Received: 1 May 2020 | Revised: 6 August 2020 | Accepted: 7 August 2020

DOI: 10.1002/jdn.10059

RESEARCH ARTICLE

INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE

WILEY

Single exercise stress reduces central neurotrophins levels and adenosine A_1 and A_2 receptors expression, but does not revert opioid-induced hyperalgesia in rats

Liciane Fernandes Medeiros[1,2] | **Éllen Almeida Nunes**[1,3] | **Bettega Costa Lopes**[1,3] | **Andressa de Souza**[1,2,4] | **Angélica Regina Cappellari**[5] | **Joice Soares de Freitas**[1] | **Isabel Cristina de Macedo**[1] | **Jonnsin Kuo**[1] | **Stefania Giotti Cioato**[1] | **Ana Maria de Oliveira Battastini**[6] | **Wolnei Caumo**[4] | **Iraci L. S. Torres**[1,3,4]

[1]Laboratório de Farmacologia da Dor e Neuromodulação: Investigações Pré-clínicas, Centro de Pesquisa Experimental (CPE), Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil

[2]Programa de Pós-Graduação em Saúde e Desenvolvimento Humano, Universidade La Salle, Canoas, Brazil

[3]Programa de Pós-Graduação em Ciências Biológicas: Fisiologia, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

[4]Programa de Pós-Graduação em Medicina: Ciências Médicas, Faculdade de Medicina, UFRGS, Porto Alegre, Brazil

[5]Programa de Pós-Graduação em Biologia Celular e Molecular, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, Brazil

[6]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, ICBS, UFRGS, Porto Alegre, Brazil

Correspondence
Iraci L. S. Torres, Laboratório de Farmacologia da Dor e Neuromodulação: Investigações Pré-clínicas, Centro de Pesquisa Experimental (CPE), Hospital de Clínicas de Porto Alegre (HCPA), Rua Ramiro Barcelos, 2350, 2nd floor, Porto Alegre, RS 90035-003, Brazil.
Email: iltorres@hcpa.edu.br

Funding information
This study was supported by Hospital de Clínicas de Porto Alegre (#140425)

Abstract

Background: This study assessed the effects of an acute stress model upon the long-term hyperalgesia induced by repeated morphine administration in neonatal rats. We also evaluated neurotrophins and cytokines levels; expressions of adenosine and acetylcholine receptors, and acetylcholinesterase enzyme at the spinal cord.

Material and methods: Male Wistar rats were subjected to morphine or saline administration from P8 to P14. Thermal hyperalgesia and mechanical hyperesthesia were assessed using the hot plate (HP) and von Frey (vF) tests, respectively, at postnatal day P30 and P60. After baseline measurements, rats were subjected to a single exercise session, as an acute stress model, at P30 or P60. We measured the levels of BDNF and NGF, interleukin-6, and IL-10 in the cerebral cortex and the brainstem; and the expression levels of adenosine and muscarinic receptors, as well as acetylcholinesterase (AChE) enzyme at the spinal cord.

Results: A stress exercise session was not able to revert the morphine-induced hyperalgesia. The morphine and exercise association in rats induced a decrease in the neurotrophins brainstem levels, and A_1, A_{2A}, A_{2B} receptors expression in the spinal cord, and an increase in the IL-6 cortical levels. The exercise reduced M2 receptors expression in the spinal cord of naive rats, while morphine prevented this effect.

Liciane Fernandes Medeiros and Éllen Almeida Nunes are first authors.

Conclusions: Single session of exercise does not revert hyperalgesia induced by morphine in rats; however, morphine plus exercise modulate neurotrophins, IL-6 central levels, and expression of adenosine receptors.

KEYWORDS
adenosine receptors, biomarkers, exercise, hyperalgesia, morphine, neonate rats

1 | INTRODUCTION

Morphine-induced hyperalgesia has been investigated in pre-clinical and clinical settings and involves several mechanisms, including opioidergic (Roeckel et al., 2017) and non-opioidergic signaling (Holtman & Wala, 2005; Rozisky, 2016, 2011). These changes imply an altered neurochemistry signaling, to impaired firing pattern between afferent neurons and descending modulation pathways in important centers to control nociceptive information (Chu, Zheng, Loh, & Law, 2008; DuPen, Shen, & Ersek, 2007; Juif et al., 2016). Overall, it can trigger a modulation in physiological responses, mainly in neonates, once their immature nociceptive system is developing (Fitzgerald, 2005).

Our previous studies reinforce such findings, the neonatal morphine exposure induces long-term changes in nociceptive pathways, promoting hyperalgesia in the formalin test (Rozisky, 2016, 2011), thermal hyperalgesia, mechanical allodynia and hyperalgesia in adult life (Nunes et al., 2017), and changes the morphine response in adult life (Rozisky et al., 2008). Also, repeated morphine exposure during neonatal life decreased brain-derived-neurotrophic factor (BDNF) and neurotrophic growth factor (NGF) levels in the cerebral cortex of rats (Nunes et al., 2017). Proinflammatory cytokine expression, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), were up-regulated in the spinal cord of rats after chronic morphine treatment, as well as the BDNF levels (Ferrini et al., 2013).

Evidence suggests that a single session of exercise can elicit acute stress responses in the body (Hackney, 2006), as stress-induced analgesia (SIA). SIA refers to a reduced nociceptive response after acute stress exposure; which is mediated by descending pain-inhibitory circuits and may be an indicator of adequate centrally mediated pain control (Jennings, Okine, Roche, & Finn, 2014). The benefits of exercise on the nociceptive response were characterized by the involvement of the opioid system (Hoeger Bement & Sluka, 2005; Koltyn, 2000; Stagg et al., 2011). Furthermore, some studies also described the role of exercise upon different neurotransmitters, e.g., adenosinergic (Sawynok, 2016), and muscarinic systems (Heo et al., 2014).

Considering all exposed before, the aim of this study was to determine the effects of a single exercise session, as an acute stress model, upon the hyperalgesia induced by neonatal morphine exposure indexed by the thermal and mechanical nociceptive response at P30 and P60 in rats. Additionally, we assessed neurotrophic factors (BDNF and NGF), and cytokines (IL-6 and IL-10) in the cerebral cortex and brainstem levels (at P32 and P62), and the spinal cord expressions of A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors, M2 acetylcholine receptor, and of acetylcholinesterase enzyme (P62).

2 | MATERIALS AND METHODS

Male Wistar rats ($n = 62$; at P8) were divided into two groups: saline-control and morphine, and housed in polypropylene cages (49 cm × 34 cm × 16 cm) with sawdust-covered flooring in a controlled environment (22 ± 2°C; 12/12 hr light–dark cycle; water and chow ad libitum). At birth, litters were culled to eight pups per dam (Rozisky et al., 2008, 2013), and were weaned at 21 days of age. Only the males were assessed in the study. All experiments were approved by the Institutional Animal Care and Use Committee (protocol No. 140425) and met the ARRIVE guidelines (Kilkenny & Altman, 2010).

2.1 | Pharmacological treatment

Each rat received saline (control group) or morphine (5 μg, s.c. in the mid-scapular area) (Rozisky et al., 2008, 2013). One milliliter of morphine sulphate (Dimorf® 10 mg/ml, purchased from Cristália; Brazil) was diluted in 9 ml of 0.9% NaCl (saline) daily.

2.2 | Exercise protocol

At P30 or P60, rats were exposed to a single exercise session, as an acute stress model, which consisted of running on a rats' treadmill at a moderate intensity (12 m/min for 20 min). The rats were put in the treadmill turned off for 5 min during three days before the test day (Brust, Corbell, Al-Nakkash,

Babu, & Broderick, 2014), and no electric shock or physical stimulus was used.

2.3 | Experimental design

From P8 to P14, male pups rats received morphine or saline. After baseline nociceptive measurements, rats were subjected to a single session of exercise at P30 or P60 or sedentary. Thermal hyperalgesia was assessed by hot plate test: baseline (P30 or P60), 1 and 24 hr after exercise. Mechanical hyperesthesia was assessed using von Frey (vF) test: baseline (P30 or P60), and 24 hr after the exercise session (P31 or P61). Rats were euthanized by decapitation at 24 hr after the last behavioral measurements, at P32 or P62, for structures collection (Figure 1).

