Regular physical activity reduces the percentage of spinally projecting neurons that express mu-opioid receptors from the rostral ventromedial medulla in mice

Kathleen A. Sluka*, Jessica Danielson, Lynn Rasmussen, Sandra J. Kolker

Abstract

Introduction: Regular physical activity/exercise is an effective nonpharmacological treatment for individuals with chronic pain. Central inhibitory mechanisms, involving serotonin and opioids, are critical to analgesia produced by regular physical activity. The rostral ventromedial medulla (RVM) sends projections to the spinal cord to inhibit or facilitate nociceptive neurons and plays a key role in exercise-induced analgesia.

Objective: The goal of these studies was to examine if regular physical activity modifies RVM-spinal cord circuitry.

Methods: Male and female mice received Fluoro-Gold placed on the spinal cord to identify spinally projecting neurons from the RVM and the nucleus raphe obscurus/nucleus raphe pallidus, dermorphin-488 into caudal medulla to identify mu-opioid receptors, and were immunohistochemically stained for either phosphorylated-N-methyl-D-aspartate subunit NR1 (p-NR1) to identify excitatory neurons or tryptophan hydroxylase (TPH) to identify serotonin neurons. The percentage of dermorphin-488-positive cells that stained for p-NR1 (or TPH), and the percentage of dermorphin-488-positive cells that stained for p-NR1 (or TPH) and Fluoro-Gold was calculated. Physically active animals were provided running wheels in their cages for 8 weeks and compared to sedentary animals without running wheels. Animals with chronic muscle pain, induced by 2 intramuscular injections of pH 4.0, were compared to sham controls (pH 7.2).

Results: Physically active animals had less mu-opioid-expressing neurons projecting to the spinal cord when compared to sedentary animals in the RVM, but not the nucleus raphe obscurus/nucleus raphe pallidus. No changes were observed for TPH.

Conclusions: These data suggest that regular exercise alters central facilitation so that there is less descending facilitation to result in a net increase in inhibition.

Keywords: Pain, Muscle, Exercise, Physical activity, Opioid, Serotonin

1. Introduction

Regular physical activity/exercise is an important and effective nonpharmacological treatment for individuals with chronic pain,3,8,10,28 and large population studies show that people who are physically active have a lower incidence of chronic pain.36,37,72 In parallel, prior studies show that regular physical activity prevents development of chronic muscle pain, activity-induced pain, and neuropathic pain in animal models.4,6,21,38,40,60 Central inhibitory mechanisms, involving serotonin and opioids, are critical to the analgesia produced by regular physical activity. Specifically, there are increases in endogenous opioids in the rostral ventromedial medulla (RVM) and the periaqueductal gray with regular exercise, and blockade of opioid receptors systemically or supraspinally (RVM and periaqueductal gray) reduces the analgesic effects of regular physical activity in animal models of chronic muscle pain and neuropathic pain.6,40,62 Furthermore, regular physical activity increases serotonin and decreases the serotonin transporter, whereas systemic depletion of serotonin prevents the analgesia in animal models of chronic muscle pain and neuropathic pain.5,6,40 These data show that central inhibitory pathways, including the RVM, are important components of exercise-induced analgesia.

Prior data show that there is an increase in phosphorylated-N-methyl-D-aspartate (NMDA) subunit pR1 (ser 831) (p-NR1) in the RVM and caudal medulla (nucleus raphe obscurus/nucleus raphe pallidus [NRO/NRP] in response to repeated injections of acidic saline in sedentary mice and that regular physical activity prevents development.
this increase.\textsuperscript{50} The RVM contains 3 populations of neurons: ON cells, OFF cells, and neutral cells.\textsuperscript{18} The ON cells are facilitation neurons that when activated enhance pain. Experimentally, ON cells express mu-opioid receptors (MOR), are directly inhibited by MOR agonists, and removal of MOR-expressing neurons in the RVM with dermorphin-saporin prevents the development of hyperalgesia to nerve injury.\textsuperscript{18,24,25,48,50} The RVM and caudal medulla also contain serotonergic cells, proposed to be neutral cells.\textsuperscript{51,52} However, increases in serotonin in the RVM produces analgesia, and blockade of serotonin receptors or the serotonin transporter in sedentary animals are analgesic.\textsuperscript{24,29} Furthermore, there are interactions between the serotonin and opioid systems within the RVM.\textsuperscript{25} Specific to exercise-induced analgesia, blockade of opioid receptors prevents the exercise-induced reductions in the serotonin transporter.\textsuperscript{40} Thus, ON-cells, MOR, and serotonin in the RVM are key components of endogenous inhibition.

The RVM sends projections to the spinal cord to inhibit or facilitate nociceptive neurons in the dorsal horn. Prior studies show that nearly 60\% of spinaly projecting RVM neurons respond to mu-opioid agonists, 40\% of spinally projecting neurons express the serotonin marker tryptophan hydroxylase (TPH), and a subpopulation of spinally projecting neurons expression MOR.\textsuperscript{42} However, it is unclear how exercise modulates the circuitry in the RVM. The goal of these studies was to examine the ability of regular physical activity to modify the RVM-spinal cord circuitry. We hypothesized that there would be a reduction in spinally projecting mu-opioid-expressing neurons to the spinal cord, a reduction in p-NR1 in mu-opioid-expressing neurons, and no change in TPH-projecting spinal neurons.

