Regular physical activity prevents development of chronic muscle pain through modulation of supraspinal opioid and serotonergic mechanisms

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Abstract

Introduction: It is generally believed that exercise produces its effects by activating central opioid receptors; there are little data that support this claim. The periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) are key nuclei in opioid-induced analgesia, and opioids interact with serotonin to produce analgesia.
Objectives: The purpose was to examine central inhibitory mechanisms involved in analgesia produced by wheel running.
Methods: C57/Black6 mice were given access to running wheels in their home cages before induction of chronic muscle hyperalgesia and compared with those without running wheels. Systemic, intra-PAG, and intra-RVM naloxone tested the role of central opioid receptors in the antinociceptive effects of wheel running in animals with muscle insult. Immunohistochemistry for the serotonin transporter (SERT) in the spinal cord and RVM, and pharmacological blockade of SERT, tested whether the serotonin system was modulated by muscle insult and wheel running.
Results: Wheel running prevented the development of muscle hyperalgesia. Systemic naloxone, intra-PAG naloxone, and intra-RVM naloxone reversed the antinociceptive effect of wheel running in animals that had received muscle insult. Induction of chronic muscle hyperalgesia increased SERT in the RVM, and blockade of SERT reversed the hyperalgesia in sedentary animals. Wheel running reduced SERT expression in animals with muscle insult. The serotonin transporter in the superficial dorsal horn of the spinal cord was unchanged after muscle insult, but increased after wheel running.
Conclusion: These data support the hypothesis that wheel running produced analgesia through central inhibitory mechanisms involving opioidergic and serotonergic systems.

Keywords: Pain, Analgesia, Exercise, Physical activity, Serotonin, Opioid, Periaqueductal gray, Rostral ventromedial medulla, Serotonin transporter, SERT, Supraspinal

1. Introduction

People who are physically active are less likely to develop chronic musculoskeletal pain.[48,49,111] Similarly, previous studies by us and others show that prior physical activity prevents development of hyperalgesia in animal models of muscle and neuropathic pain.[6,29,77,94] Thus, these data suggest that the underlying mechanisms by which physical activity prevents development of pain are key factors for the transition from acute to chronic pain.

Although it is generally believed that exercise produces its effects by activating central opioid receptors, there are little data that support this claim. In healthy human subjects, a single exercise task increases beta-endorphin and produces analgesia that is blocked by naloxone.[37,82] In uninjured animals, regular exercise produces tolerance at mu-opioid receptors, and analgesia is blocked by opioid receptor antagonists.[37,44,60,62,97,98] In animals with neuropathic pain, treadmill exercise started after injury, increases endogenous opioids in the periaqueductal gray (PAG) (β-endorphin) and the rostral ventromedial medulla (RVM) (met-enkephalin), and both systemic and intracerebroventricular naloxone reverses the antinociception.[101] In animals with chronic muscle pain, treadmill running starting after development of hyperalgesia produces antinociception that is reversed by systemic naloxone.[5] Furthermore, in animals with nerve injury, the serotonin transporter (SERT) is increased, and serotonin is decreased in the RVM, both of which are normalized by treadmill running starting after injury,[6] and systemic serotonergic depletion prevents this exercise-induced analgesia.[6,63] These studies suggest that opioids and serotonin play a role in

analgesia produced by exercise in human subject and animals with and without pain, and suggest that central pain inhibitory pathways underlie this analgesia.

The RVM mediates hyperalgesia after tissue insult, as well as opioid-induced analgesia, and PAG-mediated opioid analgesia.[9,16,25,31,36,71,74,102–106,108] For example, systemic morphine analgesia is reduced by lesions or opioid receptor blockade in the PAG,[20,22] and analgesia produced by activation of the PAG is prevented by opioid receptor blockade or lesions of the RVM.[4,25,27,46,79,100,110] Further opioid receptors and opioid peptides are located in the RVM, and activation of opioid receptors in the RVM produces analgesia.[1,24,36,39,43,72,73] Furthermore, serotonergic RVM neurons receive input from endogenous opioid peptides and project to the spinal cord to produce analgesia, or hyperalgesia, depending on the receptor activated.[63] In the RVM, microinjection of 5-HT produces analgesia, and mice lacking central 5-HT neurons show greater inflammatory hyperalgesia.[56,57,113] Furthermore, systemic serotonergic depletion, or blockade of serotonin receptors in the RVM, prevents analgesia from morphine injected systemically or into the PAG.[11,45,81] These data show a critical role for the PAG and RVM in endogenous inhibition, that this, analgesia involves opioids and serotonin.

The purpose of the current study was to examine the central opioid sites involved in the prevention of chronic muscle pain by regular physical activity, if SERT expression in the RVM and spinal cord is modulated by regular physical activity, and if SERT mediates the hyperalgesia in animals with chronic muscle pain.

2. Material and methods

2.1. Sedentary vs physically active mice

All experiments were approved by the Animal Care and Use Committee at the University of Iowa. Male (n = 42) and female (n = 30) C57BL/6 mice were used for these experiments. Sedentary and naive mice were housed in their home cage with food and water. Physically active mice had free access to running wheels in their home cages for 8 weeks, and activity, in km/d, was recorded. Mice were housed individually. Running wheels were removed from home cages after 8 weeks, before induction of the chronic muscle pain model. We previously showed that 8 weeks of running wheel activity prevents development of hyperalgesia in this model that lasts for 8 days after stopping the running wheel activity.[93]

2.2. Noninflammatory chronic muscle pain

The model of chronic muscle pain was induced by 2 injections of unbuffered pH 4.0 saline (20 μL) into the left gastrocnemius muscle 5 days apart while the mouse was anesthetized with 4% isoflurane, as previously published.[93] This insult produces enhanced mechanical sensitivity of the paw and muscle bilaterally without tissue damage.[18,93]

2.3. Behavior testing

Mice were acclimated for 2×/day for 2 days prior to behaviour testing procedures. This included acclimation to a gardener's glove used to immobilize the animals, acclimation to the wire mesh in small Lucite cubicles, and acclimation to the grip force test. All experiments were performed with the experimenter blinded to the group.

Muscle withdrawal thresholds were tested by applying a pair of calibrated forceps to the gastrocnemius muscle as previously described and validated.[89,107] Mice were placed in a gardener's glove, and one hindlimb was extended. A pair of forceps was applied perpendicularly to the middle of the gastrocnemius muscle belly until the animal withdrew the hindlimb. Tests were performed bilaterally and 3 trials averaged.

