Animal Study

Neonatal Sleep Restriction Increases Nociceptive Sensitivity in Adolescent Mice

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Disclaimer: There was no external funding in the preparation of this manuscript. Conflict of interest: Each author certifies that he or she, or a member of his or her immediate family, has no commercial association (i.e., consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted manuscript.

Manuscript received: 04-13-2017
Revised manuscript received: 07-14-2017
Accepted for publication: 09-01-2017

Free full manuscript: www.painphysicianjournal.com

Background: Sleep loss in infants may have a negative effect on the functional and structural development of the nociceptive system. We tested the hypothesis that neonatal sleep restriction induces a long-term increase of pain-related behaviors in mice and that this hypersensitivity occurs due to changes in the neuronal activity of nociceptive pathways.

Objectives: We aim to investigate the effects of sleep loss in neonatal mice on pain behaviors of adolescent and adult mice in a sex-dependent manner. We also analyzed neuroanatomical and functional changes in pain pathways associated with behavioral changes.

Study Design: An experimental animal study.

Setting: A basic sleep research laboratory at Universidade Federal de São Paulo in Brazil.

Methods: Neonatal mice at postnatal day (PND) 12 were randomly assigned to either control (CTRL), maternal separation (MS), or sleep restriction (SR) groups. MS and SR were performed 2 hours a day for 10 days (PND 12 until PND 21). The gentle handling method was used to prevent sleep. At PND 21, PND 35, or PND 90, the mice were tested for pain-related behaviors. Their brains were harvested and immunohistochemically stained for c-Fos protein in the anterior cingulate cortex, primary somatosensory cortex, and periaqueductal gray (PAG).

Results: Neonatal SR significantly increased nociceptive sensitivity in the hot plate test in adolescent mice (-23.5% of pain threshold). This alteration in nociceptive response was accompanied by a decrease in c-Fos expression in PAG (-40% of c-Fos positive cells compared to the CTRL group). The hypersensitivity found in adolescent mice was not present in adult animals, and all mice showed a comparable nociceptive response.

Limitations: Even using a mild manipulation method, in which a minimal amount of handling was applied to maintain wakefulness, sleep deprivation was a stressful event evidenced by higher corticosterone levels.

Conclusion: Repeated exposures to sleep loss during early life were able to induce changes in the nociceptive response associated with alterations in neural activity in descending control of pain.

Key words: Brain maturation, hypersensitivity, neuronal activity, nociception, pain, periaqueductal gray, postnatal development, sleep, sleep deprivation

Pain Physician 2018; 21:E137-E148

The development of the pain system is highly responsive to experience or sensory activity (1). Noxious stimuli in infancy result in long-lasting changes in pain pathways and increased nociceptive sensitivity (2-4). Modifications in nociceptive sensitivity occur due to functional and structural changes in peripheral and central mechanisms. Abnormal neural activity in the brainstem and cortex, including the

somatosensory cortex and anterior cingulate cortex, has the potential to cause alterations in pain behavior (5,6). Cytoarchitecture changes in somatosensory areas are also associated with nociceptive sensitivity and chronic pain conditions (7). Furthermore, neonatal experiences could change pain behavior in a sex-dependent manner (8) and interfere beyond pain pathways in central areas related with emotional and motivational behaviors (9,10).

Neural activity during sleep seems to contribute to brain development, especially the maturation of sensory pathways whose development is experience-dependent (11-14). Sleep loss could be an infant experience able to interfere with neuronal activity and the maturational process of the nociceptive system. Sleep loss can lead to negative implications in the functional and structural development of pain areas leading to behavioral changes.

Evidence suggests that early life experiences induce plastic changes in the nociceptive system and that the postnatal period represents a critical phase for shaping and defining nociceptive behavior for life. In this sense, we investigated the effects of sleep loss in the second and third week of life on pain and anxiety behaviors of adolescent and adult mice in a sex-dependent manner. We also analyzed neuroanatomical and functional changes in pain pathways associated with behavioral changes. The hypothesis was that neonatal sleep restriction (SR) induces a long-term increase in nociceptive sensitivity in mice and that this hypersensitivity occurs due to changes in the neuronal activity of nociceptive pathways.

Experimental Procedures

Subjects

Adult male and female BALB/c mice from the breeding facility of our university were housed in standard polypropylene cages in a colony maintained at 22°C with a 12:12 hour light-dark cycle (lights on at 7 a.m.). Food and water were available ad libitum. Sets of 2 females and 1 male were paired for breeding. After 10 days, pregnancy was identified by the presence of a distended abdomen, and the females were individually housed and allowed to give birth to pups. The day of parturition was designated as postnatal day 0 (PND 0). On PND 3, litters were culled to a maximum of 8 pups per litter (4 males and 4 females). Litters with less than 3 pups were not included in the study. At PND 12, the litters were randomly assigned to the following groups: control (CTRL), maternal separation (MS), and SR groups. Weaning took place at PND 22, and mice were group-housed by litter and sex until adolescence (PND 35) or adulthood (PND 90). All pups in the same litter received the same treatment. The experimental protocol was in accordance with the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals (15), and it was approved by the university's ethics committee: #1960/09.

Neonatal Manipulations

SR Group

The gentle handling method was used to prevent sleep. The method involves keeping the animals awake by shaking the cage, switching pups from one cage to another (litter not mixed), introducing novel objects in the cage, or touching the animals with the hand or a brush, when they show signs of sleep onset. Sleep was behaviorally detected by motor activity quiescence, eye closure, and myoclonic twitching. The gentle handling method is effective in producing sleep deprivation in neonatal rodents, being able to eliminate up to 91% of total sleep (16). Animals were sleep restricted by gentle handling for 2 hours (from 8 a.m. to 10 a.m.) from PND 12 until PND 21. This postnatal period was chosen due to the easy recognition of sleep and wakefulness behaviors parameters, as the eye opening and the increased mobility that occurs around PND 11–12 (17). Furthermore, the functional maturation of peripheral and central areas responsible for pain awareness seems to occur only after PND 7 (1,18). The 10-day protocol (PND 12 until PND 21) allowed us to conduct the SR in a longer period within the critical phase for shaping and defining nociceptive behavior. Daily duration of gentle handling was based off of previous studies showing that a range of 70 to 140 minutes is a minimal amount of time necessary to induce a compensatory increase in sleep in the second and third postnatal week (19). Compensatory response after sleep deprivation is a marker of the protocol effectiveness. Manipulation of pups occurred in the absence of the mother; therefore, the sleep loss was associated with maternal separation. To control for possible effects of the absence of the mother, the MS group was used.