2.4 | von Frey test

Mechanical hyperesthesia was assessed as previously described (Cioato et al., 2016). The intensity of the stimulus supported up to paw withdrawal, in grams (s), was automatically recorded. Three successive recordings were measured between interval periods of 5 s and averaged. The averages were used as the final measurements and were expressed in grams (g).

2.5 | Hot plate test

Thermal hyperalgesia was assessed as described (Woolfe & Macdonald, 1944). The time in seconds between the placement of the rat and the first response (foot licking, jumping, or rapidly removing paws) was recorded as the latency of nociceptive response. The cut-off time was 20 s to avoid tissue damage.

2.6 | Tissue collection

The rats were killed at P32 and P62. The tissues (cerebral cortex and brainstem) were collected and frozen at −80°C. For PCR, the spinal cord was removed, immersed in QIAzol, and snapped frozen for posterior analysis at P62.

2.7 | Neurochemical assays

Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for quantifying IL-6, IL-10, BDNF, and NGF levels on the cerebral cortex and brainstem were performed using monoclonal antibodies specific (R&D Systems, United States). The data were expressed in pg/mg of protein (Bradford, 1976).

2.8 | RT-PCR and qPCR assays

Total RNA the spinal cord was isolated with QIAzol Lysis Reagent in accordance with the manufacturer's instructions. The cDNA was synthesized with SuperScript® III (Invitrogen—Life Technologies™) from 1μg of total RNA in a final volume of 20 μl with an oligodT primer in accordance with the manufacturer's instructions. The specific primers used for qPCR are described in Table 1. All experiments were performed in triplicate. The melting curve and standard curves were measured for each primer set and cDNA sample in order to verify reaction efficiency.

2.9 | Statistical analysis

Two-way mixed ANOVA followed by Bonferroni was used for nociceptive tests (morphine and exercise). Three-way ANOVA/Bonferroni was performed to compare neurochemical levels between groups, considering age, morphine, and exercise. Expression levels were analyzed by one-way ANOVA/Tukey test. The data were expressed as the mean ± standard error of the mean (S.E.M) and considered significant at $p < .05$. SPSS 20.0 for Windows was used.

3 | RESULTS

3.1 | Thermal hyperalgesia

There was an interaction between time and exercise (two-way mixed ANOVA/Bonferroni, $p < .05$, $F_{(2,56)} = 7.769$, $n = 8$; Figure 2a) at P30; the exercise decreased the paw withdrawal latency 24 hr after exercise in relation to previous measures. In addition, there was an interaction between morphine and

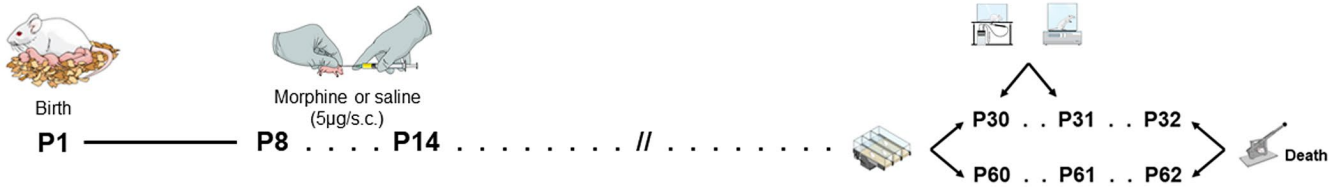

FIGURE 1 Experimental design [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Primer sequences

Primer sequence		Fragment size (bp)
A1 F	5′-ATT GCT GTG GAT CGA TAC C-3′	100
A1 R	5′-GAA TCC AGC AGC CAG CTA T-3′	
A2A F	5′-GCA GAG TTC CAT CTT TAG C-3′	100
A2A R	5′-CGC CCT CAC ACC TGT CA-3′	
A2B F	5′-TCC ATC TTT AGC CTC TTG G-3′	100
A2B R	5′-TC CTC TTG CTC GTG TTC-3′	
A3 F	5′-CTG CGA GTC AAG CTG AC-3′	100
A3 R	5′-GTC CCA CCA GAA AGG ACA-3′	
AChE F	3´-AAC TAC ACC GTG GAG GAG AGA-5´	
AChE R	3´-CCA TCC CCA CTC CAA TAC CAC-5´	
Chrm2 F	3´-CCT ACC CAG TTA AGC GGA CCA C-5´	
Chrm2 R	3´-TGC CAG AAG AGA ATG GCT GG-5´	
β2-microglobulin F	3´-TCC TGG CTC ACA CTG AAT TC-5´	
β2-microglobulin R	3´-CTT TGT GGA TAA ATT GTA TAG CA-5´	
β-actin F	5′-GGT CAT CAC TAT CGG CAA T-3′	100
β-actin R	5′-GAA TGT AGT TTC ATG GAT GC-3′	

Note: These primers listed were used to RT-PCR and real time-PCR. Melting curve analysis was performed to determine the specificity for each real-time PCR reaction.

