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Chronic sleep restriction increases pain sensitivity over time in a periaqueductal gray and nucleus accumbens dependent manner

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ABSTRACT

Painful conditions and sleep disturbances are major public health problems worldwide and one directly affects the other. Sleep loss increases pain prevalence and severity; while pain disturbs sleep. However, the underlying mechanisms are largely unknown. Here we asked whether chronic sleep restriction for 6 h daily progressively increases pain sensitivity and if this increase is reversed after two days of free sleep. Also, whether the pronociceptive effect of chronic sleep restriction depends on the periaqueductal grey and on the nucleus accumbens, two key regions involved in the modulation of pain and sleep-wake cycle. We showed that sleep restriction induces a pronociceptive effect characterized by a significant decrease in the mechanical paw withdrawal threshold in rats. Such effect increases progressively from day 3 to day 12 remaining stable thereafter until day 26. Two consecutive days of free sleep were not enough to reverse the effect, not even to attenuate it. This pronociceptive effect depends on the periaqueductal grey and on the nucleus accumbens, since it was prevented by their excitotoxic lesion. Complementarily, chronic sleep restriction significantly increased c-Fos protein expression within the periaqueductal grey and the nucleus accumbens and this correlates with the intensity of the pronociceptive effect, suggesting that the greater the neural activity in this regions, the greater the effect. These findings may contribute not only to understand why painful conditions are more prevalent and severe among people who sleep poorly, but also to develop therapeutic strategies to prevent this, increasing the effectiveness of pain management in this population.

1. Introduction

Pain conditions and sleep disorders are major public health problems worldwide (Appleton et al., 2018; Murphy et al., 2017) and there is a clear bidirectional relationship between them. There is no doubt that pain impairs sleep (Artner et al., 2013; Karaman et al., 2014) and different types of sleep impairment increase pain sensitivity (Okifuji and Hare, 2011). However, the underlying mechanisms are largely unknown.

The reason why sleep disorders are great predictors of pain development (Mork and Nilsen, 2012; Okifuji and Hare, 2011) may rely on the ability of sleep loss to disrupt endogenous pain modulation, as suggested by clinical findings (Paul-Savoie et al., 2012; Tiede et al., 2010). In fact, several brain regions that play a key role in pain modulation, such as the ventrolateral periaqueductal gray (vlPAG) (Fields, 2004), and the Nucleus Accumbens (NAc), (Gear et al., 1999), also contribute to control sleep-wake cycle (Lu et al., 2006; Oishi et al., 2017; Weber et al., 2018). Since most sleep disorders are characterized by impairment mainly in rapid eye movement (REM) sleep (Brown et al., 2012; Naiman, 2017), we have performed REM sleep deprivation (REM-SD) in rats to provide a mechanistic basis for these clinical observations. According to our previous data, REM-SD increases nociceptive responses by disrupting the PAG-RVM (periaqueductal gray – rostral ventral medulla) descending pain modulation system (Tomim et al., 2016) as well as by increasing NAc adenosinergic activity and by decreasing NAc dopaminergic activity (Sardi et al., 2018).

However, insomnia (Calhoun et al., 2014) and restriction of sleep time due to occupational or recreational reasons have become increasingly frequent in the modern society (Owens et al., 2014). In

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order to mimic this decrease in sleep duration in laboratory animals, some types of gentle stimulation have been used to keep them awake for long periods of time. Like selective REM-SD (Damasceno et al., 2009; Nascimento et al., 2007; Sardi et al., 2018; Tomim et al., 2016; Wei et al., 2013), sleep restriction (SR) has been associated with increased pain sensitivity in both humans (Okifuji and Hare, 2011; Tiede et al., 2010) and animals (Alexandre et al., 2017). However, the underlying mechanisms are largely unknown and many unanswered questions remain. For example: Does the pronociceptive effect of sleep restriction increase over time? If yes, when does it reach the maximum intensity? Are two days of normal sleep (mimicking free sleep on weekends) enough to normalize pain sensitivity? Is the pronociceptive effect of chronic sleep restriction also dependent on the PAG and on the NAc? If yes, the neuronal activity within these regions increases with chronic sleep restriction? This study aimed to answer these questions.

2. Materials and methods

2.1. Animals

The experiments were performed in male Wistar rats (270–300 g). The animals were housed five per cage in a room with controlled 12:12-h light/dark cycle and temperature (23 °C ± 2), with free access to food and water. All animal experimental procedures and protocols were approved by the Committee on Animal Research of the Federal University of Parana, Brazil, and followed the guidelines of the Ethics Standards of the International Association for the Study of Pain in animals (Zimmermann, 1983).

2.2. Stereotaxic surgery and NMDA lesion

In this study we have focused in the ventrolateral column of the PAG (vlPAG) and in the core region of the NAc for two reasons; first, both vlPAG (Fields, 2004; Reynolds, 1969) and NAc core (Gear et al., 1999; Gear and Levine, 2011; Tobaldini et al., 2014) have key roles in pain modulation. vlPAG for example, controls the descending pain modulation system in an opioid dependent manner (Fields, 2004). Second, previous work from our lab have implicated either vlPAG or NAc core (as well as NAc shell) in the pronociceptive effect of REM-sleep deprivation (Sardi et al., 2018; Tomim et al., 2016).

Stereotaxic surgery was performed 20 days before experiments under general anesthesia (ketamine, 60 mg/kg and xylazine, 10 mg/kg, i.p.). NMDA (N-Methyl-D-aspartic acid; from Sigma, St Louis, MO, USA) or its vehicle was microinjected during the surgery procedure under the following coordinates: NAc - from bregma: anteroposterior +1.3 mm; lateral ± 1.8 mm; and dorsoventral−7.2 mm; PAG - from lambda: anteroposterior 0 mm; lateral−2 mm; dorsoventral−5.4 mm in a 18° angle (Paxinos and Watson, 2007).

