

Gentle handling temporarily increases c-Fos in the substantia nigra pars compacta

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Dopaminergic neurotransmission is involved in the regulation of sleep. In particular, the nigrostriatal pathway is an important center of sleep regulation. We hypothesized that dopaminergic neurons located in substantia nigra pars compacta (SNpc) could be activated by gentle handling, a method to obtain sleep deprivation (SD). Adult male C57/BL6J mice (N = 5/group) were distributed into non-SD (NSD) or SD groups. SD animals were subjected to SD once for 1 or 3 h by gentle handling. Two experiments were performed. The first determined the activation of SNpc neurons after SD, and the second examined the same parameters after pharmacologically induced dopaminergic depletion using intraperitoneal reserpine (2 mg/kg). After 1 or 3 h, SD and NSD mice were subjected to motor evaluation using the open field test. Immediately after the behavioral test, the mice were perfused intracardially to fix the brain and for immunohistochemical analysis of c-Fos protein expression within the SNpc. The open field test indicated that SD for 1 or 3 h did not modify motor behavior. However, c-Fos protein expression was increased after 1 h of SD compared with the NSD and 3-h SD groups. These immunohistochemistry data indicate that these periods of SD are not able to produce dopaminergic supersensitivity. Nevertheless, the increased expression of c-Fos within the SNpc suggests that dopaminergic nigral activation was triggered by SD earlier than motor responsiveness. Dopamine-depleted mice (experiment 2) exhibited a similar increase of c-Fos expression compared to control animals indicating that dopamine neurons are still activated in the 1-h SD group despite the exhaustion of dopamine. This finding suggests that this range (2-5-fold) of neuronal activation may serve as a marker of SD.

Key words: Sleep deprivation; Dopamine; c-Fos; Substantia nigra pars compacta; Gentle handling of mice

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Introduction

Dopamine (DA) has been identified as a key substance in the regulation of sleep-wake states (1-7). DA is critically involved in regulating complex movements and emotions (8). Therefore, altered central dopaminergic neurotransmission has been implicated in important neurological and psychiatric disorders such as Parkinson's disease and schizophrenia.

Impairment of the nigrostriatal pathway is primarily associated with Parkinson's disease (9). This pathway,

running from the dorsal striatum to the motor cortex and back to the cortex via the thalamus, plays a role in the control and initiation of voluntary movements (10). In patients with Parkinson's disease, the loss of dopaminergic cells within the substantia nigra pars compacta (SNpc) and, to a lesser degree, within the ventral tegmental area (VTA) correlates with increased diurnal sleepiness. This sleep fragmentation is exacerbated by dopaminergic D₂ receptor agonists (11,12). In contrast, clinical evidence demonstrates a transient restoration of motor control in Parkinson's disease patients during REM/paradoxical

sleep, indicating a strong participation of DA in this sleep phase (13). Additionally, evidence from animal models has established that, in rats, SNpc neurons are involved in the regulation of sleep patterns, particularly paradoxical sleep (5). Involvement of DA has been reported after paradoxical sleep deprivation (SD) (7, 14-17) and was shown to generate D₂ receptor supersensitivity (7, 18).

A robust increase in the electrophysiological activity of the dopaminergic neurons of the VTA has been documented during paradoxical sleep (19). In parallel, a specific lesion of 50% of the neurons present within the SNpc causes sleep disruptions (5) in a rodent model of Parkinson's disease. These experimental observations suggest a role for the dopaminergic system in the regulation of sleep patterns. Furthermore, dopaminergic VTA neurons appear to become maximally active via c-Fos expression during paradoxical SD, indicating that c-Fos up-regulation could be a marker of DA activity (20).

These findings support the hypothesis that dopaminergic neurons within the SNpc are selectively activated after total SD. This would suggest that dopaminergic neurons of the SNpc could be important for the generation of paradoxical sleep. To test this hypothesis, we examined c-Fos protein expression in the SNpc after two distinct experimental conditions: SD for 1 or 3 h, and SD plus reserpine-induced dopamine depletion. Additionally, behavioral motor activity was determined at each time in order to evaluate dopaminergic modulation resulting from SD and reserpine treatment.

