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Role of brainstem serotonin in analgesia produced by low-intensity exercise on neuropathic pain following sciatic nerve injury in mice

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Abstract

Physical exercise is a low-cost, safe and efficient intervention for the reduction of neuropathic chronic pain in humans. However, the underlying mechanisms for how exercise reduces neuropathic pain are not yet well understood. Central monoaminergic systems play a critical role in endogenous analgesia leading us to hypothesize that the analgesic effect of low-intensity exercise occurs through activation of monoaminergic neurotransmission in descending inhibitory systems. To test this hypothesis we induced peripheral nerve injury (PNI) by crushing the sciatic nerve. The exercise intervention consisted of low-intensity treadmill running for two weeks immediately after injury. Animals with PNI showed an increase in pain-like behaviors that were reduced by treadmill running. Reduction of serotonin (5-HT) synthesis using the tryptophan hydroxylase inhibitor PCPA prevented the analgesic effect of exercise. However, blockade catecholamine synthesis with the tyrosine hydroxylase inhibitor AMPT had no effect. In parallel, 2

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Conflict of interest statement

The authors have no conflict of interest.

weeks of exercise increased brainstem levels of the 5-HT and its metabolites (5-HIAA), decreased expression of the serotonin transporter (SERT), and increased expression of 5-HT receptors (5HT_{1B}, 2A, 2C). Lastly, PNI-induced increased in inflammatory cytokines, TNF- α and IL-1 β , in the brainstem was reversed by 2 weeks of exercise. These findings provide new evidence indicating that low-intensity aerobic treadmill exercise suppresses pain-like behaviors in animals with neuropathic pain by enhancing brainstem 5-HT neurotransmission. These data provide a rationale for the analgesia produced by exercise to provide an alternative approach to the treatment of chronic neuropathic pain.

Keywords

low-intensity treadmill exercise; peripheral nerve injury; inflammatory cytokines and serotonin

1 Introduction

Regular physical exercise has been recognized as an important approach for the general health and quality of life worldwide. In contrast physical inactivity has been identified as the fourth leading risk factor for global mortality (6% of deaths) [67] and it is now accepted as a crucial risk factor for development of many chronic diseases such as cardiovascular disease, depression, cancer and diabetes [81]. In fact, physical activity is reduced in people with chronic diseases often due to chronic pain [19], and greater physical activity is associated with a lower risk for development of chronic pain [37,38]. A study of WHO revealed that persistent pain afflicted between 5.3% and 33% of individuals resident in both developing and developed countries [27], and in the US chronic pain costs up to \$635 billion each year in medical treatment and lost productivity [1].

Neuropathic pain resulting from PNI leads to significant loss of function resulting in disability and reduced life quality [27]. The mechanisms underlying the development of neuropathic pain are complex and multifactorial. Animal models of nerve injury are characterized by hypersensitivity to mechanical and thermal stimuli and have shown a plethora of changes in both the peripheral and central nervous system (CNS) (reviewed in [11,13,51,60,66,78,80]). Central brainstem pathways play a critical role in both the inhibition and facilitation of nociceptive behavior. Specifically, the midbrain periaqueductal gray matter (PAG) projects directly to the rostral ventromedial medulla (RVM) and the A₆/A₇ noradrenergic nucleus in the pons [3,32,43,47,49,56,60]. In fact, it has been established that the RVM, which contains neurons that express serotonin (5-HT), can facilitate the neuropathic pain caused by nerve injury (for review see [59,60]). Similarly, the A₆/A₇ noradrenergic cells groups in the pons also increased the potency and efficacy of α_2 -adrenergic receptor agonists in relieving neuropathic pain caused by nerve injury [4,28,43,72]. Beyond its role in modulating nociception, medullary raphe nuclei also has a key role responding motor input [23], modulating motor output with descending projections to motor neurons [31,50], and utilizes serotonergic neurotransmission to facilitate motor impulses [34,79].

Physical exercise is an important component of rehabilitative therapies and is used to decrease pain and improves function in people with chronic neuropathic pain [22,26,29,64]. In animal models, regular exercise diminishes hyperalgesia produced by inflammatory pain [36], chronic muscle pain [5,68] and chronic neuropathic pain [7,12,36,70]. Indeed, aerobic exercise activates the PAG and RVM [68,70] and evidences pointed to changes in dopaminergic, noradrenergic, and serotonergic metabolism during exercise [48], which may explain its analgesic effect. However, the underlying physiological mechanisms for physical exercise related chronic pain relief remain largely unexplored.

We therefore, hypothesized that PNI would result in central monoaminergic system changes decreasing the availability of monoamines and facilitating nociception, and that low-intensity aerobic exercise would counteract these changes. Therefore, the present study investigated, through pharmacological, behavioral, and biochemical and molecular tools the effect of low-intensity aerobic exercise on the central monoaminergic system in a mice model of chronic neuropathic pain induced by PNI.

2 Material and methods

2.1 Animals and surgical procedures

Male Swiss mice (25 to 35 g, 8-9 weeks old) were housed in a room with a constant temperature of $22\pm 2^{\circ}\text{C}$ under a 12-hour light/dark cycle with access to food and water ad libitum. After transportation to the laboratory, mice were acclimated to the room for at least 1 h before testing. All of the procedures used in the present study were approved by the Institutional Ethics Committee of the Universidade Federal de Santa Catarina (CEUA/UFSC, protocol number PP00681 and PP00745) or the University of Iowa (Iowa City, IA, USA – protocol number 1110229) and were carried out in accordance with the “Principles of Laboratory Animal Care” from National Institutes of Health publication No. 85–23.