Selective bilateral lesions were performed within the NAc core and unilateral lesions within the ventrolateral PAG by injecting NMDA dissolved in NaCl 0.9% (pH 7.2–7.4) (Jongen-Relo et al., 2002) at a dose of 5.5 μg. The rats assigned to the sham-lesion group were infused with NaCl 0.9%. Microinjections were performed through injectors (stainless steel needle, 30gauge, Misawa Medical Industry, Japan) connected with a polyethylene tubing (PE-20) in a 5 μl Hamilton syringe coupled to an injection pump (model KDS-100 kdScientific Holliston, MA, USA). Total microinjection volume was 0.3 μl infused within 2 min. Injectors were left on site for an additional period of 1 min to minimize backflow along the cannulae tract.

After surgery, the rats received dipyrone (30 mg/kg) and gentamicin (0.5 mg/kg) and were observed for 2 h after they regain consciousness. Experiments were carried out 20 days later (Jongen-Relo et al., 2002). Lesion location and extension were histologically assessed.

2.3. Chronic sleep restriction (CSR)

The animals were deprived of total sleep during six hours per day by the gentle handling method, which reduces approximately 98% of slow-wave sleep and abolishes REM sleep in rodents (Alexandre et al., 2017). This method is characterized by keeping the animals awake by inserting novel objects in their home cage (Alexandre et al., 2017), rotating and tapping on the cage (O'Hara et al., 1993) and, if necessary, by brushing their back with a soft bristle brush. This procedure was performed 5 days per week (from Monday to Friday); 6 h per day starting at the first light of day (7:00 a.m. to 1:00 p.m.), during 12 or 26 days depending on the group, with free access to food and water. After daily CSR, the animals were free for sleeping. The control groups were housed in their cages under the same conditions and in the same room, but have not had their sleep disturbed.

2.4. Mechanical paw-withdrawal test

The mechanical paw-withdrawal test (Randall and Selitto, 1957) was performed and the nociceptive threshold was used as a measure of pain sensitivity (Sardi et al., 2018; Tomim et al., 2016). The test was performed in a blinded fashion, always before the start of the CSR procedure (07:00). In this test, an increasing pressure is applied to the dorsal surface of the rat's hind paw, the nociceptive mechanical threshold is defined as the force (mean of three readings) in grams at which the rat withdrew its paw. The test was performed before experiments (basal) and repeated at different time points thereafter (every Monday, Wednesday and Friday along the experiment).

2.5. Qualitative assessment of home cage activity

Decreased activity measured by actimetry has been used as an indirect behavioral measure of sleep in both rodents (Tang and Sanford, 2002) and humans (Townhill et al., 2016). Therefore, home cage activity was monitored in order to indirectly assess the effect of CSR and NAc or PAG lesion in the sleep-wake cycle. Since social isolation affects behavioral nociceptive measures (Meng et al., 2010), the animals were maintained in standard environmental conditions (community, 4 animals per cage). This avoids an additional variable to the pain tests, but makes statistical tests unfeasible, since each experimental group of 8 animals originates data from two home cages. Therefore these data were qualitatively presented.

Passive infrared motion captors placed over the cages were connected to a computerized data acquisition system (National Instruments, Austin, TX, USA). Home cage activity was continuously monitored throughout the experiment (12 or 26 days), except when animals were being either sleep deprived or submitted to the control condition (control groups were maintained in the same room where sleep deprivation was being conducted, but rats were free to sleep). Activity records were analyzed with the LabVIEW software package.

2.6. Histological sample preparation

The rats were anesthetized (ketamine, 60 mg/kg and xylazine, 10 mg/kg, i.p.) and transcardiacally perfused with saline 0.9%, pH 7.4, followed by 4% paraformaldehyde in 0.1-Mphosphate buffer, pH 7.4. Brains were removed and immersed in paraformaldehyde at 4 °C for a week, in 30% sucrose solution for another week and stored

at −80 °C until sectioning. Eight sections (30 μm) were sliced per animal, between bregma +1.44 and + 1.20 mm for the NAc and - 8.04 and - 8.28 for the PAG.

2.7. Cresyl violet staining

Cresyl Violet, a cationic dye that stains Nissl corpuscles present in the cell body and dendrites of neurons (Ovalle, 2013) was used in order to determine the extension of NMDA lesions. The sections were mounted on gelatin-coated slices, passed through a series of ethanol solutions of descending concentration (100%, 95%, and 70% ethanol in water, 3 min each) and stained for approximately 1 min with cresyl violet (0.05% aqueous cresyl violet, 2 mM acetic acid, and 5 mM formic acid in water). After staining, sections were rinsed in water and 70% ethanol; differentiated in 95% ethanol with acetic acid; dehydrated in ascending concentrations of ethanol–xylene and cover slipped.

2.8. c-Fos immunohistochemistry

c-Fos protein is rapidly and transiently expressed in stimulated neurons in response to elevation of intracellular calcium (Coderre et al., 1993; Lerea et al., 1992). Therefore, we have quantified c-Fos expression in an attempt to indirectly estimate the effect of CSR on neuronal activation within the NAc and PAG. Free-floating sections were rinsed in 0.1 M phosphate-buffered saline (PBS) and treated with 0.5% H2O2 in 0.1 M PBS for 30 min to suppress endogenous peroxidase activity. Tissue sections were incubated overnight at 4 °C with rabbit anti-c-Fos primary antibody (#AB038; Chemicon, Temecula, CA; 1:500 in phosphate-buffered saline plus 0.3%Triton X-100) and then incubated with a biotin-conjugated secondary antibody (#PK4001; Vector Laboratories, Burlingame, CA; 1:200) for 2 h at room temperature. After several washes with phosphate-buffered saline, the antibody complex was localized using the ABC system (#PK4001; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) followed by reaction with 3,30-diaminobenzidine with nickel enhancement. The sections were then mounted on gelatin-coated slices and cover slipped after dehydration in ascending concentrations of ethanol-xylene solutions.

2.9. Quantification of excitotoxic lesion and c-Fos immunoreactive cells

The slices were digitized with a microscope scanner (Axio Imager Z2, Carl Zeiss, Jena, DE) coupled to an imaging system (Metasystems, Altlussheim, DE). Quantification of the NAc and PAG lesions and c-Fos immunoreactive (c-Fos-ir) cells was performed automatically by optical density using ImageJ 1.37c (Schneider et al., 2012) image analysis software.

