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IL1 β inhibition contributes to the anti-nociceptive effects of voluntary exercise on ischemia/reperfusion-induced hypersensitivity

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Abstract

Issues of peripheral circulation have been increasingly suggested as an underlying cause of musculoskeletal pain in many conditions, including sickle cell anemia and peripheral vascular disease. We have previously shown in our model of transient ischemia and reperfusion injury (I/R) of the forelimb that individual group III and IV muscle afferents display altered chemosensitivity and mechanical thresholds 1 d following injury. Functional alterations corresponded to increased evoked and spontaneous pain-related behaviors and decreased muscle strength and voluntary activity—all actions that echo clinical symptoms of ischemic myalgia. These behavioral and physiological changes appeared to originate in part from the action of increased interleukin 1 β (IL1 β) in the injured muscles at its upregulated interleukin 1 receptor (IL1r1) within the DRGs. Here we describe that two days of voluntary wheel running prior to I/R blocks both injury-induced IL1 β enhancement and the subsequent development of ischemic myalgia-like behaviors. Furthermore, the protective effects of 2d prior exercise on the I/R-evoked increases in pain-related behaviors were also paralleled with systemic injection of the IL1 receptor antagonist (IL1RA) during I/R. IL1RA treatment additionally prevented the I/R-induced changes in mechanical and chemical sensitivity of individual primary muscle afferents. Altogether, these data strengthen the evidence that transient ischemia and reperfusion injury sensitizes group III and IV muscle afferents via increased IL1 β in the muscles to stimulate ischemic myalgia development. Targeting IL1 β may therefore be an effective treatment strategy for this insidious type of muscle pain.

1. Introduction

Musculoskeletal pain is a widely experienced clinical issue, yet many underlying mechanisms have yet to be deciphered [10,48]. A variety of conditions can result in myalgia, and finding an effective treatment strategy for pain management may depend on the

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Conflict of interest statement

The authors declare that there is no conflict of interest.

underlying cause [10]. Exercise is commonly recommended for these patient populations, but recent clinical and animal studies have shown mixed results [3,6,10,38,41,51,54,63]. In some myalgia-generating conditions, robust exercise interventions may even be detrimental [3,43,44]. Thus determining the appropriate exercise or treatment regimen is critical.

The mechanism through which exercise modulates muscle pain may be due to altered cytokine signaling in the periphery [3,21,41]. Exercise is known to increase muscle cytokines, such as the interleukins (ILs) IL-6, IL-10, and IL1 β [7,53,56]. While many pro-inflammatory mediators may provoke peripheral sensitization, the exercise-induced upregulation of both pro- and anti-inflammatory cytokines may not always result in exercise-induced pain in healthy subjects. However, this may not be the case for patients with existing muscle pain conditions, such as ischemic myalgia, which may occur in diseases including sickle cell anemia and peripheral vascular disease [1,48,71,81].

During ischemic conditions, the affected muscle tissue is infiltrated by immune cells [47,61] that release inflammatory cytokines and growth factors to stimulate repair [8,47], similar to those that are modulated during exercise. In our mouse model of transient ischemia and reperfusion (I/R) injury, PCR array analysis of the affected muscle tissue showed significant increases in IL1 β and glial derived neurotrophic factor (GDNF), but few other changes in cytokine and/or growth factor expression [62]. I/R also stimulated the upregulation of interleukin-1 receptor 1 (IL1r1), the receptor for IL1 β , in the affected dorsal root ganglia (DRGs) [61,62]. Furthermore, I/R-induced changes in pain-related behaviors have mimicked common clinical features of ischemic myalgia [1,42,48,81], such as increased guarding, local mechanical hypersensitivity, decreased muscle strength, and decreased voluntary activity [61,62]. Afferent-specific inhibition of IL1 β /IL1r1 signaling prevented the development of myalgia-like behaviors after I/R [61].

Enhanced IL1 β /IL1r1 signaling also modulated the I/R-induced sensitization of individual group III and IV primary afferents, likely through stimulating the *de novo* upregulation of acid-sensing ion channel 3 (ASIC3) [46,61]. ASIC3 is a known contributor to mechanical [64,65,75] and chemical [5,24,52] sensation in nociceptive muscle afferents, especially in the case of muscle ischemia [24,25,49,52] as well as in many other muscle pain models [19,20,74,76,78–80]. With *ex vivo* muscle/nerve/DRG/spinal cord electrophysiology [31] of individual muscle afferents, we previously found that afferent chemo- and mechano-sensory function, as well as response phenotypes, are significantly altered within one day following I/R [61,62].