2. Materials and methods

All animal procedures were approved by the authors’ institution’s animal care and use committee at the University of Iowa and are in accordance with the National Institutes of Health guide for the care and use of laboratory animals. C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used for these experiments. Mice were housed with 12-hour light/dark cycle in University of Iowa husbandry with ad libitum access to food and water. For dermorphin-488 analysis, male (n = 10) and female (n = 11) C57/ Black 6 mice 6 to 8 weeks old (Jackson Laboratories) were used for these studies. Mice were anesthetized with 2\% to 4\% isoflurane and received either repeated injections of pH 4.0 saline (n = 14) or pH 7.2 saline into the gastrocnemius muscle (n = 7) and were either sedentary (n = 11) or physically active in running wheels (n = 10). A subpopulation of animals was analyzed for TPH. Male (n = 7) and female (n = 8) mice were used for these studies. Mice were anesthetized with 2\% to 4\% isoflurane and received either repeated injections of pH 4.0 saline (n = 8) or pH 7.2 saline (n = 7) in the gastrocnemius muscle and were either sedentary (n = 8) or physically active in running wheels (n = 7). Animals were randomly assigned to groups, and groups were evenly distributed across the data collection period. Two 20-\mu L injections of saline (pH 4.0 or pH 7.2) were given into the gastrocnemius muscle, 5 days apart, while the animal was anesthetized with 2\% to 4\% isoflurane.\textsuperscript{59} Previous data show a significant decrease in muscle and paw withdrawal thresholds bilaterally after 24 hours after 2 injections of pH 4.0, and that 8 weeks of running wheel activity prevents these decreases.\textsuperscript{5,59,60}

Physically active mice were housed individually with running wheels (Columbus Instruments) in their cages for 8 weeks, whereas sedentary mice were housed individually in home-cages without running wheels. Prior studies show there was no difference between locked wheels and those with no wheels in development of hyperalgesia and that those with locked wheels still develop hyperalgesia.\textsuperscript{51} Prior studies show that there were no sex differences in the analgesia.\textsuperscript{38}

2.1. Labeling of spinally projecting neurons

To label spinally projecting neurons, Fluoro-Gold was applied to the spinal cord 7 days before perfusion. Mice were anesthetized with isoflurane (2\%–4\%), and a laminectomy was performed to expose the lumbar spinal cord (L4-L6 region). A Fluoro-Gold (2\%) soaked piece of gel foam was applied to the surface of the L4-5 dorsal horn and left in place. Animals were sutured closed and allowed to recover for 2 days before intramuscular saline injections. Mice were continuously monitored for 5 days.

2.2. Labelling cells with mu-opioid receptors

Preliminary experiments in mice were unable to find a mu-opioid receptor antibody that showed staining selectivity for wild-type mice but not mu-opioid mice in the rostrocaudal medulla (RVM). We therefore directly injected dermorphin conjugated to a Hilyte-488 fluorescent tag into the RVM to bind and label cells expressing MOR. Dermorphin-488 is internalized after binding to MOR and thus represents cells that express MOR.\textsuperscript{1,41} Control experiments showed no dermorphin-488 signal in mu-opioid receptor knockout mice (Fig. 1A, B).

Dermorphin-488 was injected 24 hours after the second intramuscular saline injection, 60 minutes before perfusion. Mice were anesthetized with ketamine/xylazine (6 \mu L/g) and were placed into stereotaxic frame. Multiple injections of dermorphin-488 (0.2 mg/mL in 20\% DMSO, 0.2 \mu L) were made into the RVM, NRO, and NRP. A 33-gauge injector was attached to a piece PE10 tubing and a Hamilton syringe. The Hamilton syringe and tubing were filled with saline, then fluid was extruded from the tubing, a bubble was introduced into the tubing, and then 1 to 2 \mu L of dermorphin was drawn up to fill the tubing. Before each injection, a small amount of dermorphin was injected out of the end of the needle to ensure the tip was filled. Three injections of dermorphin-488 were injected directly in the rostrocaudal medulla 0.1 mm in-between each injection. Injections were made at IA = −5.5, −5.6, and −5.7 mm from bregma; ML 0 from ear bars; DV = −5.7 mm from surface. Mice remained anesthetized for 60 minutes before perfusion with 4\% paraformaldehyde. The experimenter performing the injection was blinded to group.

2.3. Tissue processing

Animals were deeply anesthetized (100 mg/kg sodium pento-barbital) and transcardially perfused with heparinized saline followed by 4\% paraformaldehyde 24 hours after the second saline injection. The brain was removed, the medulla blocked and embedded in optimal tissue cutting compound (OCT), cryopreserved in 30\% sucrose overnight, and then frozen at −20\(^\circ\)C until analysis. Serial sections were cut on a microscope at 20 \mu m and placed on slides. These sections included the nucleus raphe magnus (NRM), the nucleus raphe obscurus, and the nucleus raphe pallidus for each animal.