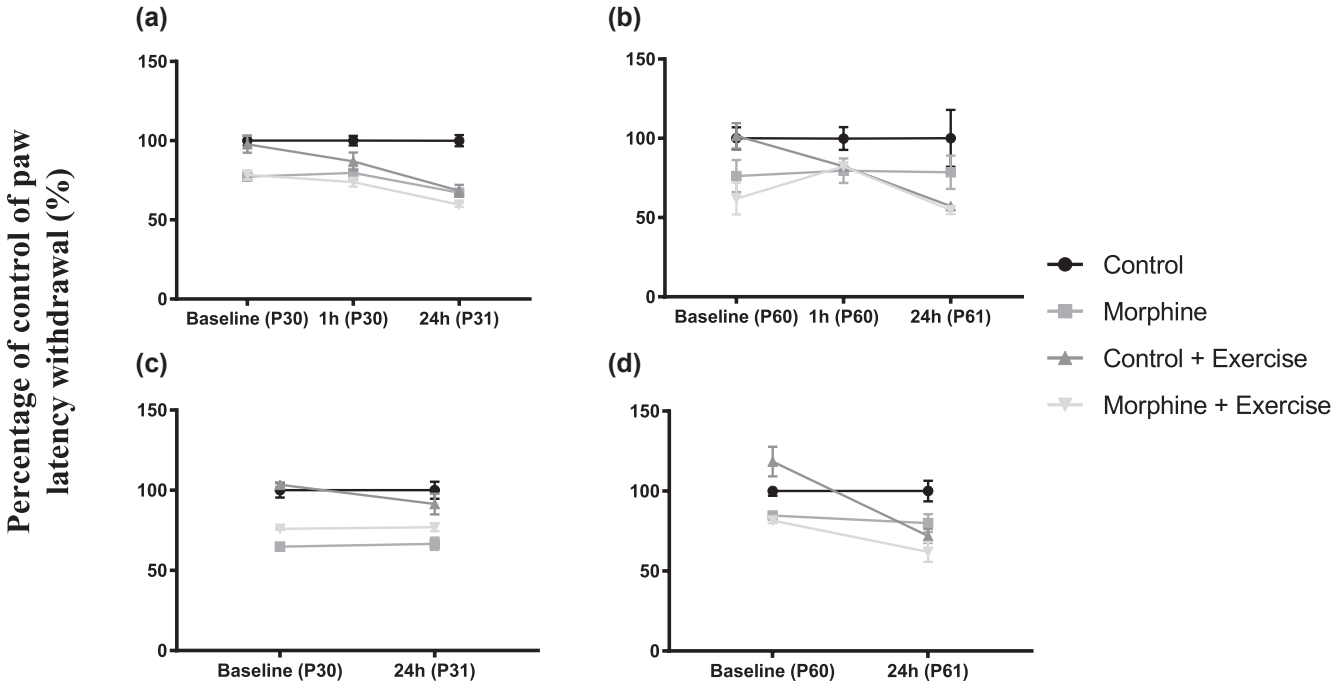

FIGURE 2 Latency of paw withdrawal assessed using Hot Plate (HP) test at baseline, 1 and 24 hr after unique exercise session assessed at P30 (a) and at P60 (b) and nociceptive response assessed by Von Frey (VF) test at baseline and 24 hr after unique exercise session assessed at P30 (c) and at P60 (d). Data presented as mean ± *SEM* of percentage of control [VF: P30 (28.97g) and P60 (39.32g); HP: P30 (2.53s) and P60 (2.62s). (a) There was an interaction between time and exercise (two-way mixed ANOVA/Bonferroni, $p < .05$, $F_{(2,56)} = 7.769$, $n = 8$). (b) There was an interaction between time and exercise (two-way mixed ANOVA/Bonferroni, $p < .01$, $F_{(2,54)} = 6.058$, $n = 7–8$). (c) There was an interaction between morphine and exercise (two-way mixed ANOVA/Bonferroni, $p < .05$, $F_{(1,28)} = 6.388$, $n = 8$). (d) There was an interaction between time, exercise, and morphine (two-way mixed ANOVA/Bonferroni, $p < .05$, $F_{(1,27)} = 5.788$, $n = 7–8$)

exercise ($p < .05$, $F_{(1,28)} = 7.233$); and main effects of morphine ($p < .001$, $F_{(1,28)} = 81.864$) and exercise ($p < .001$, $F_{(1,28)} = 21.033$).

At P60, there was an interaction between time and exercise (two-way mixed ANOVA/Bonferroni, $p < .01$, $F_{(2,54)} = 6.058$, $n = 7–8$; Figure 2b). We observed a decrease of paw withdrawal latency according to time in the groups subjected to exercise. In addition, there was main effects of time ($p < .05$, $F_{(2,54)} = 3.415$); morphine ($p < .001$, $F_{(1,27)} = 19.323$); and exercise ($p < .05$, $F_{(1,27)} = 7.754$).

3.2 | Mechanical hyperalgesia

At P30, there was an interaction between morphine and exercise (two-way mixed ANOVA/Bonferroni, $p < .05$, $F_{(1,28)} = 6.388$, $n = 8$; Figure 2c); and main effect of morphine ($p < .001$, $F_{(1,28)} = 110.100$).

At P60, there was an interaction between time, exercise, and morphine (two-way mixed ANOVA/Bonferroni, $p < .05$, $F_{(1,27)} = 5.788$, $n = 7–8$; Figure 2d); we observed a decrease of paw withdrawal latency according to time in the group submitted to exercise and morphine. There was an interaction time and exercise ($p < .001$, $F_{(1,27)} = 21.930$); and main effects of time ($p < .01$, $F_{(1,27)} = 29.237$); and morphine ($p < .001$, $F_{(1,27)} = 22.575$).

3.3 | BDNF levels

In the brainstem, there was an interaction between age, morphine, and exercise (three-way ANOVA/Bonferroni, $F_{(1,47)} = 16.012$, $p < .001$, $n = 5–8$; Figure 3), and an interaction between morphine and exercise (three-way ANOVA/Bonferroni, $F_{(1,47)} = 5.327$, $p < .03$, $n = 5–8$; Figure 3a). It is possible to observe that rats subjected to morphine in early life and subjected to one session of exercise at P60 displayed lower levels of BDNF, while exercise in the control group promoted increased BDNF levels.

In the total cerebral cortex of rats, there was an interaction between age and morphine (three-way ANOVA/Bonferroni, $F_{(1,52)} = 8.342$, $p < .01$, $n = 5–8$; Figure 3b), where morphine groups (morphine, and morphine plus exercise) presented lower levels of BDNF at P60. And, there was an interaction between morphine and exercise (three-way ANOVA/Bonferroni, $F_{(1,52)} = 4.721$, $p < .05$, $n = 5–8$).

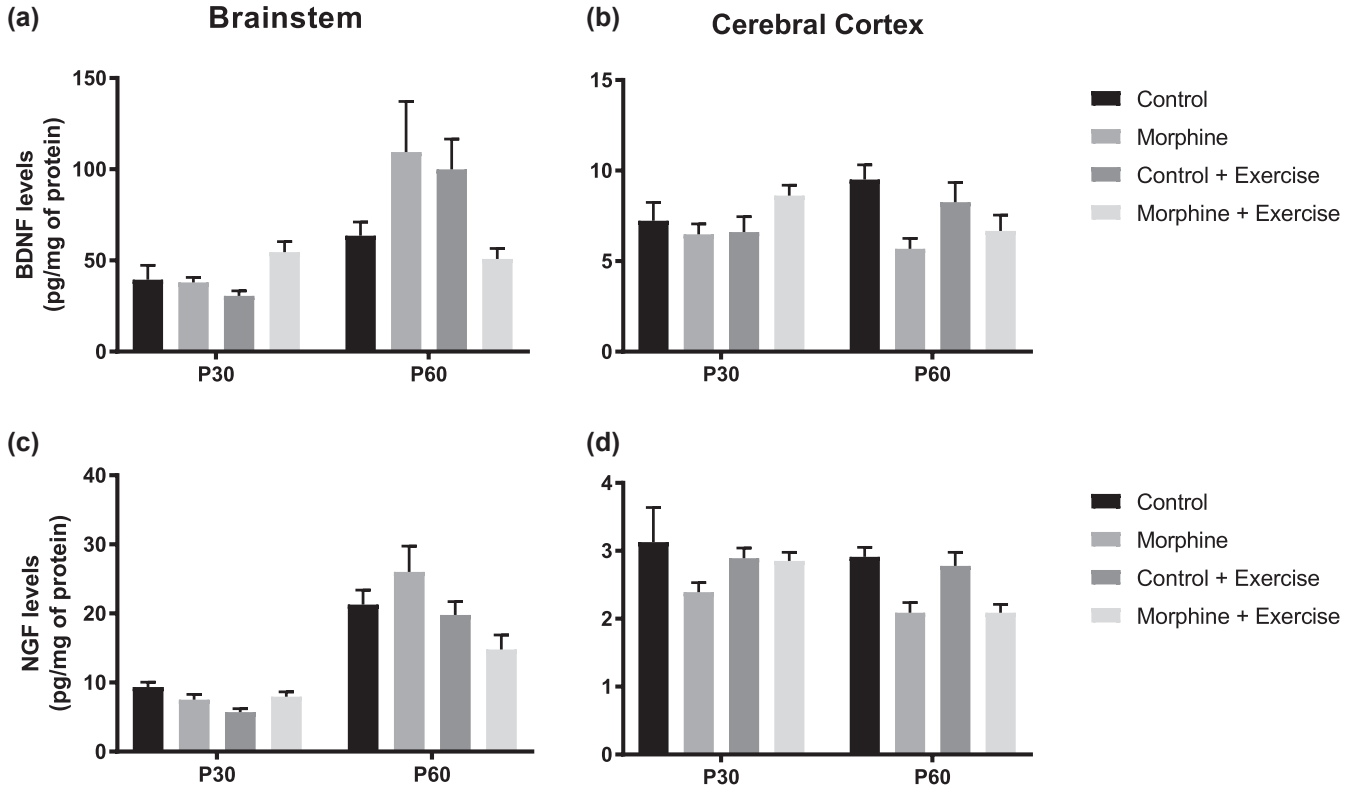

FIGURE 3 Biochemical levels of BDNF and NGF assessed 48 hr after unique exercise session at P32 and P62. Data presented as mean ± *SEM* (pg/mg of protein). (a) BDNF brainstem levels. There was an interaction between age, morphine, and exercise (three-way ANOVA/Bonferroni, $F_{(1,47)} = 16.012$, $p < .001$, $n = 5–8$). (b) BDNF cortical levels. There was an interaction between age and morphine (three-way ANOVA/Bonferroni, $F_{(1,52)} = 8.342$, $p < .01$, $n = 5–8$). (c) NGF brainstem levels. There was an interaction between age, morphine, and exercise (three-way ANOVA/Bonferroni, $F_{(1,47)} = 7.840$, $p < .01$, $n = 5–8$). (d) NGF cortical levels. There was a morphine effect (three-way ANOVA/Bonferroni, $F_{(1,52)} = 11.545$, $p < .01$, $n = 8$)