Clinical studies have shown that IL1r1-targeting drugs designed to reduce inflammation, such as the IL1 receptor antagonist (IL1RA), can also provide pain relief [22], while other preclinical reports have implicated exercise as a potential regulator of IL1 β in neuropathic pain models [9,21]. Based on these findings, we sought to determine how systemic IL1RA treatment and/or various exercise regimens could modulate the observed behavioral and/or electrophysiological effects of I/R.

2. Methods

2.1. Animals

Male Swiss Webster mice (Charles River) between 4–8 weeks of age were used for all experiments. Animals were housed in a pathogen-free, climate-controlled barrier facility in Cincinnati Children's Hospital Medical Center (CCHMC) under the supervision of institutional Veterinary Services. Mice were kept on a 12-hr light/dark cycle and allowed access to standard rodent chow and water *ad libitum*. For all injections and surgical procedures, mice were anesthetized with 2–3% isoflurane, and for terminal procedures mice were deeply anesthetized with ketamine (90mg/kg) and xylazine (10 mg/kg). No additional analgesics were administered in accordance with approved animal use protocols. All procedures were approved and monitored by the CCHMC Institutional Care and Use Committee and were compliant with NIH and AALAC standards.

2.2. Ischemia/Reperfusion Surgery

Ischemia and reperfusion (I/R) surgeries were performed as previously described [61,62] immediately following baseline behavior (BL) or 24 hours prior to sacrifice for specific *ex vivo* or molecular analyses as outlined below. Briefly, the right forelimb of an anesthetized mouse was incised and the brachial artery exposed. A 7-0 silk suture was tied around the brachial artery and the incision closed. After animals were allowed to freely recover in their home cages for 6 hours, the suture was surgically removed and forepaw allowed to reperfuse for 18 hours before any further analysis. In some experiments, a sham surgical procedure was utilized wherein a suture was placed behind the brachial artery, but not tied. As in our previous studies [61,62], data from naïve and sham animals did not differ in any assay (Table S1), and have thus been combined for ease of presentation where indicated.

2.3. Drugs

For systemic pharmacological blockade of IL1 β /IL1r1, based on previous research [6] and dose-response verification (not shown), anesthetized mice were injected intraperitoneally with 3 mg/kg interleukin-1 receptor antagonist (mouse IL1RA, ProSpec) both immediately prior to occlusion surgery and again following reperfusion. Comparison groups received I/R surgery and vehicle (H₂O), and separate control groups received IL1RA or vehicle injections alone at the same intervals. Data from naïve, sham, and injection only conditions were not statistically different in any assay (Tables S1, S2); therefore, data from these conditions was combined for ease of presentation where indicated.

2.4. RNA Isolation, Reverse Transcription, and Real-Time PCR

Analysis of relative mRNA expression was performed with quantitative real-time polymerase chain reaction (qPCR) as previously described [34,45,62]. After transcardial perfusion with a 1:1 solution of sterile 0.9% NaCl : RNAlater (Ambion), the ipsilateral forepaw muscles from age-matched naïve and I/R mice with or without 2d prior voluntary exercise exposure were excised and flash frozen in liquid nitrogen. Following tissue homogenization, RNA was isolated from muscle samples using a Qiagen RNeasy fibrous tissues kit according to the manufacturer's protocol. Concentrations of isolated RNA

samples were measured, then DNase I and Superscript II reverse transcriptase treated (Invitrogen). 20 µg of cDNA was plated in duplicate reactions with SYBR Green reagents and analyzed on a Step-One real-time PCR machine (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward and reverse primer sequences obtained from Elitt et al [14]) was used as an internal control to determine cycle time (Ct) values for all targets. Ct values for each gene were calculated by subtracting the normalized target gene Cts from those of naïve controls. 2^{-Ct} was used to determine fold change after injury and/or exercise (Applied Biosystems), which were then converted to percent change where 2-fold = 100% for ease in reporting. Primer sequences for IL1 β and GDNF [62], NGF and artemin [29], and IL-6 and tumor necrosis factor α (TNF α) [45] have been reported previously.