The response frequency to mechanical stimulation of the paw was tested as previously described.[18] The number of withdrawals to a von Frey filament (2.44, 0.4 mN) applied to the paw 5 times was assessed, followed by a 5-minute waiting period. This was repeated 10 times, averaged, and presented as percent response.

Grip strength of the hindpaw and forepaw was tested as previously described.[10] Mice were pulled by the tail until a good grip was felt to read grip force for either the hindpaw or the forepaw. An average of 5 trials was recorded.

2.4. Implantation of guide cannula

Intracerebral guide cannulae were placed in the vlPAG and RVM 2 days before the first intramuscular injection of 4.0 as previously described.[21,92] Mice were anesthetized with ketamine/xylazine and positioned in a stereotaxic head holder. The skull was exposed, and a small hole drilled for placement of guide cannulae. For the vlPAG, cannula were placed −4.1 mm from bregma, 0.4 mm from midline, and −2.6 mm from surface; for the RVM, cannula were placed −5.6 mm from bregma, 0.0 mm from midline, and −5.7 mm from surface. Cannulae were secured to the skull with 2 screws and dental cement. Mice were allowed to recover 3 to 7 days before testing. To examine the placement of the cannula, 0.2 μL methylene blue dye was injected through the cannula at the end of the experiment. After euthanasia, the brain was removed and postfixed in 10% formalin. The day before cutting, brains were transferred to 30% sucrose. Forty micrometer sections were cryosectioned through the injection site and examined under light microscopy for the placement of the cannula. Sites were verified by 2 individuals and recorded in a spreadsheet. In additional mice, guide cannula were misplaced outside the RVM, into the gigantocellularis nucleus (n = 8), and outside the vlPAG into the dorsolateral PAG (n = 2), lateral PAG (n = 1), or deep mesencephalic nucleus (n = 1).

2.5. Drugs

Naloxone hydrochloride and naloxone methiodide (Sigma-Aldrich, St. Louis, MO; 10 mg/kg, i.p.) were dissolved in 0.9% sterile saline for systemic delivery, and dosing was based on previously published data in mice.[67,68,70,109] For microinjection, naloxone hydrochloride was dissolved in saline at a concentration of 10 nmol/0.2 μL; dosing was based on previously published data in mice.[12,14] Fluoxetine doses (Sigma-Aldrich) were extrapolated from previous reports and tested in preliminary studies.[13,58] For fluoxetine, 2 nmol/0.2 μL was dissolved in 0.9% sterile saline and 20 nmol/0.2 μL in 10% 2-hydroxypropyl-B-cyclodextrin (HBC) in sterile saline. Vehicles for all experiments were 0.9% sterile saline, except for the 20 nmol/0.2 μL, which used 10% HBC in sterile saline.

2.6. Immunohistochemistry

Animals were deeply anesthetized with 150 mg/kg sodium pentobarbital and transcardially perfused with heparinized saline followed by 4% paraformaldehyde. The brainstem and lumbar spinal cord was removed, stored in 30% sucrose overnight, and frozen at −20°C. Sections were cut on a cryostat at 20 μm on to

slides. All sections were stained using an antibody to SERT (rabbit Anti-5HT Transporter; 1:1000) using standard immunofluorescent techniques as previously described.[6] Preliminary data show specificity of the antibody to SERT because removal of the primary antibody and SERT−/− mice showed no immunoreactivity.[54] On day 1, sections were blocked in 3% normal goat serum, avidin, and biotin and incubated in the primary antibody overnight at room temperature. On day 2, sections were rinsed, blocked in 3% normal goat serum, and incubated in biotinylated immunoglobulin G (IgG) for 1 hour at room temperature (1:1,000; Invitrogen, Eugene, OR). These sections were then reacted with streptavidin conjugated to Alexa Fluor 568 (Goat Anti-rabbit IgG; 1:1000) for 1 hour at room temperature. Sections were coverslipped with Vectashield (Vector Labs, Burlingame, CA) and stored until analysis. Brain and spinal cord sections for each group were stained simultaneously to avoid variability between stains across days.

Images of stained sections were taken in the Central Microscopy Facility at the University of Iowa on an Olympus BX-51 light microscope equipped with a SPOT camera (RT Slider; Diagnostic Instruments, Sterling Heights, MI). Rostral ventromedial medulla sections were identified at Bregma −6.00 mm from the Mouse Brain Atlas.[69] All sections were taken under the same condition and stored for later analysis. Using Image J software, optical density was analyzed as previously described.[38] Each picture was converted to 8-bit gray scale, calibrated independently using the "uncalibrated OD" function, and density values were analyzed per area. Ten sections of the RVM and superficial dorsal horn were quantified.

2.7. Experimental design

Experiment 1 tested whether a regular physical activity prevented development of chronic muscle hyperalgesia using the following groups: (1) sedentary with muscle insult (n = 3 M, 3 F), (2) wheel running with muscle insult (n = 4 M, 4 F), and (3) naive (n = 3 M, 3 F). Mice were tested before wheel running, and before and 24 hours after muscle insult for muscle withdrawal thresholds, paw response frequency, and grip strength. Naive mice did not receive muscle insult and did not have access to running wheels. Running wheels were stopped at the time of the first intramuscular injection.

Experiment 2 tested the role of peripheral and central opioid systems on prevention of hyperalgesia by wheel running using the following groups: (1) vehicle (n = 3 M, 2 F), (2) naloxone hydrochloride (10 mg/kg, i.p.; n = 3 M, 2 F), and (3) naloxone methiodide (10 mg/kg, i.p.; n = 2 M, 3 F). Muscle withdrawal thresholds were assessed before and 24 hours after muscle insult; and 30 and 120 minutes after injection. Running wheels were stopped at the time of the first intramuscular injection. Naloxone or vehicle was administered 24 hours after the second intramuscular injection.

Experiment 3 tested whether the PAG and RVM, central opioid sites involved in analgesia, were involved in the prevention of hyperalgesia by wheel running. For PAG, active mice with muscle insult were divided in 2 groups: (1) vehicle (saline, n = 3 M, 3 F) and (2) naloxone hydrochloride (10 nmol/0.2 µL, i.c.; n = 4 M, 3 F). For RVM, active animals with the muscle insult were divided into 2 groups: (1) vehicle (n = 3 M, 2 F) and (2) naloxone hydrochloride (10 nmol/0.2 µL, i.c.; n = 3 M, 3 F). Muscle withdrawal thresholds were assessed before and 24 hours after muscle insult, and 30 and 120 minutes after microinjection. Running wheels were stopped at the time of cannulae placement. Two days after the placement of cannulae, the first intramuscular injection of acidic saline was given. Naloxone or vehicle was administered 24 hours after the second intramuscular injection.

