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Neonatal handling and the expression of immunoreactivity to tyrosine hydroxylase in the hypothalamus of adult male rats

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Abstract

Neonatal handling has long-lasting effects on behavior and stress reactivity. The purpose of the present study was to investigate the effect of neonatal handling on the number of dopaminergic neurons in the hypothalamic nuclei of adult male rats as part of a series of studies that could explain the long-lasting effects of neonatal stimulation. Two groups of Wistar rats were studied: nonhandled (pups were left undisturbed, control) and handled (pups were handled for 1 min once a day during the first 10 days of life). At 75-80 days, the males were anesthetized and the brains were processed for immunohistochemistry. An anti-tyrosine hydroxylase antibody and the avidin-biotin-peroxidase method were used. Tyrosine hydroxylase-immunoreactive (TH-IR) neurons were counted bilaterally in the arcuate, paraventricular and periventricular nuclei of the hypothalamus in 30-µm sections at 120-µm intervals. Neonatal handling did not change the number of TH-IR neurons in the arcuate (1021 ± 206, N = 6; 1020 ± 150, N = 6; nonhandled and handled, respectively), paraventricular (584 ± 85, N = 8; 682 ± 62, N = 9) or periventricular (743 ± 118, N = 7; 990 ± 158, N = 7) nuclei of the hypothalamus. The absence of an effect on the number of dopaminergic cells in the hypothalamus indicates that the reduction in the amount of neurons induced by neonatal handling, as shown by other studies, is not a general phenomenon in the brain.

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Environmental stimuli during the neonatal period have lasting effects on emotional behavior and reactivity to stress in adult animals (1,2). Neonatal handling has been used as an experimental model to examine the mechanisms by which early environmental changes could affect neural systems, leading to stable behavioral and neuroendocrine changes (3-6). This apparently harmless procedure in infancy reduces fear in novel environments (5-7) and the secretion of corticosterone, adrenocorticotrophin and prolactin in response to stressors in adulthood (4-6). Furthermore, preliminary results from our laboratory showed that neonatal manipulation reduced the number of neurons in the medial amygdala, the prefrontal cortex (8) and the locus coeruleus (9), and decreased the number of somatostatinergic neurons in the hypothalamic periventricular nucleus

(10). These results suggest that neonatal handling can induce morphological changes in brain areas that could be the cause for the reduced stress responsiveness observed in adult animals.

Dopamine, which belongs to the catecholamine group, is an important transmitter of the central nervous system in mammals related to the control of motor functions, behavior and neuroendocrine integration (11). Dopamine plays an inhibitory role in prolactin synthesis (11), has a stimulatory role in the synthesis of corticotrophin-releasing hormone (12), and is affected by stressors (13). On the other hand, previous results from our laboratory (8-10) showed that neonatal handling is related to a reduced number of neurons in areas that modulate hypothalamic-pituitary function. On the basis of these results, the purpose of the present study was to analyze the effect of neonatal handling on the number of dopaminergic neurons in hypothalamic nuclei using immunohistochemical detection of tyrosine hydroxylase, a key enzyme in the catecholamine synthesis. The present study is part of a series concerning the determination of possible morphological changes in the central nervous system that could explain the long-lasting effects of neonatal stimulation.

Male adult Wistar rats were divided into two groups: 1) nonhandled (control) and 2) handled during the neonatal period. On the day after birth (day 1), the number of pups was standardized at 8 per mother and the handling stimulation began. The litter and the mother in their home cage were taken to a room next to the animal facility, with the same light period and temperature. The pups were separated from the mother that was kept in a cage next to them and all of them together were gently handled with fine latex gloves for 1 min. After handling, all pups were returned to the nest at the same time and then the mother was placed back in the cage. This procedure was repeated from the first to the tenth day of life. The protocol has been used in our laboratory in behavioral and neuroendocrine experiments. It is basically the same procedure that other authors use with the same behavioral and hormonal results (5,6). After weaning (at 21 days), the males were placed together in groups of 4 per cage (41 x 34 x 17 cm). All animals were maintained under a 12:12-h light/dark cycle, the dark phase beginning at 5:30 pm and with water and food *ad libitum*.

At 75-80 days, the male rats were anesthetized with sodium thiopental (40 mg/kg, *ip*) and perfused through the heart with 100 ml phosphate-buffered saline (PBS) with heparin for 20 min, followed by 200 ml of 5% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4°C for 30 min. The brains were removed and postfixed for 4 h in the same fixing solution at 4°C and then immediately cryoprotected in a 30% sucrose solution diluted in 0.1 M PBS at 4°C, frozen in a container with isopentane in dry ice and kept in the freezer at -80°C. Coronal sections of the brain, 30 µm thick, were obtained with a cryostat (HM 505E, MICROM) at -15°C and collected serially at 120-µm intervals (1:5) from the beginning to the end of the arcuate (bregma: -2.12 to -4.30 mm), paraventricular (bregma: -0.80 to -2.12 mm) and periventricular (bregma: -0.26 to -3.60 mm) nuclei of the hypothalamus identified according to an atlas of the rat brain (14). The sections were mounted on slides covered with gelatin. In order to detect cells immunoreactive to tyrosine hydroxylase, the sections were incubated for 30 min at room temperature with a monoclonal antibody (clone TH-2) from Sigma (St. Louis, MO, USA) diluted 1:500 in 0.4% PBS-Triton X-100. Positive markings were detected using the avidin-biotin-peroxidase complex method with a secondary antibody biotinylated for 30 min and with peroxidase-conjugated streptavidin for 30 min using the Dako LSAB kit of Dako Corp. (Carpinteria, CA, USA) according to its protocol (15). The chromogen used was diaminobenzidine 3,3'-tetrahydrochlo-

ride (Sigma) diluted in PBS (6 mg/10 ml). The sections were then counterstained with Mayer hematoxylin. As a negative control, the sections were processed as previously described, but in the absence of the first antibody, which was replaced with 0.4% PBS-Triton. None of the slices of the negative control presented structures immunoreactive to tyrosine hydroxylase, confirming the specificity of the first antibody.