2.5. Behavioral Analyses

All behavioral analyses were performed during morning light hours by blinded experimenters as previously described [58,61,62]. Assessments of spontaneous pain-like guarding behaviors, as well as evoked measurements of mechanical hypersensitivity (von Frey paw withdrawal) and muscle function (grip strength), were performed immediately prior to occlusion surgery (baseline, BL), 24 hours following occlusion (18 hours post reperfusion, D1), and 72 hours after the initial occlusion surgery (D3). Briefly, mice were habituated for 30 minutes in a raised acrylic chamber with a steel mesh bottom prior to assessments of forepaw guarding. Every 5 minutes for 1 hour, each mouse was given a score of 0–2 for each forepaw, where 0 = mouse firmly places paw on mesh, 1 = mouse paw is touching mesh but is not bearing full weight, and 2 = mouse holds paw completely above mesh. The average score for all 12 trials on each behavior day was used for analysis [61,62,77]. Then, to assess mechanical hypersensitivity, an increasing series of calibrated von Frey filaments (0.07–6g) were used to stimulate the plantar surface of the forepaw through the mesh of the behavioral chamber. Threshold to withdrawal was recorded for each forepaw 3 times with 5 minutes between each round, and median values were used for analysis [58,61,62]. Mice were then placed back in their home cages and individually assayed for forepaw grip strength (in g) using a grip strength meter (BioSeb). The average score of 9 trials (3 rounds of 3 with 5 min between rounds), was used for analysis [58,61,62].

2.5.1. Voluntary Wheel Running—For experimental paradigms including voluntary wheel running, mice were individually housed with standard food, water, and bedding in chambers accommodating in-cage activity wheels (Lafayette Instruments). Velocity and distance traveled were recorded by an automated wheel monitoring system (Lafayette Instruments) in hourly bins [61]. Select exercise exposed mice were placed in these cages two days prior to occlusion surgery (averaging 4197 $\pm$ 375 meters/day across groups), whereas all other wheel-exposed groups were habituated for only 15–30 minutes preceding the occlusion. Following the initial surgery, mice were housed individually in these cages for the remainder of the study, and removed only for reperfusion surgery or behavioral assays at D1 and/or D3.

2.5.2. Forced Running—Following the above BL behavioral assessments, I/R surgeries were performed. Immediately following reperfusion surgery, both I/R mice and naïve littermates were placed on a 6-lane treadmill (Columbus Instruments) at a 0° incline. After habituation to the apparatus, the speed was slowly increased from 9 to 13 m/min at 1 m/min intervals, and then maintained at 13 m/min until mice had reached a total distance of 500 m traveled in 45 min [58,70]. 24 hours following I/R surgery (D1), guarding, von Frey paw withdrawal, and grip strength assays were repeated. We have previously shown that this particular running protocol alone is not sufficient to induce pain-like behaviors in mice [58].

2.5.3. Saccharin Preference Task—In order to assess the effects of our injury and/or interventions on motivation, individually housed mice were provided two bottles, one filled with normal sterile drinking water, and the second containing a 0.1% saccharin solution [23,66,73]. During the week prior to I/R surgery, mice were exposed O/N (16 hrs) to the two bottles within their home cages on two randomly chosen nights to become acclimated to saccharin availability. The bottle positions were swapped between training sessions to reduce any confound produced by side bias, and the second session was used as a BL measurement. After completing these sessions, mice underwent I/R surgeries with or without IL1RA treatments, and then were returned to their home cage where they again had the option of drinking either the saccharin solution or standard water for 16 hours (D1). Bottles were weighed before and after each exposure night, and saccharin preference was calculated as a percentage of the volume of saccharin intake over the total volume of fluid intake [23].

2.6. Protein Isolation and Western Blotting

For each protein sample, the ipsilateral forepaw muscles of a single mouse that was intracardially perfused with an ice cold 0.9% saline solution were excised and prepared as previously described [29,30,32–35,61]. Samples were homogenized in protein lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mM pH 7.4 Tris-HCl, and protease inhibitors (pepstatin, leupeptin, and aprotinin at 1 µg/ml each, 1 mM sodium orthovanadate, and 100 µg/ml phenylmethylsulfonyl fluoride), Sigma-Aldrich], and concentrations measured by spectroscopy using the DC protein assay (BioRad). Immediately prior to electrophoresis on a 12% SDS-polyacrylamide gel, 30 µg of each sample was boiled in denaturing buffer containing SDS and β-mercaptoethanol. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) overnight at 4°C. Membranes were blocked for 60 minutes in 1:1 diluted LiCor Odyssey blocking buffer in 0.01 M PB, and treated with primary antibodies for IL1β (Goat α-IL1β, 1:2000, R&D Systems) and GAPDH (Chicken α-GAPDH, 1:2000, ProSci) in Odyssey blocking buffer containing 0.2% Tween-20 at 4°C overnight. After washing, membranes were treated with 1:1 Odyssey blocking buffer:0.01 M PB containing secondary antibodies (below), 0.2% Tween-20, and 0.01% SDS. A LiCor Odyssey Imaging System and Image Studio v3.1 software (LiCor) were used to detect secondary antibody (LiCor) binding of infrared dye-conjugated donkey anti-goat (1:15,000) at 800 nm and donkey anti-chicken at 680 nm (1:20,000) with consistent settings between runs. Immunoreactive bands were analyzed by densitometry using NIH Image J software and optical density quantified as fold change normalized to GAPDH (mean ± SEM).