3.4 | NGF levels

In the brainstem, there was an interaction between age, morphine, and exercise (three-way ANOVA/Bonferroni, $F_{(1,47)} = 7.840$, $p < .01$, $n = 5–8$; Figure 3c). Interestingly, it was observed a reduction in the brainstem NGF levels at P60 in morphine plus exercise group; similar effect observed in brainstem BDNF levels. In addition, there was a main effect of exercise (three-way ANOVA/Bonferroni, $F_{(1,47)} = 10.486$, $p < .01$, $n = 5–8$; Figure 3c), decreasing NGF levels; and the main effect of age (three-way ANOVA/Bonferroni, $F_{(1,47)} = 109.178$, $p < .001$, $n = 5–8$; Figure 3c), increasing NGF levels with the age increase.

Also, we observed the main effects of morphine in the NGF cortical levels (three-way ANOVA/Bonferroni, $F_{(1,52)} = 11.545$, $p < .01$, $n = 8$; Figure 3d), with reduced NGF levels in the morphine groups.

3.5 | IL-6 levels

In the brainstem, there was a main effect of age (three-way ANOVA/Bonferroni, $F_{(1,47)} = 51.719$, $p < .001$, $n = 6–8$; Figure 4a); IL-6 levels increased according to age increase, at least until P60. While in the cerebral cortex, it was observed an interaction between morphine and exercise (three-way ANOVA/Bonferroni, $F_{(1,52)} = 4.021$, $p < .05$, $n = 5–8$; Figure 4b), thus morphine plus exercise increased IL-6 levels in relation to morphine group.

3.6 | IL-10 levels

In the brainstem and the cerebral cortex, there was a main effect of age (three-way ANOVA/Bonferroni, $F_{(1,47)} = 59.322$, $p < .001$, and $F_{(1,52)} = 4.608$, $p < .05$, respectively, $n = 5–8$; Figure 4c and d), IL-10 levels increase according to age increase, at least until P60.

3.7 | A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors in spinal cord

According to our data, there was a reduction in the A_1 expression receptor in the morphine plus exercise group in relation to morphine sedentary group (one-way ANOVA, $p < .05$, $F_{(3,11)} = 3.253$, $n = 4$, Figure 5). It was observed a reduction in the A_{2A} expression receptor in the morphine plus exercise group in relation to other groups (one-way ANOVA, $p < .01$, $F_{(3,10)} = 11.84$, $n = 4$, Figure 5). Interestingly, the morphine

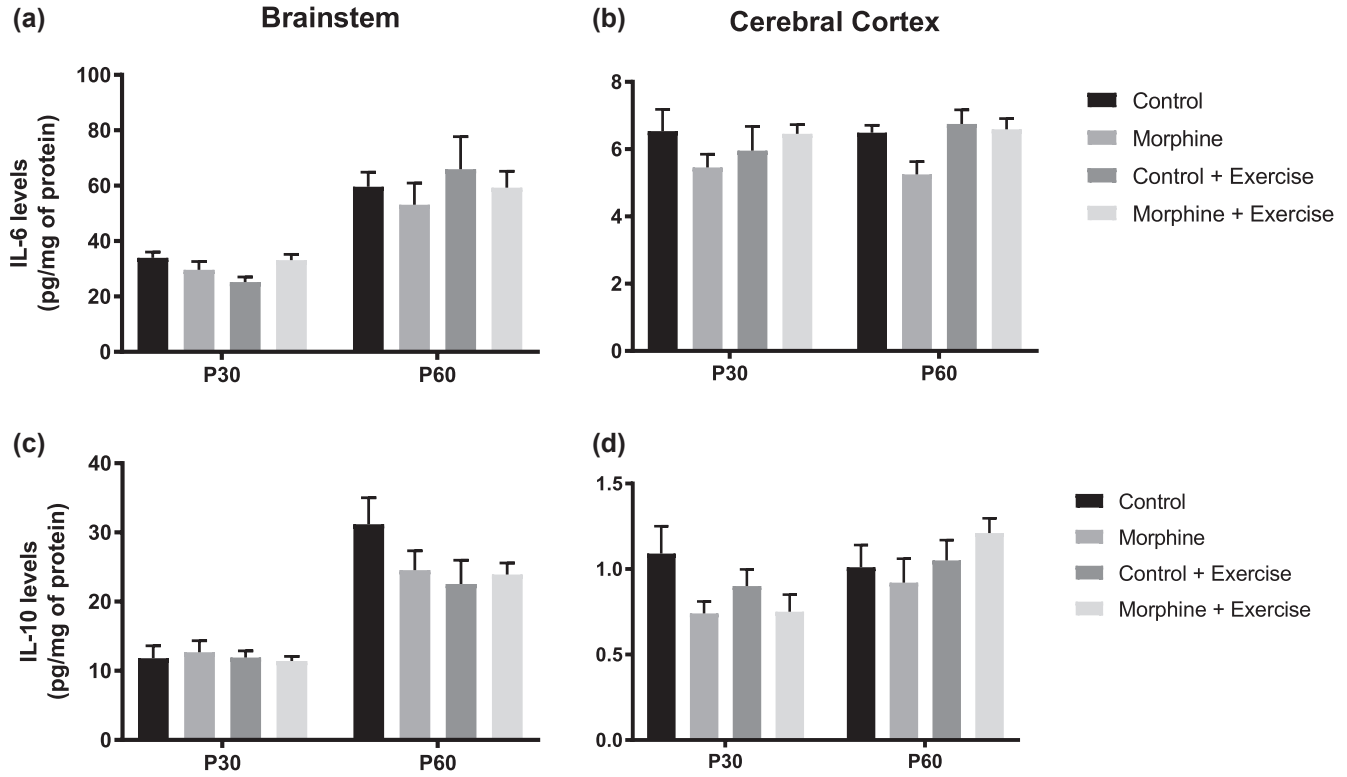

FIGURE 4 Biochemical levels of IL-6 and IL-10 assessed 48 hr after unique exercise session at P32 and P62. Data presented as mean ± *SEM* (pg/mg of protein). (a) IL-6 brainstem levels. There was an age effect (three-way ANOVA/Bonferroni, $F_{(1,47)} = 51.719$, $p < .001$, $n = 6–8$). (b) IL-6 cortical levels. There was an interaction between morphine and exercise (three-way ANOVA/Bonferroni, $F_{(1,52)} = 4.021$, $p < .05$, $n = 5–8$). (c) IL-10 brainstem levels. There was an age effect (three-way ANOVA/Bonferroni, $F_{(1,47)} = 59.322$, $p < .001$, $n = 5–8$). (d) IL-10 cortical levels. There was an age effect (three-way ANOVA/Bonferroni, $F_{(1,52)} = 4.608$, $p < .05$, $n = 5–8$)