2.4. Immunohistochemistry

Sections from all animals were then immunohistochemically stained for phosphorylated-NR1 (p-NR1) according to previously published procedures.\textsuperscript{60} Sections were incubated overnight in
the primary antibody, p-NR1 (Millipore Cat# ABN99, RRID: AB_10807298, 1:500 dilution), followed the next day by 1-hour incubation with biotinylated goat anti-rabbit (Jackson ImmunoResearch Labs Cat# 111-066-144, RRID:AB_2337970, 1:200) and then 1-hour incubation in streptavidin-Alexa 648 conjugate Alexa 647-conjugate (Thermo Fisher Scientific Cat# S-21374, RRID:AB_2336066, 1:500). We previously determined that downregulation of NMDA receptors in the RVM reduces p-NR1 staining showing specificity of staining. A second set of slides was used to examine TPH staining as a marker for serotonin neurons using the following staining protocol. Tryptophan hydroxylase is an enzyme that converts l-tryptophan into the serotonin precursor L-5-hydroxytryptophan and is used as a marker of serotonin-expressing cells including RVM neurons. Sections were incubated overnight in the primary antibody, anti-TPH/Tyrosine Hydroxylase/Phenylalanine Hydroxylase (Millipore Cat# MAB5278, RRID:AB_2207684, 1:1000), followed the next day by 1-hour incubation with the secondary IgG Alexa-647 (Jackson ImmunoResearch Labs Cat# 111-066-144, RRID:AB_2338917, 1:500). Removal of the primary antibody eliminated TPH staining in the tissue. The person performing the immuno-histochemistry was blinded to group.

### 2.5. Data analysis

All images were mapped to Paxinos and Franklin Mouse atlas\textsuperscript{47} to define the region for quantification. A template for each bregma was made to outline the nuclei within the RVM and NRO/NRP to guide capturing of images to allow standardizing areas counted based on location (Fig. 2). Low-power phase images (4x; light microscope) were collected to identify location of the section on each slide and use as a landmark for taking higher-magnification images (Fig. 2). The quantification of the RVM included the NRM and the gigantocellularis pars alpha. The caudal medulla included the nucleus raphe obscurus and the nucleus raphe pallidus.

Sections with dermorphin-488 staining were imaged with confocal microscopy (Confocal Zeiss 710) for dermorphin-488 (argon laser), Fluoro-Gold (diode laser), and p-NR1 (helium laser) or TPH (helium neon laser) immunoreactivity. A 20x power was used for imaging p-NR1-stained sections, and a 10x power was used for imaging TPH-labeled sections. Images were built in ImageJ to form a composite image of all 3 filters. All cells in dermorphin-488 labeled sections were counted separately for each filter as positively or negatively labeled. All cells were counted by 1 of 2 individuals for each animal, and data were summarized for each individual animal. Both investigators were blinded to group. The 2 investigators who counted the cells were trained by the same investigator, and interrater reliability of the counts was determined to be $r > 0.9$. We then calculated the percentage of dermorphin-488-positive cells that stained for p-NR1 (or TPH), Fluoro-Gold, and for p-NR1 (or TPH) and Fluoro-Gold.

We used a 2-by-2 factorial design and tested for effects of the injury condition (pH 4, pH 7.2), the effects of exercise condition (sedentary, active), and an interaction between the injury and exercise condition using a 2-way analysis of variance. The percent of dermorphin-488-positive cells that expressed Fluoro-Gold, p-NR1, or TPH, or Fluoro-Gold+p-NR1, or TPH was analyzed and reported. For comparison, we also calculated the number of Fluoro-Gold-labeled cells that expressed either p-NR1 or TPH. Data are expressed as the mean with S.E.M. for each condition: injury or exercise condition for the rostral medulla and caudal medulla.

### 3. Results

Because ON-cells in the RVM facilitate pain and removal of ON-cells reduces hyperalgesia after nerve injury,\textsuperscript{35,50} we examined whether there were differences in ON-cells in the RVM after chronic pain and after exercise. To label ON-cells, we micro-injected the mu-opioid agonist dermorphin-488 into the NRM, NRO, and NRP in anesthetized animals. Fluorescent microscopic imaging shows strong labeling for dermorphin-488 in the RVM and the NRO/NRP around the sites of injection (Fig. 1).

Because prior studies show alterations in p-NR1 in the NRM, NRO, and NRP in a chronic muscle pain model that is modulated by physical activity,\textsuperscript{60} we immunostained the tissue for p-NR1. Physical activity was induced by provided running wheels to mice; mice averaged 6.1 ± 8.6 km/d. Nearly all dermorphin-
positive cells stained for p-NR1. To determine whether there were differences in the population of ON-cells that project to the spinal cord, we placed Fluoro-Gold in the dorsal horn of the spinal cord to retrogradely label medullary cells that project to the dorsal horn. A subpopulation of dermorphin-positive cells projected to the spinal cord.

In the p-NR1 staining groups, we counted 282 ± 43 cells per animal for dermorphin-488 and 219 ± 34 cells for Fluoro-Gold in the RVM, and an average of 60 ± 9 cells for dermorphin-488 and 54 ± 7 cells for Fluoro-Gold in the NRO/NRP. Three animals did not have labeled dermorphin-488 cells in the NRO/NRP and were excluded from analysis of the NRO/NRP (male runner pH 4.0, female runner pH 4.0, male sedentary pH 4.0). The majority of dermorphin-488-positive cells in the RVM and the NRO/NRP were positively labeled with p-NR1 (Fig. 3). A subpopulation of dermorphin-488-positive cells projected to the spinal cord from the RVM (69 ± 11%, mean ± SD) in sedentary animals. There was a significant reduction in spinally projecting cells positive for NR1 (FG⁺/p-NR1⁺). Similar to the RVM, the majority of dermorphin-488-positive cells in the NRO/NRP also stained for p-NR1 and approximately 60% of these cells projected to the spinal cord (Fig. 3). However, there was no effect of activity, chronic pain status, or an interaction between activity and chronic pain status in the NRO/NRP (Table 1).