2.10. Statistical analysis

Data from nociceptive tests were analyzed by repeated-measures (time) analysis of variance (ANOVA) with sleep condition (CSR or control procedure) as between-subject factor for naïve animals and sleep condition and treatment (NMDA lesion or sham-lesion) as between-subject factors for all other groups. Data from histological analysis of the excitotoxic lesion with the NMDA were analyzed by two-way ANOVA with sleep condition and treatment (NMDA lesion or sham-lesion) as factors. Data from c-Fos i immunoreactivity were analyzed by one-way ANOVA. The correlation between c-Fos immunoreactivity and nociceptive threshold was determined by the Pearson's Correlation test. All post hoc contrasts, when appropriate, were performed using Tukey's test ($p < 0.05$). The software SigmaPlot® (Systat Software, San Jose, CA, USA) was used to perform data analysis and graphical representation. Data are plotted in figures as mean ± SEM. The number of animals in each group ranges from 6 to 8, except data from c-FOS expression which were obtained with 4–5 animals per group.

3. Results

3.1. The pronociceptive effect of chronic sleep restriction and its temporal evolution

Chronic sleep restriction (CSR) for six hours daily progressively increased nociceptive response, as demonstrated by the decrease in mechanical nociceptive paw-withdrawal threshold (Fig. 1, repeated-measures (time) ANOVA – sleep condition (CSR or control procedure): $F(1,14) = 248.15$, $p < 0.001$; sleep condition x time: $F(11,154) = 16.208$, $p < 0.001$. Post hoc analysis using Tukey's test indicated that CSR decreased mechanical nociceptive threshold during the overall experiment, $p < 0.003$). Only two previous days of sleep restriction were sufficient to significantly increase the nociceptive response, as demonstrated by the decrease in mechanical nociceptive paw-withdrawal threshold in the third experimental day (within subject comparison in the CSR group, $p = 0.001$). The nociceptive response kept increasing progressively until the twelfth day, when it reached its maximum level, not changing significantly thereafter (within subject comparison in the CSR group, $p = 0.999$ for days 12 vs. 26). Important, along this chronic experiment, the animals were three times allowed to sleep freely for two consecutive days (indicates in figures as FS). Each two-day-periods of free sleep was neither enough to normalize pain sensitivity, nor to even significantly change the nociceptive response (within subject comparison in the CSR group, $p = 0.460$; $p = 0.999$; $p = 0.705$, respectively).

Qualitative assessment of home cage activity performed throughout the 26 days of the experiment suggests that general motor activity was decreased in chronically sleep restricted animals during the dark phase, which is compatible with an increase in

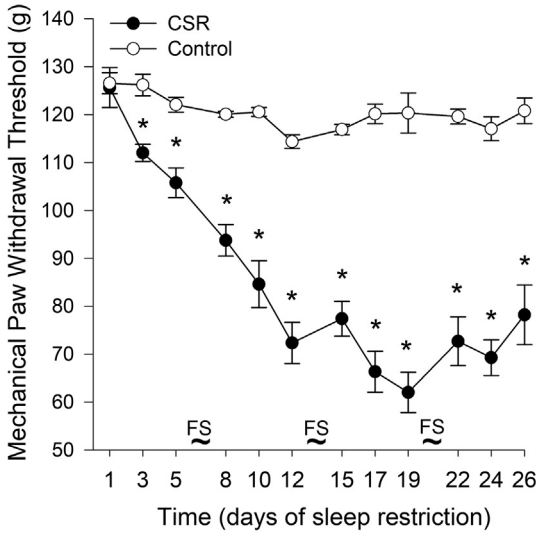

Fig. 1. The pronociceptive effect of chronic sleep restriction. The mechanical nociceptive threshold decreased progressively from day 3 to day 12 when it stabilized and no longer significantly changed until day 26. The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the control group (repeated-measures ANOVA followed by the Tukey's post hoc test, $p < 0.05$). In this and in subsequent figures data are presented as mean ± SEM. The number of animals in each group ranges from 4 to 8 per group. See methods for additional details regarding data presentation and analysis. CSR = chronic sleep restriction; FS = free sleep, it indicates the two-day-periods of free sleep.

sleep time (Supplementary Figure 1A).

3.2. The role of the nucleus accumbens in the pronociceptive effect of chronic sleep restriction

Excitotoxic NAc lesion induced by NMDA prevented the pronociceptive effect of CSR (Fig. 2A, repeated-measures (time) ANOVA – sleep condition (CSR or control procedure): $F(1,19) = 1195.7$, $p < 0.0001$; treatment (NMDA or sham-lesion): $F(1,19) = 957.71$, $p < 0.0001$; sleep condition x treatment: $F(1,19) = 1257.1$, $p < 0.0001$; sleep condition x treatment x time: $F(5,95) = 68.872$, $p < 0.0001$; Tukey test $p < 0.001$) and significantly decreased the number of the NAc neurons (Fig. 2B, two-way ANOVA – sleep condition (SD or control procedure): $F(1,14) = 1.805$, $p = 0.20$; treatment (NMDA or sham-lesion): $F(1,14) = 27.878$, $p < 0.001$; sleep condition x treatment: $F(1,14) = 1.029$, $p = 0.32$; Tukey test $p < 0.01$). Representative images of lesioned tissue clearly indicate a decreased number of neurons within the NAc (Fig. 2C). Schematic anatomical reconstruction of the lesions shows their location and extension (Fig. 2D).