Surgical procedures to induce PNI were performed under deep anesthesia that was induced with a premixed solution containing ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and maintained using isoflurane (1 to 2% in 100% O_2). The sciatic nerve with its three major branches was exposed through a gluteal muscle-splitting incision in the right thigh. The nerve was crushed 1 cm above its trifurcation, once for 30 s, with 2-mm-wide forceps, as described previously [8]. The skin incision was closed with a 4-O Ethilon suture and each animal was allowed to recover for 3 days before exercise training. The sham groups were subjected to the same surgical procedures, but the sciatic nerve was not crushed. A summary of the time line and experimental protocols is depicted in Fig. 1A and B.

2.2 Low-intensity aerobic treadmill running

The animals were run on a treadmill (EXER-6M, Columbus Instruments, Columbus, OH, USA or Athletic Advanced 2, Joinville, SC, Brazil) for 30 minutes at a speed of 10 m/min with no inclination five days per week, for two weeks as described previously by Bobinski et al. [7]. We previously determined the intensity of training by measuring blood lactate concentration and establishing the maximal blood lactate steady state (MLSS), and thus this exercise protocol is interpreted as low-intensity since mice in our study performed the

exercise 10 m/min corresponded to 75% of MLSS [7]. Animals were subjected to 6-days familiarization on the treadmill for 10 minutes/day at a speed of 10 m/min with no inclination, before the experimental protocol began. Treadmill training began on the third postoperative day (Fig. 1A and B). The animals in the sham-operated (Sham/Saline/Sedentary, Sham/PCPA/Sedentary or Sham/Sedentary) and sciatic nerve crushed (Crushed/Saline/Sedentary, Crushed/PCPA/Sedentary, Crushed/AMPT/Sedentary or Crushed/Sedentary) groups were handled and placed on a treadmill with no motion at the same times as the exercised groups.

2.3 Mechanical sensitivity of the hindpaw

Mechanical sensitivity was evaluated as the withdrawal response frequency to 10 applications of a 0.4 g of von Frey filament (VHF Stoelting, Chicago, IL, USA). Mice were placed individually in clear plexiglass boxes ($9 \times 7 \times 11$ cm) on an elevated wire mesh platform to allow access to the ventral surface of the hindpaw. The number of paw withdrawals was recorded and expressed as a percentage of the withdrawal response [7]. Measurements were assessed before sciatic nerve crushing and 24h after physical exercise from the 3rd to 14th postoperative day (Fig. 1A and B). An increased number of withdrawals or increased frequency of withdrawal was interpreted as mechanical hyperalgesia.

2.4 Involvement of the monoaminergic system

To investigate the serotonergic system involvement in the analgesia produced by exercise animals were pretreated with para-chlorophenylalanine methyl ester dissolved in saline (PCPA, 100 mg/kg, i.p., Sigma Chemical Company, St Louis, MO, USA). PCPA inhibits the enzyme tryptophan hydroxylase (Tph), a rate-limiting enzyme in the biosynthesis of 5-HT, that results in a functional 5-HT synthesis blockade. Control animals received saline injections instead of PCPA (NaCl 0.9%, 10 ml/kg, i.p.). PCPA or saline was administered once a day for 4 consecutive days before beginning the exercise program and again on days 11-14 postoperatively. Prior studies show that repeated application of PCPA results in reduction of 5-HT in the central nervous system [20, 55]. The current study confirmed decreases in 5-HT in the brainstem using high performance liquid chromatography (HPLC) analysis (see below). Mechanical sensitivity was assessed 24 h after exercise on days 5, 7 and 14 postoperatively (Fig. 1A). For this experiment, the following groups were used: i) Sham/Saline/Sedentary (n=8); ii) Sham/Saline/Exercised (n=8); iii) Sham/PCPA/Sedentary (n=8); iv) Sham/PCPA/Exercised (n=8); v) Crushed/Saline/Sedentary (n=8); vi) Crushed/Saline/Exercised (n=8); vii) Crushed/PCPA/Sedentary (n=8) and viii) Crushed/PCPA/Exercised (n=8).

In a separate series of experiments on the 7th postoperative day, in order to investigate the possible contribution of catecholaminergic system to the exercise-induced analgesia, mice were pretreated with a single dose of alpha-methyl-para-tyrosine, dissolved in saline (AMPT, 100 mg/kg, i.p., Sigma Chemical Company, St Louis, MO, USA). AMPT is a tyrosine hydroxylase (TH) inhibitor that is a rate-limiting enzyme in the biosynthesis of catecholamines and results in a functional reduction in NE and dopamine (DA) [20, 55]. Control animals received saline (NaCl 0.9%, 10 ml/kg, i.p.). AMPT or saline was given 3.5 h before a single exercise session. Mechanical sensitivity was measured 0.5, 1, 2 and 24

hours after exercise (Fig. 1B). For this experiment, the following groups were used: i) Crushed/Saline/Sedentary (n=8); ii) Crushed/Saline/Exercised (n=8); iii) Crushed/AMPT/Exercised (n=8); iv) Crushed/AMPT/Sedentary (n=6); and sham-operated group vii) Sham/Saline/Sedentary (n=7). After this experiment, we observed that treatment of animals with AMPT was unable to interfere with the analgesia induced by exercise, subsequently the following experiments were concentrated in the effect of physical exercise toward the serotonergic system.

2.5 Levels of 5-HT and its metabolite by HPLC with Electrochemical Detection (ED)

On the 15th postoperative day, 24 hours after last exercise session (Fig. 1A and B), mice from all groups listed above were deeply anesthetized with isoflurane (2 to 3% in 100% O₂), euthanized by decapitation to remove the brainstem for subsequent HPLC-ED analysis of monoamines. Samples were frozen on dry ice and stored at -80°C until preparation. Tissue samples were homogenized with an ultrasonic cell disrupter (Sonics) in 0.1 M perchloric acid containing sodium metabisulfite 0.02% and internal standard. After centrifugation at 10,000 × g for 30 min, 4 °C, 20 µl of the supernatant was injected into the chromatograph. To examine the 5-HT and its metabolite, the same groups described in the section 2.4 were used.

