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Research report

Contact with infants modulates anxiety-generated *c-fos* activity in the brains of postpartum rats

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ABSTRACT

The postpartum period is associated with many behavioral changes, including a reduction in anxiety, which is thought to be necessary for mothers' ability to appropriately care for infants. In laboratory rats, this reduction in anxiety requires recent contact with pups, but areas of the brain where infant contact influences neural activity to reduce anxiety are mostly unknown. We examined *c-fos* expression in lactating rats whose pups were removed for 4 h to increase mothers' anxiety, or not removed to maintain low anxiety in mothers, followed by exposure to the anxiogenic stimuli of either brief handling or handling followed by exposure to an elevated plus maze. Control animals had their litters removed or not, but no further stimulation. A large number of neural sites traditionally implicated in regulating anxiety in male rats were examined, and similar to what is found in male rats, most showed increased Fos expression after handling and/or elevated plus-maze exposure. Litter presence before testing affected Fos expression due to handling or elevated plus-maze exposure only in the ventral bed nucleus of the stria terminalis, dorsal and ventral preoptic area, ventromedial hypothalamus, lateral habenula, and supramammillary nucleus. Contrary to expectations, prior litter presence was associated with more Fos expression in most of these sites after handling and/or elevated plus-maze stimulation, and only after such stimulation. These sites may be of particular importance for how sensory inputs from infants modulate anxiety and other mood states during the postpartum period.

1. Introduction

The postpartum period is a physically and emotionally demanding time of the reproductive cycle, and requires the coordination of numerous behavioral and physiological changes that maintain maternal homeostasis and promote offspring development. Reduced anxiety is one component of these changes, and occurs in both postpartum women and laboratory rats [for review see 43]. This reduction is critical for normal mother–infant interactions and infant development, because human or rodent mothers with high anxiety display altered or even completely aberrant maternal care [1,3,5,6,14,46–48,53,57,61,87–89,95].

The postpartum reduction in anxiety requires physical contact with offspring. In lactating women and rats, either suckling or non-suckling contact with infants can produce an anxiolytic effect [19,33,42]. Contact with infants must be recent, at least in rats, because anxiety-related behaviors in dams reverts back to the high levels found in diestrous virgin females if the litter is absent for even a few hours [42,59].

Areas of the brain affected by recent physical contact with infants to reduce anxiety in their mothers have not been explored in detail. Previous studies using peripheral or intracerebroventricular injections of receptor antagonists implicate numerous neurochemical systems, including GABA, norepinephrine, prolactin, and oxytocin [22,31,32,62,83,86], but do not address the specific sites of action. Lesion studies have been few, but reveal that the paraventricular hypothalamus is not involved in dams' exploration of a novel environment or freezing in response to a startling noise [64], and that destruction of the ventrocaudal periaqueductal gray further decreases dams' already low anxiety [45].

To explore where in the brain recent contact with pups might alter neural function in response to an anxiogenic experience, we examined *c-fos* expression in the brains of postpartum rats with low or high anxiety after they were subjected to brief handling or handing followed by exposure to an elevated plus maze. Low- and high-anxiety states were generated by permitting or preventing dams' recent contact with pups, respectively. The literature on the neural regulation of anxiety in females at any stage of the reproductive cycle is relatively small. Therefore, to help determine sites to be analyzed, we turned to the large literature on the neural control of anxiety in males, which implicates sites including the midline cortex, numerous areas of the hypothalamus, bed nucleus of the stria

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terminalis, septum, hippocampus, amygdala, and periaqueductal gray [9,10,52]. Parsimony suggests that the fundamental neural networks mediating anxiety-related behaviors are similar between the sexes, but that males and females are susceptible to different endocrine and sensory influences, including those resulting from physical contact with infants.