Experiment 4 tested the involvement of the serotonin system in the chronic muscle pain model and modulation by wheel running. Using animals from experiment 1, SERT, expression in the RVM, and superficial dorsal horn using immunohistochemistry in sedentary, physically active, and naive mice was assessed.

Experiment 5 tested whether pharmacological blockade of SERT in the RVM, in animals with muscle insult, mimicked the behavioural effects of exercise. Sedentary mice were implanted with guide cannula in RVM, induced with chronic muscle pain model, and randomly divided as follows: (1) Saline (n = 5 M, 1 F), (2) Saline with 10% HBC (n = 3 M, 2 F), (3) Fluoxetine (2 nmol/0.2 µL) (n = 4 M, 2 F), and (4) Fluoxetine (20 nmol/0.2 µL) (n = 3 M, 3 F). Muscle withdrawal thresholds were tested before and 24 hours after muscle insult; and 30 and 120 minutes after microinjection.

2.8. Statistical analysis

A repeated-measures analysis of variance was performed to test for changes in withdrawal thresholds of the muscle, the response frequency to mechanical stimulation of the paw, and grip force to examine for differences between groups and across time. This was followed by post hoc testing with a Tukey's test for differences between groups. Experiment 1 had 3 groups (sedentary, wheel running, and naive) and 3 time periods (baseline, before insult, and after insult). Experiment 2 had 3 groups (vehicle, naloxone hydrochloride, and naloxone methiodide) and 4 time periods (before and after muscle insult, 30 minutes and 1 hour after drug). Experiment 3 had 2 groups per site (vehicle and naloxone hydrochloride) and 4 time periods (before and after muscle insult, 30 minutes and 1 hour after drug). Experiment 5 had 5 groups (saline, saline with 10% HBC, fluoxetine 2 nmol, fluoxetine 20 nmol, missed sites) and 4 time periods before and after muscle insult, 30 minutes and 1 hour after drug). We report within-subjects effects, within-subjects contrasts, and between-subjects effects for group for each experiment in **Table 1**. Post hoc analysis examined for individual differences between groups with Tukey's test. For immunohistochemistry in experiment 4, a 1-way analysis of variance tested for differences between groups for the changes in SERT expression which had 3 groups (sedentary, wheel running, and naive) and 1 time period followed by post hoc analysis for group differences with Tukey's test. All data are mean ± SEM, and $P \leq 0.05$ is considered significant.

3. Results

3.1. Physical activity prevents development of chronic muscle hyperalgesia

On average, mice ran 2.46 ± 0.87 km/d over the 8-week time period with a range from 0.86 km/d to 9.48 km/d. There was a significant overall difference in muscle withdrawal thresholds for time, an interaction between time and group, and a significant difference between groups (**Table 1**). Specifically, 24 hours after induction of the chronic muscle pain model, the sedentary mice showed significantly lower muscle withdrawal thresholds when compared with the physically active mice (ipsilateral: $P = 0.0001$, contralateral: $P = 0.02$) and the naive mice (ipsilateral: $P = 0.0001$; contralateral: $P = 0.0001$) (**Fig. 1A, B**).

For response frequency of the paw, there was a significant overall difference for time, an interaction between time and

Table 1

Statistical results for repeated-measures analysis of variance for experiments 1, 2, 3, and 5.