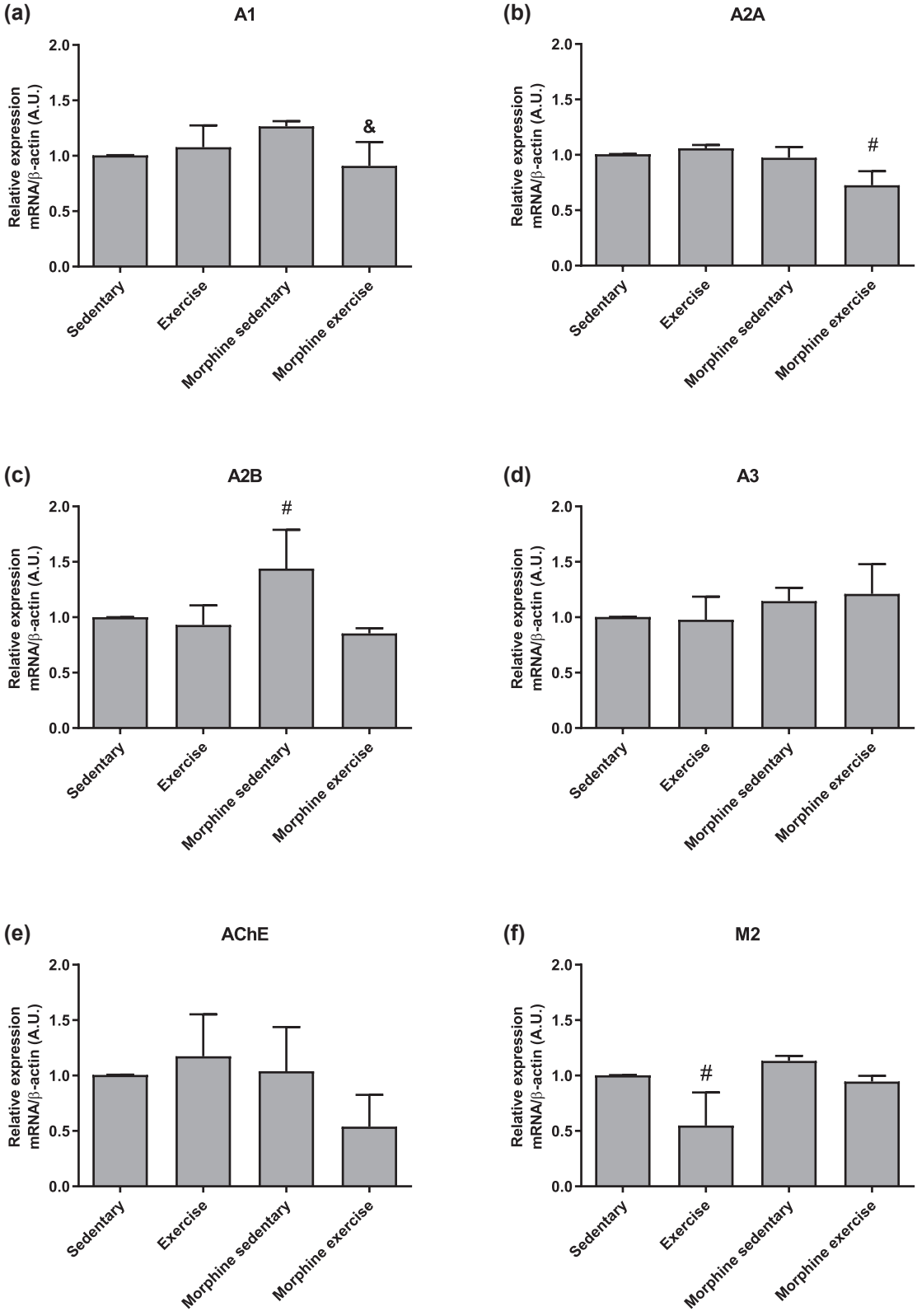

FIGURE 5 Analysis of mRNA expression–mRNA expression of A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors in addition to acetylcholinesterase (AChE) and muscarinic receptor 2 (Chrm2) were evaluated by qPCR. Quantitative analysis of the relative expression was evaluated by qPCR and used β-actin gene expression as an internal control for normalization of the expression levels. Data were analyzed by one-way ANOVA, followed by the Tukey post hoc test. The experiments were performed three times in triplicate. & different from the morphine sedentary group ($p < .05$). # different from other groups($p < .05$)

sedentary group presented higher A_{2B} expression receptors; however, the exercise was able to reduce this expression in the morphine plus exercise group at sedentary group levels (one-way ANOVA, $p < .01$, $F_{(3,12)} = 7.056$, $n = 4$, Figure 5). In relation to the A3 receptor, no significant differences between the groups were found at its expression (one-way ANOVA, $p > .05$, $F_{(3,11)} = 1.555$, $n = 4$, Figure 5).

3.8 | Expression of acetylcholinesterase (AChE) enzyme and M2 acetylcholine receptors in the spinal cord

In relation to AChE enzyme expression, no significant differences were found among groups (one-way ANOVA, $p > .05$, $F_{(3,12)} = 3.188$, $n = 4$, Figure 5). The M2 receptor expression was reduced by exercise in relation to other groups (one-way ANOVA, $p < .05$, and $p < .01$, $F_{(3,10)} = 10.10$, $n = 4$, Figure 5).

4 | DISCUSSION

In the current study, we showed that neonate rats subjected to repeated morphine exposure displayed mechanical and thermal hyperalgesia in adult life. In addition, we showed that acute stress induced by a single session of exercise did not revert the hyperalgesia, and in contrast to our SIA hypothesis, it triggered delayed hyperalgesia in the saline group. Also, the exercise association to early morphine decreased the brainstem levels of BDNF and NGF according to postnatal day analyzed; and reversed the reduction in the IL-6 levels in the cerebral cortex induced by morphine treatment. Moreover, morphine plus exercise reduced the expression receptors A_1, A_{2A}, A_{2B} in the spinal cord. And, exercise reduced M2 receptors expression in naive rats; while morphine prevented this effect.

It is interesting to note that the mechanisms of morphine-induced hyperalgesia are linked to changes in different systems, as opioidergic and glutamatergic (Suarez-Roca, 2006), downstream pathways (Song, Wu, & Zuo, 2015), and inflammatory mediators (Borghi et al., 2015), characterizing complex alterations. In the current study, we have tested a single session of exercise to the relief the hyperalgesia; however, we failed to show that. The rational to test one session of exercise as analgesic upon pain behavior was based on previous studies that have shown that single session of the exercise was able to trigger antinociceptive effect (Galdino & Romero, 2014a, 2014b; King-Himmelreich et al., 2017).

A close relationship between exercise and opioid signaling has been described. Previous study revealed that low-intensity exercise activates opioid receptors to reduce hyperalgesia in rats with chronic musculoskeletal pain (Hoeger Bement & Sluka, 2005). And it increases endogenous opioid content in brainstem regions related to pain modulation; and these effects are reversed by opioid receptor antagonists (Stagg et al., 2011). Despite exercise acts in different pathways, it is possible that this close relationship may contribute to no analgesic effect found in our findings.

In contrast to our SIA hypothesis, we also noted a decrease in the nociceptive threshold in the control group subjected to one session of exercise. It is known that exercise may produce hypoalgesia or hyperalgesia according to the protocol applied, brain areas activated and different timepoints assessing the after-effects (Lima, Abner, & Sluka, 2017), mainly, in preclinical settings, where the animals are, sometimes, subjected to a forced exercise. Our previous study corroborates that rats subjected to chronic restraint-induced hyperalgesia displayed analgesic response after forced swimming, as an acute stress model, but not after 3 hr restraint (Da Silva Torres et al., 2003). However, we have shown that regular aerobic treadmill exercise presents an analgesic effect in a neuropathic pain model in rats (Lopes et al., 2020). In this context, rats showed delayed hyperalgesia induced by the single exercise stress protocol.

In the literature, we have found studies showing a hyperalgesic effect triggered by exercise. For example, low-intensity exercise triggered hypernociceptive behavior in rats, achieving the peak 4 hr after exercise (Lana, Paulino, & Gonçalves, 2006). Also, a single bout of fatiguing exercise increases hyperalgesia in sedentary animals (Gregory, Brito, Fusaro, & Sluka, 2016; Gregory, Gibson-Corley, Frey-Law, & Sluka, 2013). In the same line, a single exercise session exacerbates pain by increasing phosphorylation of NMDA receptors, and modulate important centers in nociception (Lima et al., 2017; Fields, Malick, & Burstein, 1995). We might not rule out the central and peripheral events, i.e., the role of metabolites peripherally released in response to exercise in the skeletal muscle may contribute to the exercise-induced hyperalgesia, linked to inflammation and delayed-onset muscle soreness.

