	8 $\pm$ 2	4 $\pm$ 2	PPD
Total Nursing	78 $\pm$ 4	80 $\pm$ 4	PPD	86 $\pm$ 3	90 $\pm$ 3	-
On Nest	100 $\pm$ 3	105 $\pm$ 3	-	109 $\pm$ 1	112 $\pm$ 1	PPD
Nesting	2 $\pm$ 1	3 $\pm$ 1	-	2 $\pm$ 1	3 $\pm$ 1	PPD
Licking Pups	12 $\pm$ 2	13 $\pm$ 2	PPD	12 $\pm$ 1	11 $\pm$ 2	PPD, PPD x Lesion
Self-Grooming	9 $\pm$ 2	10 $\pm$ 2	PPD	9 $\pm$ 1	6 $\pm$ 1	Lesion
Feeding	5 $\pm$ 1	5 $\pm$ 1	-	6 $\pm$ 1	4 $\pm$ 1	-
Exploring	4 $\pm$ 1	4 $\pm$ 1	-	3 $\pm$ 0	3 $\pm$ 0	-
Inactive	7 $\pm$ 2	3 $\pm$ 2	-	0 $\pm$ 0	1 $\pm$ 0	-
Nest score	2.6 $\pm$ 0	2.4 $\pm$ 0	-	2.4 $\pm$ .1	2.4 $\pm$ .1	PPD
<i>Retrieval Tests</i>						
Latency to retrieve 1 st pup (s)	8 $\pm$ 1	7 $\pm$ 1	-	6 $\pm$ 2	11 $\pm$ 3	-
Latency to retrieve 8 th pup (s)	80 $\pm$ 14	81 $\pm$ 14	-	83 $\pm$ 12	86 $\pm$ 15	-
Latency to hover over pups (s)	106 $\pm$ 16	115 $\pm$ 16	-	104 $\pm$ 11	122 $\pm$ 15	-
Number pups retrieved	8 $\pm$ 0	8 $\pm$ 0	-	8 $\pm$ 0	8 $\pm$ 0	-

Table 3

Anxiety-like behaviors (Mean $\pm$ SEM) in an elevated plus maze (EPM) and light-dark box displayed by dams receiving prepartum or postpartum Anti-SERT-SAP or control infusion into the DR.

	Prepartum Control	Prepartum Lesion	Postpartum Control	Postpartum Lesion
<i>Elevated plus maze</i>				
Total time in open arms (s)	128 $\pm$ 21	95 $\pm$ 16	97 $\pm$ 8	94 $\pm$ 15
% Time in open arms	26 $\pm$ 5	18 $\pm$ 3	29 $\pm$ 4	25 $\pm$ 5
Total entries into open arms	13 $\pm$ 2	10 $\pm$ 1	14 $\pm$ 1	10 $\pm$ 2
% Entries into open arms	42 $\pm$ 2	38 $\pm$ 2	42 $\pm$ 2	39 $\pm$ 3
Total entries into closed arms	17 $\pm$ 2	16 $\pm$ 1	16 $\pm$ 1	14 $\pm$ 2
Total arm entries	30 $\pm$ 3	26 $\pm$ 2	27 $\pm$ 2	25 $\pm$ 3
<i>Light-dark box</i>				
Total entries into light chamber	3 $\pm$ 1	4 $\pm$ 1	4 $\pm$ 1	5 $\pm$ 1
Total stretches into light chamber	14 $\pm$ 3	13 $\pm$ 2	18 $\pm$ 2	22 $\pm$ 3
Frequency of rears in light chamber	11 $\pm$ 3	13 $\pm$ 2	9 $\pm$ 2	8 $\pm$ 3
Latency to 1 st exit light chamber (s)	5 $\pm$ 1	5 $\pm$ 1	7 $\pm$ 1	5 $\pm$ 1
Latency to 1 st reenter light chamber	264 $\pm$ 80	266 $\pm$ 60	322 $\pm$ 62	226 $\pm$ 67
Total time in light chamber (s)	44 $\pm$ 14	60 $\pm$ 12	55 $\pm$ 14	49 $\pm$ 14

Table 4

Aggressive and non-aggressive behaviors (Mean $\pm$ SEM) displayed by dams receiving prepartum or postpartum Anti-SERT-SAP or control infusion into the DR.

	Prepartum Control	Prepartum Lesion	Postpartum Control	Postpartum Lesion
Attack latency (s)	155 $\pm$ 51	303 $\pm$ 55	209 $\pm$ 54	249 $\pm$ 81
Average attack bout duration (s)	2 $\pm$ 1	1 $\pm$ 0	6 $\pm$ 1	2 $\pm$ 1 *
Total attack duration (s)	27 $\pm$ 9	4 $\pm$ 1 *	36 $\pm$ 12	11 $\pm$ 4
Aggressive grooming duration (s)	1 $\pm$ 1	6 $\pm$ 4	1 $\pm$ 1	0 $\pm$ 0
Lateral threat duration (s)	6 $\pm$ 3	3 $\pm$ 2	19 $\pm$ 9	14 $\pm$ 6
Sniff intruder duration (s)	52 $\pm$ 4	69 $\pm$ 11	60 $\pm$ 5	67 $\pm$ 16
Attack Frequency	8 $\pm$ 2	2 $\pm$ 1 *	5 $\pm$ 1	3 $\pm$ 1
Bite Frequency	1 $\pm$ 1	0 $\pm$ 0	1 $\pm$ 1	1 $\pm$ 1
Pin Frequency	5 $\pm$ 2	1 $\pm$ 0 *	5 $\pm$ 2	3 $\pm$ 1
Kick Frequency	1 $\pm$ 1	0 $\pm$ 0	5 $\pm$ 2	5 $\pm$ 2

*
 $p < 0.05$