Because RVM and the NRO/NRP are key serotonin nuclei, and increased serotonin in the RVM can reduce pain,6,40,52 we examined whether there were differences in TPH immunoreactivity in dermorphin-488-positive cells. Figure 4 shows representative images of dermorphin-488, TPH immunoreactivity, and Fluoro-Gold in the RVM. In this group, we counted an average 107 ± 10 cells positive for TPH, 166 ± 31 cells positive for dermorphin-488, and 256 ± 26 cells positive for Fluoro-Gold per animal in the RVM, and 22 ± 4 cells positive for TPH, 45 ± 10 cells positive for dermorphin-488, and an average of 36 ± 5 cells positive for Fluoro-Gold in the NRO/NRP. For the RVM, there was minimal colocalization between TPH and dermorphin-488 (≈10%), and between TPH, dermorphin-488, and Fluoro-Gold (≈5%). A greater proportion (≈20%-30%) of dermorphin-488-positive cells were labeled for TPH.
in the NRO/NRP, and ≈10% of these projected to the spinal cord (Fig. 5). There were no significant differences between conditions (activity level, chronic pain status) or an interaction between activity level and chronic pain status for TPH positive staining in the RVM (Table 2).

4. Discussion

The current study showed that physically active animals have less dermorphin-488-positive neurons, but not TPH-positive neurons, projecting to the spinal cord when compared to sedentary animals in the RVM, but not the NRP/NRO. These data are consistent with prior studies showing regular exercise produces analgesia through endogenous opioid systems in the RVM and spinal cord. Because MOR are purported to be ON-cells and facilitate nociception, these data suggest that there is less descending facilitation from the RVM in physically active mice.

Classically, the RVM is thought to modulate nociception, whereas the NRO/NRP are thought to regulate motor function; however, there is overlap of function between these 2 regions with some neurons in the NRO/NRP responding to nociceptive input and some in the RVM responding to motor input. Both nuclei express MOR and p-NR1, contain serotonergic neurons, and after expression of p-NR1 and the serotonin transporter after chronic muscle pain and exercise, and blockade of NMDA receptors in both nuclei is antinociceptive.

### Table 1

| Site                  | Activity Status      | Pain Status      | Activity*Pain Interaction |
|-----------------------|----------------------|------------------|---------------------------|
| Dermorphin+/p-NR1+    | NRM                  | F_{1,20} = 0.001, P = 0.98 | F_{1,20} = 0.48, P = 0.5   | F_{1,20} = 0.001, P = 0.24 |
| Dermorphin+/FG+       | NRM                  | F_{1,20} = 14.4, P = 0.001 | F_{1,20} = 0.05, P = 0.83   | F_{1,20} = 0.04, P = 0.84   |
| Dermorphin+/FG+/p-NR1+| NRM                  | F_{1,20} = 12.7, P = 0.002 | F_{1,20} = 0.05, P = 0.82   | F_{1,20} = 0.006, P = 0.94   |
| Dermorphin+/p-NR1+    | NRO/NRP             | F_{1,17} = 0.60, P = 0.45 | F_{1,17} = 0.93, P = 0.35   | F_{1,17} = 0.76, P = 0.4     |
| Dermorphin+/FG+       | NRO/NRP             | F_{1,17} = 0.83, P = 0.38 | F_{1,17} = 0.05, P = 0.82   | F_{1,17} = 0.83, P = 0.38   |
| Dermorphin+/FG+/p-NR1+| NRO/NRP             | F_{1,17} = 0.15, P = 0.24 | F_{1,17} = 0.05, P = 0.82   | F_{1,20} = 0.99, P = 0.33   |

Significant changes are highlighted in bold.
positive cells were labeled for TPH agreeing with prior literature. Sedentary animals, and approximately 10% of dermorphin-488-positive cells from the RVM projected to the spinal cord in NRP. The current study showed approximately 60% mu-opioid-horn to the RVM, but still showing some projections to NRO and showing the majority of Fluoro-Gold projections from the dorsal activity. If exercise released opioids at synapses primarily on spinally in the RVM reverses the analgesia produced by running wheel studies show that acute blockade of opioid receptors with naloxone (which could maintain or increase opioid receptor expression). Consistent with increased opioid tone in the RVM, prior expression to decrease the number of functional opioid receptors in structural change in the connections. Functionally, exercise could increase the opioid tone in the RVM altering mu-opioid receptor expression to decrease the number of functional opioid receptors in neurons. Consistent with increased opioid tone in the RVM, prior studies show that acute blockade of opioid receptors with naloxone in the RVM reverses the analgesia produced by running wheel activity. If exercise released opioids at synapses primarily on spinally projecting neurons, a lower number of cells would internalize dermorphin-488, and thus be reflected as a reduction in spinally projecting MOR. It should be noted that once internalized, a receptor can be tagged for either degradation (which would decrease opioid receptor expression) or for reinsertion to the plasma membrane (which could maintain or increase opioid receptor expression).