Endogenous levels of 5-HT and their metabolite 5-hydroxyindoleacetic acid (5-HIAA) were assayed by reverse-phase HPLC with ED. The system consisted of a Synergi Fusion-RP C-18 reverse-phase column (150 × 4.6 mm i.d., 4 µm particle size) fitted with a 4 × 3.0 mm pre-column (SecurityGuard Cartridges Fusion-RP); an electrochemical detector (ESA Coulochem III Electrochemical Detector) equipped with a guard cell (ESA 5020) with the electrode set at 350 mV and a dual electrode analytical cell (ESA 5011A); a LC-20AT pump equipped with a manual Rheodyne 7725 injector with a 20 µl loop (Shimadzu Corporation, Kyoto, Japan). The column was maintained inside a temperature-controlled oven (25 °C).

The cell contains two chambers in series: each chamber includes a porous graphite coulometric electrode, a double counter electrode and a double reference electrode. Oxidizing potentials were set at 100 mV for the first electrode and at 450 mV for the second electrode. Dopamine and metabolites were detected at the second electrode. The mobile phase, used at a flow rate of 1 ml/min, had the following composition: 20 g citric acid monohydrated, 200 mg octane-1-sulfonic acid sodium salt, 40 mg ethylenediaminetetraacetic acid (EDTA), 900 ml HPLC-grade water. The pH of the buffer running solution was adjusted to 4.0 then filtered through a 0.45 µm filter. Methanol was added to give a final composition of 10% methanol (v/v). The peak areas of the external standards were used to quantify the sample peaks. Total protein content was measured in the supernatant using the method of Bradford and all results were expressed as pg/g of protein. Serotonin turnover rate was also calculated.

2.6 Immunohistochemistry to SERT on Medullary Raphe Nuclei

Immunohistochemistry for the SERT in the medullary raphe nuclei was performed in the following groups: i) Sham/Sedentary (n=5); ii) Crushed/Sedentary (n=4) and iii) Crushed/Exercised (n=5). On the 15th postoperative day, 24 hours after last exercise session (Fig.

1A), mice were deeply anesthetized (100 mg/kg, sodium pentobarbital) and transcardially perfused with heparinized saline followed by freshly prepared 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline. The brainstem was removed and post-fixed in 4% PFA overnight (4°C), transferred to 30% sucrose in PBS (phosphate-buffered saline) (4°C) for 24h, and then the RVM was blocked and frozen in cryomolds embedded in optimal cutting temperature compound (OCT, Tissue-Tek®, Fisher Scientific). Serial cross sections of 20 µm from medullary raphe nuclei were cut on a cryostat and placed on slides.

Sections from all animals were processed and simultaneously immunostained for SERT using standard protocols. Briefly, sections were blocked with 10% normal goat serum (NGS) followed by a standard Avidin/Biotin blocking protocol (Vector Labs, CA, USA). Sections were then incubated overnight with primary antibody against SERT (Rabbit anti-5-HT transporter; ImmunoStar, 1:500). On the second day, sections were incubated in the secondary antibody incubation (Biotinylated Goat anti-Rabbit IgG, Invitrogen, 1:1000, 1h) followed by Streptavidin-Alexa Fluor® 568 conjugate (Life Technologies, 1:500, 1h). All antibodies and Streptavidin were diluted in 1% NGS with 0.05% Triton-X 100 and 1:100 sodium azide. Slides were cover slipped with Vectashield (Vector Labs, CA, USA).

BioRad Laser Sharp 2000 was used to capture the images. The location captured was chosen visually using a 20 × objective lens. All images were taken under the same conditions and pictures were stored for off-line analysis. One picture in each section in the following nuclei: Raphe Pallidus (RPa), Raphe Magnus (RMg), and Raphe Obscurus (ROb); and 3 sections per animal per site were randomly chosen and added to give one number per site per animal. There were a total of 14 animals with 4-5 animals per group). Each evaluated nucleus was outlined, and the number of pixels occupied by immunoreactive cells was measured using Image J 1.24 software (NIH). Specifically, each picture was first converted to eight-bit gray scale, and then it was calibrated independently using the “uncalibrated OD” function with pixel values ranging from 0 to 255 as we previously published [30]. The density values represent pixels per area and were expressed as arbitrary units.

2.7 Real-time quantitative PCR (qPCR)

Gene expression analysis of the 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_{3A}), SERT, Tph enzyme was performed as previously described [46]. In a separate series of experiments on the 15th postoperative day, 24 hours after last exercise session (Fig. 1A), mice from following groups: i) Sham/Sedentary, ii) Sham/Exercised; iii) Crushed/Sedentary and iv) Crushed/Exercised (n= 8-10 per group), were deeply anesthetized with isoflurane (2 to 3% in 100% O₂), euthanized by decapitation and the brainstem was dissected, immediately frozen on dry ice and stored at -80°C until RNA extraction. Total RNA was extracted using the TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich, MO, USA). cDNA was synthesized using iScript cDNA synthesis kit (BioRad, CA, USA). Subsequent qPCR was performed using a CFX96 Real-time PCR (BioRad, CA, USA) and iQ SYBR Green Supermix (BioRad, CA, USA). Relative quantification of gene expression was analyzed via the 2^{-Ct} method, using β-actin gene expression to normalize the data. The genes chosen for qPCR analyses and their respective NCBI references, primers, fragment size and references are described in Table 1.

