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Research article

Comparison of c-Fos expression in brain regions involved in maternal behavior of virgin and lactating female mice

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HIGHLIGHTS

- Pup presentation induced more VTA expression of c-Fos in dams than in virgin females.
- Expressionof c-Fos is suppressed in the BNST of lactating females.
- Expression of c-Fos in the MPOA is constitutively high in lactating females.
- GABAergic neurons project from the MPOA to the BNST.

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ABSTRACT

Maternal care is indispensable for the survival of mammalian offspring. Although virgin female mice avoid pups, they actively display maternal behavior after parturition. To determine which brain regions are involved in the qualitative differences observed in the responses of virgin and lactating females to pups, we compared the expression of c-Fos, which is a marker of neuronal activation, in brain regions involved in regulating maternal behavior. Pup presentation increased the number of c-Fos-positive cells in both the ventrotegmental area (VTA) and nucleus accumbens to a greater extent in lactating females than in virgin females. The bed nucleus of striaterminalis (BNST), which innervates VTA neurons to regulate both aversive and rewarding responses, showed increased number of c-Fos-positive cells following pup presentation in virgin females, butnotin lactating females. On the other hand, the number of c-Fos-positive cells in the medial preoptic area (MPOA) increased in both virgin and lactating females. The number of c-Fos-positive cells in lactating females not presented with pups was high and similar to that in virgin females presented with pups. Moreover, c-Fos-positive GABAergicneurons projecting from the MPOA to the BNST was confirmed using a retrograde tracer Fluorogold in lactating females. Our results indicate that constitutive GABAergic modulation projecting from the MPOA may suppress the activity of BNST neurons and prevent avoidance responses to pups in lactating females.

1. Introduction

Maternal care, including lactation, pup licking, and grouping, is fundamental to the survival of mammalian offspring. In rodents, nulliparous females initially lack maternal behavior or display incomplete behavior, typically avoiding newborn pups, which sometimes includes infanticide [1]. However, female mice actively care for newborn pups soon after parturition. Thus, the response to pups is qualitatively different before and after parturition.

The mesolimbic dopamine system is a neuronal pathway in which dopaminergic neurons in the VTA project to the nucleus accumbens (NAc). Rewarding stimuli increase the activity of dopaminergic neurons in the VTA, whereas aversive stimuli mostly inhibit activity [2,3]. Conversely, modulation of dopaminergic neuron activity can induce avoidance or depression-like behavior [4,5]. Maternal behavior is a naturally rewarding behavior [6]. Interaction with pups increases dopamine release in the NAc of dams [7,8]. Additionally, electrical or neurotoxic lesions of the VTA or NAc [9,10], or injection of a dopamine receptor antagonist into the NAc [11] inhibits maternal behavior in dams. Thus, the mesolimbic dopamine system is crucial for the display of maternal behavior. However, research has not yet examined how VTA neurons respond

Abbreviations: BNST, bed nucleus of striaterminalis; CRF, corticotropin-releasing factor; LDTg, laterodorsal tegmental nucleus; MPOA, medial preoptic area; NAc, nucleus accumbens; PPTg, tegmental pedunculopontine nucleus; TH, tyrosine hydroxylase; VTA, ventrotegmental area.

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to pup presentation in virgin females. Dopaminergic neurons in the VTA may show different responses to pups when virgin and lactating females are compared.

Hormonal priming during pregnancy is crucial for the formation of mother-infant relationship [17]. Hormones such as progesterone, estradiol, and oxytocin cause functional changes in various regions of the brain [17]. The BNST and MPOA, from which neurons project to the VTA [12–14], are also regulated by those hormones during pregnancy, and are critical for maternal behavior [15,16]. There is also reciprocal innervation between the BNST and MPOA, and lesions to the lateral connection between the BNST and MPOA disrupt maternal behavior [18]. Although interactions between the BNST and MPOA are required for maternal behavior, neural pathways connecting them have not yet been characterized. Changes in these brain regions during pregnancy may also alter the interactions between these brain areas, and may therefore affect activity of dopaminergic neurons in the VTA.

In the present study, we compared the expression of c-Fos which is a marker of neuronal activation, in the VTA, NAc, BNST, and MPOA between virgin and lactating females. We hypothesized that GABAergic projections from the MPOA to the BNST are required for changing female responses to pups during pregnancy.

2. Materials and methods

2.1. Animals

BALB/cAJcl mice (Clea Japan, Tokyo, Japan, $N = 47$) were housed in shredded paper bedding with a pellet diet (Clea Japan) and water available ad libitum. The temperature ($22 \pm 2\,^{\circ}C$) and humidity ($35\% \pm 5\%$) in the laboratory were kept constant and a 12:12 h light:dark cycle was maintained with lights on at 7:00. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23; revised 1985) and were approved by the Animal Research Committee of the Obihiro University of Agriculture and Veterinary Medicine.