It is also possible that physical activity changes the phenotype of RVM neurons, so there are less ON-cells. A change in phenotype in the RVM is supported by prior literature, which showed that neutral cells adopted both ON-cell and OFF-cell phenotypes after inflammation resulting in increases in both ON-cells and OFF-cells. Exercise can alter neuron phenotype as evidenced by a prior study showing fast, but not slow or intermediate, motor neurons increase expression of glutamate after exercise training. Alternatively, exercise could enhance synaptogenesis of OFF-cell projections in the spinal cord, purported to inhibit nociception, which would result in a lower proportion of ON-cells. In support, there is a substantial body of literature showing cell proliferation, increased synaptic densities, and synaptogenesis in the hippocampus after exercise. For example, in the hippocampus, blockade of MOR reduces exercise-induced synaptogenesis, suggesting endogenous opioids can promote synaptogenesis. This is in contrast to exogenous opioids, such as morphine, where chronic administration reduces synaptogenesis in the hippocampus. It is unclear if the effects of opioids on synaptogenesis extend to endogenous opioids in the spinal cord and antinociceptive pathways. Previous studies show there are increases in p-NR1 in sedentary animals after muscle insult in the RVM and NRO/NRP that did not occur in physically active animals, however, in the current study, there were no changes in expression of p-NR1 in the mu-opioid-expressing cells. In fact, the majority of mu-opioid receptor-expressing cells also expressed p-NR1 regardless of conditions. If there were decreases in the overall expression of mu-opioid receptor-

### Table 2

| Site                  | Activity Status | Pain Status | Activity*Pain Interaction |
|-----------------------|-----------------|-------------|---------------------------|
| Dermorphin+/TPH+      | NRM             | F1,14 = 1.0, P = 0.33 | F1,14 = 0.01, P = 0.91, F1,13 = 0.39, P = 0.54 |
| Dermorphin+/FG+ +TPH+ | NRM             | F1,14 = 3.9, P = 0.08 | F1,14 = 0.76, P = 0.4, F1,13 = 0.14, P = 0.71 |
| Dermorphin+/TPH+      | NRO/NRP         | F1,14 = 0.11, P = 0.74 | F1,14 = 0.11, P = 0.74, F1,14 = 0.001, P = 0.97 |
| Dermorphin+/FG+ +TPH+ | NRO/NRP         | F1,14 = 0.12, P = 0.73 | F1,14 = 0.12, P = 0.73, F1,13 = 0.31, P = 0.79 |

TPH, tryptophan hydroxylase.
expressing cells with exercise, the p-NR1 decreases would not be detectable, consistent with the current data.

The RVM both facilitates and inhibits noiceptive information through descending input to the spinal cord. ON-cells facilitate pain and increase their firing in response to noxious stimuli, whereas OFF-cells inhibit pain and decrease their firing in response to noxious stimuli. There is generally a balance between inhibition and excitation. Peripheral injury can shift the balance such that ON-cell activity outweighs OFF cell activity after tissue injury.7,34,35,71 Further evidence shows that removal of ON-cells in the RVM with a dermorphin-saporin conjugate reduces hyperalgesia after nerve injury and in visceral pain, reduces MOR protein and mRNA, and DAMGO analgesia. 12,50,56,73 These data support the notion that ON-cells facilitation hyperalgesia.

Tryptophan hydroxylase-expressing neurons are reported to be neutral cells, 51,52 but spinal serotonin can either facilitate or inhibit nociception.46 There is substantial evidence suggests that supraspinal serotonergic input to the spinal cord has facilitatory effects on spinal neurons after tissue injury, primarily through its action on 5-HT3 receptors.23,45,56,63 However, electrical stimulation of the RVM produces analgesia, releases serotonin, and 5-HT-induced nociception is blocked by 5-HT1 antagonists.31,33,55,61 Furthermore, endogenous analgesia produced by transcutaneous electrical nerve stimulation or joint mobilization is prevented by spinal blockade of 5-HT1 or 5-HT2 receptor antagonists.54,57 It has also previously been shown that there are increases in expression of the serotonin transporter in the RVM and NRO/NRP after muscle insult6,40; however, the current study does not show changes in TPH expression after muscle insult or regular exercise. Prior studies, using electrophysiology, show a functional change in phenotype of RVM neurons. Neutral cells, thought to be TPH+ cells, developed ON-cell or OFF-cell-like properties after peripheral inflammation,44 whereas ON-cells and OFF-cells develop new responsiveness to innocuous mechanical stimuli after nerve injury.15 The current study, using an anatomical approach, suggests that muscle insult does not change phenotype of ON-cells (MOR+) or neutral-cells (TPH+), despite playing a significant role in the development and maintenance of chronic muscle pain.15,16,65

There are several limitations with the current study. We did not use stereological analysis of labeled cells, but rather counted profiles. We recognize counting profiles could result in an overrepresentation of larger cells and underrepresentation of smaller cells.14 Another limitation is our inability to determine whether the number of MOR were changed in these sections because we used a direct injection of dermorphin-488 to label mu-opioid-positive neurons, and thus were not able to accurately label all cells within a single session due to the variability in the injection and diffusion of the dermorphin-488. This was necessary because we were unable to obtain a specific antibody that showed adequate staining in a wild-type mouse compared to a mu-opioid receptor mouse. In 3 animals, we were did not find labeling of MOR+ cells in the NRO/NRP, which reduced the number of animals per group. It is likely in these animals, injections of dermorphin-488 did not localize to the NRO/NRP.

In summary, we show that regular exercise reduces the number of putative ON-cells cells that project to the spinal cord. These data suggest that regular exercise alters central facilitation.
so that there is less descending facilitation to result in a net increase in inhibition. This change in the balance between inhibition and facilitation could explain why regular exercise is protective against the development chronic pain.

Disclosures
The authors have no conflicts of interest to declare.