Material and Methods

Subjects

All experiments were conducted in accordance with National Institutes of Health (USA) guidelines for the care and use of animals and with an animal protocol approved by the Universidade Federal de São Paulo Ethics Committee for Animal Experimentation (#0628/08). Male C57BL/6J mice weighing 25-35 g at the beginning of the experiments were used. Mice were housed 5 per cage and maintained under standard laboratory conditions (22 ± 2°C, 12-h light/dark cycle, lights on at 7:00 am) with food and water provided *ad libitum*.

Experimental design

Experiment 1. This experiment was designed to determine the possible activation of SNpc neurons after 1 and 3 h of SD. Fifteen mice were distributed equally into three groups: non-sleep deprived (NSD), sleep deprived for 1 h (1-h SD) and sleep deprived for 3 h (3-h SD). The SD groups were handled gently for 1 or 3 h once while the NSD

group was allowed to sleep. After the periods of SD, the animals were submitted to an open field test and histological evaluations.

Experiment 2. This experiment challenged the dopaminergic system by administering reserpine (2 mg/kg, intraperitoneally) to deplete neuronal stores of DA. Twenty-five mice were distributed equally into five groups: saline NSD, saline SD, reserpine NSD, reserpine 1-h SD, and reserpine 3-h SD. Motor and histological evaluations were performed similarly to Experiment 1.

Open field test

In order to assess general motor behavior, the open field test was applied immediately after the SD periods. The animal was placed in the center of a circular arena (40 cm in diameter, divided into 19 squares, and surrounded by a 40-cm high wall) and observed for 5 min. Locomotion frequencies at the periphery (in the 12 squares adjacent to the wall) and in the center (in the seven squares not adjacent to the wall) were quantified by counting the number of inter-square lines crossed. An entry into a square was counted once the mouse had entered a new square with all four paws. Immobility time (number of seconds of lack of movement during testing) during the trial and rearing frequency (number of times the animals stood on their hind legs) were also recorded. The apparatus was washed with 5% ethanol before behavioral testing to eliminate possible bias due to odors left by previous mice.

c-Fos immunohistochemistry

Mice were deeply anesthetized with ketamine immediately after the behavioral test and were intracardially perfused with saline followed by 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed from the skulls and were immersed for 1 week in formaldehyde at 4°C. Subsequently, the brains were placed in 30% sucrose solution for 48 h before sectioning. Four series of 30-µm coronal sections were cut on a cryostat from the caudal diencephalon to the caudal midbrain. Sections were incubated overnight at 4°C with rabbit anti-c-Fos primary antibody (1:500 in PBS plus 0.3% Triton X-100; antibody catalogue #AB038, Chemicon, USA). Sections were then incubated with a biotin-conjugated secondary antibody (1:200; catalogue #S-1000, Vector Laboratories, USA) for 2 h at room temperature. After several washes with PBS, the antibody complex was localized using the ABC system (Vectastain ABC Elite kit, catalogue #PK6101, Vector Laboratories) followed by reaction with 3,3'-diaminobenzidine with nickel enhancement. Slides were then dehydrated in increasing ethanol concentrations and cleared in xylene. An adjacent series was stained with cresyl violet to serve

as a reference series for cytoarchitectural purposes.

Quantification of dopaminergic neurons

In order to estimate the number of immunoreactive c-Fos neurons in the midbrain, the cells were manually counted by means of a reticular field. A neuroanatomical map was constructed to locate the dopaminergic areas in the ventral midbrain (21,22) and to identify the SNpc neurons expressing c-Fos (Figure 1). Counts were done on 8-10 tissue sections (one in four series), and an average count per section was determined for each animal. The selected areas were digitized with a DP71 digital camera (Olympus Optical Co., Japan) using an Olympus BX50 microscope.