3.3. The role of the periaqueductal gray in the pronociceptive effect of chronic sleep restriction

Excitotoxic PAG lesion induced by NMDA prevented the pronociceptive effect of CSR (Fig. 3A, two-way repeated measures ANOVA – sleep condition (SD or control procedure): $F(1,19) = 445.12$, $p < 0.0001$; treatment (NMDA or sham-lesion): $F(1,19) = 361.56$, $p < 0.0001$; sleep condition x treatment: $F(1,19) = 392.52$, $p < 0.0001$; sleep condition x treatment x time: $F(5,95) = 103.00$, $p < 0.0001$; and Tukey test $p < 0.001$) and significantly decreased the number of the PAG neurons (Fig. 3B, two-way ANOVA – sleep condition (SD or control procedure): $F(1,14) = 0.0311$, $p = 0.86$; treatment (NMDA or sham-lesion): $F(1,14) = 15.312$, $p = 0.002$; sleep condition x treatment: $F(1,14) = 0.546$, $p = 0.47$; Tukey test $p < 0.04$). Representative images of lesioned tissue clearly indicate a decreased number of neurons within the PAG (Fig. 3C). Schematic anatomical reconstruction of the lesions shows their location and extension (Fig. 3D).

3.4. Chronic sleep restriction increases c-Fos expression in the nucleus accumbens and in the periaqueductal gray

Chronic sleep restriction for 12 days ($p < 0.001$), significantly increased c-Fos expression within the NAc. Excitotoxic PAG lesion did not change c-Fos expression within the NAc either in control or in sleep restricted animals (Fig. 4A, one-way ANOVA – between groups: $F(3, 14) = 21.823$, $p < 0.001$). Representative images clearly indicate increased c-Fos expression within the NAc with 12 days of CSR (Fig. 4B). There is a strong negative correlation between

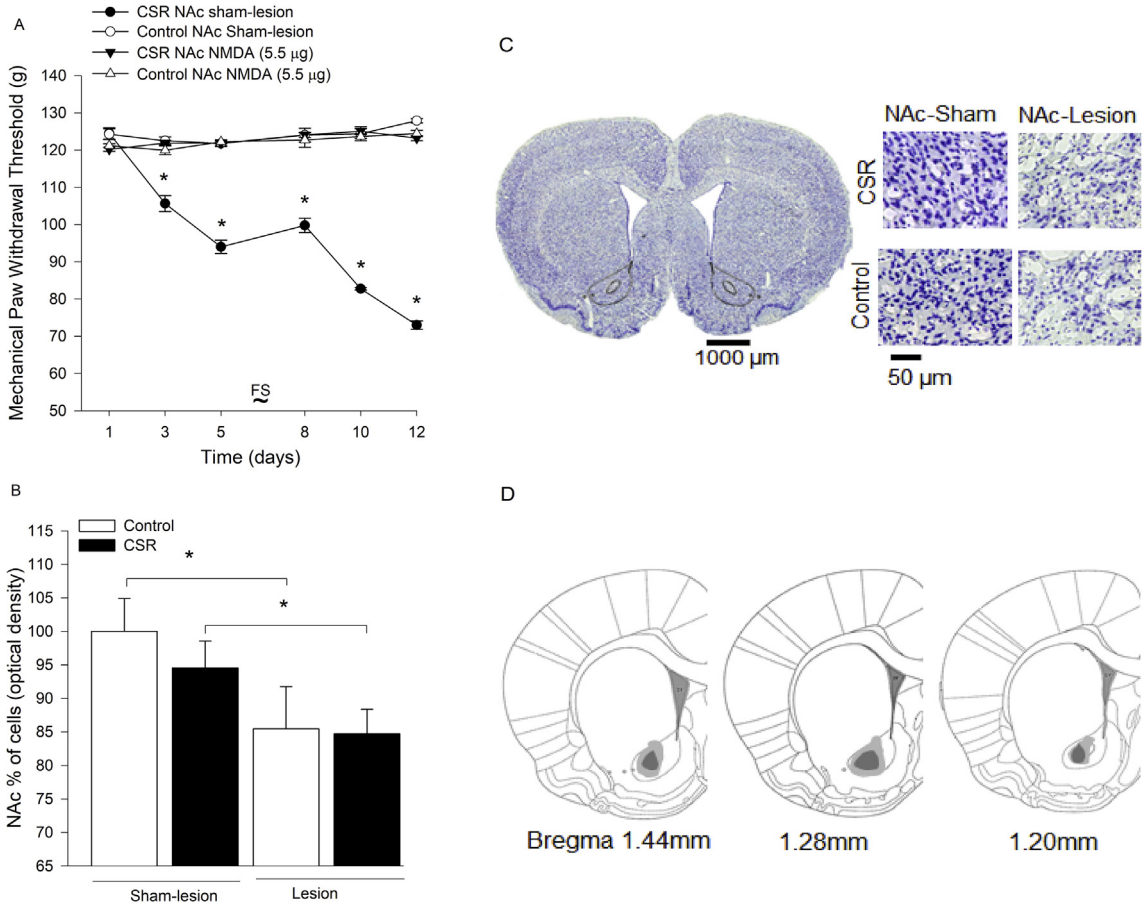

Fig. 2. The role of the nucleus Accumbens in the pronociceptive effect of chronic sleep restriction. A-The excitotoxic lesion of the NAc with NMDA (5.5 μg) prevented the pronociceptive effect of CSR. The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the other groups (repeated-measures ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **B-** The administration of NMDA into the NAc significantly decreased the number of local neurons, characterizing the excittoxic lesion. The symbol "*" indicates a significantly lower number of neurons (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **C-** Representative images of the NAc lesion. **D-** Anatomical reconstruction of the NAc lesions based on the atlas of Paxinos and Watson (2007).