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References
[1] Arttamangkul S, Alvarez-Maubecin V, Thomas G, Williams JT, Grandy DK. Binding and internalization of fluorescent opioid peptide conjugates in living cells. Mol Pharmacol 2000;58:1570–80.
[2] Bertuzzi M, Chang W, Ampatzis K. Adult spinal motoneurons change their neurotransmitter phenotype to control locomotion. Proc Natl Acad Sci USA 2018;115:E9926–33.
[3] Bidonde J, Busch AJ, Bath B, Mlosojlevic S. Exercise for adults with fibromyalgia: an umbrella systematic review with synthesis of best evidence. Curr Rheumatol Rev 2014;10:45–79.
[4] Bobinski F, Teixeira JM, Sluka KA, Santos ARS. Interleukin-4 mediates opioid pain after sciatic nerve injury in mice. PAIN 2015;156:2595–606.
[5] Bobinski F, Teixeira JM, Sluka KA, Santos ARS. Interleukin-4 mediates the analgesia produced by low-intensity exercise in mice with neuropathic pain. PAIN 2018;159:437–50.
[6] Brito RG, Rasmussen LA, Sluka KA. Regular physical activity prevents development of chronic muscle pain through modulation of supraspinal opioid and serotonergic mechanisms. Pain Rep 2017;2:e2618.
[7] Budai D, Khasabov SG, Mantyh PW, Simone DA. NK-1 receptors to GABAergic neurons in the central nervous system, including the brainstem, raphe nuclei, and substantia gelatinosa from the rostral ventromedial medulla revealed by antinociceptive brainstem circuits. J Comp Neurol 1998;392:528–47.
[8] Busch AJ, Barber KA, Overend TJ, Peloso PM, Schachter CL. Exercise for adults with fibromyalgia: an umbrella systematic review with synthesis of best evidence. Curr Rheumatol Rev 2014;10:45–79.
[9] Busch AJ, Webber SC, Brachaniec M, Bidonde J, Bello-Haas VL, Danyliv AD, Overend TJ, Richards RS, Sawant A, Schachter CL. Exercise therapy for fibromyalgia. Curr Pain Headache Rep 2011;15:358–67.
[10] Busch AJ, Webber SC, Richards RS, Bidonde J, Schachter CL, Schafer LA, Danyliv A, Sawant A, Dal Bello-Haas V, Rader T, Overend TJ. Resistance exercise training for fibromyalgia. Cochrane Database Syst Rev 2013;12:CD010884.
[11] Cai YG, Wang W, Hou YY, Pan ZZ. Optogenetic activation of brainstem serotonergic neurons induces persistent pain sensitization. Mol Pain 2014;10:70.
[12] Cao F, Chen SS, Yan XF, Xiao XP, Liu XL, Yang SB, Xu AJ, Gao F, Yang H, Chen ZJ, Tian YK. Evaluation of side effects through selective ablation of the mu opioid receptor expressing descending nociceptive facilitatory neurons in the rostral ventromedial medulla with dermorphin-saporin. Neurotoxology 2009;30:1096–106.
[13] Carlson JD, Maurice JJ, Martensson ME, Heinricher MM. Sensitization of pain-modulating neurons in the rostral ventromedial medulla after peripheral nerve injury. J Neurosci 2007;27:13222–31.
[14] Coggleshall RE, Lekan HA. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. J Comp Neurol 1996;364:6–15.
[15] da Silva LFS, DeSantana JM, Sluka KA. Activation of NMDA receptors in the brainstem, rostral ventromedial medulla, and nucleus reticularis gigantocellularis mediates mechanical hyperalgesia produced by repeated intramuscular injections of acidic saline in rats. PAIN 2010;11:378–87.
[16] da Silva LFS, Waldner RY, Davidson BL, Wilson SP, Sluka KA. Changes in expression of NMDA-NR1 receptor subunits in the rostral ventromedial medulla modulates pain behaviors. PAIN 2010;151:155–61.
[17] Eisich A, Barrot M, Schad CA, Self DW, Nestler EJ. Opiates inhibit neurogenesis in the adult rat hippocampus. Proc Natl Acad Sci U S A 2000;97:7579–84.
[18] Fields HL, Basbaum AI, Heinricher MM. Central nervous system mechanisms of pain modulation. In: McMahon SB, Koltzenburg M, editors. Textbook of pain. Philadelphia: Elsevier; 2006. p. 125–142.
[19] Francisco A, Low SA, Sypek EJ, Christensen AJ, Sotoudeh C, Beier KT, Ramakrishnan C, Ritola KD, Shant-Naeini R, Deisseroth K, Deil SP, Malenka RC, Luo L, Hartman AW, Scherrer G. A brainstem-spinal cord inhibitory circuit for mechanical pain modulation by GABA and enkephalin. Neuron 2017;93:822–39 e826.
[20] Goodchild CS, Guo Z, Freeman J, Gent JP, 5-HT spinal antinociception involves mu opioid receptors: cross tolerance and antagonism studies. Br J Anaesth 1997;78:563–9.
[21] Grace PM, Fabisiak TJ, Green-Fulghum SM, Anderson ND, Strand KA, Kwilasz AJ, Galler EL, Walker FR, Greenwood BN, Maier SF, Flesher M, Watkins LR. Prior voluntary wheel running attenuates neuropathic pain. PAIN 2016;157:2012–23.
[22] Gregory NS, Gibson-Corley K, Frey-Law L, Sluka KA. Fatigue-enhanced hyperalgesia in response to muscle insult: induction and development occur in a sex-dependent manner. PAIN 2013;154:2668–76.
[23] Guo W, Miyoshi K, Dubner R, Gu M, Li M, Liu J, Yang J, Zou S, Ren K, Noguchi K, Wei F. Spinal 5-H13 receptors mediate descending facilitation and contribute to behavioral hypersensitivity via a reciprocal neuron-glial signaling cascade. Mol Pain 2014;10:33.
[24] Hammond DL, Nelson V, Thomas DA. Intrathecal methysgeride antagonizes the antinociception, but not the hyperalgesia produced by microinjection of baclofen in the ventromedial medulla of the rat. Neurosci Lett 1998;244:93–6.
[25] Heinricher MM, Fields HL, Central nervous system mechanisms of pain modulation. In: McMahon SB, Koltzenburg M, Tracey I, Turk DC, editors. Melzack and Wall’s textbook of pain. Philadelphia: Elsevier, 2013. p. 129–142.
[26] Heinricher MM, Morgan MM, Fields HL. Direct and indirect actions of morphine on medial neurons that modulate nociception. Neurosci 1992;46:533–49.
[27] Heinricher MM, Morgan MM, Tortorici V, Fields HL. Disinhibition of off-cells and antinociception produced by an opioid action within the rostral ventromedial medulla. Neurosci 1994;63:279–88.
[28] Høegger Berment MK, Sluka KA. Exercise-induced hypalgesia: an Evidence-based review. In: Sluka KA, editor. Pain mechanisms and management for the physical Therapist. Philadelphia: Wolters Kluwer, 2016. p. 177–202.
[29] Inasee M, Nakahama H, Otsuki T, Fang JZ. Analgesic effects of serotonin microinjection into nucleus raphe magnus and nucleus raphe dorsalis evaluated by the monosodium urate (MSU) tonic pain model in the rat. Brain Res 1987;244:205–11.
[30] Jones SL, Light AR. Serotonergic medullary raphespinal projection to the lumbar spinal cord: an anatomicoimmunohistochemical study. J Comp Neurol 1990;297:267–82.
[31] Jones SL, Light AR. Serotonergic medullary raphespinal projection to the lumbar spinal cord in the rat: a retrograde immunohistochemical study. J Comp Neurol 1992;322:599–610.
[32] Kaluzhyne AE, Wessendorf MW. Relationship of mu- and delta-opioid receptors to GABAergic neurons in the central nervous system, including antinociceptive brainstem circuits. J Comp Neurol 1998;392:528–47.
[33] Kato G, Yasaka T, Kato T, Funke H, Mizuno M, Iwamoto Y, Yamamura M. Direct GABAergic and glycineergic inhibition of the substantia gelatinosa from the rostral ventromedial medulla revealed by in vivo patch-clamp analysis in rats. J Neurosci 2006;26:1787–94.
[34] Khasabov SG, Brink TS, Schupp M, Noack J, Simone DA. Changes in response properties of rostral ventromedial medulla neurons during prolonged inflammation: modulation by neurokinin-1 receptors. Neuroscience 2012;224:225–48.
[35] Kincaid W, Neubert MJ, Xu M, Kim CJ, Heinricher MM. Role for medullary pain facilitating neurons in secondary thermal hyperalgesia. J Neurophysiol 2006;95:33–41.
[36] Landmark T, Romundstad P, Borchgrevink PC, Kaasa S, Dale O. Associations between recreational exercise and chronic pain in the general population: evidence from the HUNT 3 study. PAIN 2011;152:258–67.
[37] Landmark T, Romundstad PR, Borchgrevink PC, Kaasa S, Dale O. Longitudinal associations between exercise and pain in the general population—the HUNT pain study. PLoS One 2013;8:e65279.
Leung A, Gregory NS, Allen LA, Sluka KA. Regular physical activity prevents chronic pain at altering resident muscle macrophage phenotype and increasing IL-10 in mice. PAIN 2016;157:79.