2.8 Measurement of pro-inflammatory cytokine levels in the brainstem by Enzyme Linked Immunosorbent Assay (ELISA)

Twenty-four hours after the last exercise session on 15th postoperative day (Fig. 1A), mice were deeply anesthetized with isoflurane (2 to 3% in 100% O₂) and sacrificed by decapitation. Mice into the same groups described in the section 2.7 were analyzed (n=8). The brainstem was removed and homogenized with a glass homogenizer (Dounce Tissue Grinders, Omni International, Kennesaw, GA, USA) in a PBS solution containing: Tween 20 (0.05 %), phenylmethylsulphonyl fluoride (PMSF) 0.1 mM, ethylenediaminetetraacetic acid (EDTA) 10 mM, Aprotinin 2 ng/ml and benzamethonium chloride 0.1 mM. The homogenates were transferred to 1.5 mL Eppendorfs tubes, centrifuged at 3000 × g for 10 min at 4 °C, and the supernatant obtained was stored at -70 °C until further analyses. Total protein content was measured in the supernatant using the method of Bradford. Sample aliquots of 100 µl were used to measure the tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) levels using mouse cytokine ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. The levels of cytokines were estimated by interpolation from a standard curve by colorimetric measurements at 450 nm (correction wavelength 540 nm) in an ELISA plate reader (Berthold Technologies – Apollo 8 – LB 912, KG, Germany). All results were expressed as pg/mg of protein.

2.9 Statistical analyses

Results are presented as the mean ± S.E.M. for each group. Mechanical sensitivity was compared using two-way analysis of variance with repeated measures followed by post hoc Bonferroni's test when appropriate. Biochemical, molecular and immunohistochemistry assessments were performed using a one-way ANOVA followed by Student-Newman-Keuls test when appropriate. P values less than 0.05 were considered to be indicative of significance.

3 Results

3.1 Low-intensity exercise after PNI decreased mechanical sensitivity, but its effect is prevented by PCPA, but not by AMPT pretreatment

There were no differences between animals assigned to the crush nerve injury and sham-operated groups in their baseline (preoperative) responsiveness to the 0.4 g force applied to their paw (Fig. 2A, B and C). After PNI there was a significant increase in the number of withdrawals in the Crushed/Sedentary animals when compared to Sham/Sedentary animals from the 3rd to 14th day post-crush ($P < 0.001$, Fig. 2A), indicating that the PNI produced marked and sustained mechanical sensitivity in mice. Treadmill exercise produced a significant reduction in the number of responses to repeated mechanical stimulation of the paw from the 5th to 14th day after nerve injury (Crushed/Exercise) when compared to the Crushed/Sedentary group ($P < 0.001$, Fig. 2A).

To test for a role of 5-HT in the analgesia produced by exercise, we reduced 5-HT systemically by treating mice with PCPA prior to treadmill exercise. The brainstem 5-HT content was markedly decreased in PCPA pretreated mice, when compared with the groups that did not receive PCPA (i.e. Sham/Saline/Exercised group with Sham/PCPA/Exercised

group, $P < 0.05$ and Crushed/Saline/Exercised group with Crushed/PCPA/Exercised group, $P < 0.001$) (Fig. 2D). Pretreatment of nerve injury mice with PCPA (100 mg/kg, i.p.) significantly prevented the analgesic effect of treadmill exercise (Crushed/PCPA/Exercised) when compared with the control group (Crushed/Saline/Exercised) on 5 ($P < 0.01$), 7 ($P < 0.01$) and 14 days ($P < 0.001$) after nerve injury (Fig. 2B).

In order to investigate the involvement of the catecholaminergic system in the analgesic effect observed, we reduced NE and DA with AMPT, a tyrosine hydroxylase (TH) inhibitor that is a rate-limiting enzyme in the biosynthesis of catecholamines, delivered systemically as previously described [20, 55]. Interestingly, blockade of TH with AMPT (100 mg/kg, i.p.) (Crushed/AMPT/Exercised) did not prevent the analgesic effect of treadmill exercise 0.5, 1, 2 and 24 hours after exercise when compared with the Crushed/Saline/Exercised group ($P > 0.05$, results not shown). A sedentary group pretreated with AMPT (100 mg/kg, i.p.; Crushed/AMPT/Sedentary) was used as a control to verify the possible effect of the drug *per se*; there was no significant difference when compared with Crushed/Saline/Sedentary group ($P > 0.05$, results not shown). Despite the fact that AMPT pretreatment did not reverse the analgesia produced by exercise, we have focused on the involvement of the serotonergic system in the physical exercise effect.

3.2 Low-intensity exercise after PNI increased 5-HT and 5-HIAA content, but its effect is prevented by PCPA pretreatment

HPLC analysis of 5-HT content (Fig. 2D) in the brainstem of nerve injury mice (Crushed/Saline/Sedentary) was significantly decreased when compared with sham-operated mice (Sham/Saline/Sedentary) ($P < 0.05$). Interestingly, treadmill exercise significantly increased brainstem 5-HT (Sham/Saline/Exercised and Crushed/Saline/Exercised) when compared to sham or crushed sedentary group, respectively (Sham/Saline/Sedentary or Crushed/Saline/Sedentary) ($P < 0.001$). Similar to 5-HT, 5-HIAA content was reduced by nerve injury (Crushed/Saline/Sedentary) when compared to the sham group (Sham/Saline/Sedentary) ($P < 0.01$). Treadmill exercise (Crushed/Saline/Exercised) significantly increased brainstem 5-HIAA content to levels similar to Sham/Saline/Sedentary group and was significantly increased from the nerve injured group (Crushed/Saline/Sedentary) ($P < 0.01$). Besides, treadmill exercise significantly increased 5-HIAA content also in sham animals (Sham/Saline/Exercised) when compared to Sham/Saline/Sedentary mice ($P < 0.01$). In addition, 5-HIAA content was decreased when sham or crushed mice were treated with PCPA (i.e. Sham/Saline/Exercised group compared with Sham/PCPA/Exercised group, $P < 0.05$ and Crushed/Saline/Exercised group compared with Crushed/PCPA/Exercised group, $P < 0.001$; Fig. 2E).

