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Research report

Neonatal handling alters the structure of maternal behavior and affects mother–pup bonding

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HIGHLIGHTS

- Neonatal handling affects maternal care and alters mother–pups relationship.
- Handling desynchronizes mother–pup interactions by changing maternal behavior sequence.
- Neonatal handling induces sex-dependent changes in the nest odor preference.
- Handling affects CREB and BDNF levels in pup's olfactory bulb, in a sex-specific manner.
- Results suggest a differential olfactory learning and preference for nest odor in pups.

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ABSTRACT

During early life, a mother and her pups establish a very close relationship, and the olfactory learning of the nest odor is very important for the bond formation. The olfactory bulb (OB) is a structure that plays a fundamental role in the olfactory learning (OL) mechanism that also involves maternal behavior (licking and contact). We hypothesized that handling the pups would alter the structure of the maternal behavior, affect OL, and alter mother–pup relationships. Moreover, changes in the cyclic AMP-response element binding protein phosphorylation (CREB) and neurotrophic factors could be a part of the mechanism of these changes. This study aimed to analyze the effects of neonatal handling, 1 min per day from postpartum day 1 to 10 (PPD 1 to PPD 10), on the maternal behavior and pups' preference for the nest odor in a Y maze (PPD 11). We also tested CREB's phosphorylation and BDNF signaling in the OB of the pups (PPD 7) by Western blot analysis. The results showed that handling alters mother–pups interaction by decreasing mother–pups contact and changing the temporal pattern of all components of the maternal behavior especially the daily licking and nest-building. We found sex-dependent changes in the nest odor preference, CREB and BDNF levels in pups OB. Male pups were more affected by alterations in the licking pattern, and female pups were more affected by changes in the mother–pup contact (the time spent outside the nest and nursing).

1. Introduction

In mammals, the impact of leaving the safe uterine environment and facing many unfamiliar environmental stimuli and risks requires the protection of a caregiver. During the neonatal period, the mother is an interface between the newly born mammal and the environment and can powerfully shape infant development [1]. Early life is a critical phase for the nervous system, when the brain undergoes functional organization, neuronal proliferation, migration and differentiation, gliogenesis and myelination [2]. More than 50 years of study have explored the implications of changes in maternal behavior on neonatal programming and its persistent consequences on behavioral and neurochemical outcomes later in life [3–12].

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During early life, mother and infant establish a very close relationship. Auditory and visual sensory systems are heavily implicated in this process but during the neonatal period, olfactory learning is a key factor for the attachment establishment, especially in small-brain mammals such as rodents [13–19]. In rats, the pups learn how to identify the mother through a process similar to the classic paradigm of conditioning, involving tactile stimulation from the dam (unconditioned stimulus) and the dam's odor (conditioned stimulus) [16,17,19–21]. The olfactory bulb (OB) and the locus coeruleus (LC) are important structures in the olfactory learning mechanism [14,22–24], enabling the rat pup, born deaf and blind, to direct its behavior toward the mother [23]. Tactile stimulation can activate pups' LC, which increases Noradrenaline (NA) in the olfactory bulb mitral cells [22]. This association activates a chain of events in the mitral neurons of the pup's OB increasing the phosphorylation of CREB (cyclic AMP-response element binding protein), which is responsible for the transcription of a variety of factors (including brain derived neurotrophic factor [BDNF]) that lead to biochemical and morphological changes in memory formation [14,25–29]. BDNF appears to be a key factor in olfactory association learning [30]. BDNF gene expression increases in response to several stimuli, including neurotransmitters signaling and CREB phosphorylation [31]; for a review, see [32] and is critical in the OB morphologic development [33–38].

Neonatal handling is an experimental procedure that involves brief maternal separation and tactile stimulation, which is extensively used to investigate the effects of early life interventions on behavioral and endocrine alterations. This repeated disruption in the mother–pup relationship reduces fear [39], alters HPA axis (Hypothalamic-Pituitary-Adrenal) response to a variety of stressors [40–44] and may also affect social behaviors and fertility in both male and female rats [45–48]. In addition to the behavioral and neuroendocrine aspects of these changes, neonatal handling alters the brain plasticity and neurotrophic signaling, thus producing long-lasting structural changes [49–51].