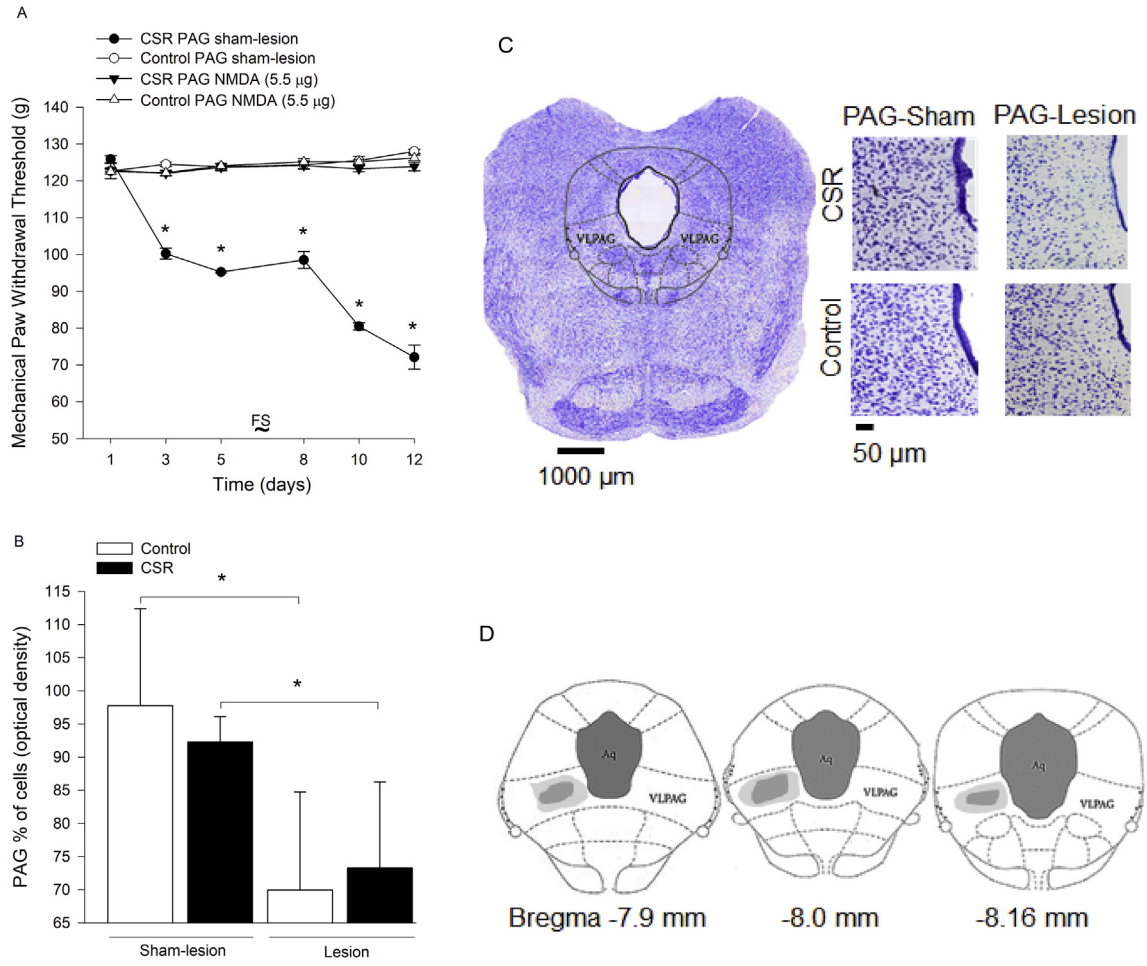

Fig. 3. The role of the periaqueductal grey in the pronociceptive effect of chronic sleep restriction. A- The excitotoxic lesion of the PAG with NMDA (5.5 μg) prevented the pronociceptive effect of CSR. The symbol "*" indicates a mechanical nociceptive threshold significantly lower than that of the other groups (repeated-measures ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **B-** The administration of NMDA into the PAG significantly decreased the number of local neurons, characterizing the excittoxic lesion. The symbol "*" indicates a significantly lower number of neurons (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **C-** Representative images of the PAG lesion. **D-** Anatomical reconstruction of the PAG lesions based on the atlas of Paxinos and Watson (2007).

nociceptive threshold and c-Fos expression within the NAc in animals with no lesion (Fig. 4C $r = -0.915$; $p = 0.00143$). When the PAG is lesioned, this correlation is no longer observed (Fig. 4D $r = -0.548$; $p = 0.101$).

Chronic sleep restriction for 12 days ($p < 0.001$) significantly increased c-Fos expression within the PAG. Excitotoxic NAc lesion did not change c-Fos expression within the PAG in sleep restricted animals ($p = 0{,}996$), but significantly increased it in control ones ($p = 0{,}002$) (Fig. 5A, one-way ANOVA – between groups: $F(3, 15) = 28.569$, $p < 0.001$). Representative images clearly indicate increased c-Fos expression within the PAG with 12 days of CSR (Fig. 5B). There is a strong negative correlation between nociceptive threshold and c-Fos expression within the PAG in animals with no lesion (Fig. 5C $r = -0.910$; $p = 0.00168$). When the PAG is lesioned, this correlation is no longer observed (Fig. 5D $r = -0.100$; $p = 0.769$).

Qualitative assessment of home cage activity performed throughout the 12 days of the experiment suggests that neither NAc nor PAG lesion affect general motor activity in chronically sleep restricted animals (Supplementary Figure 1B) or control animals (Supplementary Figure 1C).

4. Discussion

This study demonstrated that sleep restriction for 6 h daily induces a pronociceptive effect that increases progressively from day 3 to day 12 remaining stable thereafter. Repeated two-day-periods of free sleep were neither enough to normalize pain sensitivity, nor to even attenuate the increased nociceptive response. The pronociceptive effect of CSR depends on both the NAc and the PAG, since it was prevented by the excitotoxic lesion of any one of them. Complementarily, CSR significantly increased c-Fos protein expression within the NAc and the PAG and this correlates with the intensity of the pronociceptive effect, suggesting that the greater the neural activity in these regions, the greater the pronociceptive effect.