| Experiment	Outcome	Side	Within-subjects effects		Within-subjects contrasts		Between-subjects effects
			TIME	TIME × GROUP	TIME	TIME × GROUP	GROUP
Experiment 1, effects of running wheel	Muscle withdrawal threshold	Ipsilateral	$F_{2,34} = 46.4$, $P = 0.0001$	$F_{4,32} = 15.2$, $P = 0.0001$	$F_{1,17} = 102.2$, $P = 0.0001$	$F_{2,17} = 19.8$, $P = 0.0001$	$F_{2,17} = 24.9$, $P = 0.0001$
		Contralateral	$F_{2,34} = 36.3$, $P = 0.0001$	$F_{4,32} = 12.8$, $P = 0.0001$	$F_{1,17} = 77.7$, $P = 0.0001$	$F_{2,17} = 25.5$, $P = 0.0001$	$F_{2,17} = 9.9$, $P = 0.001$
	Paw response frequency	Ipsilateral	$F_{2,34} = 251.2$, $P = 0.0001$	$F_{4,32} = 67.5$, $P = 0.0001$	$F_{1,17} = 329.5$, $P = 0.0001$	$F_{2,17} = 81.2$, $P = 0.0001$	$F_{2,17} = 425.7$, $P = 0.0001$
		Contralateral	$F_{2,34} = 344$, $P = 0.0001$	$F_{4,32} = 80.5$, $P = 0.0001$	$F_{1,17} = 424.3$, $P = 0.0001$	$F_{2,17} = 92.7$, $P = 0.0001$	$F_{2,17} = 71.4$, $P = 0.001$
	Grip force	Forepaw	$F_{2,34} = 15.4$, $P = 0.0001$	$F_{4,32} = 0.821$, $P = 0.52$	$F_{1,17} = 36.2$, $P = 0.0001$	$F_{2,17} = 0.49$, $P = 0.62$	$F_{2,17} = 0.96$, $P = 0.40$
		Hindpaw	$F_{2,34} = 1.7$, $P = 0.20$	$F_{4,32} = 1.6$, $P = 0.20$	$F_{1,17} = 0.692$, $P = 0.42$	$F_{2,17} = 5.0$, $P = 0.02$	$F_{2,17} = 0.06$, $P = 0.94$
Experiment 2, systemic naloxone	Muscle withdrawal threshold	Ipsilateral	$F_{3,36} = 18.7$, $P = 0.0001$	$F_{6,36} = 3.8$, $P = 0.005$	$F_{1,12} = 17.7$, $P = 0.001$	$F_{2,12} = 9.8$, $P = 0.003$	$F_{2,12} = 8.2$ $P = 0.006$
		Contralateral	$F_{3,36} = 20.9$, $P = 0.0001$	$F_{6,36} = 2.1$, $P = 0.07$	$F_{1,12} = 20.7$, $P = 0.001$	$F_{2,12} = 3.2$, $P = 0.08$	$F_{2,12} = 1.6$ $P = 0.25$
Experiment 3, periaqueductal gray naloxone	Muscle withdrawal threshold	Ipsilateral	$F_{3,33} = 53$, $P = 0.0001$	$F_{3,33} = 18.3$, $P = 0.0001$	$F_{1,11} = 63.4$, $P = 0.0001$	$F_{1,11} = 23.1$, $P = 0.001$	$F_{1,11} = 37.2$, $P = 0.001$
		Contralateral	$F_{3,33} = 22.3$, $P = 0.0001$	$F_{3,33} = 17.6$, $P = 0.0001$	$F_{1,11} = 27.3$, $P = 0.0001$	$F_{1,11} = 19.0$, $P = 0.001$	$F_{1,11} = 42.3$, $P = 0.0001$
Experiment 3, rostral ventromedial medulla naloxone	Muscle withdrawal threshold	Ipsilateral	$F_{3,27} = 14.6$, $P = 0.0001$	$F_{3,27} = 5.8$, $P = 0.003$	$F_{1,9} = 12.0$, $P = 0.007$	$F_{1,9} = 14.5$, $P = 0.004$	$F_{1,9} = 4.6$, $P = 0.05$
		Contralateral	$F_{3,27} = 6.7$, $P = 0.002$	$F_{3,27} = 8.3$, $P = 0.0001$	$F_{1,9} = 14$ $P = 0.004$	$F_{1,9} = 8.9$, $P = 0.02$	$F_{1,9} = 10.8$, $P = 0.009$
Experiment 5, rostral ventromedial medulla fluoxetine	Muscle withdrawal threshold	Ipsilateral	$F_{3,63} = 116$, $P = 0.0001$	$F_{12,63} = 9.9$, $P = 0.0001$	$F_{1,21} = 257$, $P = 0.0001$	$F_{4,21} = 3.7$, $P = 0.02$	$F = {}_{4,21} = 3.0$, $P = 0.04$
		Contralateral	$F_{3,63} = 95.2$, $P = 0.0001$	$F_{12,63} = 8.2$, $P = 0.0001$	$F_{1,21} = 170.6$, $P = 0.0001$	$F_{4,21} = 9.2$ $P = 0.0001$	$F_{4,21} = 1.3$, $P = 0.3$
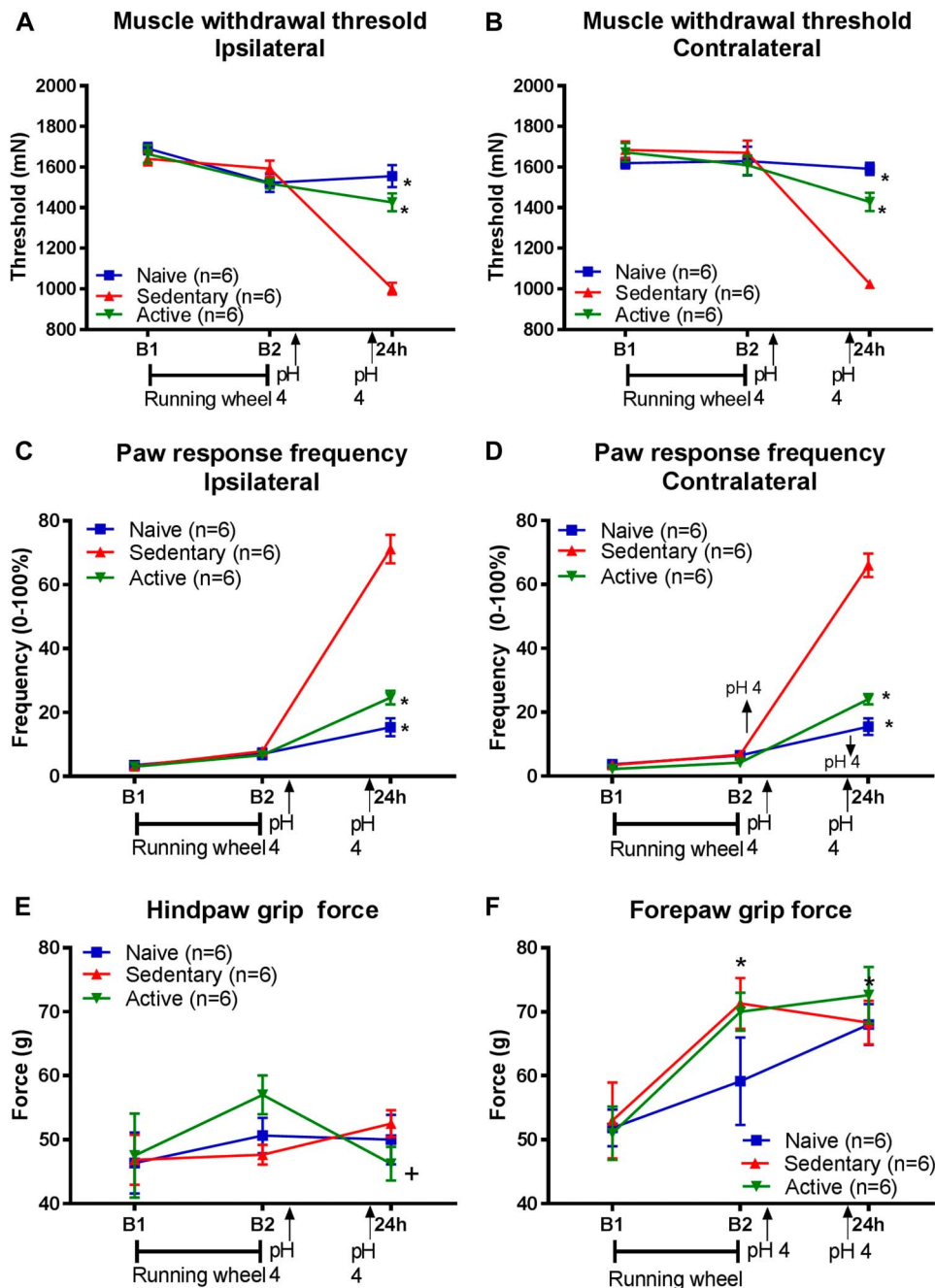
Figure 1. Long-term running wheel activity prevents development of hyperalgesia produced by repeated acid injections. Graphs show the muscle withdrawal threshold (A and B), paw response frequency (C and D), and grip force (E and F) before (B1) and after 8 weeks of running wheel (B2), and 24 hours after the second injection of acidic saline for naive (blue), sedentary (red), and running wheel (active, green) groups. The withdrawal threshold and paw response frequency were significantly lower in sedentary animals after the second acid injection when compared with the naive or physically active groups bilaterally (*$P < 0.05$). The hind paw grip force was significantly greater in the active group compared with the naive group for the forepaw and significantly less after the second acidic saline injection for the active group (*, $P < 0.05$) compared with the time after running wheel (+, $P < 0.05$). Data are the mean ± SEM.