Fig. 2F show that treadmill exercise (Crushed/Saline/Exercised) inhibited the increase in the turnover ratio of 5-HIAA to 5-HT, when compared with Crushed/Saline/Sedentary mice ($P < 0.05$) or Crushed/PCPA/Exercised group ($P < 0.05$), indicating that there is a reduction of 5-HT metabolism in mice subject to treadmill exercise in the brainstem. However, Sham/Saline/Exercised group showed increase in turnover ratio, when compared with Sham/Saline/Sedentary mice ($P < 0.01$) (Fig. 2F).

3.3 Low-intensity exercise decreases SERT staining in the medullary raphe nuclei after PNI in mice

Since low-intensity exercise can promote changes in the brainstem serotonergic system we tested if PNI and the low-intensity exercise would modify the SERT in the medullary raphe. Fig. 3A shows photomicrographs with representative images of SERT immunohistochemical staining in the RMg, ROb and RPa in the medulla for sham mice, nerve injured mice (Crushed/Sedentary), and those mice that performed treadmill exercise (Crushed/Exercised). Quantitative analysis shows an increased SERT staining density in the ROb ($P < 0.01$), RMg ($P < 0.05$) and RPa ($P < 0.05$) in the nerve injured, Crushed/Sedentary group compared with Sham/Sedentary group (Figs. 3B, C and D, respectively). Interestingly, low-intensity exercise (Crushed/Exercised) counteracts the increase of SERT staining density induced by PNI in all medullary raphe nuclei (ROb, $P < 0.05$; RMg, $P < 0.05$ and RPa, $P < 0.05$; Figs. 3B, C and D, respectively), and was able to normalize the SERT staining density with no significant difference compared to Sham/Sedentary mice.

3.4 Low-intensity exercise decreased SERT and does not change Tph2 mRNA expression in the brainstem after PNI

In order to confirm and extend the data related to decrease SERT density staining in the medullary raphe nuclei by low-intensity exercise, we also quantified the SERT mRNA expression in the brainstem by qPCR. Fig. 4A shows that low-intensity exercise (Crushed/Exercised group) counteracts the increase in SERT mRNA expression in the nerve injured mice (Crushed/Sedentary, $P < 0.01$) and there was no difference between Crushed/Exercised and Sham/Sedentary groups. Furthermore, mice from sham-operated group that performed treadmill exercise (Sham/Exercised) showed a marked decreased in mRNA expression of SERT when compared with Sham/Sedentary group ($P < 0.001$).

Because 5-HT content in the brainstem was increased in exercised mice, we measured if it was related to increase in Tph2 enzyme RNA levels. Interestingly, qPCR analyses show no significant difference among nerve injury groups (Crushed) for Tph2 mRNA expression ($P = 0.5075$, however, sham-operated mice subject to exercise (Sham/Exercised) showed a reduction for Tph2 mRNA expression when compared with Sham/Sedentary group ($P < 0.05$; Fig. 4B).

3.5 Low-intensity exercise increased 5-HT receptors mRNA expression after PNI

Since there were alteration in 5-HT levels and SERT expression in the brainstem, we evaluated the gene expression of the 5-HT receptors in the brainstem by qPCR. There was a significant increase in mRNA expression of the 5-HT_{2A} and 5-HT_{2C} receptors in nerve injured mice (Crushed/Sedentary) when compared with Sham/Sedentary group ($P < 0.01$ and $P < 0.05$, respectively; Fig. 5C and D). Treadmill exercise after PNI (Crushed/Exercised) increased the mRNA expression of the 5-HT_{1B} ($P < 0.05$), 5-HT_{2A} ($P < 0.05$) and 5-HT_{2C} ($P < 0.05$) receptors when compared with nerve-injured mice (Crushed/Sedentary) (Figs. 5B, C and D, respectively). Furthermore, treadmill exercise after PNI (Crushed/Exercised) increased the mRNA expression of the 5-HT_{1A} ($P < 0.01$) receptor when compared with Sham/Sedentary group (Fig. 5A), however was not statistically significant when compared with nerve-injured mice (Crushed/Sedentary)(Fig. 5A).

Interestingly, qPCR analyses showed that sham-operated mice subject to exercise (Sham/Exercised group) significantly increased the mRNA expression of the 5-HT_{3A} and 5-HT₇ receptors when compared with Sham/Sedentary group ($P < 0.001$ and $P < 0.01$, respectively; Fig. 5E and F). Sham-operated and exercised group also showed increased mRNA expression of the 5-HT_{1B} receptor when compared with Sham/Sedentary group, however was not statistically significant ($P > 0.05$, Fig. 5B).

3.6 Low-intensity exercise counteracted the increased pro-inflammatory cytokines levels in the brainstem after PNI

Figure 6 shows that PNI (Crushed/Sedentary group) increased the levels of pro-inflammatory cytokines TNF- α ($P < 0.05$) and IL-1 β ($P < 0.01$) in the brainstem when compared to the Sham/Sedentary group (Figs. 6A and B). A control sham-operated group (Sham/Exercised) was used to observe the effect of exercise *per se* and showed no difference to Sham/Sedentary group in both cytokines measured. Besides, the concentrations of TNF- α and IL-1 β were decreased after two-weeks of treadmill exercise (Crushed/Exercised) when compared to the Crushed/Sedentary group ($P < 0.05$), there were no differences in these cytokines levels when compared to Sham/Sedentary group (Figs. 6A and B).