MS Group

The MS protocol was performed for 2 hours (from 8 a.m. to 10 a.m.) from PND 12 until PND 21. The litters were removed from dams and placed in separate cages,

on a heating pad, in the same room as the SD protocol. During the separation period, food and water were unavailable, the pups were not handled, and could sleep. During the manipulations of the SD and MS groups, the CTRL litters remained undisturbed with their dams in the colony room.

Behavioral Parameters

Nociceptive Sensitivity

Hot Plate Test

Mice were placed singly on a hot plate apparatus (Ugo Basile S.R.L., Monvalle VA, Italy) that consists of a temperature-controlled metal surface (53 ± 1°C) encased by a cylindrical Plexiglas chamber. A timer was started when all 4 paws were in contact with the surface. The latency to withdraw the paw (licking either a hind paw or forepaw) to avoid thermal stimulus was measured in seconds, at which point the timer was stopped, and the mouse was immediately removed from the hot plate. A 60-second cut-off latency was determined for non-responsive animals (20). The mice were exposed to the hot plate only once. The hot plate test measures the nociceptive threshold to a noxious thermal stimulus and indicates acute nociceptive sensitivity. Reduced threshold represents higher sensitivity. Paw licking as a response to the hot plate exposure can induce c-Fos expression in supraspinal areas (21,22).

Formalin Test

Formaldehyde (37%; synth) was diluted with 0.9% NaCl to obtain 2% formalin (≈ 0.74% formaldehyde). The mouse was gently restrained and 10 µL of formalin was injected subcutaneously into the dorsal hind paw of the mouse using an insulin syringe (U-100) with a 26-gauge needle. The mouse was then observed in a chamber, and the behavior was recorded for 30 minutes, starting immediately after the formalin injection. The noxious stimulus induces a biphasic pattern of nociceptive response: an early phase (nociceptive phase) that is the result of the direct chemical stimulation of nociceptors and the late phase (inflammatory phase) resulting from an inflammatory process in the injured tissue (23). The nociceptive phase was scored 0–5 minutes after the injection of formalin, and the inflammatory phase was scored 15–30 minutes after the injection. The nociceptive response to each phase was defined by the amount of time the animal spent lifting or licking the injected paw. More time spent lifting or licking the injected paw indicates higher nociceptive sensitivity. The formalin test is a valid and reliable model to analyze the acute and tonic nociceptive sensitivity in response to a persistent noxious stimulus.

Anxiety-Like Behavior - Elevated Plus Maze Test

To analyze anxiety-like behavior, the mice were submitted to the elevated plus maze test. The elevated plus maze consists of 4 arms, 2 opposing open arms (28 x 8 cm) and 2 opposite closed arms (28 x 8 x 14 cm), connected by a central platform (8 x 8 cm). The maze was elevated to a height of 48 cm above the floor. The mice were placed on the center platform facing the closed arm, and the sessions were tape-recorded for 5 minutes. The total number of entries into the open and closed arms, the percentage of time (in relation to the total time in the maze) spent in each arm, the locomotion (number of entries into the open and closed arms), and the risk assessment behavior (number of head dipping and stretch-attend posture) were measured. Anxiety behavior is determined by the increased closed arm time and/or closed arm entries (24).

In all behavioral tests, the investigator that conducted and analyzed the experiments was unaware of the treatment received by the animals. For this, a second investigator was responsible to take the mice to the behavioral room in a random order and to identify samples in a numerical order for posterior analysis (videos and histological data).

Hormonal Analysis

Corticosterone

Immediately after decapitation, trunk blood was collected in plastic tubes containing EDTA. We used a small funnel to facilitate the blood collection into the tubes. Blood was centrifuged (3,500 rpm/15 min) at 4°C to obtain samples of plasma. Plasma corticosterone concentrations (intra-assay coefficient 7.1%) were assayed using a double antibody radioimmunoassay method specific for rats and mice using a commercial kit (MP Biomedicals, Santa Ana, CA). The sensitivity of the assay was 0.25 ng/mL.

Histological Preparations

Ninety minutes after the hot plate test, the mice were anesthetized with 10% chloral hydrate and perfused intracardiacally with 0.9% saline and 4% paraformaldehyde in 0.1M NaHPO4 buffer (pH 7.4). The brains were postfixed overnight in paraformaldehyde at 4°C,

cryoprotected in 30% sucrose in 0.02 M KHPO4 during 48 hours (changed solution after 24 hours), frozen and stored at -80°C. Brains were coronally sectioned into 30 µm thick slices in a cryostat (Leica Biosystems, Wetzlar, Germany) at -20°C and serially stored in 4 compartments. One series was stained with cresyl violet for cytoarchitecture analyses. The other 3 series were used for immunohistochemical procedures.

Cytoarchitecture Analyses – Cortical Thickness

Coronal sections were stained with cresyl violet. All brains were qualitatively examined (10–40x magnification) and only histological sections with clear distinction between layers were used in the analysis. Layers boundaries were visibly defined by changes in cell size and density in a 10x magnification, with the exception of boundaries between layers II and III. Quantitative analysis of total cortical thickness and the thickness of layers I, II/III, IV, V, and VI were measured using NIH ImageJ program (10x magnification). We sampled the primary somatosensory cortex in the right side of the brain hemisphere, based on the neuroanatomical Mouse Brain Atlas (25). We used landmarks such as the anterior commissure, the position and shape of lateral ventricle, and corpus callosum as the base for the sampling.