group, and a significant difference between groups (**Table 1**). Specifically, 24 hours after induction of the chronic muscle pain model, the sedentary mice showed a greater frequency of response to repeated mechanical stimulation of the paw bilaterally when compared with the physically active mice (ipsilateral, contralateral: $P = 0.0001$) and naive mice (ipsilateral, contralateral: $P = 0.0001$) (**Fig. 1C, D**).

The forepaw showed a significant difference in grip force across time, but no interaction between time and group, and

no group differences (**Table 1**). In the active group, there were significant increases in grip force after 8 weeks of wheel running ($P = 0.02$, paired to-test) that were maintained 24 hours after induction of the chronic muscle pain model ($P = 0.0001$). The hindpaw grip force showed a significant interaction between time and group, but no difference for time (**Table 1**). In the active group, hindpaws showed significant decreases in force after induction of the chronic pain model ($P = 0.001$) when compared with the value 8 weeks after wheel running (**Fig. 1E, F**).

3.2. Central opioids mediate analgesia produced by wheel running

As opioids have been reported to mediate the analgesic effects of exercise,[5,101] we tested if systemic naloxone reversed the analgesic effects of wheel running 24 hours after muscle insult. We compared this to the peripherally restricted opioid antagonist, naloxone methiodide, to examine contributions of peripheral opioid receptors. There was a significant overall difference for time, an interaction between time and group, and a significant difference between groups (**Table 1**). The naloxone-treated group was significantly lower than the saline-treated group (ipsilateral: $P = 0.01$; contralateral $P = 0.001$). Naloxone

methiodide had no effect on the analgesia and was not different from the saline-treated group (ipsilateral: $P = 0.94$; contralateral: $P = 0.50$) (**Fig. 2A, B**).

Because systemic naloxone, but not naloxone methiodide, reversed the analgesic effects of regular physical activity, we tested whether central sites were involved in the antinociception. Because the PAG also uses opioids to produce analgesia,[25] we tested whether blockade of opioid receptors in the PAG reversed the antinociceptive effects of wheel running. Microinjection of naloxone hydrochloride into the vlPAG 24 hours after muscle insult reversed the antinociceptive effects of wheel running. There was a significant overall difference for time, an interaction between time and group, and a significant difference between
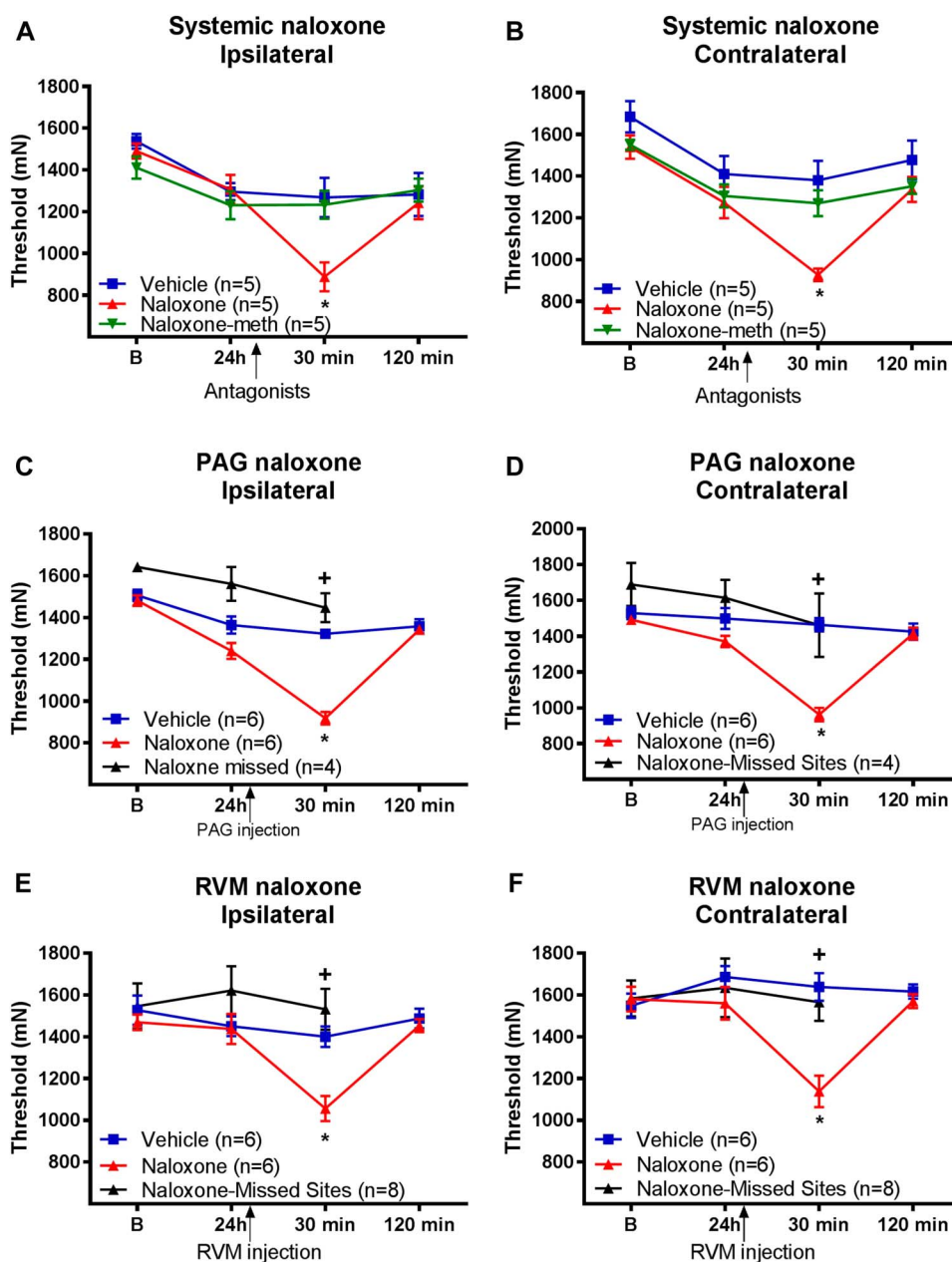