4 Discussion

Although several studies have demonstrated positive effects of treadmill exercise on nerve regeneration and functional recovery after peripheral nerve injury [10,45,65], its effects on neuropathic pain symptoms and the mechanisms involved in these effects are virtually unknown. Previously, we showed that low-intensity exercise after PNI reduced mechanical and cold sensitivity, and reduced proinflammatory cytokines (TNF- α and IL-1 β) at the nerve injury site and the spinal cord [7]. In the current study, we confirm previous data showing the analgesic effect of treadmill exercise in nerve injury, and extend results showing involvement of serotonergic neurotransmission in the analgesia produced by treadmill exercise. Our main findings were that low intensity exercise: 1) produced analgesia that was prevented by reduction of 5-HT but not catecholamines levels; 2) increased 5-HT and 5-HIAA content, and reduced 5-HT turnover in the brainstem; 3) decreased SERT protein and mRNA in the brainstem; 4) increased 5-HT receptor (5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C}) mRNA expression in the brainstem, finally 5) decreased pro-inflammatory cytokines TNF- α and IL-1 β levels in the brainstem. We propose that chronic mechanical sensitivity after PNI reflects an inadequate 5-HT neurotransmission in the brainstem which increases SERT expression, decreases 5-HT production, and alters 5-HT receptors expression. The data strongly suggest that low-intensity exercise produces analgesia through enhancement of 5-HT neurotransmission in the brainstem. This likely involves our endogenous analgesic mechanisms mediated by descending inhibitory pain pathways originating at higher CNS sites including the medullary raphe nuclei in the brainstem.

Strong evidence supports the idea that behavioral manifestations of chronic pain require active participation of supraspinal sites. The increased responses to mechanical or cold, but not to noxious thermal stimulation in rats with PNI or hind paw inflammation is abolished

by transection of the thoracic spinal cord [6,73,74]. The RVM consisting of the RMg and the adjacent ventral reticular formation is an important pain modulator because inactivation of this region with a local anesthetic reduces the hyperalgesia in animals with nerve injury [59]. Neurons in RVM project along the spinal dorsolateral funiculus to terminate in the dorsal horn, where they inhibit nociceptive transmission. Previous studies suggest that exercise produces analgesia through activation of pain inhibition pathways in the CNS. Specifically, in animals with nerve injury, regular exercise enhances opioid peptides in the PAG and RVM, and the blockade of supraspinal opioids prevents the analgesia [70]. Further, exercise reduces the enhanced phosphorylation of NMDA receptors in the RVM, a measure of enhanced facilitation [68]. Interestingly, the caudal medullary raphe nuclei, i.e., the ROb and RPa, which modulate motor output, are also involved in exercise-mediated effects showing increased activation, measured by c-fos expression, in response to exercise [69]. The medullary raphe neurons, also produce 5-HT, project to the deep dorsal horn of the spinal cord and counteract to noxious input [31,50]. Thus, the medullary raphe nuclei may be an important link between exercise and pain relief through the serotonergic system.

In the CNS the efficiency of serotonergic signaling is regulated by the release of 5-HT, the activity of the neurotransmitter synthesizing enzyme (Tph2), the negative modulatory autoreceptors 5-HT_{1A}R and 5-HT_{1B}R, and a selective re-uptake system which transports released 5-HT back into serotonergic neurons by SERT (for review see [40]). Several studies show that activation of the medullary serotonergic nuclei inhibits noxious stimuli-induced responses [62,79]. Moreover, physical exercise modifies 5-HT neurotransmission in different CNS regions. For example, motor activity and exercise induce an increase in 5-HT in the ventral and dorsal horns of the spinal cord [79], increase 5-HT concentration in rat spinal cord, brainstem, midbrain, cerebral cortex, and hippocampus [9,16,24,25], and enhance the metabolism of 5-HT in the cerebral cortex [16]. The current study demonstrated for the first time that the analgesic effect of low-intensity physical exercise in animals with PNI was abolished by reduction of 5-HT and 5-HIAA content in the brainstem. Jacobs et al. [34] suggest the analgesia produced by 5-HT in the medulla might be stimulated by motor activity. In this regard, 5-HT-induced analgesia could be secondary to increased serotonergic activity in the medullary raphe nuclei due motor activity. Interestingly, despite increased 5-HT content, Tph2 mRNA, a rate-limiting enzyme in the biosynthesis of 5-HT, was not changed in sedentary or exercised mice after PNI, which suggests increased translation of Tph2 does not mediated the increased 5-HT content in the brainstem. However, the exercise was able to reduce the Tph2 mRNA in sham-operated group. Alternatively, alterations in enzymatic activity, alterations in SERT, or changes in autoreceptors 5-HT_{1A}R and 5-HT_{1B}R directly alter 5-HT content [40].

Interestingly, we show that PNI increases SERT in the medullary raphe nuclei, and this increase is reversed by low-intensity exercise. SERT is a key player in 5-HT signaling regulating the uptake of 5-HT into the presynaptic neuron at synapse playing a critical role in the duration and intensity of 5-HT activity [40]. Consequently drugs which elevate serotonergic signaling are used as pharmacotherapies for neuropathic pain [18,53]; selective 5-HT re-uptake inhibitors (SSRIs) bind to SERT, block 5-HT uptake and thereby elevate concentrations of extracellular 5-HT. Thus, decreased SERT mRNA and protein in exercised

mice are consistent with reported effects of the clinical use of SSRIs and could explain raphe SERT expression decreased [41,42,75].