Immunohistochemistry – c-Fos Expression

Brain sections were rinsed in 0.1 M Tris buffer, incubated for 48 hours at 4°C in a solution of anti-c-Fos antibody (1:20,000, Calbiochem, San Diego, CA), 0.1 M Tris, 0.3% Triton X-100, 2% normal goat serum. After incubation, sections were rinsed, incubated for 120 minutes at room temperature in goat biotinylated anti-rabbit IgG (1:400, Vector Labs, Burlingame, CA), 0.1 M Tris, 0.3% Triton X-100, rinsed, and exposed for 90 minutes at room temperature to avidin-biotin-complex solution (Kit ABC VectaStain Elite, Vector Labs) in 0.1 M Tris. The peroxidase complex was visualized using the chromogen diaminobenzidine 3,3-tetrachloride Kit with nickel (DAB, Vector Labs). Brain sections were rinsed and mounted onto gelatin-coated slides and dehydrated through alcohol to xylol. Only brains with quality in the fixation and immunohistochemistry staining processes were used in the analysis.

Sections of primary somatosensory cortex (S1, S1FL, and S1HL, from bregma 1.18 to -1.06 mm), anterior cingulate cortex (ACC1 and ACC2, from bregma 1.18 to -0.46 mm), and periaqueductal grey region (PAG, from bregma -3.64 to -4.16mm) were photographed using an Olympus light microscope (10x magnification) (Olympus, Southborough, MA). For each animal, 2 sections from PAG: 6 from S1, S1FL, and S1HL and 8 sections from ACC1 and ACC2 were independently analyzed and quantified using NIH ImageJ. The total area of brain region was measured, and all cells above a threshold determined for each brain were counted as c-Fos immunopositive. The data were expressed as the neuronal density of immunopositive cells (cells/mm^2).

The investigator that analyzed the histological and immunohistochemistry slices was unaware of the treatment that the animals had received.

Statistical Analysis

Behavioral data and corticosterone concentration were evaluated using 2-way analysis of variance (ANOVA), with group and sex as the main factors. Based on behavioral outcomes, histological analyses of c-Fos expression and cortical thickness were evaluated using one-way ANOVA (group as the main factor). Post-hoc comparisons were performed using the Tukey's test. The Pearson correlation test was performed to evaluate the relationship between corticosterone levels and nociceptive response. All analyses were conducted in a blind-way regarding group and sex. Results were expressed as the mean ± SEM. The level of significance was set at $P < 0.05$. All analyses were carried out using SPSS Version 20.0 (IBM Corporation, Armonk, NY).

Experimental Procedures

Experiment 1: Long-term effects of neonatal SR on nociceptive sensitivity.

On PND 12, all mice were numbered, and the litters were assigned to 1 of 3 groups: CTRL, MS, or SR. The MS and SR groups were performed 2 hours a day for 10 days (PND 12 until PND 21). The CTRL litters were left undisturbed with their mothers in the colony room. Neonatal manipulations (SR and MS) were conducted by the same researcher during the entire protocol. After the manipulations, the mice were submitted to the nociceptive test and then euthanized for plasma collection (PND 21). The second group remained with their dams until PND 22, when they were group-housed by litter and sex until adolescence (PND 35) or adulthood (PND 90). As showed in Fig. 1A, at each developmental timepoint (PND 21, PND 35, and PND 90), different groups of mice were tested for nociceptive behavior (hot plate test). At PND 35 or PND 90, after the hot plate test, the mice were perfused intracardiacally, and the brains