2. Materials and methods

2.1. Subjects

Subjects were 55 Long-Evans female rats born and raised in our colony, but descended from rats purchased from Harlan Laboratories (Indianapolis, IN). After weaning at 21 days old, subjects were housed in clear polypropylene cages (48 cm × 28 cm × 16 cm) with woods shavings for bedding in groups of 2–3 littermates per cage. Beginning at 70 days old, females' estrous cycles were monitored daily with a vaginal impedance meter (Fine Science Tools, Foster City, CA), and females in proestrus placed overnight with a Long-Evans male from our colony. Mating was confirmed the next morning by copulatory plugs underneath the males' cages. Mated females were removed from males' cages and rehoused with one or two other recently inseminated females until 4–5 days before the expected day of parturition. Females were then individually housed and remained so with their litters throughout the remainder of the experiment. Litters were culled to four males and four females within 24 h after birth. The day of birth was assigned as day 0 postpartum. Food (Purina Rat Chow) and water were continuously available, lights were on a 12:12 light/dark cycle with onset at 0800 h daily, and the ambient temperature was ∼22 °C.

2.2. Experimental design and behavioral testing

Dam and litter manipulation occurred between 0900 and 1000 h on day 7 or 8 postpartum. To minimize disturbance before testing, anyone other than the experimenter was prohibited from entering the colony room on the day of testing. We used a between-subjects, 2-factor design with litter presence before testing as one factor (litter removed or not removed) and subsequent anxiogenic stimulation as the other factor (no stimulation, handling, or handling followed by exposure to an elevated plus maze). This resulted in six groups with final samples sizes of 8 animals in each group; an additional 7 dams fell off the elevated plus-maze and were immediately removed from the study. Dams whose pups were not removed had the experimenter's hand placed in their home cage, and the pups picked up and immediately replaced, to control for perturbation caused by longer-term litter removal in females from the other condition. Four hours after litters were removed or not removed, experimental dams received one of two anxiogenic experiences—brief handling or handling followed by a 10-min exposure to an elevated plus maze (see immediately below).

Handled subjects were carefully carried across the hallway from our colony room to a 10 ft × 10 ft testing room illuminated by a single 100-W bulb. Subjects were removed from their cage and very briefly suspended by their tail over an elevated plus maze. Subjects were immediately returned to their home cage, which contained the pups or not, and the cage then returned to the colony room.

For groups receiving exposure to an elevated plus maze, dams were carried in their home cage to the same testing room described above. The elevated plus maze was elevated 50 cm from the floor, and made of black plastic with four arms emerging from a 10 cm × 10 cm center square [68]. Arms measured 50 cm × 10 cm, two of which were open, while the other two had 40-cm-high walls. Illumination was ∼28 lx on the open arms and ∼2 lx at the end of enclosed arms. Each subject was removed from their home cage and placed in the central square of the maze facing an open arm and released. The home cage was then moved to an adjacent room. A Panasonic low-light-sensitive video camera relayed images of the plus maze from a mirror suspended above the maze to a Panasonic videocassette recorder and monitor in an adjacent room. An experimenter simultaneously recorded subjects' behavior with a computerized data-acquisition system that allowed recording the time spent in the open arms and closed arms, and the frequency of entries into each arm. An entry was recorded when the female placed her head and both front paws into an arm. Time spent in none of the arms was scored as time spent in the center square. After testing, dams were gently removed from the maze, and returned to their home cage (which contained the pups or not), which was carried back to the colony. The elevated plus maze was cleaned with 70% ethanol and dried between subjects.

Groups of control dams had their pups removed in the morning, or not removed, but these dams were not handled or exposed to the plus maze. They instead remained in the colony room, unmanipulated in their home cages with or without their pups until sacrifice.

2.3. Immunocytochemistry

One hour after experimental dams were handled or exposed to the elevated plus maze, or 5 h after the morning litter manipulation for the unstimulated controls, subjects were overdosed with sodium pentobarbital and perfused through the heart with 150 ml saline followed by 150 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffered saline (PBS, pH 7.4). This time point was based on the maximal Fos protein expression in the rat brain following exposure to an elevated plus maze [17]. Brains were removed and postfixed overnight in 4% paraformaldehyde/PBS. On the following day, brains were placed in a 20% sucrose/PBS solution until sectioning. Each brain was cut into 35 μm sections and stored in a sucrose-based cryoprotectant at −20 °C until immunocytochemical processing.