A mechanism that could explain the reduction of SERT density could be reduction of brainstem proinflammatory cytokines in exercised mice that are normally increased after PNI. We previously demonstrated that PNI increased spinal cord and sciatic nerve IL-1 β and TNF- α levels and low-intensity exercise reduced this increase [7]. Here we extend these data demonstrating that physical exercise counteracts the increase of IL-1 β and TNF- α levels in the brainstem in nerve crushed sedentary animals. Longer-term exposure to either IL-1 β [63] or TNF- α [52] increased 5-HT uptake through an increase in SERT expression in JAR choriocarcinoma cells which is consistent with the increased SERT density in the medullary raphe in PNI mice in the current study. Both TNF- α and IL-1 β mRNA and protein are present in brainstem pain-modulatory pathways specific CNS neuronal populations [15,33], and receptors for these cytokines have differential expression patterns in brain [15,21,35,44,76]. Exposure of neurons to inflammatory cytokines results in diverse neuronal and behavioral effects. For example, in guinea-pig brain slice preparations, IL-1 β induces a rapid reduction in the firing rates of neurons in serotonergic dorsal raphe [44]. TNF- α enhances both intrinsic activity and the surface density of SERT, and can increase SERT trafficking through protein kinase (PK) C or PKG-related mechanisms [61,82]. The current study showed that low-intensity exercise decreases SERT expression in the medullary raphe nuclei suggesting that reduced brainstem IL-1 β and TNF- α levels in exercised mice after PNI could reduce pre-synaptic SERT which in turn increase 5-HT availability in the synapse. TNF- α also inhibits NE release in a manner dependent on α -2 adrenergic receptor activation [33]. Albeit catecholamines (NE and DA) are not necessary for the analgesic effect of the exercise mice with PNI, once again increased TNF- α levels after PNI could inhibit NE release and exercise could counteract this reduction. Further, inflammatory cytokines could directly modulate 5-HT activity by enhancing synthesis, altering trafficking of receptors or transmitters, or changing channel conductance.

Numerous studies suggest an inhibitory role for serotonergic transmission from RVM to spinal cord dorsal horn, and the subtypes of the 5-HT receptors associated with this modulation are investigated mainly at spinal level [17,54]. However, serotonergic fibers and terminals are found throughout RVM [71], as are a number of 5-HT receptor subtypes [39,57,58,77]. The current study shows that PNI increases brainstem 5-HT_{2A} and 5-HT_{2C} mRNA expression what could be a compensatory mechanism in an attempt to enhance serotonergic neurotransmission after PNI. Since low-intensity exercise increases 5-HT neurotransmission in the brainstem it is reasonable that brainstem 5-HT receptors are involved in the anti-hyperalgesic effect of exercise; however, which subtypes mediate this analgesia are still unknown. The current study showed that exercise increased brainstem 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} mRNA expression in mice with PNI. Furthermore, the exercise was also effective in increasing the expression of mRNA for 5-HT_{3A} and 5-HT₇ receptors in the brainstem of sham-operated animals. Although the increase gene expression may not reflect increased protein or function, we hypothesize that increased 5-HT receptor expression is required for the 5-HT action of low-intensity exercise-induced analgesia.

In summary, the present study demonstrates that PNI produces mechanical sensitivity, reduces brainstem 5-HT and 5-HIAA content, increases SERT density in the medullary raphe nuclei, increases brainstem TNF- α and IL-1 β levels and increases expression of 5-HT receptors. Furthermore, we found that low-intensity exercise suppresses pain behavior by enhancing brainstem 5-HT neurotransmission through increases in 5-HT and 5-HIAA content, 5-HT receptor expression (5HT- 1_B , $2A$, $2C$), and decreases SERT expression and inflammatory cytokines levels (TNF- α and IL-1 β) (see Fig. 7). These findings could have direct therapeutic applications to reduce neuropathic chronic pain after PNI and could provide a basis for new approaches to treating nerve injury.

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Figure 1.

Schematic representations of the experimental design and protocol timeline for Serotonergic (A) and Catecholaminergic (B) Systems Investigation. (A and B) Days -7 to -1 indicate the time before crushing surgery; Days -7 to -2 indicate the familiarization time on treadmill running; Day 0 indicates the day of crushing surgery. (A) PCPA mice treatment was performed from day 0 up to 3, and again from day 11 up to 14 after crushing, and the mechanical sensitivity (von Frey test) tested by days -1, 5, 7 and 14. On day 15 after crushing, mice were sacrificed and samples tissues were collected to HPLC, ELISA, IH and qPCR. (B) AMPT mice treatment was performed by day 7 and the mechanical sensitivity tested on days -1, 7 and 8. vF, von Frey test; IH, immunohistochemistry.

Figure 2.

Effect of exercise after sciatic nerve crushing and role of the serotonergic system in analgesia produced by exercise after PNI. (A) Nerve injury resulted in a significant increase in the number of withdrawals in the sedentary nerve injured animals (Crushed/Sedentary) when compared to sedentary sham-operated animals (Sham/Sedentary) from the 3rd to 14th day post-crushing; treadmill running reduced this increased in Crushed/Exercised mice from 5th to 14th day after induction of PNI post-crushing. The measures were performed from day 3 to 14 post-crushing, 24 h after each exercise session (### $P < 0.001$ as compared with Sham/Sedentary group; * $P < 0.05$ and ** $P < 0.01$ as compared with Sham/Exercised group; ^{lll} $P < 0.001$ as compared with Crushed/Sedentary group). (B) Reduced mechanical sensitivity after nerve injury in Crushed/Exercised mice is reversed by PCPA from 5th to 14th day post-crushing, as detected by von Frey test (** $P < 0.01$ and *** $P < 0.001$ as compared with Crushed/Saline/Exercised group; ^{lll} $P < 0.001$ as compared with Crushed/Saline/Sedentary group; $n = 8$ mice per group). (C) Sham-operated control groups show no difference between them. (D and E) HPLC analyses of 5-HT and its metabolite 5-HIAA in the brainstem of mice. Low-intensity aerobic exercise significantly increased the levels of (D) 5-HT and (E) 5-HIAA, it was reverse by pretreatment with PCPA in sham-operated (Sham) and nerve injury (Crushed) mice (# $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ when compared with Sham/Saline/Sedentary group; ^l $P < 0.05$, ^{ll} $P < 0.01$ and ^{lll} $P < 0.001$ when compared with Crushed/Saline/Sedentary group; * $P < 0.05$ and *** $P < 0.001$ when compared Crushed/Saline/Exercised with Crushed/PCPA/Exercised group; $n = 8$ mice per group). (F) 5-HT turnover rate detected by ratio of 5-HIAA/5-HT. Low-intensity aerobic

exercise inhibited the increase in the turnover ratio in the brainstem of peripheral nerve injured mice, on the other hand, in sham-operated mice the exercise increased 5-HT turnover ([#] $P < 0.05$ when compared with Sham/Saline/Sedentary group; [†] $P < 0.05$ when compared Crushed/Saline/Exercised with Crushed/Saline/Sedentary group; $n = 8$ mice per group). Data are the mean $\pm$ SEM from each experimental group.