Liang H, Wang S, Francis R, Whan R, Watson C, Paxinos G. Distribution of raphespinal fibers in the mouse spinal cord. Mol Pain 2015;11:42.

Lima LV, DeSantana JM, Rasmussen LA, Sluka KA. Short-duration physical activity prevents the development of activity-induced hyperalgesia through opioid and serotoninergic mechanisms. PAIN 2017;158:1697–710.

Macey TA, Ingram SL, Bobeck EN, Hegarty DM, Aicher SA, Attarmangkul S, Morgan MM. Opioid receptor internalization contributes to demorphin-mediated antinociception. Neuroscience 2010;168:543–50.

Martelli S, Vaughan CW, Schnell SA, Wessendorf MW, Christie MJ. Rostral ventromedial medulla neurons that project to the spinal cord express multiple opioid receptor phenotypes. J Neurosci 2002;22:10847–55.

Mason P. Contributions of the medullary raphe and ventromedial reticular region to pain modulation and other homeostatic functions. Annu Rev Neurosci 2001;24:737–77.

Miki K, Zhou QQ, Guo W, Guan Y, Terayama R, Dubner R, Ren K. Changes in gene expression and neuronal phenotype in brain stem pain modulatory circuitry after inflammation. J Neurophysiol 2002;87:750–60.

Miranda A, Peles S, McLean PG, Sengupta JN. Effects of the 5-HT3 receptor antagonist, alosetron, in a rat model of somatic and visceral hyperalgesia. PAIN 2006;126:54–63.

Ossipov MH, Morimura K, Porreca F. Descending pain modulation and chronification of pain. Curr Opin Support Palliat Care 2014;8:143–51.

Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates. Academic Press, 2001.