In our previous study, data have shown that neonatal morphine administration triggered changes in the hippocampal BDNF levels (Rozisky et al., 2013). It is possible to observe that prenatal morphine exposure caused a reduction in the hippocampal BDNF levels in the female, but not male rats, and over 30 days of postnatal, exercise or enriched environment was able to restore the BDNF levels at adult age (Ahmadalipour, Ghodrati-Jaldbakhan, Samaei, & Rashidy-Pour, 2018). Here, we observed that a single session of exercise in the morphine group decreased BDNF and NGF levels in the brainstem without effects in the cerebral cortex. However, the exercise increased BDNF levels in the saline group, suggesting a state- and structure-dependent effect. Both neurotrophins (BDNF and NGF) are involved with peripheral or central sensitization in different chronic pain conditions (Pezet & McMahon, 2006). In this context, our current findings corroborate previous studies regarding the role of central neurotrophins in the long-lasting effect of morphine; also, how exercise was able to modulate them.

Our previous study has shown the effects of morphine exposure in neonatal age decreased ADP hydrolysis and increase the expression levels of E-NTPDase 1 in the spinal cord (Rozisky et al., 2010). Here, we observed that rats subjected to early morphine plus a single session of exercise (P60) decreased A_1R, $A_{2A}R$, and $A_{2B}R$ expression levels, while those only subjected to early morphine exposure showed an increase in the spinal cord $A_{2B}R$ expression levels, which could be related to hyperalgesia induced by early morphine exposure. Corroborating this hypothesis, a previous study showed an analgesic effect induced by adenosine $A_{2B}R$ antagonist in two independent models of chronic pain (Hu et al., 2016). However, we cannot discard a possible interference of delayed time for biochemical analysis as the behavioral assessments were performed in a different time frame. While spinal adenosine A_1 receptor activation inhibits pain-related behavior (Aley, Green, & Levine, 1995; Goldman et al., 2010); acute activation of peripheral A_{2A} receptors increased the pain-related behavior (Li et al., 2010). Thus, exercise applied upon morphine rats may trigger an imbalance in the spinal cord adenosine receptors; which may contribute to no analgesic effect observed in rats with morphine-induced hyperalgesia. However, in the current study, mRNA levels in the spinal cord were measured, not directly the membrane receptor expression.

In this study, it was observed downregulation of M2 receptors, which might be attributed to an oversupply or a shuttle of acetylcholine for large motor neurons that innervate skeletal muscle during exercise (Welton, Stewart, Kerr, & Maxwell, 1999), as well as, for their vasodilatory effects on smooth vasculature after exercise. Effects of acetylcholine on muscarinic and/or nicotinic receptors are implied on antinociception mediated by inhibitory G-Protein-Coupled Receptors (GPCRs) in the spinal cord. The analgesic mechanisms provided by systemic morphine administration involves the release of acetylcholine in the spinal cord (Chen & Pan, 2001). Also, it is possible to suggest that the downregulation of M2 receptors triggered by exercise can be the result of an imbalance between the muscarinic receptors' spinal cord levels; and this effect was prevented by early morphine administration.

Regarding the ontogenic effect, in the current study, we found an increased central level of IL-6 and IL-10 with age. It is important to note that similar results were found in our previous data that have shown an age effect upon different biomarker levels (Nunes et al., 2017).

In conclusion, our findings corroborate data from the literature that have shown the repeated exposure of morphine in the neonatal life induces hypernociceptive behavior at the adult age of rats. Despite a single session of exercise that does not revert this behavior, the association of exercise and morphine modulates expression levels of receptors, such as A_1R, $A_{2A}R$, $A_{2B}R$; as well as IL-6 levels in the central nervous system, as summarized in Figure 6.

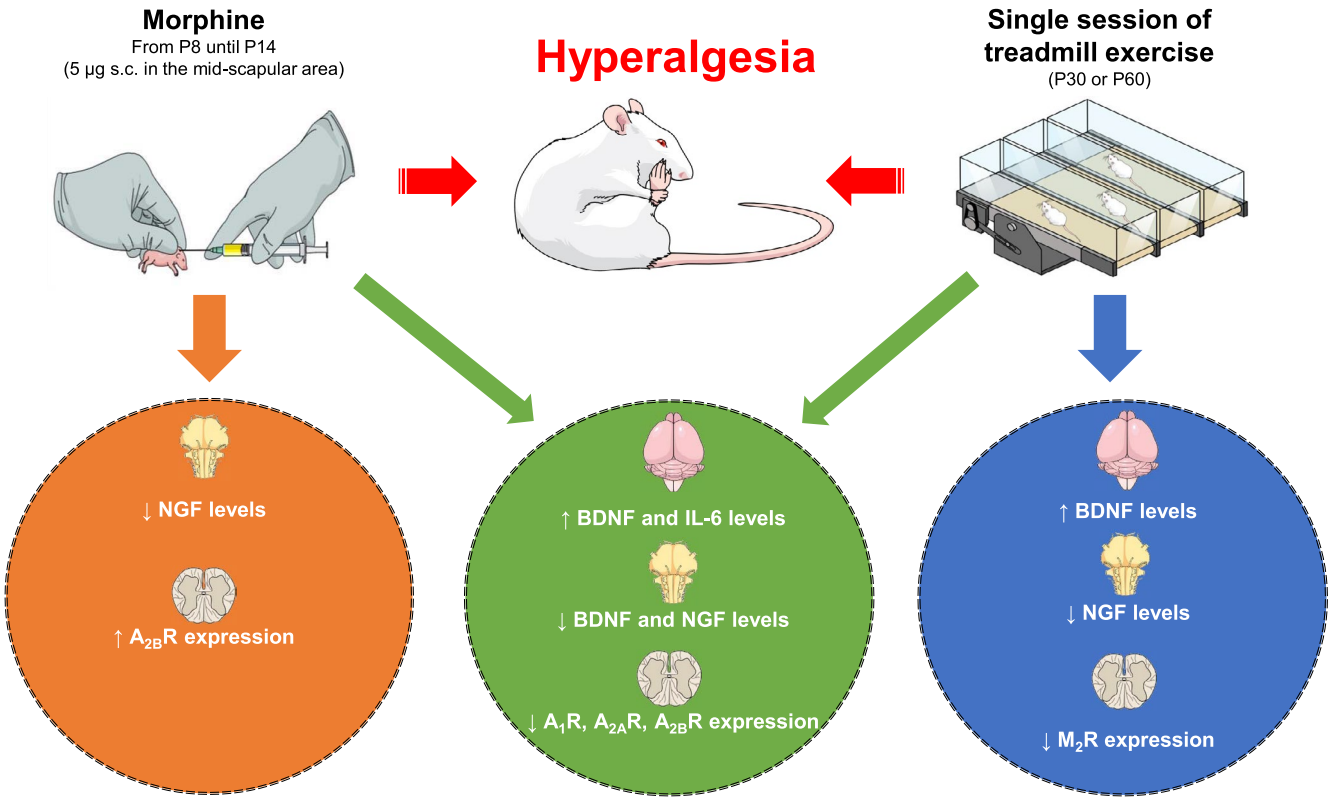

FIGURE 6 Schematic representation of the findings [Colour figure can be viewed at wileyonlinelibrary.com]

ACKNOWLEDGEMENTS

The research was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development—CNPq (Dr. I.L.S. Torres; Dr. W.Caumo; J.S. Freitas); Committee for the Development of Higher Education Personnel—CAPES (E.A. Nunes; B.C. Lopes); Hospital de Clínicas de Porto Alegre—GPPG (I.L.S.Torres—Grant 140425). PROPESq/UFRGS; FAPERGS/PRONEM (ILS Torres –Grant 11/2050-3).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Liciane Fernandes Medeiros: Conceptualization, Methodology, Writing-Original draft preparation. Éllen Almeida Nunes: Conceptualization and Methodology. Bettega Costa Lopes: Writing-Reviewing and Editing. Andressa de Souza, Angélica Regina Cappellari, Joice Soares de Freitas, Jonnsin Kuo, Stefania Giotti Cioato: Methodology, Data curation. Isabel Cristina de Macedo: Conceptualization. Ana Maria de Oliveira Battastini, Wolnei Caumo: Writing-Reviewing and Editing. Iraci L. S. Torres: Supervision, Writing-Reviewing, and Editing.