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Figure 3.

(A) Photomicrographs of immunohistochemistry in the Medullary Raphe Nuclei show the effect of low-intensity aerobic exercise on SERT staining after PNI in mice. Optical density quantification in the Raphe Pallidus nucleus (RPa) (B), in the Raphe Magnus nucleus (RMg) (C), and in the Raphe Obscurus nucleus (ROb) (D). Data are the mean $\pm$ SEM from 4-5 animals per group. [#] $P < 0.05$, ^{##} $P < 0.01$ when compared to Sham/Sedentary group; ^{*} $P < 0.05$ and ^{**} $P < 0.01$ when compared to Crushed/Sedentary group. Scale bar = 50 μ m.

A Serotonin Transporter (SERT) **B Tryptophan hydroxylase 2 (Tph2)** **Figure 4.**

SERT and Tph2 gene expression analysis in the brainstem. (A) Low-intensity aerobic exercise counteracts the increased SERT mRNA expression, after peripheral nerve injury in mice. Low-intensity exercise performed in sham-operated mice (Sham/Exercised group) decrease SERT compared to Sham/Sedentary group. (B) Peripheral nerve injury (Crushed/Sedentary group) or low-intensity aerobic exercise performed after nerve injury (Crushed/Exercised group) does not change Tph2 mRNA expression. However, low-intensity exercise performed in sham-operated mice (Sham/Exercised group) decrease Tph2 mRNA

expression when compared to Sham/Sedentary group. Data are the mean $\pm$ SEM from experiments using 8-10 mice per group. # $P < 0.05$, ### $P < 0.001$ when compared to Sham/Sedentary group; ** $P < 0.01$ when compared to Crushed/Sedentary group.

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Figure 5.

Gene expression analyzes in the brainstem of 5-HT_{1A} (A), 5-HT_{1B} (B), 5-HT_{2A} (C), 5-HT_{2C} (D), 5-HT_{3A} (E), and 5-HT₇ (F) receptors shows the effect of low-intensity aerobic exercise after peripheral nerve injury (Crushed group) or in sham-operated (Sham group) mice. Data are the mean $\pm$ SEM from experiments using 8-10 mice per group. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ as compared to Sham/Sedentary group; * $P < 0.05$ as compared with Crushed/Sedentary group.

Figure 6.

ELISA shows that low-intensity aerobic exercise counteracts the increased proinflammatory cytokines levels in mice subjected to peripheral nerve injury. Levels of IL-1 β (A) and TNF- α (B) in the brainstem of sham-operated (Sham) or nerve injury mice (Crushed), subjects to two-weeks of exercise or sedentary. Data are the mean $\pm$ SEM from 8 animals per group. # P < 0.05 and ## P < 0.01 compared to Sham/Sedentary group; * P < 0.05 and ** P < 0.01 compared to Crushed/Sedentary group.

Figure 7.

Schematic representation of the low-intensity exercise effects on serotonergic neurotransmission in the brainstem nuclei after sciatic nerve injury. We suggest that low-intensity exercise suppresses pain behavior by enhancing brainstem 5-HT neurotransmission through increasing 5-HT, 5-HIAA content, 5-HT receptor expression (5HT-_{1B}, _{2A}, _{2C}) and decreasing SERT expression and 5-HT turnover ratio.

Table 1

Genes for qPCR analyses and their respective NCBI reference, primers, fragment size and references.

Gene name	NCBI gene reference	Forward/Reverse primers sequence 5'-3'	Fragment size	Reference
5-HT1A receptor	NM_008308	GGATGTTTTCCTGTCTGGT/CACAAAGGCCTTTCCAGAACT	121bp	Bibancos et al., 2007.
5-HT1B receptor	NM_010482	TCACATGGCCATTCTTGTACT/CAGTTTGTGGAACGCTTGTT	112bp	Bibancos et al., 2007.
5-HT2A receptor	NM_172812	AGAAACCCCAATTCACCATAGC/ATCCTGTAGCCCGAAGACTG	119bp	Bibancos et al., 2007.
5-HT2C receptor	NM_008312	AGCAGTGCCTAGTCCTGTG/CTTTGCTCCCTCAGTCCAAT	132bp	Bibancos et al., 2007.
5-HT3A receptor	NM_013561	CTTCCCTTTTGATGTGTCAG/CCACTCGCCCTGATTTATG	139bp	Bibancos et al., 2007.
5-HT7 receptor	NM_008315	TTCTGCAACGTCTTCATCG/ATTCTGCCTCACGGGGTA	126bp	Bibancos et al., 2007.
5-HT transporter	NM_010484	TGCAGATCCATCAGTCAAAAGG/AATGTAAAGGGAAGGTGGCTG	161bp	PrimerQuest® program, IDT, Coralville, USA. Retrieved 12 December, 2012. http://www.idtdna.com/Scitools .
Tryptophan hydroxylase 2	NM_173391	GGAAAGTATTTTGTGGATGTGGC/AGTCGGGTAGAGTTTGGAGAG	128bp	PrimerQuest® program, IDT, Coralville, USA. Retrieved 12 December, 2012. http://www.idtdna.com/Scitools .
Actin β	NM_007393.3	TGGAATCCTGTGGCATCCATGA/AATGCCTGGGTACATGGTGGTA	122bp	Martins-Silva et al., 2011