All cell bodies immunoreactive to tyrosine hydroxylase (positive marking) in each sampled section of a nucleus were counted on both sides of the brain. This quantification was performed separately by two observers, using a light microscope (Zeiss, Oberkochen, Germany) with a 40X lens and a 10X eyepiece with a 1-mm^2 reticule, which permitted an easier count. The total estimated number of neurons immunoreactive to tyrosine hydroxylase in each nucleus studied was calculated according to the formula Nt = ns x P, where Nt is the total number of estimated units in the structure, ns the number of units counted in all sampled sections of the nucleus, and P the period that the sections were sampled (16). An average of the number of cells counted by the two observers was calculated. The results are reported as means (± SEM) of the total estimated number of positively marked cells in each structure and were compared between the experimental groups using the Student *t*-test. The level of significance was set at $P<0.05$.

When adult male rats submitted to neonatal handling (1 min per day during the first 10 days of life) were compared to nonhandled animals, no differences were observed in the number of neurons immunoreactive to tyrosine hydroxylase in the arcuate nucleus (6 animals per group), in the paraventricular nucleus (N = 8 in the nonhandled and N = 9 in the handled group, respectively), or in the periventricular nucleus (7 animals per group) (Figure 1).

Previous studies (1-3) have shown that several procedures which stimulate pups during the neonatal period appear to affect the animal's reaction to stress stimulation in adult life, but not the basal quantities of hormones (5,6) or the number of corticotroph cells in the pituitary (17). Similarly, our results showed that neonatal handling induced no change in the number of neurons immunoreactive to tyrosine hydroxylase in hypothalamic nuclei of animals that were not submitted to new stressful stimuli before the cell count. However, we cannot rule out

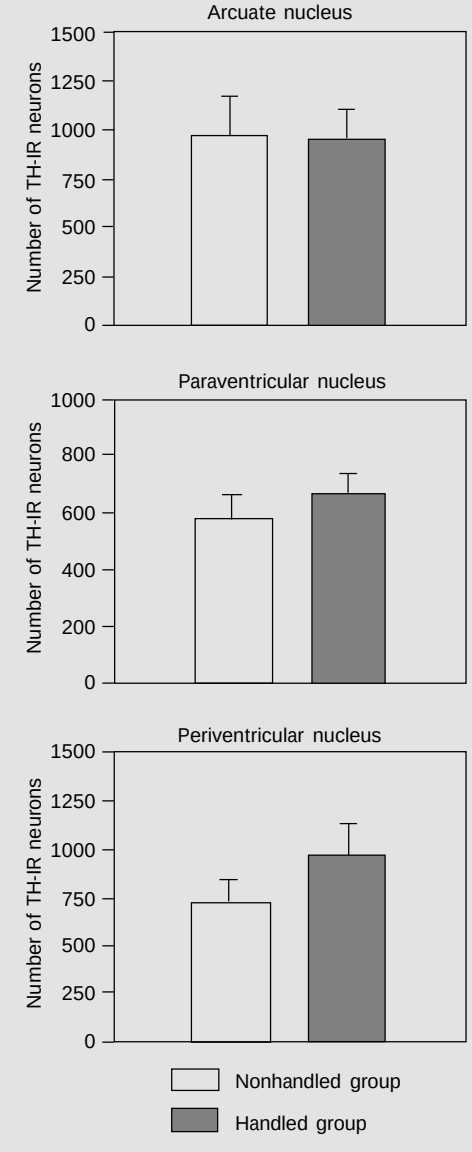

Figure 1. Effect of neonatal handling (1 min per day) during the first 10 days of life on the number of neurons immunoreactive to tyrosine hydroxylase (TH-IR) in the arcuate (N = 6 in each group), paraventricular (N = 8 in the nonhandled group and N = 9 in the handled group) and periventricular (N = 7 in each group) nuclei of the hypothalamus of adult (75-80 days old) male rats. Each bar represents the mean ± SEM of the estimated total number of TH-IR neurons in bilateral counts.

a possible modification in the amount of dopamine produced by these neurons, since studies to assess dopamine expression were not performed.

Adult rats that were handled in infancy present a less marked increase in corticosterone, corticotrophin and prolactin levels when faced with a stressing stimulus as compared with nonmanipulated animals (18,19). This may be occurring due to a reduction in the stimulatory role of the dopaminergic system, since some studies have shown that the stimulation of D1 and D2 dopaminergic receptors excites the neurons that produce corticotrophin-releasing hormone in the paraventricular nucleus of the hypothalamus (12). On the other hand, a previous study has suggested that the dopaminergic system is not related to the regulation of the response to stress (20). Thus, the change in the corticosterone response promoted by neonatal handling may be related to changes in pathways other than the dopaminergic one.

Although previous studies have reported reduction in the number of somatostatinergic neurons in the hypothalamic periventricular nucleus (10) and in the total number of neurons in the medial amygdala nucleus and in the prefrontal cortex of neonatally handled animals (8), the present study, using the same neonatal stimulation procedure, showed no significant change in the number of neurons immunoreactive to tyrosine hydroxylase in the hypothalamus. These results indicate that the reduction in the number of neurons induced by neonatal stimulation, as previously shown, is not a general phenomenon in the brain.

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