To prepare the lactating females, each virgin female mouse was housed together with a male mouse in a plastic cage ($182\,mm \times 260\,mm \times 128\,mm$; $l \times w \times h$) for 10 days, after which females were housed individually. The day that parturition was confirmed was defined as parturition day 0. The primiparous females with 5–8 litter size were subjected to the experiments, and completed the experimental tasks 3–5 days after the parturition.

2.2. Behavioral testing

All behavioral tests were performed between 15:00 and 19:00. Virgin females or lactating females with their pups were housed individually in the test cage ($225\,mm \times 338\,mm \times 140\,mm$; $l \times w \times h$) for at least 1 day before testing. The pups (three to five days old) were transferred from their home cage into another cage 6 h before testing. During testing, three pups were individually deposited into each corner of the test cage except the one closest to the nest. Virgin or lactating females were presented with unfamiliar or biological pups, respectively. During a subsequent 10 min observation, the following data were recorded: the latency to the female touching a pup, retrieval of the pups into the nest, and crouching over the pups. The retrieving and crouching behaviors are defined in our previous report [19]. If a virgin female injured a pup, the test was stopped.

2.3. Immunohistochemistry

Virgin or lactating females were presented with pups following the same procedures as for the behavioral test. After 1 h, the mice were intracardially perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS under isoflurane anesthesia. After postfixation, the brains were sectioned coronally at 40 μm thickness (Microslicer, DTK-1000, Dosakaem, Kyoto, Japan). According to the brain atlas [20], two sections were selected from each of the VTA, NAc, BNST, or MPOA at the following coordinates: 3.16 mm and 3.28 mm posterior to bregma for the VTA, 1.18 mm and 1.34 mm anterior to bregma for the NAc, 0.10 mm and 0.22 mm posterior to bregma for the BNST, 0.02 mm anterior and 0.10 mm posterior to bregma for the MPOA. According to the procedures reported previously [19], the sections were immunostained with the antibodies described in supplementary data. Images were obtained with a confocal laser-scanning microscope (C2+, Nikon, Tokyo, Japan), and then c-Fos-positive cells were counted in the bilateral areas of each section of the captured images (Supplementary Fig. 1). Mean values across the two sections were calculated for each subject.

2.4. Injection of fluorogold into the BNST

The lactating females were positioned using a stereotaxic apparatus (NARISHIGE, Tokyo, Japan) under 2,2,2-tribromoethanol (1.25 mg/10 g body weight, Sigma–Aldrich, St. Louis, USA) anesthesia. Using a 10 μl microsyringe (ITO, Fuji, Japan), 0.1 μl of 2% Fluorogold (Setareh Biotech LLC, Eugene, OR, USA) dissolved in sterile saline was injected into the right BNST at bregma-relative coordinates: posterior, 0.9 mm, right, 0.7 mm, and depth, 4.2 mm. After three days, brains were collected and sectioned, followed by the immunostaining with the antibodies described in Supplementary data.

2.5. Statistical analyses

All statistical analyses were performed using SPSS 16.0 software (SPSS Inc. Chicago, IL, USA). For the behavioral test, the latency to the lactating females touching the pups was analyzed using Student's *t*-test. The number of lactating females that retrieved or crouched over the pups was analyzed using Fisher's exact probability test. The number of c-Fos-positive cells was analyzed using two-way analysis of variance with the factors: parturition vs. pup exposure. According to whether parturition and pup exposure interact, the data from the VTA, BNST, and MPOA were analyzed using Bonferroni's tests, and the data from the NAc core and shell were analyzed using Tamhane tests. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Behavioral tests

There was no difference between groups in the latency to touching the pups (virgin females: 10.9 ± 9.2 s, lactating females: 9.5 ± 7.7 s). Eight of the nine lactating females crouched over and retrieved all three pups to the nest. However, four of the six virgin females did not retrieve any pups and two of the virgin females injured the pups by biting them. Although two virgin females retrieved one of the three pups to the nest, they did not crouch over the pup. The number of pups retrieved to the nest was therefore greater for the lactating females than for the virgin females (lactating females: 2.8 ± 0.2, virgin females: 0.3 ± 0.5, $p < 0.001$).

3.2. The analyses of c-Fos expression

In the VTA, there was no difference in the number of cells expressing tyrosine hydroxylase (TH), which is an enzyme necessary for dopamine synthesis, and in the expression level of TH