Statistical analysis

Differences in cell counts and behavioral data were assessed by ANOVA followed by the Tukey test. Differences were considered to be significant if $P < 0.05$. Data are reported as mean \pm SEM.

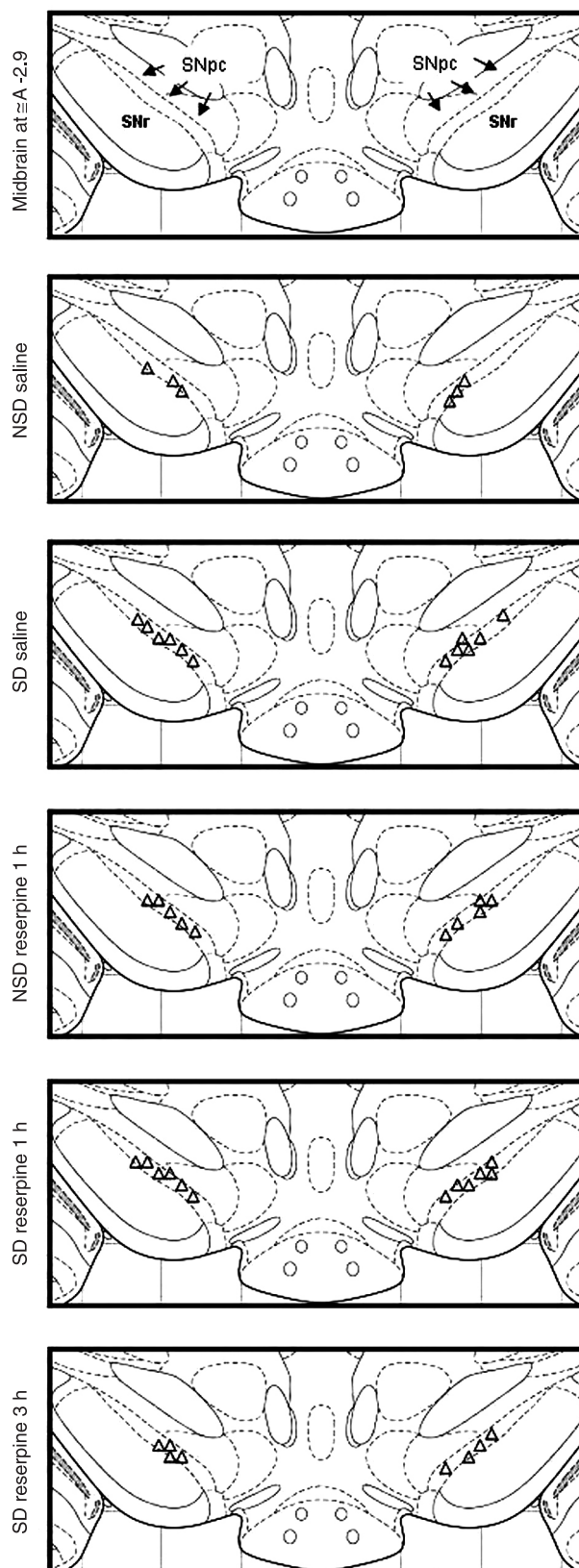
Results

In the open field test there was no statistical difference in total locomotion between 1-h SD, 3-h SD, and NSD (control). Rearing frequency, a motor parameter correlated with exploratory behavior, was also not different for 1-h SD and 3-h SD compared with the NSD group. Likewise, the immobility time was not statistically different for the 3 groups.

Bilateral quantitative analyses showed that the 1-h SD group displayed a significant increase in c-Fos-immunoreactive neurons ($F_{(7,22)} = 7.615$; $P < 0.01$) compared with the NSD and 3-h SD groups (Figures 1 and 2A). While 1-h SD displayed c-Fos induction within the SNpc in comparison to NSD, the 3-h SD group did not.