Figure 2. Central opioid receptors in periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) mediate the analgesia produced by wheel running. Muscle withdrawal thresholds for animals treated with naloxone systemically (A and B), into the PAG (C and D) and into the RVM (E and F) for both the ipsilateral and contralateral sides. All animals were given running wheels for 8 weeks before induction of the chronic muscle pain model. Thirty minutes after injection of naloxone systemically, or into the PAG or RVM, there was a significant decrease in the withdrawal thresholds of the muscle (*, $P < 0.05$) when compared with animals that received vehicle. Missed injection sites at the level of the PAG or RVM show significantly greater withdrawal thresholds compared with those injected with naloxone into the PAG or RVM (+, $P < 0.05$). Data are the mean ± SEM.

groups (**Table 1**). Specifically, the withdrawal thresholds of the muscle were significantly lower in the physically active mice that received naloxone in the PAG when compared with the saline group (ipsilateral, contralateral: $P = 0.0001$) 30 minutes after injection (**Fig. 2C, D**). This effect was temporary, and withdrawal thresholds were not significantly different from the saline group 2 hours after induction of the hyperalgesia (ipsilateral: $P = 0.7$; contralateral: $P = 0.52$) (**Fig. 2C, D**). Furthermore, 4 animals had cannulae misplaced outside the vlPAG into the dlPAG, lPAG and deep mesencephalic nucleus. In these animals, the withdrawal threshold remained unchanged after microinjection of naloxone hydrochloride, and was significantly greater than in those injected with naloxone into the vlPAG (**Fig. 2C, D**) ($P < 0.05$).

Because the RVM plays a significant role in analgesia, receives projections from the PAG, and uses opioid receptors to produce analgesia,[25] we tested whether opioid receptors in the RVM were involved in the analgesic effect of wheel running after muscle insult. Microinjection of naloxone into the RVM 24 hours after induction of the chronic muscle pain model reversed the antinociceptive effects of regular physical activity. There was a significant overall difference for time, an interaction between time and group, and a significant difference between groups (**Table 1**). Specifically, after microinjection of naloxone into the RVM, muscle withdrawal thresholds were significantly lower than those that received saline (ipsilateral: $P = 0.002$; contralateral: $P = 0.001$) (**Fig. 2E, F**). The effect was temporary, and

antinociception returned 2 hours after naloxone injection (vs vehicle; ipsilateral: $P = 0.54$; contralateral: $P = 0.36$) (**Fig. 2E, F**). In 8 mice, naloxone hydrochloride was injected into the gigantocellularis nucleus (Gi). In these animals, there was no change in withdrawal thresholds after microinjection, and these were significantly different than withdrawal thresholds from animals injected with naloxone into the RVM (**Fig. 2E, F**) ($P < 0.05$).

3.3. Physical activity modulates serotonin transporter expression

Because there is an interaction between the serotonergic and opioidergic systems in the RVM,[11,45] we tested whether there were changes in SERT in the RVM. **Figures 3A and D** shows SERT staining density in the nucleus raphe magnus (NRM) 24 hours after muscle insult in sedentary animals compared with those that had performed wheel running and naive controls. After muscle insult, there were significant differences in the density of SERT immunoreactivity in the NRM ($F_{2,18} = 13.1$, $P = 0.0001$), with sedentary mice showing an increase in SERT density 24 hours after muscle insult when compared with naive controls ($P = 0.009$). Furthermore, SERT density in the physically active mice was significantly lower than that in sedentary mice 24 hours after muscle insult ($P = 0.0001$), but not different from naive mice ($P = 0.35$).
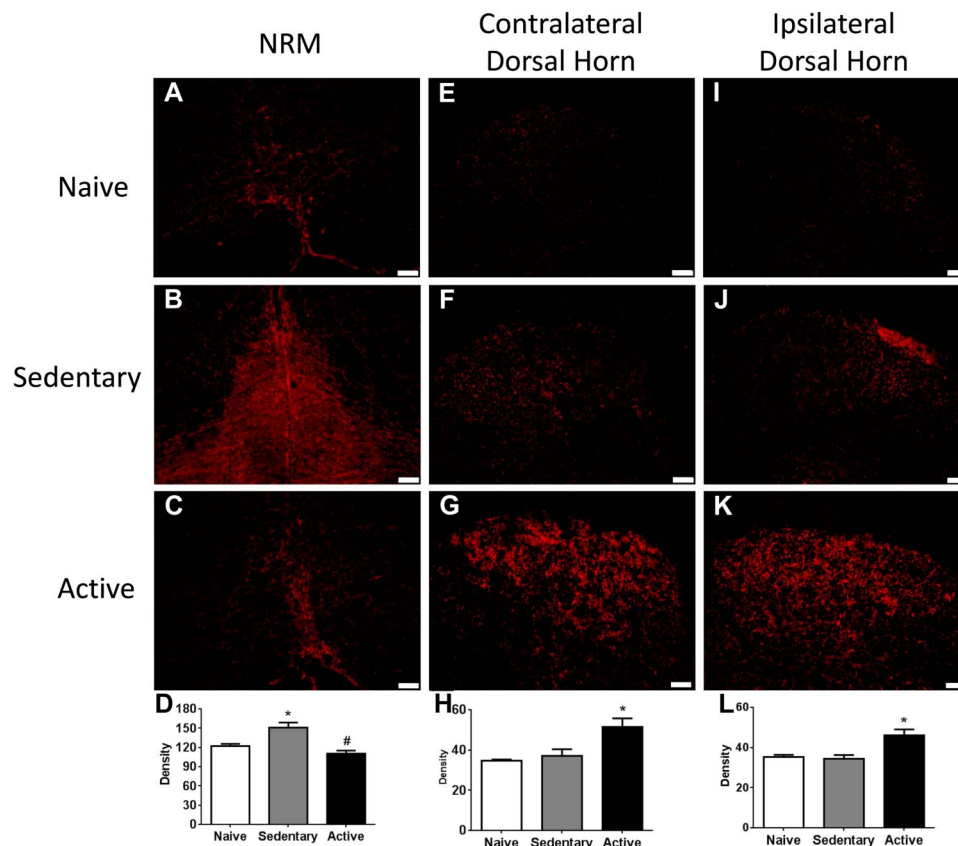