These findings largely extended a limited literature about the ability of sleep loss to increase pain sensitivity and the underlying mechanisms. The majority of the studies on this field have used selective REM sleep deprivation in animals (Damasceno et al., 2009; Nascimento et al., 2007; Sardi et al., 2018; Tomim et al., 2016; Wei et al., 2013). However, nowadays people worldwide sleep less than they would like or need due to occupational/recreational reasons or insomnia disorders (Calhoun et al., 2014; Owens et al., 2014). Understanding to what extent and how sleep restriction impacts pain sensitivity would certainly contribute to increase the effectiveness of pain management in this population. In order to advance our understanding of this issue, we have used a four week protocol in which the animals were prevented from

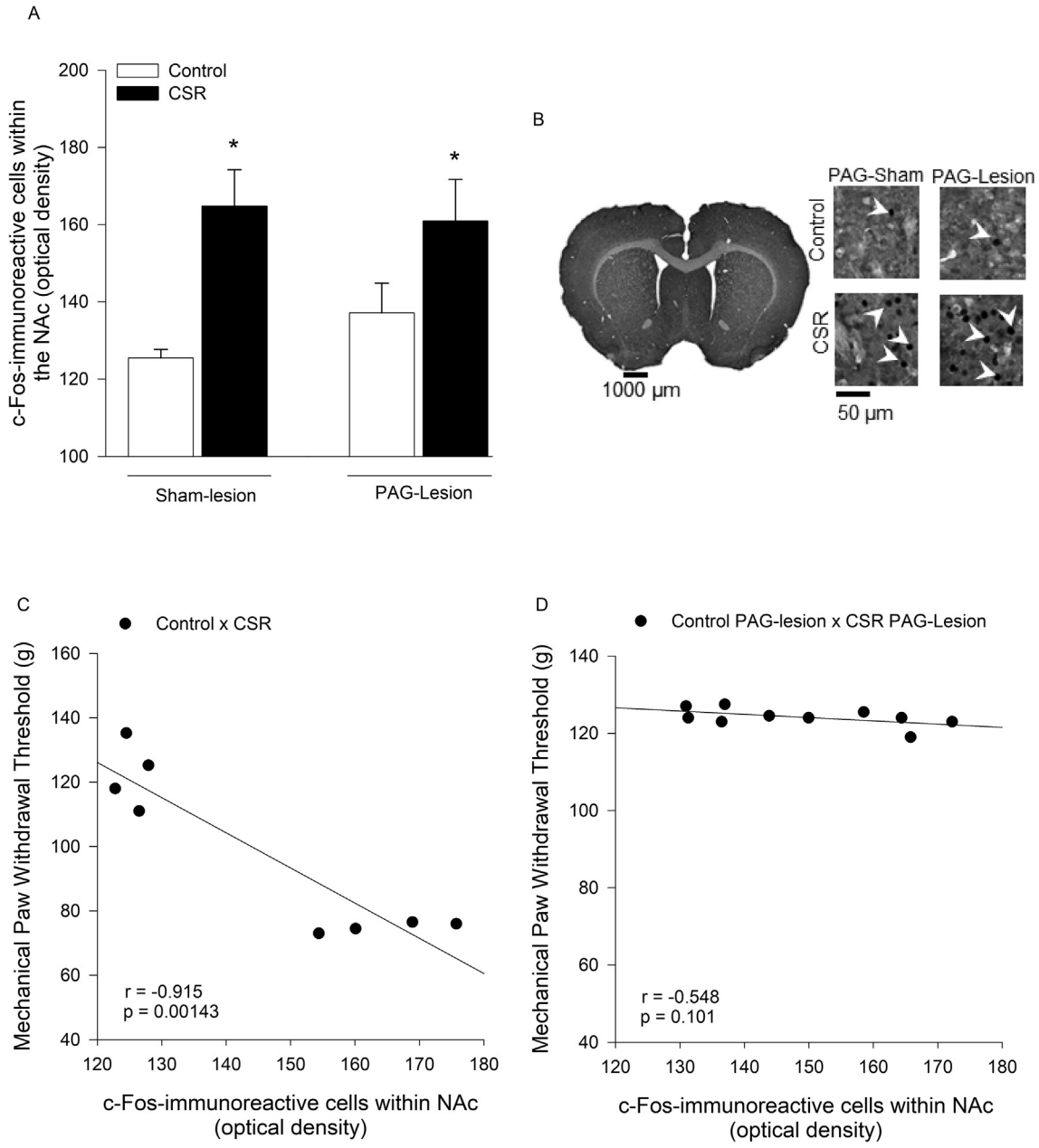

Fig. 4. The effect of chronic sleep restriction on c-Fos protein expression within the nucleus Accumbens. A- CSR significantly increased c-Fos expression within the NAc and this is not affected by PAG lesion. The symbol "*" indicates a significantly higher number of c-Fos-immunoreactive cells within the NAc (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **B-** Representative images of c-Fos immunoreactive cells (indicated by arrows) within the NAc. **C-** There is a strong negative correlation between mechanical paw withdrawal threshold and c-Fos-immunoreactive cells within the NAc of non-lesioned animals (Pearson's Correlation test). **D-** In animals with PAG lesion this correlation is lost.

sleeping during the first six hours of the light phase, when sleep pressure is highest in rodents. Although similar procedures for sleep restriction in both animals (Alexandre et al., 2017) and humans (Tiede et al., 2010) have already been associated with increased pain sensitivity, this study extend these previous ones, in part, by showing the temporal evolution of the pronociceptive effect of CSR and its unchanged persistence after two days of free sleep (Fig. 1). Our data showed a progressive increase in nociceptive responses, demonstrated by the decrease in mechanical nociceptive threshold, until day 12, after which the nociceptive response stabilized and significant changes were no longer observed until day 26, when the experiment ended. Important, along this four-week chronic experiment, three two-day-periods of free sleep were designed in order to mimic free sleep during weekends. Their complete inability to restore normality in pain sensitivity suggests that extended periods of sleep recovery are demanded to reverse the effects of CSR. From a translational perspective, this suggests that the impact of sleep loss on the prevalence and intensity of pain conditions is even greater than could be supposed, since free sleep on weekends may not be enough to reverse the changes in pain processing imposed during the working week.