ETHICAL APPROVAL

The animal protocol was approved by the Institutional Animal Care and Use Committee (protocol N°140425).

DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the reasonable request.

ORCID

Liciane Fernandes Medeiros https://orcid.org/0000-0002-6842-7241
Éllen Almeida Nunes https://orcid.org/0000-0003-3860-0020
Bettega Costa Lopes https://orcid.org/0000-0002-8090-0273
Andressa de Souza https://orcid.org/0000-0002-6608-4695
Angélica Regina Cappellari https://orcid.org/0000-0002-8202-609X
Joice Soares de Freitas https://orcid.org/0000-0002-7861-1691
Isabel Cristina de Macedo https://orcid.org/0000-0001-6215-1371
Jonnsin Kuo https://orcid.org/0000-0002-1890-2921
Stefania Giotti Cioato https://orcid.org/0000-0002-1854-0713
Ana Maria de Oliveira Battastini https://orcid.org/0000-0001-6068-3278
Wolnei Caumo https://orcid.org/0000-0002-5083-4658

REFERENCES

Ahmadalipour, A., Ghodrati-Jaldbakhan, S., Samaei, S. A., & Rashidy-Pour, A. (2018). Deleterious effects of prenatal exposure to morphine on the spatial learning and hippocampal BDNF and long-term potentiation in juvenile rats: Beneficial influences of postnatal treadmill exercise and enriched environment. *Neurobiology of Learning and Memory*, *147*, 54–64. https://doi.org/10.1016/j.nlm.2017.11.013

Aley, K. O., Green, P. G., & Levine, J. D. (1995). Opioid and adenpsine peripheral antinociception are subject to tolerance and withdrawal. *Journal of Neuroscience*, *15*(12), 8031–8038. https://doi.org/10.1523/jneurosci.15-12-08031.1995

Borghi, S. M., Pinho-Ribeiro, F. A., Zarpelon, A. C., Cunha, T. M., Alves-Filho, J. C., Ferreira, S. H., … Verri, W. A. (2015). Interleukin-10 limits intense acute swimming-induced muscle mechanical hyperalgesia in mice. *Experimental Physiology*, *100*(5), 531–544. https://doi.org/10.1113/EP085026

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Analytical Biochemistry*, *72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brust, K. B., Corbell, K. A., Al-Nakkash, L., Babu, J. R., & Broderick, T. L. (2014). Expression of gluconeogenic enzymes and 11β-hydroxysteroid dehydrogenase type 1 in liver of diabetic mice after acute exercise. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *7*, 495–504. https://doi.org/10.2147/DMSO.S70767

Chen, S. R., & Pan, H. L. (2001). Spinal endogenous acetylcholine contributes to the analgesic effect of systemic morphine in rats. *Anesthesiology*, *95*(2), 525–530. https://doi.org/10.1097/00000542-200108000-00039

Chu, J. I., Zheng, H., Loh, H. H., & Law, P.-Y. (2008). Morphine-induced μ-opioid receptor rapid desensitization is independent of receptor phosphorylation and β-arrestins. *Cellular Signalling*, *20*(9), 1616–1624. https://doi.org/10.1016/j.cellsig.2008.05.004

Cioato, S. G., Medeiros, L. F., Marques Filho, P. R., Vercelino, R., de Souza, A., Scarabelot, V. L., … Torres, I. L. S. (2016). Long-lasting effect of transcranial direct current stimulation in the reversal of hyperalgesia and cytokine alterations induced by the neuropathic pain model. *Brain Stimulation*, *9*(2), 209–217. https://doi.org/10.1016/j.brs.2015.12.001

da Silva Torres, I. L., Cucco, S. N. S., Bassani, M., Duarte, M. S., Silveira, P. P., Vasconcellos, A. P., … Ferreira, M. B. C. (2003). Long-lasting delayed hyperalgesia after chronic restraint stress in rats—Effect of morphine administration. *Neuroscience Research*, *45*(3), 277–283. https://doi.org/10.1016/S0168-0102(02)00232-8

DuPen, A., Shen, D., & Ersek, M. (2007). Mechanisms of opioid-induced tolerance and hyperalgesia. *Pain Management Nursing : Official Journal of the American Society of Pain Management Nurses*, *8*, 113–121. https://doi.org/10.1016/j.pmn.2007.02.004

Ferrini, F., Trang, T., Mattioli, T.-A., Laffray, S., Del'Guidice, T., Lorenzo, L.-E., … De Koninck, Y. (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl- homeostasis. *Nature Neuroscience*, *16*(2), 183–192. https://doi.org/10.1038/nn.3295

Fields, H. L., Malick, A., & Burstein, R. (1995). Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. *Journal of Neurophysiology*, *74*(4), 1742–1759. https://doi.org/10.1152/jn.1995.74.4.1742

Fitzgerald, M. (2005). The development of nociceptive circuits. *Nature Reviews Neuroscience*, *6*(7), 507–520. https://doi.org/10.1038/nrn1701

Galdino, G., Romero, T., da Silva, J. F. P., Aguiar, D., de Paula, A. M., Cruz, J., … Perez, A. (2014). Acute resistance exercise induces antinociception by activation of the endocannabinoid system in rats. *Anesthesia and Analgesia*, *119*(3), 702–715. https://doi.org/10.1213/ANE.0000000000000340

Galdino, G., Romero, T. R. L., Silva, J. F. P., Aguiar, D. C., de Paula, A. M., Cruz, J. S., … Perez, A. C. (2014). The endocannabinoid system mediates aerobic exercise-induced antinociception in rats. *Neuropharmacology*, *77*, 313–324. https://doi.org/10.1016/j.neuropharm.2013.09.022

Goldman, N., Chen, M., Fujita, T., Xu, Q., Peng, W., Liu, W., … Nedergaard, M. (2010). Adenosine A1 receptors mediate local anti-nociceptive effects of acupuncture. *Nature Neuroscience*, *13*(7), 883–888. https://doi.org/10.1038/nn.2562

Gregory, N. S., Brito, R. G., Fusaro, M. C. G. O., & Sluka, K. A. (2016). ASIC3 is required for development of fatigue-induced hyperalgesia. *Molecular Neurobiology*, *53*(2), 1020–1030. https://doi.org/10.1007/s12035-014-9055-4

Gregory, N. S., Gibson-Corley, K., Frey-Law, L., & Sluka, K. A. (2013). Fatigue-enhanced hyperalgesia in response to muscle insult: Induction and development occur in a sex-dependent manner. *Pain*, *154*(12), 2668–2676. https://doi.org/10.1016/j.pain.2013.07.047

Hackney, A. C. (2006). Stress and the neuroendocrine system: The role of exercise as a stressor and modifier of stress. *Expert Review of Endocrinology & Metabolism*, *1*(6), 783–792. https://doi.org/10.1586/17446651.1.6.783

Heo, Y.-M., Shin, M.-S., Lee, J.-M., Kim, C.-J., Baek, S.-B., Kim, K.-H., & Baek, S.-S. (2014). Treadmill exercise ameliorates short-term memory disturbance in scopolamine-induced Amnesia Rats. *International Neurourology Journal*, *18*(1), 16–22. https://doi.org/10.5213/inj.2014.18.1.16

Hoeger Bement, M. K., & Sluka, K. A. (2005). Low-intensity exercise reverses chronic muscle pain in the rat in a naloxone-dependent manner. *Archives of Physical Medicine and Rehabilitation*, *86*(9), 1736–1740. https://doi.org/10.1016/j.apmr.2005.03.029