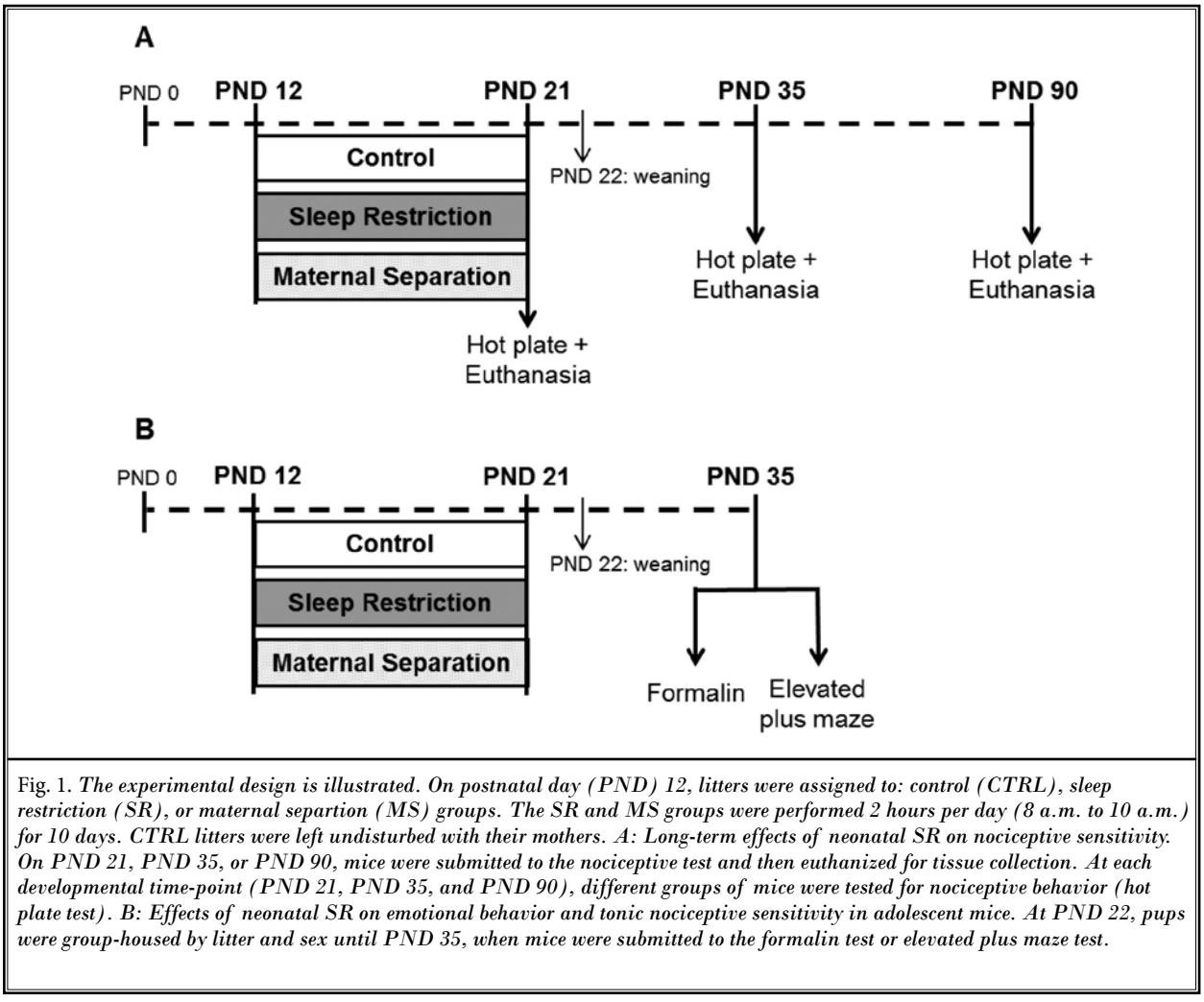

Fig. 1. *The experimental design is illustrated. On postnatal day (PND) 12, litters were assigned to: control (CTRL), sleep restriction (SR), or maternal separtion (MS) groups. The SR and MS groups were performed 2 hours per day (8 a.m. to 10 a.m.) for 10 days. CTRL litters were left undisturbed with their mothers. A: Long-term effects of neonatal SR on nociceptive sensitivity. On PND 21, PND 35, or PND 90, mice were submitted to the nociceptive test and then euthanized for tissue collection. At each developmental time-point (PND 21, PND 35, and PND 90), different groups of mice were tested for nociceptive behavior (hot plate test). B: Effects of neonatal SR on emotional behavior and tonic nociceptive sensitivity in adolescent mice. At PND 22, pups were group-housed by litter and sex until PND 35, when mice were submitted to the formalin test or elevated plus maze test.*

were removed for histological analysis, as previously described. We conducted the cytoarchitecture analyses and immunohistochemical staining for c-Fos protein only in the age that nociceptive response was changed by neonatal sleep restriction. Mice manipulations and the behavioral tests were performed during the light portion of the light-dark cycle.

Experiment 2: Effects of neonatal SR on emotional behavior and tonic nociceptive sensitivity in adolescent mice.

The adolescence time-point was used to explore the effects of neonatal sleep loss on the anxiety behavior and nociceptive sensitivity in response to a persistent noxious stimulus. Neonatal mice were randomly assigned to the same groups previously described and manipulations were performed similar to those in experiment 1. At PND 22, pups were group-housed by litter and sex until PND 35, when mice were submitted to the formalin test or elevated plus maze test. One male and one female were randomly selected from each litter for behavioral tests. Each animal was only used for one test (Fig. 1B).

Results

Hot Plate Test

The analysis of nociceptive response in PND 21 mice demonstrated no effect of neonatal manipulation, sex, or their interactions on thermal sensitivity. The paw withdrawal threshold in the hot plate test was similar between subjects (Fig. 2A). As opposed to acute analysis, the ANOVA revealed the effect of neonatal sleep loss on nociceptive response in adolescent mice

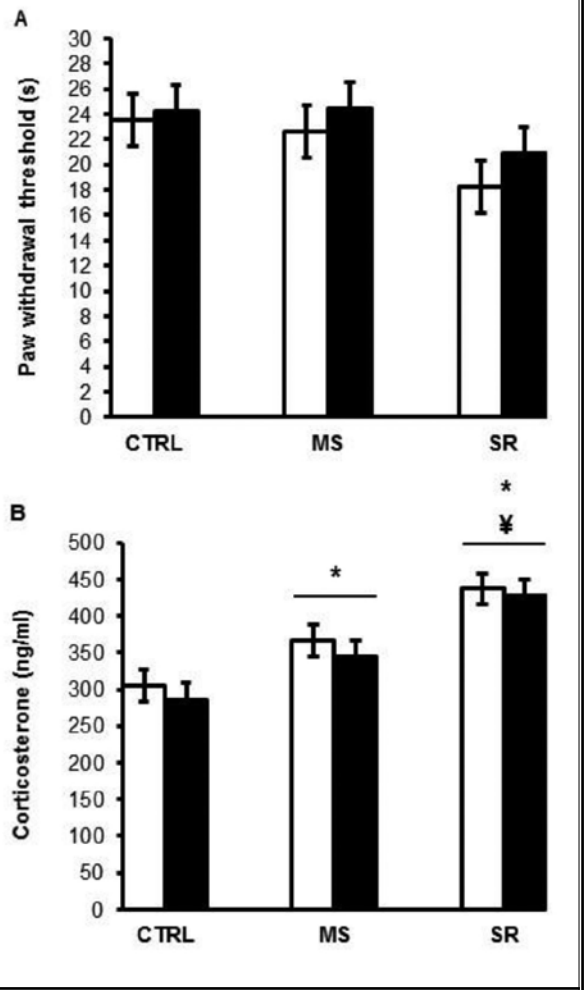

Fig. 2. *Effect of neonatal manipulations on the hot plate test (A) and corticosterone concentrations (B) in control (CTRL, n = 10 males and 9 females), maternal separation (MS, n = 10/sex), and sleep restriction (SR, n = 10/sex) groups at postnatal day 21. The white bars represent male mice, and the black bars represent female mice. Data are shown as mean ± S.E. * vs. CTRL; ¥ vs. MS; P < 0.05.*