The mechanisms by which sleep loss increases pain sensitivity (Alexandre et al., 2017; Nascimento et al., 2007; Okifuji and Hare, 2011; Sardi et al., 2018; Schuh-Hofer et al., 2013; Tomim et al., 2016) and the prevalence of pain conditions (Mork and Nilsen, 2012; Paul-Savoie et al., 2012; Tiede et al., 2010) are largely

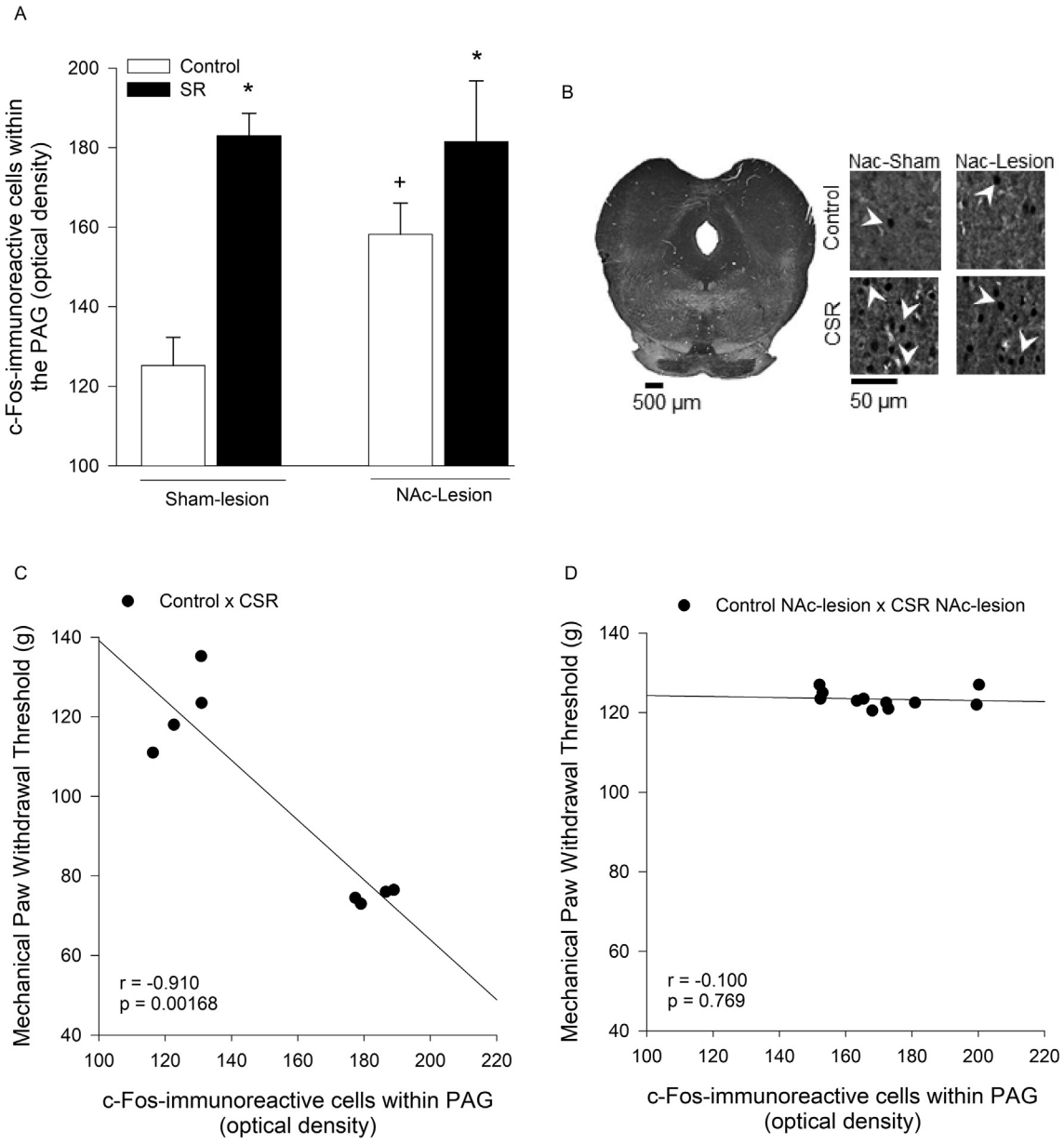

Fig. 5. The effect of chronic sleep restriction on c-Fos protein expression within the periaqueductal grey. A-c-Fos expression within the PAG was significantly increased by CSR or NAc lesion. The significantly higher number of c-Fos-immunoreactive cells within the PAG is indicated by the symbol "*" comparison with all other groups and "+" comparison with control animals (two-way ANOVA followed by the Tukey's post hoc test, $p < 0.05$). **B-** Representative images of c-Fos immunoreactive cells (indicated by arrows) within the PAG. **C-** There is a strong negative correlation between mechanical paw withdrawal threshold and c-Fos-immunoreactive cells within the PAG of non-lesioned animals (Pearson's Correlation test). **D-** In animals with NAc lesion this correlation is lost.

unknown. This study also advances our understanding in this area by showing that the potent pronociceptive effect of CSR depends on both the NAc and the PAG. To our knowledge no previous studies have investigated the central mechanisms underlying the pronociceptive effect of CSR, being this the first evidence of specific brain nuclei mediating such effect. The experimental support to this evidence was provided by data showing that the excitotoxic lesion of either the NAc (Fig. 2) or of the PAG (Fig. 3) prevented the pronociceptive effect induced by CSR. The indirect estimation of neural activity by c-Fos expression within the NAc and the PAG further support their role in such effect. The increased c-Fos expression in both the NAc (Fig. 4A and B, two bars/images at left) and the PAG (Fig. 5A and B, two bars/images at left) of chronic sleep restricted animals is consistent with their increased activity in response to CSR. In fact, findings from nociceptive activity and c-Fos expression correlate well in non-lesioned animals. The higher the nociceptive response (decrease in mechanical nociceptive threshold), the greater the expression of c-Fos either in the NAc (Fig. 4C) or in the PAG (Fig. 5C). As expected, the high significant correlation between nociceptive response and c-Fos expression is lost in animals with lesion either in the PAG (Fig. 4D) or in the NAc (Fig. 5D). This is because although the excitotoxic lesion of the PAG prevented the pronociceptive effect of CSR, it did not change the increased c-Fos expression within the NAc of chronic sleep restricted animals (Fig. 4A and B, two bars/images at right). Similarly, the excitotoxic lesion of the NAc prevented the pronociceptive effect of CSR without affecting the increased c-Fos expression within the PAG of chronic sleep restricted animals (Fig. 5A and B, two bars/images at