Figure 3. Wheel running modulates expression of serotonin transporter (SERT) in the RVM and spinal cord. Representative photomicrographs of the nucleus raphe magnus (NRM, A-C) and spinal cord dorsal horn (ipsilateral: E-G; contralateral: I-K) from naive, sedentary, and active animals immunohistochemically stained for SERT. In the NRM, there was an increase in staining density in the sedentary animals (B and D) when compared with the naive (A and D) that was reduced in the exercised group (C and D). Quantitation of the density of staining showed a significant increase in staining density in the sedentary group in the NRM (*, $P < 0.05$) that was significantly reduced in the physically active animals (#, $P < 0.05$). For the dorsal horn, there was minimal immunohistochemical staining for SERT in the superficial dorsal horn in naive and sedentary mice. In the physically active animals, there was an increase in staining density in the superficial dorsal horn bilaterally. Quantification showed a significant increase in staining density in the active group when compared with sedentary or naive mice (*, $P < 0.05$). Data are the mean ± SEM.

Because the RVM projects to the spinal cord, and serotonin plays a role in pain inhibition in the spinal cord,[3,20,25] we tested whether there were differences in SERT in the spinal cord. **Figures 3E and L** shows the SERT staining density in the spinal cord 24 hours after muscle insult in sedentary animals and those that performed 8 weeks of wheel running compared with naive controls. There was a significant difference in the density of immunoreactivity for SERT bilaterally in the spinal cord dorsal horn (ipsilateral: $F_{2,18} = 9.9$, $P = 0.002$; contralateral: $F_{2,18} = 7.4.1$, $P = 0.005$). There was no significant difference between sedentary mice in SERT density in the spinal cord 24 hours after muscle insult when compared with naive controls (ipsilateral: $P = 0.97$; contralateral: $P = 0.88$). However, in animals that performed wheel running and received muscle insult, SERT density was significantly greater bilaterally than naive (ipsilateral: $P = 0.006$; contralateral: $P = 0.007$) or sedentary mice 24 hours after muscle insult (ipsilateral: $P = 0.003$; contralateral: $P = 0.02$).

3.4. Blockade of serotonin transporter reverses hyperalgesia

Because there were increases in SERT in the RVM after muscle insult and regular physical activity decreases these SERT increases, we tested whether blockade of SERT in the RVM of sedentary animals with muscle insult mimicked the effects of wheel running. Fluoxetine was microinjected into the RVM 24 hours after induction of the model. For these experiments, there was a significant difference in withdrawal thresholds of the muscle, a significant overall difference of time, an interaction between time and group, and a significant difference between groups (**Table 1**). Muscle withdrawal thresholds from the group that received 20 nmol fluoxetine were significantly greater than those from the group that received saline (ipsilateral: $P = 0.017$; contralateral: $P = 0.001$), 2 nmol fluoxetine (ipsilateral: $P = 0.0001$), and HBC-vehicle (ipsilateral: $P = 0.0001$; contralateral: $P = 0.004$) 15 minutes after injection (**Fig. 4**). Fluoxetine effects were temporary, and by 2 hours, there were no significant differences between groups. In 3 animals that received 2 nmol dose of fluoxetine, the cannula were misplaced and withdrawal thresholds were decreased bilaterally (ipsilateral: 1172 + 43 mN; contralateral: 1246 + 15.6 mN).

4. Discussion

The current study showed that blockade of opioid receptors in the RVM and PAG reversed the antinociception produced by long-term wheel running before muscle insult. These data are in agreement with prior studies in healthy human subjects showing an increase in systemic β-endorphin, and blockade of analgesia with systemic naloxone, after high-intensity exercise.[7,15,33,82] In animals without tissue injury, systemic naloxone reduces analgesia produced by strength training, treadmill exercise, and long-term running wheel activity.[26,53,86,101] Furthermore, long-term wheel running also decreased analgesia to exogenously applied mu-opioid agonists,[44,60,61] suggesting that opioid tolerance had developed. Thus, prior studies in human subjects and animal without tissue injury, clearly show a role for opioids in analgesia using not only high-intensity exercise, but also lower-intensity tasks like wheel running.

More recent studies evaluated effects of opioid antagonism on exercise-induced analgesia in animal models of pain. In these studies, systemic naloxone reversed analgesia produced by moderate- to low-intensity exercise started after development of hyperalgesia in animals with neuropathic pain, chronic muscle pain, and in those that exercised before induction of acetic
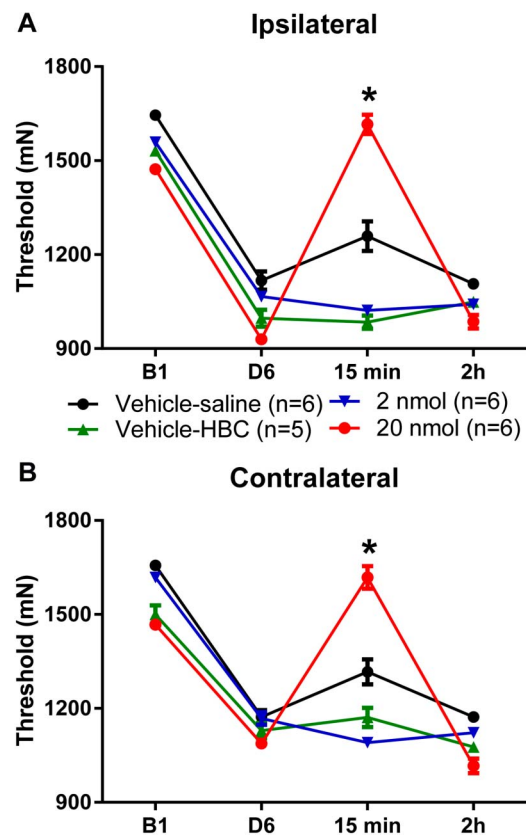
Figure 4. Blockade of serotonin transporter reverses hyperalgesia produced by repeated acid injections. Graphs represent muscle withdrawal thresholds before and 24 hours after the second acid injection in sedentary animals. The decrease in withdrawal threshold of the paw induced by repeated acid injection was reversed 15 minutes after microinjection of 20 nmol/0.2 μL of fluoxetine (*, $P < 0.05$) for both the ipsilateral (A) and contralateral (B) hindlimbs. By 2 hours after injection, the hyperalgesia returned to preinjection thresholds. Data are the mean ± SEM.

acid–induced pain.[5,59,62,83,101] In parallel, animals with neuropathic pain that perform moderate intensity treadmill exercise, after nerve injury, show increases β-endorphin in the PAG and met-enkephalin in the RVM, and supraspinal blockade of opioids (intracerebroventricularly) prevents the exercise-induced analgesia.[101] On the other hand, blockade of peripheral opioid receptors has no effect on exercise-induced analgesia in animals with neuropathic pain[101]; the current study shows similar results.