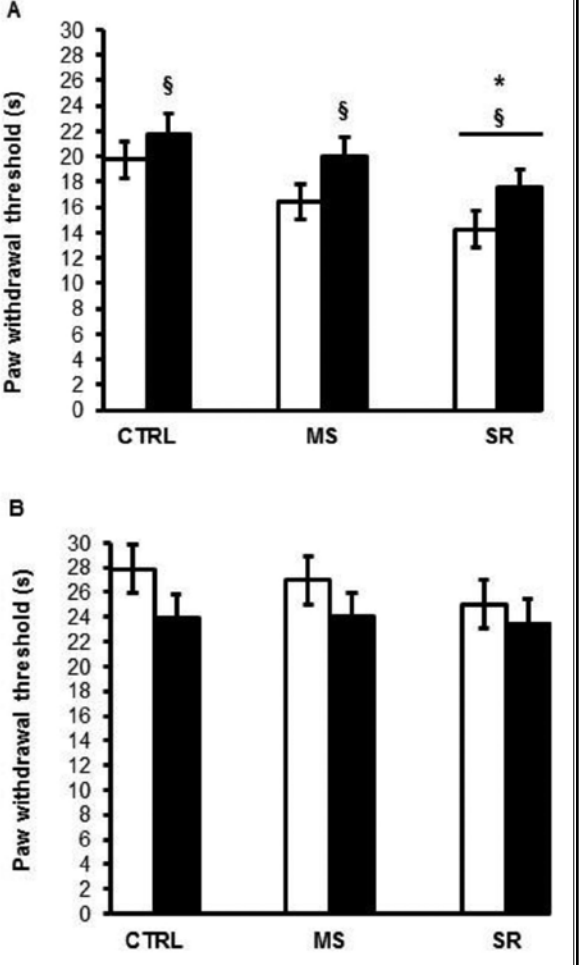

Fig. 3. *Effect of neonatal manipulations on the hot plate test in control (CTRL), maternal separation (MS), and sleep restriction (SR) groups at postnatal day 35 (A: CTRL, n = 11 males and 9 females; SR, n = 12 males and 11 females; MS, n = 11 males and 12 females) and postnatal day 90 (B: CTRL, n = 5/sex; SR, n = 5/sex; MS, n = 5/sex). The white bars represent male mice, and the black bars represent female mice. Data are shown as mean ± S.E. * vs. CTRL; § vs. Male; P < 0.05.*

(F(2,60) = 5.21; $P < 0.01$; $\eta^2 = 0.14$; power = 0.81). The SR increased the nociceptive sensitivity in both male and female mice as compared with the CTRL group at PND 35 (15.8 vs. 20.6 sec; $P < 0.01$; Fig. 3A). Female mice showed higher paw withdrawal threshold than male, regardless of neonatal manipulation (average of 19.7 vs. 16.7 sec; $P < 0.05$). Changes in pain sensitivity were not found in adulthood (Fig. 3B).

Corticosterone Levels

Corticosterone response after noxious stimulus showed that neonatal manipulation was able to increase the corticosterone levels in PND 21 mice, as shown in Fig. 2B (F(2,53) = 19.98; $P < 0.001$; $\eta^2 = 0.43$; power = 1.00). The post hoc analysis revealed that MS elevated the hormonal stress response compared to the CTRL group ($P < 0.05$). Moreover, SR mice exhibited higher concentrations of corticosterone than the CTRL (432.7 vs. 295.5 ng/mL; $P < 0.001$) and MS groups (432.7 vs. 355 ng/mL; $P < 0.01$). Pearson's correlation analysis between corticosterone levels and the paw withdrawal threshold revealed a negative correlation (r = -0.26; R^2: 0.06, $P < 0.05$).

Histological Analysis

The histological measures were analyzed only in brain tissue of adolescent mice (PND 35), due to the main effect of SR in nociceptive sensitivity in this developmental time-point and without sex effect.

Cytoarchitecture analyses of primary somatosensory cortex of adolescent mice showed no effect of neonatal manipulations in the total thickness, as well as the thickness in the cortical layers I, II/III, IV, V, and VI (Fig. 4). The lack of significant difference in the cortical morphology reveals that neonatal sleep loss was unable to induce structural changes in the neocortex.

The immunohistochemistry analysis of c-Fos expression, a neuronal activity marker, showed functional changes in supraspinal nociceptive areas in response to a noxious stimulus. We found a decrease in neuronal density of c-Fos positive cells in the PAG of adolescent mice submitted to neonatal SR ($F(2,23) = 3.564$; $P < 0.05$; $\eta^2 = 0.23$; power = 0.6). The SR group showed a reduction in immunopositive cells in the PAG after the hot plate test compared to the CTRL group (Fig. 5). No significant changes were found in the ACC and S1 areas (Table 1).