right). One possible explanation for the extinction of the effect and the maintenance of c-Fos expression is that the pronociceptive effect depends on the integrity of the underlying neural circuitry, thus the lesion of one nucleus in the circuitry is able to prevent the overall effect without necessarily affecting CSR-induced changes in neural activity of other nuclei. Worthy of note is also the fact that the NAc lesion significantly increased c-Fos expression in the PAG of control (not sleep restricted) animals (Fig. 5A, compare the white bars). This may result from decreased inhibitory activity from the NAc to the PAG, since efferent activity from the NAc is predominantly GABAergic (Mogenson et al., 1983) and some of them project directly to the PAG (Zhang et al., 2013).

The NAc, in the ventral striatum, is a key component of the mesolimbic dopaminergic system, with recognized role in the modulation of both pain (Gear and Levine, 2011; Tobaldini et al., 2014) and sleep wake cycle (Oishi and Lazarus, 2017). Sleep pressure is believed to increase the NAc efferent activity in order to inhibit wake-promoting nuclei in the brainstem and hypothalamus (Lazarus et al., 2013). We have recently demonstrated that increased NAc activity mediates the pronociceptive effect of acute selective REM sleep deprivation (Sardi et al., 2018). The present study extends this previous one by showing that the pronociceptive effect of CSR, which develops progressively over 12 days, is also dependent on the NAc, where c-Fos expression correlates with the intensity of such effect. Acute selective REM sleep deprivation is a worldwide used method to mimic the effects of several sleep disturbances that affect primarily REM sleep (Brown et al., 2012; Naiman, 2017). On the other hand, restriction of total sleep by some hours per day is supposed to mimic the decrease in sleep time due to occupational or recreational reasons (Iacovides et al., 2017). Together, the present study and that previous one suggest that sleep loss, no matter the model used, increases nociceptive responses by increasing the NAc activity, which is in accordance with the suggested pronociceptive role of this nucleus (Gear and Levine, 2011). Important, sleep loss is known to increase the prevalence of chronic pain conditions (Mork and Nilsen, 2012; Okifuji and Hare, 2011) and recent evidences from both human (Baliki et al., 2012) and animal (Dias et al., 2015; Miranda et al., 2015) studies support a key role of the NAc in pain chronification. Therefore, the NAc may be a major player of pain chronification in patients who sleep poorly.

The PAG is the center of the most known mechanism for pain modulation, the PAG-RVM descending system. In this system, inputs from multiple forebrain regions are integrated within the vlPAG, which projects to the rostral ventromedial medulla (RVM), from where descending pathways target the dorsal horn to facilitate or inhibit nociceptive transmission (Millan, 2002). In addition to its key role in pain modulation, the PAG also contributes to control sleep-wake cycle (Lu et al., 2006). PAG dopaminergic neurons induce wakefulness (Lu et al., 2006), while its GABAergic neurons suppresses REM sleep and consolidate NREM sleep (Weber et al., 2018). We have previously demonstrated that acute selective REM sleep deprivation increases pain by increasing the pain facilitatory activity of the PAG-RVM descending system (Tomim et al., 2016). The present study shows that the pronociceptive effect of CSR also depends on the PAG and that local c-Fos expression correlates with the intensity of such effect. Therefore, sleep loss in response to either CSR or acute REM sleep deprivation appears to increase pain by disrupting the way by which the PAG controls pain processing. The key role of the PAG in pain processing has been consistently demonstrated by several studies showing that the intensity of acute pain (Bee and Dickenson, 2008; Burgess et al., 2002) and the development of chronic pain (Granovsky, 2013; Martel et al., 2013) are strongly influenced by changes in the PAG activity. Therefore, an increase in pain facilitatory activity from the PAG may lead to the increased pain intensity and vulnerability associated to sleep loss.

One limitation of this study is that we could not determine whether and how CSR and the NAc or PAG lesion affects sleep-wake cycle. A qualitative measurement of home cage activity by actimetry performed throughout the experiment suggested that CSR increases sleep time during the dark phase (Supplementary Figure S1A), while the lesion of neither NAc nor PAG seems to have affected home cage motor activity either in chronically sleep restricted (Supplementary Figure S1B) or control (Supplementary Figure S1C) animals. Another limitation of this study is that we have focused in the core region of the NAc and in the ventrolateral column of the PAG, so that we could not determine if the role of NAc and PAG in the pronociceptive effect of CSR is limited to these specific regions. We believe that it is not for the NAc, since our previous study demonstrated that both the core and shell regions of the NAc contribute to the pronociceptive effect of REM-sleep deprivation (Sardi et al., 2018). However, we believe that the contribution of PAG is limited to its ventrolateral column, because this and not the adjacent columns of the PAG controls the descending pain modulation system (Basbaum and Fields, 1979; Behbehani, 1995), which is implicated in the pronociceptive effect of REM-sleep deprivation (Tomim et al., 2016). Future studies would contribute to overcome the limitations of the present study definitively supporting or refuting our suggestions.

In summary, this study showed that the daily restriction of sleep time progressively increases pain sensitivity, which is not reversed by two consecutive days of free sleep. The pronociceptive effect of CSR depends on the NAc and the PAG, where the neural activity, indirectly estimated by c-fos expression, correlates with the intensity of such effect. Both the NAc and the PAG have key roles in pain chronification and in the modulation of pain sensitivity. Since it is known that chronic pain conditions are more prevalent (Appleton et al., 2018; Karaman et al., 2014; Mork and Nilsen, 2012; Murphy et al., 2017; Okifuji and Hare, 2011) and pain sensitivity is greater (Okifuji and Hare, 2011; Paul-Savoie et al., 2012) among people who sleep poorly, these findings may contribute to understanding why this happens and how it can be prevented.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.06.022.

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