The motor behavior analysis demonstrated that the reserpine NSD, reserpine 1-h SD and reserpine 3-h SD groups exhibited similar and significant reductions ($P < 0.001$) in locomotor frequency compared with the saline SD group ($F_{(4,20)} = 8.094$; $P < 0.01$; Figure 3A). Similarly, rearing frequency was reduced in the reserpine NSD,

Figure 1. Computerized atlas section through the midbrain (at -2.9 mm from bregma). Dopaminergic c-Fos-ir neurons were mapped in the SNpc in representative animals from the groups depicted in the figure. Each triangle represents a cluster of 5 neurons. SNpc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; c-Fos-ir = immunoreactive c-Fos; SD = sleep deprivation; NSD = non-SD.



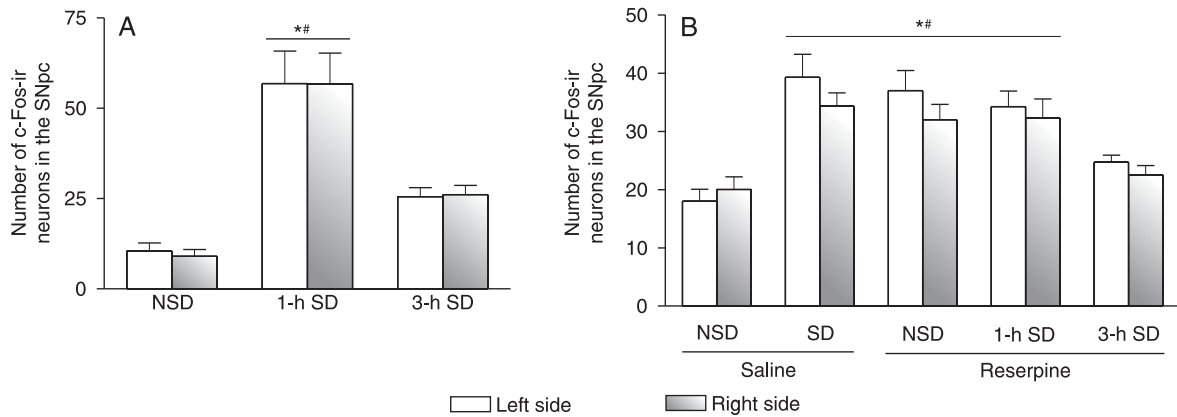


Figure 2. Sleep deprivation (SD) for 1 h produced immediate c-Fos induction within the substantia nigra pars compacta (SNpc). *A*, Bilateral quantitative analyses of c-Fos-ir neurons for the SD and non-SD (NSD, control) groups; *B*, bilateral quantitative analyses of c-Fos-ir neurons for the SD and NSD groups treated with saline or reserpine (2 mg/kg). Data are reported as mean \pm SEM for 5 mice in each group. * $P < 0.001$ compared to the NSD group; # $P < 0.01$ compared to the 3-h SD group. Data were analyzed by ANOVA followed by the Tukey test. c-Fos-ir = immunoreactive c-Fos.