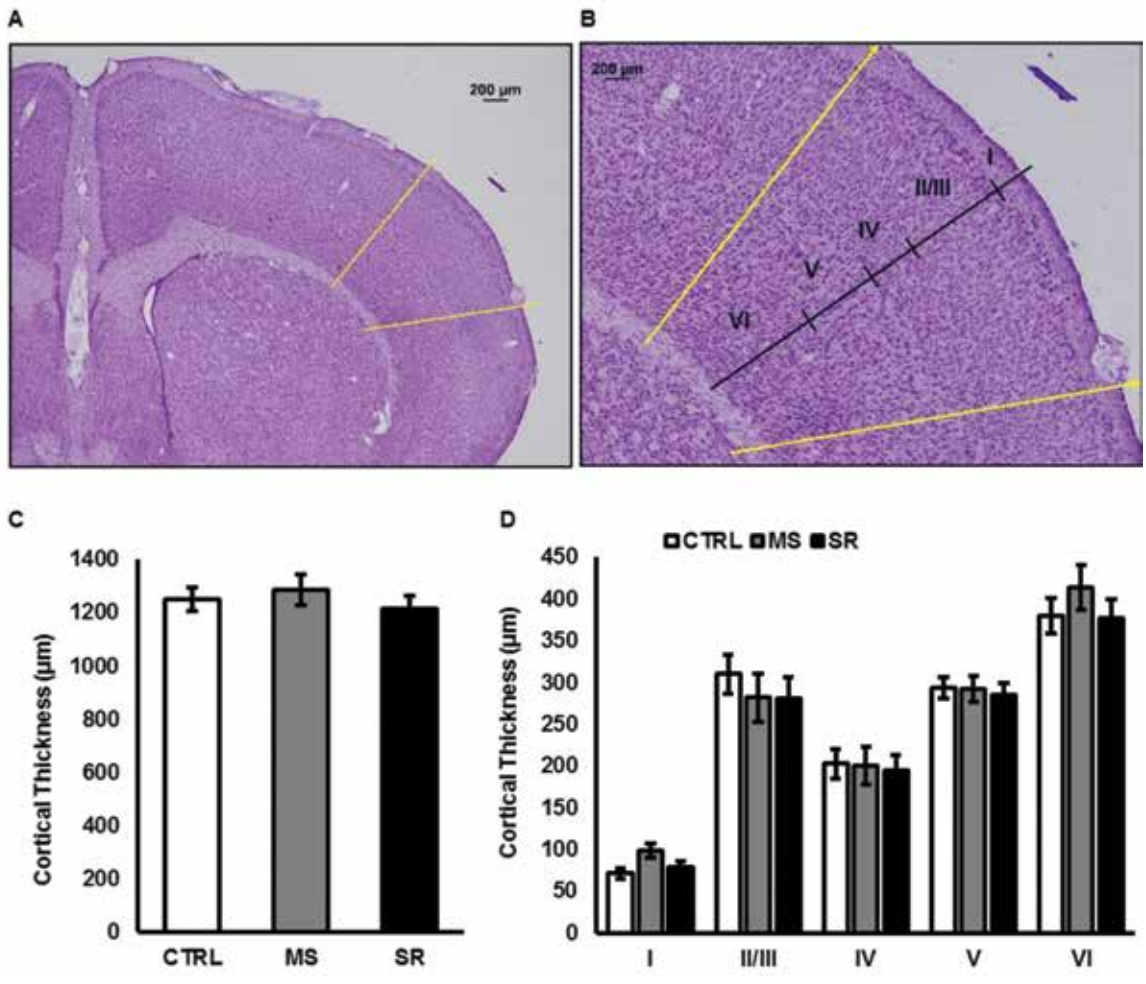

Fig. 4. *Effect of neonatal manipulations on cortical thickness of adolescent mice (postnatal day 35). A-B: Representative photomicrographs showing coronal sections of primary somatosensory cortex stained with cresyl violet. Cortical layers were identified based on differences in cytoarchitecture (B). Bregma 1.18mm; 4x magnification (A); 10x magnification (B); Scale bar: 200 µm. C: Total cortical thickness of the primary somatosensory cortex in control (CTRL, n = 8), maternal separation (MS, n = 5) and sleep restriction (SR, n = 7) groups. D: Cortical thickness across the 6 layers in CTRL (white bars), MS (gray bars), and SR (black bars) mice. Data are shown as mean ± S.E.*

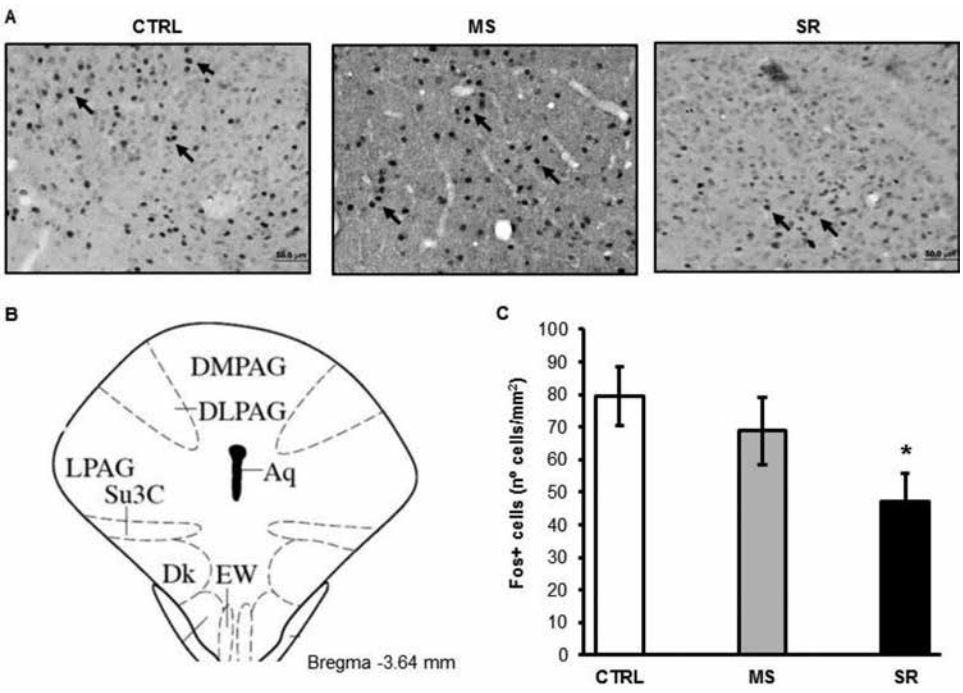

Fig. 5. *Effect of neonatal manipulations on neuronal density of c-Fos positive cells in the periaqueductal grey (PAG) of adolescent mice (postnatal day 35) following the hotplate test. A: Representative photomicrographs of c-Fos positive cells (arrows) in the PAG in control (CTRL), maternal separation (MS), and sleep restriction (SR) groups. Forty-times magnification; Scale bar: 50 μm. B: Coronal representative image of the PAG nucleus used in the analysis: dorsolateral (DLPAG), dorsomedial (DMPAG), and lateral (LPAG). C: Number of c-Fos positive cells in the PAG in CRTL (n = 9), MS (n = 7), and SR (n = 10) groups. Data are shown as mean ± S.E. * vs. CTRL; P < 0.05.*

Table 1. *Neuronal density of c-Fos positive cells in adolescent mice (postnatal day 35) following the hot plate test.*

	ACC1	ACC2	S1	S1FL	S1HL	PAG
CTRL	55.6 ± 13.5	47.6 ± 10.1	17.2 ± 4.0	45.6 ± 6.6	60.7 ± 7.2	79.6 ± 9.0
MS	58.7 ± 15.4	56.9 ± 11.5	19.8 ± 4.6	44.9 ± 7.5	60.5 ± 8.1	68.8 ± 10.2
SR	61.4 ± 12.8	43.7 ± 9.2	20.2 ± 3.8	36.5 ± 6.3	40.4 ± 6.8	47.0 ± 8.5 *

CTRL: control (n = 9); MS: maternal separation (n = 7); SR: sleep restriction (n = 10). ACC1 and ACC2: anterior cingulate cortex subregions 1 and 2; S1, S1FL, S1HL: primary somatosensory cortex subregions 1, forelimb and hindlimb, respectively; PAG: periaqueductal grey. Mean ± S.E. *vs. CTRL, $P < 0.05$.