Every third section through the brain was immunocytochemically processed similar to that previously described [39]. Free floating sections were rinsed between steps, with the exception of after blocking with normal goat serum, three times in Trisma-buffered saline (TBS; pH 7.6). Sections were first incubated in a 0.1% sodium borohydride/TBS solution for 15 min, 1% H_2O_2/TBS for 10 min, 20% normal goat serum with 0.3% Triton X-100 in TBS for 45 min, and 2% goat serum with 0.3% Triton X-100/TBS solution containing rabbit polyclonal anti-*c-fos* primary antibody (1:10,000; Santa Cruz Biotechnology, T-4037) at 4 °C for 48 h. Tissue was then incubated for 1 h in biotinylated anti-rabbit secondary antibody (1:500; Vector Laboratories, Burlingame, CA) in 2% goat serum, a solution of avidin–biotin complex (Vectastain Elite; Vector Laboratories) for 60 min, and then a solution of 3,3′-diaminobenzidine (DAB), nickel ammonium sulfate, H_2O_2, in TBS. Sections were rinsed with TBS, mounted onto microscope slides, lightly counterstained with Neutral Red, dehydrated, and coverslipped.

2.4. Microscopic analysis

Slides were randomized and coded so that experimenters were blind to group designation. Fos-IR cells were quantified at 200× magnification with the aid of a reticle placed on one ocular lens on a Nikon E400 microscope. Brain sites analyzed are shown in Fig. 1, and a few additional areas were examined but are not reported because there was no or very low Fos immunoreactivity in any of the groups (*i.e.*, CA3, medial habenula, and medial mammillary nucleus). For each site, Fos-IR cells were counted bilaterally within a standardized area from one section for each subject, and sections were chosen based on their correspondence to the appropriate plate from Swanson's atlas of the rat brain [81]. Cells with any level of immunoreactivity were quantified by a single observer (C.D.S.).

2.5. Data analyses

Arm entries lasting <1 s were considered keystroke mistakes and removed from data analyses. The brain of one handled dam that remained with her pups was not analyzable, and she was removed from the study. One dam that had her pups removed before exposure to the elevated plus maze was found to be an outlier in the number of Fos-IR cells in the VMH (Dixon's outlier test, $p < 0.05$), and was removed from the analysis of that site. Behavior in the elevated plus maze was analyzed with independent *t*-tests. The number of Fos-IR cells in each neural site was analyzed by a 2 × 3 ANOVA using pup condition (pups present or pups absent before testing) and test condition (no stimulation, handling, or handling followed by exposure to an elevated plus maze) as factors. Statistical significance for main effects and interactions was indicated by $p \leq 0.05$. Main effects were followed by Bonferroni-corrected post hoc analysis with $p \leq 0.016$ indicating statistically significant differences between groups. Pearson's *r* was used to examine correlations between Fos expression and behavior of the two groups of dams exposed to the elevated plus-maze.

3. Results

3.1. Elevated plus-maze behavior

Similar to our previous results [42], removal of the pups 4 h before testing increased dams' anxiety-related behaviors in the elevated plus maze compared to dams allowed physical contact with pups until testing. Dams with their pups removed spent a significantly lower percentage of time in the open arms of the elevated plus maze ($t_{14} = 2.36$, $p \leq 0.05$; Fig. 2A), and tended to have a lower percentage of entries made into open arms ($t_{14} = 1.84$, $p = 0.087$; Fig. 2B). The raw duration of time spent in the open arms was also reduced by litter removal (89 ± 20 vs. 33 ± 11 s; $t_{14} = 2.41$, $p \leq 0.04$), and the raw number of entries into open arms tended to be reduced (9 ± 2 vs. 5 ± 1; $t_{14} = 2.11$, $p = 0.054$). In contrast, the duration of time spent in the closed arms was somewhat higher for dams whose pups had been removed (445 ± 22 vs. 493 ± 18 s; $t_{14} = 1.71$, $p = 0.10$). Removal of the litter did not significantly affect dams' total time spent in the arms of the maze (open plus closed; 526 ± 9 vs. 534 ± 6 s; $t_{14} = 0.77$, $p \geq 0.45$), the number of entries into closed arms (11 ± 2 vs. 14 ± 1; $t_{14} = 1.43$, $p \geq 0.17$), or the total number of arm entries—although this tended to be lower for dams who had their pups removed (23 ± 2 vs. 16 ± 3; $t_{14} = 1.94$, $p = 0.07$).