Persson AI, Naylor AS, Jonsdottir IH, Nyberg F, Eriksson PS, Thorlin T. Differential regulation of hippocampal progenitor proliferation by opioid receptor antagonists in running and non-running spontaneously hypertensive rats. Eur J Neurosci 2004;19:1847–55.

Pierce TL, Wessendorf MW. Immunocytochemical mapping of endomorphin-2-immunoreactivity in rat brain. J Chem Neuroanat 2000;18:181–207.

Porreca F, Burgess SE, Gardell LR, Vanderah TW, Malan TP, Ossipov MH, Lappi DA, Lai J. Inhibition of neuropathic pain by selective ablation of brainstem medullary cells expressing the mu-opioid receptor. J Neurosci 2001;21:5281–8.

Potrebic SB, Fields HL, Mason P. Serotonin immunoreactivity is contained in one physiological cell class in the rat rostral ventromedial medulla. J Neurosci 1994;14:1655–65.

Potrebic SB, Mason P, Fields HL. The density and distribution of serotoninergic appositions onto identified neurons in the rat rostral ventromedial medulla. J Neurosci 1995;15:3273–83.

Prouty EW, Chandler DJ, Waterhouse BD. Neurochemical differences between target-specific populations of rat dorsal raphe projection neurons. Brain Res 2017;1675:28–40.

Radhakrishnan R, Sluka KA. Spinal muscarinic receptors are activated during low or high frequency TENS-induced antihyperalgesia in rats. Neuropharmacology 2003;45:1111–19.

Shnauss C, Hammond DL, Ochi JW, Yaksh TL. Pharmacological effects of the antiinociceptive effects of serotonin in the rat spinal cord. Eur J Pharmacol 1983;90:349–57.

Sikandar S, Bannister K, Dickinson AH. Brainstem facilitations and descending serotonergic controls contribute to visceral nociception but not pregabalin analgesia in rats. Neurosci Lett 2012;519:31–6.

Skyba DA, Radhakrishnan R, Rohliwig JJ, Wright A, Sluka KA. Joint manipulation reduces hyperalgesia by activation of monoamine receptors but not opioid or GABA receptors in the spinal cord. PAIN 2003;106:199–208.

Sluka KA, Danielson J, Rasmussen L, Dasilva LF. Exercise-induced pain requires NMDA receptor activation in the medullary raphe nuclei. Med Sci Sports Exerc 2012;44:420–7.

Sluka KA, Kalra A, Moore SA. Unilateral intramuscular injections of acidic saline produce a bilateral, long-lasting hyperalgesia. Muscle Nerve 2001;24:37–46.

Sluka KA, O’Donnell JM, Danielson J, Rasmussen LA. Regular physical activity prevents development of chronic pain and activation of central neurons. J Appl Physiol 2013;114:725–33.

Sorkin LS, Mcadoo DJ, Willis WD. Raphe magnus stimulation induced antinociception in the cat is associated with release of amino acids as well as serotonin in the lumbar dorsal horn. Brain Res 1993;618:95–106.

Stagg NJ, Mata HP, Ibrahim MM, Henrikson EL, Porreca F, Vanderah TW, Philip MT Jr. Regular exercise reverses sensory hypersensitivity in a rat neuropathic pain model: role of endogenous opioids. Anesthesiology 2011;114:940–8.

Suzuki R, Dickinson A. Spinal and supraspinal contributions to central sensitization in peripheral neuropathy. Neurosignals 2005;14:175–81.

Terayama R, Dubner R, Ren K. The roles of NMDA receptor activation and nucleus reticularis gigantocellularis in the time-dependent changes in descending inhibition after inflammation. PAIN 2002;97:171–81.

Tillu DV, Gehbart GF, Sluka KA. Descending facilitatory pathways from the RVM initiate and maintain bilateral hyperalgesia after muscle insult. PAIN 2006;136:321–9.

van Praag H, Mata RP, Djunaidy NT, Serrano-Pepin L, Christie MJ, Shors TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A 1999;96:13427–31.

van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 1999;2:266–70.

van Zastrow M. Regulation of opioid receptors by endocytic membrane traffic: mechanisms and translational implications. Drug Alcohol Depend 2010;108:166–71.

Wasinski F, Batista AO, Bader M, Araujo RC, Klump P, Bradykynin B2 receptor is essential to running-induced cell proliferation in the adult mouse hippocampus. Brain Struct Funct 2018;223:5901–7.

Wei H, Pertoavaara A, MK-801, an NMDA receptor antagonist, in the rostroventromedial medulla attenuates development of neuropathic symptoms in the rat. Neuroreport 1999;10:2933–7.

Xu M, Kim CJ, Neubert MJ, Heinricher MM. NMDA receptor-mediated activation of medullary pro-nociceptive neurons is required for secondary thermal hyperalgesia. PAIN 2007;127:253–62.

Zhang R, Chomistek AK, Dimitrakoff JD, Giovannucci EL, Willett WC, Meigs JB,rewrite R, Zhao L, Buring JE, Hu FB, Manson JE, Jacobs EE, Jacobs Jr HH, Hsia J, Sampson L, Land YM. Exercise is associated with lower risk of type 2 diabetes in Chinese American women: the China Women’s Health Study. Am J Epidemiol 2014;180:418–29.

Zhang W, Gardell S, Zhang D, Xie XJ, Agnes RS, Badghisi H, Hruby VJ, Rance N, Ossipov MH, Vanderah TW, Porreca F, Lai J. Neuroactive pain is maintained by brainstem neurons co-expressing opioid and cholecystokinin receptors. Brain 2009;132:775–87.