Formalin Test

The formalin test was performed to analyze the nociceptive tonic behavior of adolescent mice in the presence of a persistent injury. An intraplantar injection of 2% formalin caused comparable nociceptive sensitivity in all mice as indicated by a non-significant difference for the time spent lifting or licking the hind paw in both phases of pain behavior (Fig. 6).

Elevated Plus Maze Test

As demonstrated in Table 2, a significant effect of sex for both % time in the closed arm ($F(1,33) = 4.83$; $P < 0.05$; $\eta^2 = 0.12$; power = 0.48) and risk assessment behavior ($F(1,33) = 5.16$; $P < 0.05$; $\eta^2 = 0.13$; power = 0.59) was found. Female mice spent less time in the closed arm (144.1 vs. 229.9 sec) and showed higher frequency of head dipping and stretch-attend posture than male mice. No main effects of neonatal condition or group x sex interaction were found in behavioral parameters measured in the task.

Discussion

In the present study, we employed a prolonged SR protocol in a critical period for the development of the

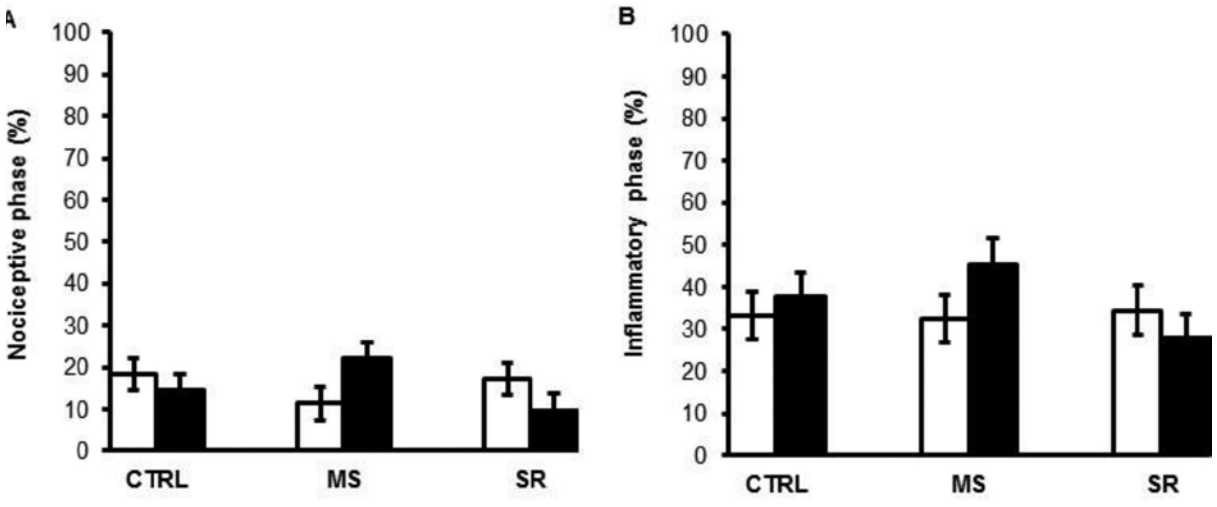

Fig. 6. *Time spent lifting or licking the injected paw (pain behavior) induced by formalin injection in PND 35. A: Nociceptive phase (0–5 min). B: Inflammatory phase (15–30 min). CTRL = control group (n = 6/sex); MS = maternal separation group (6/sex); SR = sleep restriction group (n = 6 males and 5 females). The white bars represent male mice, and the black bars represent female mice. Data are shown as mean ± S.E.*

Table 2. *Behavioral response of adolescent mice (postnatal day 35) in the elevated plus maze test.*

	CTRL		MS		SR	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Time spent (%)						
Closed arms	79.8 ± 15.3	52.2 ± 15.3*	72.6 ± 16.5	47.7 ± 15.3*	76.9 ± 16.5	43.5 ± 16.5*
Open arms	14.0 ± 13.2	25.0 ± 13.2	18.0 ± 14.3	27.0 ± 13.2	15.4 ± 14.3	30.2 ± 14.3
Number of entries						
Closed arms	1.4 ± 0.3	1.2 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.0 ± 0.3	0.5 ± 0.3
Open arms	0.7 ± 0.2	0.7 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.3 ± 0.2	0.5 ± 0.2
Locomotion	2.1 ± 0.3	2.0 ± 0.3	1.3 ± 0.3	1.0 ± 0.3	1.5 ± 0.3	1.7 ± 0.3
Risk assessment	8.80 ± 2.4	18.5 ± 2.4*	12.5 ± 2.6	17.5 ± 2.4*	11.8 ± 2.6	11.0 ± 2.6*

CTRL: control (n = 7/sex); MS: maternal separation (n = 6 males and 7 females); SR: sleep restriction (n=6/sex). Mean ± S.E. *vs. Male; $P < 0.05$. n = 6-7/group/sex

nociceptive system and investigated the effects on nociceptive sensitivity at different time-points. The main findings were the increased thermal sensitivity shown by adolescent mice (reduction of 23.5% of pain threshold) associated with a decrease in neuronal activity in PAG, the supraspinal area related with inhibitory pain control.

Nociception is an essential component of the pain system and is modulated by genetic and environmental interactions. Despite evidence demonstrating heritability of nociceptive response (26,27), the contributions of environmental factors to determine and predispose an individual to pain conditions has been increasingly investigated. Infant experience may contribute to variability in noxious stimuli sensitivity and induce marked and long-lasting changes on the developing pain system (4,28-30). Disruption of mother pup interactions, the classical model of early life stress in rodents, results in visceral hypersensitivity and increased c-Fos expression in central pain areas in adulthood (29). Green et al (30) found a reduced mechanical nociceptive threshold in adult rats submitted to a neonatal limited bedding stress model. This behavior was associated with changes in the functional plasticity of the nociceptors. Our data

demonstrated no main effect on adult mice, and the increased sensitivity in adolescence, 2 weeks after normal sleep time opportunity, indicates the short-lasting effect of neonatal sleep loss.