Holtman, J. R., & Wala, E. P. (2005). Characterization of morphine-induced hyperalgesia in male and female rats. *Pain*, *114*(1), 62–70. https://doi.org/10.1016/j.pain.2004.11.014

Hu, X., Adebiyi, M. G., Luo, J., Sun, K., Le, T.-T., Zhang, Y., … Xia, Y. (2016). Sustained elevated adenosine via ADORA2B promotes chronic pain through neuro-immune interaction. *Cell Reports*, *16*(1), 106–119. https://doi.org/10.1016/j.celrep.2016.05.080

Jennings, E. M., Okine, B. N., Roche, M., & Finn, D. P. (2014). Stress-induced hyperalgesia. *Progress in Neurobiology*, *121*, 1–18. https://doi.org/10.1016/j.pneurobio.2014.06.003

Juif, P.-E., Salio, C., Zell, V., Melchior, M., Lacaud, A., Petit-Demouliere, N., … Poisbeau, P. (2016). Peripheral and central alterations affecting spinal nociceptive processing and pain at adulthood in rats exposed to neonatal maternal deprivation. *European Journal of Neuroscience*, *44*(3), 1952–1962. https://doi.org/10.1111/ejn.13294

Kilkenny, C., & Altman, D. G. (2010). Letter to the Editor, *8*(January), 377–378. https://doi.org/10.1371/journal.pbio.1000412

King-Himmelreich, T. S., Möser, C. V., Wolters, M. C., Schmetzer, J., Schreiber, Y., Ferreirós, N., … Niederberger, E. (2017). AMPK contributes to aerobic exercise-induced antinociception downstream of endocannabinoids. *Neuropharmacology*, *124*, 134–142. https://doi.org/10.1016/j.neuropharm.2017.05.002

Koltyn, K. F. (2000). A review. *29*(2), 85–98.

Lana, C., Paulino, C. A., & Gonçalves, I. D. (2006). Influence of low and high intensity physical exercise on hypernociception threshold and other parameters of rats. *Revista Brasileira De Medicina do Esporte*, *12*(5), 248–254.

Li, L., Hao, J. X., Fredholm, B. B., Schulte, G., Wiesenfeld-Hallin, Z., & Xu, X. J. (2010). Peripheral adenosine A2A receptors are involved in carrageenan-induced mechanical hyperalgesia in mice. *Neuroscience*, *170*(3), 923–928. https://doi.org/10.1016/j.neuroscience.2010.07.045

Lima, L. V., Abner, T. S. S., & Sluka, K. A. (2017). Does exercise increase or decrease pain? Central mechanisms underlying these two phenomena. *Journal of Physiology*, *595*(13), 4141–4150. https://doi.org/10.1113/JP273355

Lopes, B. C., Medeiros, L. F., Silva de Souza, V., Cioato, S. G., Medeiros, H. R., Regner, G. G., … Torres, I. L. S. (2020). Transcranial direct current stimulation combined with exercise modulates the inflammatory profile and hyperalgesic response in rats subjected to a neuropathic pain model: Long-term effects. *Brain Stimulation*, *13*(3), 774–782. https://doi.org/10.1016/j.brs.2020.02.025

Nunes, E. A., Medeiros, L. F., Freitas, J. S., Macedo, I. C., Kuo, J., Souza, A., … Torres, I. L. S. (2017). Morphine exposure during early life alters thermal and mechanical thresholds in rats. *International Journal of Developmental Neuroscience*, *60*, 78–85. https://doi.org/10.1016/j.ijdevneu.2016.12.008

Pezet, S., & McMahon, S. B. (2006). Neurotrophins: Mediators and modulators of pain. *Annual Review of Neuroscience*, *29*(1), 507–538. https://doi.org/10.1146/annurev.neuro.29.051605.112929

Roeckel, L.-A., Utard, V., Reiss, D., Mouheiche, J., Maurin, H., Robé, A., … Gaveriaux-Ruff, C. (2017). Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide. *Scientific Reports*, *7*(1), 1–15. https://doi.org/10.1038/s41598-017-11120-4

Rozisky, J. R., da Silva, R. S., Adachi, L. S., Capiotti, K. M., Ramos, D. B., Bogo, M. R., … Torres, I. L. D. S. (2010). Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats. *European Journal of Pharmacology*, *642*(1–3), 72–76. https://doi.org/10.1016/j.ejphar.2010.05.044

Rozisky, J. R., Dantas, G., Adachi, L. S., Alves, V. S., Ferreira, M. B. C., Sarkis, J. J. F., & Torres, I. L. D. S. (2008). Long-term effect of morphine administration in young rats on the analgesic opioid response in adult life. *International Journal of Developmental Neuroscience*, *26*(6), 561–565. https://doi.org/10.1016/j.ijdevneu.2008.05.005

Rozisky, J. R., Laste, G., de Macedo, I. C., Santos, V. S., Krolow, R., Noschang, C., … Torres, I. L. S. (2013). Neonatal morphine administration leads to changes in hippocampal BDNF levels and antioxidant enzyme activity in the adult life of rats. *Neurochemical Research*, *38*(3), 494–503. https://doi.org/10.1007/s11064-012-0941-8

Rozisky, J. R., Medeiros, L. F., Adachi, L. S., Espinosa, J., de Souza, A., Neto, A. S., … da Silva Torres, I. L. (2011). Morphine exposure in early life increases nociceptive behavior in a rat formalin tonic pain model in adult life. *Brain Research*, *1367*, 122–129. https://doi.org/10.1016/j.brainres.2010.10.041

Rozisky, J. R., Scarabelot, V. L., Oliveira, C. D., Macedo, I. C. D., Deitos, A., Laste, G., … Torres, I. L. S. (2016). Melatonin as a potential counter-effect of hyperalgesia induced by neonatal morphine exposure. *Neuroscience Letters*, *633*, 77–81. https://doi.org/10.1016/j.neulet.2016.08.027

Sawynok, J. (2016). Adenosine receptor targets for pain. *Neuroscience*, *338*, 1–18. https://doi.org/10.1016/j.neuroscience.2015.10.031

Song, L., Wu, C., & Zuo, Y. (2015). Melatonin prevents morphine-induced hyperalgesia and tolerance in rats: Role of protein kinase C

and N-methyl-D-aspartate receptors. *BMC Anesthesiology*, *15*(1), 1–8. https://doi.org/10.1186/1471-2253-15-12

Stagg, N. J., Mata, H. P., Ibrahim, M. M., Henriksen, E. J., Porreca, F., Vanderah, T. W., & Philip Malan, T. (2011). Regular exercise reverses sensory hypersensitivity in a rat neuropathic pain model: Role of endogenous opioids. *Anesthesiology*, *114*(4), 940–948. https://doi.org/10.1097/ALN.0b013e318210f880

Suarezroca, H., Silva, J., Arcaya, J., Quintero, L., Maixner, W., & Pineruashuhaibar, L. (2006). Role of μ-opioid and NMDA receptors in the development and maintenance of repeated swim stress-induced thermal hyperalgesia. *Behavioural Brain Research*, *167*(2), 205–211. https://doi.org/10.1016/j.bbr.2005.09.006

Welton, J., Stewart, W., Kerr, R., & Maxwell, D. J. (1999). Differential expression of the muscarinic m2 acetylcholine receptor by small and large motoneurons of the rat spinal cord. *Brain Research*, *817*(1–2), 215–219. https://doi.org/10.1016/S0006-8993(98)01208-6

Woolfe, G., & Macdonald, A. D. A. (1944). The evaluation of the analgesic action of pethidine hydrochloride (demerol). *Journal of Pharmacology and Experimental Therapeutics*, *80*, 300–307. Retrieved from http://jpet.aspetjournals.org/content/80/3/300.abstract

How to cite this article: Medeiros LF, Nunes ÉA, Lopes BC, et al. Single exercise stress reduces central neurotrophins levels and adenosine A_1 and A_2 receptors expression, but does not revert opioid-induced hyperalgesia in rats. *Int J Dev Neurosci*. 2020;80:636–647. https://doi.org/10.1002/jdn.10059