Previous studies from our laboratory have shown that the handling procedure reduces the pup's preference for the nest odor in a sex depend way [45,48]. This lack of preference may be due to an alteration in the olfactory learning mechanisms; changes in the NA activity and CREB's phosphorylation in the OB of 7-day-old rat pups suggest that this hypothesis could be right [48]. BDNF is the perfect candidate to test this hypothesis since it is implicated in plasticity, dendritic branching, neuronal survival, migration and differentiation and axonal competition in this area during the neonatal period [33–38].

Other studies using early handling show that neurotrophic factors like BDNF play a key role in the establishment of these changes and also point out sex dependent changes [52–54]. However, is still not clear if the handling procedure could affect the neurotrophic signaling in the olfactory bulb of rat pups and if these changes could also present a sex dependent pattern like in other brain areas.

Therefore, our hypothesis is that neonatal handling alters the daily pattern of maternal behavior components beyond the licking component, and that these changes are part of the mechanism that alters BDNF gene expression through sex-dependent modifications in CREB phosphorylation and production in the pup's OB. For that, we analyzed the effects of handling on CREB phosphorylation and BDNF levels in the olfactory bulb of 7-day-old rat pups to verify whether the alterations in CREB phosphorylation are transient or are translated into protein alterations (changes in BDNF levels), which would indicate a more prolonged effect. Finally, expecting to associate the changes in maternal behavior with the pups' social behavior, we also analyzed the pups' social behavior with the nest odor preference test on PPD 11 to confirm whether the biochemical alterations in the olfactory bulb would affect social behavior already in early life.

2. Experimental procedures

2.1. Animals

Pregnant female Wistar rats were brought from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil) to the animal room in our laboratory. Approximately 7 days before delivery, the females were housed individually, and the presence of the pups was checked twice daily. Birth was considered to be day 0, and on postpartum day 1 (PPD 1), the number of pups was culled to 8 per dam by randomly removing a few pups while ensuring minimal contact with the remaining rats, the sex of the pups were not considered in this procedure. All of the animals were maintained on a 12-h light/dark cycle with the lights on at 6 a.m. The room temperature was $22 \pm 1\,^{\circ}C$, and water and food (Rodent chow, Nutrilab, Colombo, Brazil) were available at all times. Cage bedding was not changed from PPD 0 to 10. The experiments were performed in accordance with the National Institutes of Health (NIH) and Colégio Brasileiro de Experimentação Animal (COBEA) guidelines. These guidelines were designed to minimize the discomfort of animals and were approved by the Ethics in Research Committee of Federal University of Rio Grande do Sul (Process CEP/UFRGS nos. 2007937 and 19759) and followed Brazilian legislation.

2.2. Neonatal handling

Pups were handled for 1 min per day from PPD 1 to PPD 10 for behavioural studies while for Western blot analysis this procedure lasted until PPD 7. First, the home cage containing the mother and the litter was moved to a quiet room next to the animal facility and were given the same light period and temperature as described above. Then, the mother was removed from the home cage and placed into another cage. The experimenter gently handled all of the pups at the same time using both hands, covered with fine latex gloves, for 60 s. No apparent harm was inflicted to the pups; they were simply touched. After handling, all of the pups were taken to the nest at the same time, and the mother was placed back inside the home cage. The home cage was then returned to the animal facility room and left undisturbed until the same time the next day. The pups were handled during the light period of the daily photoperiod cycle (10:00–12:00) at a distance of approximately 100 cm from the mother. The total time of the mother–infant separation was approximately 90 s [39,45–48,50].

2.3. Experiments and groups

In the first experiment, we analyzed the effect of neonatal handling on maternal behavior. Litters were divided into 2 groups based on the handling procedure: the non-handled group or control group (NH, $n = 9$), in which the pups were left undisturbed with their mother during the first 10 postnatal days, and the repeatedly handled group (H, $n = 9$), in which the pups were handled as described above from PPD 1 to 10.

In the second experiment, the litters that originated from the experiment one (NH, $n = 9$; H, $n = 9$) were used for the odor of the nest preference test to analyze the social behavior of the pups on the PPD 11.

In the third experiment, the molecular mechanism in the OB related to maternal behavior was analyzed on PPD 7. Previous studies showed changes in the monoaminergic system after the handling procedure on that day [48]. A total of 48 pups (24 males and 24 females) from the two experimental groups described above (NH; $n = 12$ from each sex and H; $n = 12$ from each sex) were divided into 4 subgroups based on the time of tissue collection for Western blot analysis ($n = 6$ of each sex in all groups). Two samples of tissue