The consequences of early life experiences are mainly explored in adult life, and limited evidence reports the effects at different developmental time-points. Vega-Avelaira et al (4) demonstrated that noxious stimulus in PND 10 failed to trigger hypersensitivity over the first 3 weeks after injury, unlike the same injury applied in adulthood. The hypersensitivity only emerged during adolescence (PND 30). Similar results were found in our study. Sleep deprivation protocols conducted in adult mice results in reduced nociceptive threshold in the hot plate test immediately after sleep loss (31). Acute analysis of neonatal SR showed no effect on nociceptive threshold, and the increased sensitivity occurs only at PND 35. The absence of hypersensitivity at PND 21 could be explained by the phenomenon known as stress-induced analgesia (32). Increased corticosterone in pups after sleep deprivation by gentle handling was previously reported (16). In our study, SR mice presented higher stress hormone concentrations compared with the CTRL and MS groups. Stress hormone could have masked acute changes in nociceptive response. However, the weak correlation between paw withdrawal threshold and corticosterone concentrations indicated that corticosterone levels poorly explain our result. Another explanation, also reported in a previous study (4), is the delayed pain onset due to early life adverse events. Adolescence could be a sensitive age for the onset of pain behaviors induced by infant experiences.

Functional and structural changes in pain pathways could cause abnormal nociceptive behaviors. Adverse events in infancy may impair inhibitory control of noxious information producing increased nociceptive sensitivity (33-35). Adolescent rats submitted to a neonatal inflammatory injury showed hyperalgesic behavior in the formalin test, associated with attenuated c-Fos expression in the PAG (35). Our data indicated that mice exposed to neonatal SR exhibited lower paw withdrawal threshold and reduction of c-Fos positive cells in the PAG in response to the hot plate test. The PAG is a key supraspinal area in the pain-inhibitory circuitry and descending projections from PAG modulate spinal nociceptive processing and behavioral response (36). Reduction of neural activity in the PAG indicated that SR decreased the descending modulation to a noxious stimulus. The sustained wakefulness in the SR condition increases neural activation in the brainstem nucleus (37), and this excitability could possibly impair the maturation pattern of descending pain pathways. In fact, changes in brainstem neural activity in the first 3 weeks of life could modify inhibitory circuitry maturation favoring pain behavior (33).

Absence of differences in nociceptive response in adulthood indicates that the increased sensitivity induced by neonatal SR was a short-lived effect. Unlike in adolescence, adult mice submitted to SR showed similar paw withdrawal threshold in the hot plate test. Despite the lack of functional and structural analysis at this time-point (PND 90), the findings in adolescent mice could explain adult behaviors. Chronic pain conditions may produce morphological changes in pain pathways, mainly in the somatosensory cortex (7,38). The similarity in the cortical morphology of the S1 area in adolescent mice indicates that neonatal sleep loss was unable to induce structural changes in the neocortex. Absence of structural modifications could explain why the hypersensitivity did not persist until adulthood. Furthermore, adolescent mice showed comparable pain behaviors in the formalin test, a model more closely resembling clinical cases of chronic pain due to the persistent noxious stimulation. The affective/emotional component of pain processing, evaluated by anxiety-like behavior, was also unimpaired by neonatal sleep loss. Our data demonstrated that neonatal sleep restriction increased nociceptive sensitivity and changed pain circuitry response in a stimulus-dependent manner and that these alterations were not maintained in adulthood. The change in nociceptive pathways induced by infant sleep loss may have been reversed to enable an adequate behavior repertoire in adult life.

Sex difference is an important aspect to be investigated in pain studies. In our study, the behavioral responses were not sex-dependent. Similar to our data, Boissé et al (39) found no sex effect in pain behaviors of adult rats submitted to neonatal injury. However, Burke et al (8) reported that only female rats submitted to early life stress developed changes in nociceptive response. Sex-dependent alteration was also observed in behaviors associated with pain response (40). Sex differences in pain response are commonly investigated in the adulthood, and limited studies explore the effect of sex in young subjects. Despite divergences in sex effect in pain-related behaviors, inclusion of male and female subjects and the analysis of sex-differences in pain research are important questions to deepen our knowledge of this complex phenomenon.

The analysis of chronic impairments of neonatal sleep loss was previously investigated only using a pharmacological or instrumental approach, and it focused on rapid eye movement sleep deprivation (41-43). Our group was the first that used gentle handling to prevent neonatal sleep and to measure long standing behavioral effects (44). The present study corroborates with previous data by our group (44) and others (45) showing the effect of a proper sleep duration early in life for behavior development. However, a few limitations of this study need to be addressed. The change in nociceptive response was not statistically different from the MS group. It is possible that our results reflect the accumulation of adverse manipulations (the sum of sleep deprivation associated to MS), not only sleep loss per se. Even using a mild manipulation method, in which a minimal amount of handling was applied to maintain wakefulness, the higher corticosterone levels evidence a possible interaction of these factors. Furthermore, it is important to highlight that part of the protocol occurred in the critical period for somatosensory cortex plasticity, which reaches from PND 0 until PND 16 (46). The minimum amount of tactile stimulation during the MS and RS protocols could be another factor to interact with results.

Animal models provide an opportunity to investigate developmental changes in response to infant experience. Our data suggest that repeated exposures to sleep loss during the early life period could be an adverse experience for the immature brain. This study adds evidence of a scarce literature about the relation between sleep and pain in early life.

Acknowledgments

Authors' contributions: study design (PA, MLA), data acquisition and analysis (PA, CAOC), and drafting manuscript and critical revision (PA, CAOC, MGMO, ST, MLA). All authors give final approval of the manuscript. Associação Fundo de Incentivo à Pesquisa (AFIP) and São Paulo Research Foundation (FAPESP #10/50130-0 to PA, #12/17619-0 to CAOC) supported the study. ST and MLA are recipients of National Council for Scientific and Technological Development (CNPq). The authors report no financial conflicts of interest.

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