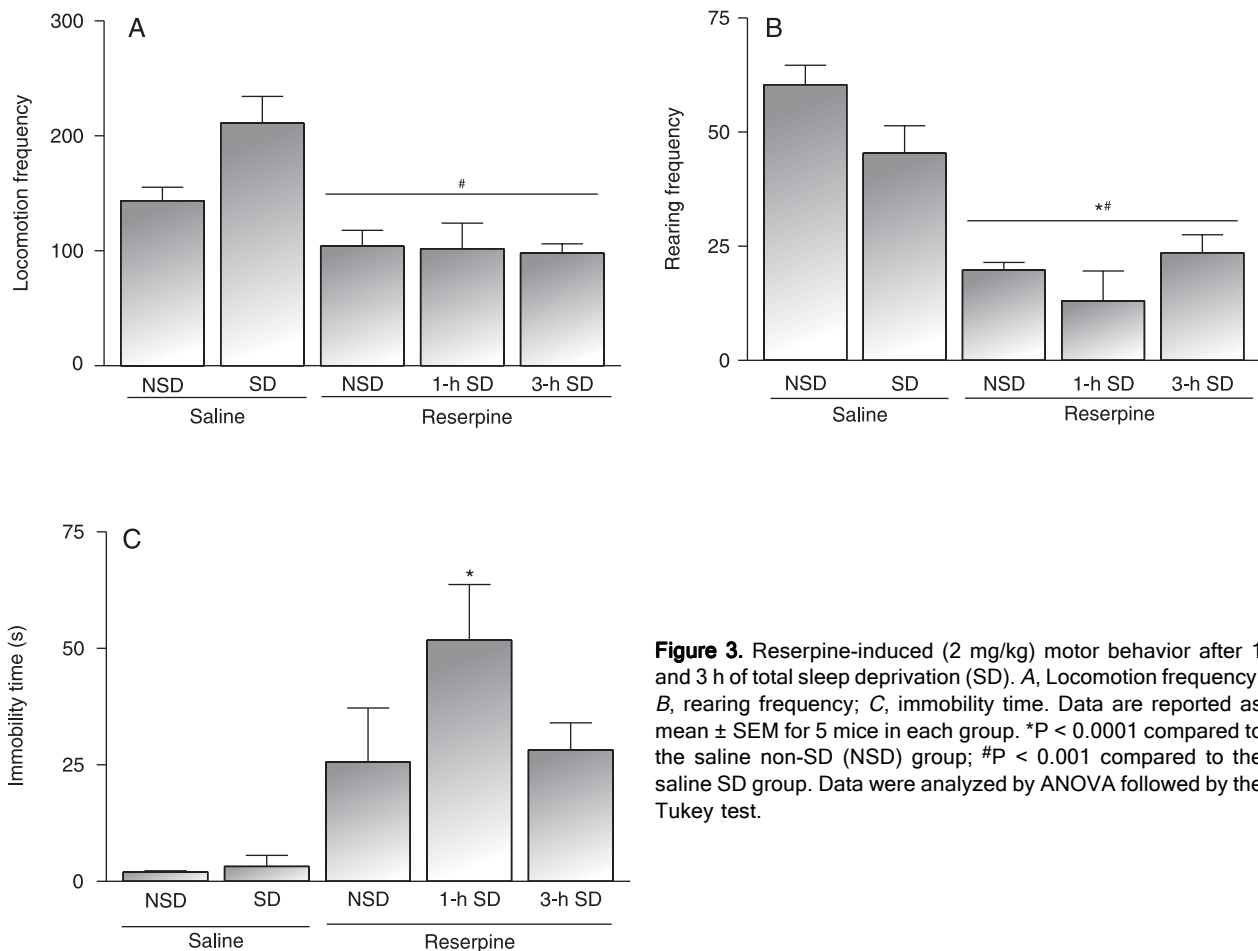


Figure 3. Reserpine-induced (2 mg/kg) motor behavior after 1 and 3 h of total sleep deprivation (SD). *A*, Locomotion frequency; *B*, rearing frequency; *C*, immobility time. Data are reported as mean \pm SEM for 5 mice in each group. * $P < 0.0001$ compared to the saline non-SD (NSD) group; # $P < 0.001$ compared to the saline SD group. Data were analyzed by ANOVA followed by the Tukey test.

reserpine 1-h SD and reserpine 3-h SD groups compared with the saline NSD ($P < 0.001$) and saline SD groups ($P < 0.01$; $F_{(4,20)} = 16.752$; $P < 0.0001$; Figure 3B). In contrast, immobility time was significantly increased ($P < 0.01$) in the reserpine 1-h SD group compared with the saline NSD group ($F_{(4,20)} = 6.659$; $P < 0.01$; Figure 3C).

More c-Fos protein was detected in the saline SD, reserpine NSD and reserpine 1-h SD groups than in the saline NSD group ($F_{(9,20)} = 6.776$; $P < 0.001$; Figures 1 and 2B). Moreover, c-Fos expression was also increased compared with the reserpine 3-h SD group ($P < 0.001$; Figures 1 and 2B).