The current study also showed that naloxone in either the PAG or the RVM reversed the antinociception produced by regular physical activity, and this effect was temporary. This would suggest that exercise does not prevent central sensitization that occurs after repeated acid injections, but that endogenous opioids produced by exercise decrease nociceptive sensitivity. While the PAG and RVM are involved in pain inhibition, they are also involved in pain facilitation, particularly in chronic musculoskeletal pain models.[21,102] It is therefore also possible that the increases in endogenous opioids in PAG and RVM are part of a parallel and independent pathway masking the ongoing central sensitization. Thus, increases in central opioids, within the PAG and RVM, mediate the analgesia by regular exercise through a modifiable mechanism.

Serotonin is a major neurotransmitter found in endogenous inhibitory pathways including the RVM and spinal cord and plays a significant role in analgesia. Classical studies show that increasing 5-HT in the NRM, by direct microinjection of 5-HT or

a reuptake inhibitor, produces analgesia.[40,56,57] By contrast, blockade of 5-HT2B receptors in the NRM prevents fear-induced analgesia,[19] and morphine-induced analgesia.[45,56] These data suggest that, in the RVM, increases in serotonin are analgesic, decreases in serotonin are hyperalgesic, and serotonergic analgesia involves the opioidergic system. Serotonin transporters control extracellular levels of 5-HT, thereby regulating serotonergic transmission.[51] The current study showed that increases in SERT in the RVM were observed 24 hours after induction of the chronic muscle pain model, suggesting increased removal of released serotonin. These studies are in agreement with our prior study showing decreased 5-HT, and increases in SERT, in the NRM in animals with neuropathic pain.[6] We extended these prior studies by showing that pharmacological blockade of SERT in the RVM reverses hyperalgesia in this model, providing a physiological role for SERT in the hyperalgesia that develops in chronic muscle pain. Depletion of serotonin with p-chlorophenylalanine prevents exercise-induced analgesia in healthy animals and in those that exercised after the development of with neuropathic pain,[6,62] supporting the proposal that an increase in serotonin mediates the analgesia by exercise. Indeed, both our current and prior studies[6] show decreases in SERT expression in the RVM in exercised animals, whether exercising before or after injury, suggesting regulation of SERT expression by regular exercise. It is unknown, whether the decreases in SERT are specific to animals with hyperalgesia, or whether these changes are observed in uninjured animals that exercise. Serotonin transporter surface expression is dynamically regulated by intracellular signalling pathways. Protein kinase C phosphorylation increases SERT internalization, and p-38 phosphorylation increases surface expression.[50,51,76,78] Thus, SERT expression in chronic pain conditions may be dynamically modulated by regular physical activity through the release of endogenous opioids.

Serotonin fibers in the spinal cord originate in the RVM,[8] and stimulation of the PAG or RVM increases 5-HT spinally.[34,99] The current study shows no change in SERT immunoreactivity in the spinal cord after the induction of the chronic muscle pain model, suggesting similar levels of serotonin in the chronic muscle pain model. However, physically active mice show increased SERT in the superficial dorsal horn, suggesting that there is less serotonin available in the spinal cord in exercised animals. The literature on spinal serotonin is varied, with some studies showing a role for serotonin in inhibition and others in facilitation of nociception.[28,30,32,41,84] Whether serotonin is inhibitory or excitatory in the spinal cord depends on the serotonin receptor activated, the animal model used, or the analgesic modality. Illustrating the variability, intrathecal injection of methysergide, a 5-HT2 antagonist, reduces hyperalgesia in nerve injured mice[80] and prevents analgesia by electrical stimulation of the RVM, morphine injected into the RVM or PAG, and transcutaneous electrical nerve stimulation.[2,35,41,75] Furthermore, spinal blockade of 5-HT1 receptors reverses hyperalgesia produced by inflammation[111] and prevents analgesia produced by electrical stimulation of the RVM or by joint manipulation.[23,88] Thus, future studies will need to investigate whether the increases in SERT in the spinal cord play a functional role in exercise-induced analgesia.

The chronic muscle pain model used in the current study produces bilateral hyperalgesia of the muscle and paw, visceral hyperalgesia, and anxiety-like and depressive-like behaviors.[55,65,84,93] Once it develops, hyperalgesia is maintained by central mechanisms[93] that include sensitization of spinal dorsal horn neurons manifested by bilateral expansion of receptive fields,[95] and activation of central facilitatory pathways in the PAG, RVM, and spinal cord.[17,38,87,102] The chronic muscle pain model

has a similar pharmacological profile to fibromyalgia, a centrally mediated clinical pain condition, with analgesic effects using centrally acting drugs, eg, reuptake inhibitors, but not peripherally acting drugs, eg, nonsteroidal anti-inflammatory drugs.[47,64,66,96] Thus, this animal model produces widespread pain similar to that observed in fibromyalgia that involves sensitization of central nervous system pathways.[91]

In conclusion, long-term voluntary wheel running produces analgesia through activation of endogenous inhibitory mechanisms that include opioids and serotonin, confirming several long-standing theories for how exercise produces analgesia. We propose that exercise resets the nervous system, so that subsequently painful stimuli normally perceived as painful in sedentary individuals, are not perceived as painful in active individuals. We suggest that there is a balance between inhibition and excitation in the central nervous system that favours excitation in sedentary individuals resulting in development of pain, and favours inhibition in physically active individuals preventing development of chronic pain. Exercise has the capacity to increase endogenous inhibition, which is reduced in individuals with chronic pain,[42,52,90] and thus may be a therapy that can alter central processing of nociception.

Disclosures

Acknowledgments

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