Discussion

The findings presented here indicate that 1 h of SD can temporarily induce c-Fos expression in the SNpc. The same magnitude of induction was not observed after 3 h of the same manipulation. We did not observe statistically significant differences in motor behavior between the groups analyzed. These data indicate that 1 or 3 h of SD were not able to produce dopaminergic supersensitivity, a well-documented consequence of longer periods of paradoxical SD (7,18,23). Nevertheless, the increased expression of c-Fos within the SNpc suggests that the dopaminergic nigral activation is triggered earlier by SD than is motor responsiveness. In fact, reserpine-induced DA depletion showed that DA neurons were still activated in the 1-h SD group despite DA exhaustion.

Evidence from the literature indicates that 3-6 h of SD produces massive induction of c-Fos protein in several brain areas: medial preoptic area, anterior and posterior cortex, paraventricular thalamic nuclei, amygdala, caudate putamen and laterodorsal tegmental nucleus (24). Moreover, a more prolonged period of SD (50 h) followed by 3 h of sleep rebound resulted in an important up-regulation of c-Fos protein in dopaminergic areas such as the VTA (20). For this reason, the increased dopaminergic neuronal activity during a period of elevated paradoxical sleep concentration strongly suggests that DA is an important regulator of paradoxical sleep. Moreover, this DA activation was detected predominantly within the confines of the SNpc, reinforcing the suspected role of this area in paradoxical sleep generation (5).

Characteristically, neuronal activation mediated by c-Fos levels can be increased within 0.5 h of a discrete stimulus. Activation typically peaks in 1-2 h, then gradually declines over the next few hours (25,26). Contributing to this decline is the fact that increases in c-Fos protein levels inhibit further transcription of *c-fos* mRNA (26). Immediate early gene products observed after 3 h of SD might,

therefore, reflect the cumulative neuronal response to slowly increasing "sleep debt" over the preceding 0.5-3 h (24).

Our results are in accordance with recent studies suggesting the importance of dopaminergic areas, especially VTA and SNpc, in the promotion and maintenance of sleep, particularly paradoxical sleep (5,6,20). Our data suggest that the dopaminergic activation elicited by 1 h of SD indicates participation of the SNpc in the triggering of REM sleep. This activation did not provoke a behavioral motor change, although longer periods of SD can do so (7,23). Moreover, the neuronal population resident within the SNpc has an important compensatory capacity, allowing it to overcome different degrees of damage. These neurons also respond to and can be rescued by different pharmacological treatments (27-29). For this reason, we suggest that the dopaminergic nigrostriatal system is more susceptible to sleep-related manipulations than are motor functions. Notably, nigrostriatal responses are dependent on the degree and duration of stimulus.

Reserpine-induced DA depletion is known to produce consistent motor deficits (30), suggesting that it is a reliable animal model of Parkinson's disease (31). The motor behavior observed in the present study corroborated that of previous research (31). In addition, SD did not reverse the motor impairment caused by reserpine, supporting the data from the first experiment showing that 1 or 3 h of total SD did not produce dopaminergic supersensitivity resulting in detectable behavioral changes. Regarding neuronal activation, DA-depleted mice exhibited a similar increase of c-Fos expression compared to non-DA-depleted mice. This result suggests that neuronal activation occurs independently of DA. Nonetheless, the reserpine NSD group displayed a similar increase in nigral c-Fos expression, indicating that neither SD nor DA depletion is completely responsible for the SNpc c-Fos up-regulation.

Nevertheless, adjacent gamma-aminobutyric acid (GABA)-synthesizing neurons in both the VTA and SNpc are activated after SD. Thus, DA neuronal activity does not appear to be suppressed by local GABAergic activity during SD. Instead, it might be altered by GABAergic inhibition as well as cholinergic excitation. These inputs might allow DA neurons to become maximally active during SD and thereby contribute to the unique physiological and cognitive aspects of that state (21).

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