2.7. Ex Vivo Recording

Electrophysiology was performed as previously described using our *ex vivo* forepaw muscles/median and ulnar nerves/DRG/spinal cord recording preparation [31,58,61,62]. Briefly, mice were deeply anesthetized with ketamine/xylazine (see above) and intracardially perfused with ice cold oxygenated artificial cerebrospinal fluid (ACSF), then the right forepaw, median and ulnar nerves, C7/C8/T1 DRGs, and hemisected spinal cord were dissected out in continuity. To allow for direct probing of muscle receptive fields (RFs) of individual neurons in the C7–T1 DRGs, the forepaw skin was removed, and then the preparation was transferred to a recording dish where the ACSF was slowly warmed to 35°C. Suction electrodes on the median and ulnar nerves provided orthograde search stimuli to isolate electrically responsive afferents, and those with RFs in the muscle were first isolated with a concentric electrode. These afferents were then characterized by applying an increasing series of von Frey filaments (0.07g to 10g), cold (0°C) and hot (53°C) physiological saline, and finally, oxygenated “low” (15mM lactate, 1mM ATP, pH 7.0) and “high” (50mM lactate, 5mM ATP, pH 6.6) metabolite mixtures to their receptive fields to assess mechano-, thermo- and chemo- sensitivity, respectively. ATP was added to the metabolite solutions immediately before application to prevent degradation. Group III and IV afferents were not significantly different in our samples and were combined for analysis, and cells recorded at the beginning of an *ex vivo* recording session did not differ from those recorded near the termination of recording. A total of 93 cells were characterized using *ex vivo* recording in the IL1RA + I/R (54 cells from 11 mice) and vehicle (H₂O) + I/R conditions (39 cells from 11 mice) here and compared to previously obtained [61,62] naïve data (n=120 cells). Data obtained from the electrophysiological preparations were analyzed offline using Spike2 software. For each cell, the peak instantaneous frequency (IF) of each response was calculated to approximate the individual cell’s maximum potential of firing to a given stimulus [40]. Mechanical thresholds (when detected) and percentage of fiber types responding to our various stimuli were also obtained in our groups.

2.8. Immunocytochemistry and Cell Counting

Immunohistochemistry was performed on C8 DRGs from naïve, IL1RA+I/R, and H₂O+I/R conditions as previously described [31,58,61,62]. Briefly, gelatin-embedded DRG sections (45 µm) were treated with 10 mM citrate buffer (pH 6.0) at 72°C for antigen retrieval, blocked in Tris-buffered saline with 10% bovine serum albumin and 0.1% Triton X-100 (Sigma-Aldrich), incubated overnight with primary antibodies for acid-sensing ion channel 3 (Guinea Pig anti-ASIC3, 1:2000, Millipore) and P2X3 (Rabbit anti-P2X3, 1:2000, ThermoFisher), and then fluorescently labeled with corresponding secondary antibodies (anti-guinea pig AF647 and anti-rabbit AF594, both 1:400, Jackson ImmunoResearch). Stained sections were mounted with Fluoro-Gel (Electron Microscopy Sciences) on gelatin-coated permafrost slides and stored at room temperature. All labeling was characterized on a Leica confocal using sequential scanning and Z-stacks of individual, non-overlapping DRG sections were scanned at 3 µm intervals for quantification. As previously described [29,58,61], cell counts were performed on 20 µm optical sections generated from these Z-stacks of individual DRGs of 3–4 different mice per condition to estimate total numbers of ASIC3-positive, P2X3-positive, and ASIC3/P2X3 co-expressing cells. Counts were performed on cells containing nuclei using Neurolucida software to prevent counting cells

twice. The average number of positive cells per each DRG was obtained by averaging counts from its three non-overlapping sections, and the average counts per each condition were used for analysis.

2.9. Statistical Analysis

Data are represented as mean $\pm$ SEM, or as percent of cells tested for *ex vivo* frequency data. All statistical analysis was performed using SigmaPlot 13.0, and data that did not adhere to a normal distribution were transformed before proceeding with parametric statistics or analyzed with a non-parametric analogue. For Western blot, the optical densities of IL1 β relative to an internal GAPDH control for each sample were compared via ANOVA with Holm-Sidak. Two-way RM ANOVA (time $\times$ condition) or three-way ANOVA (time $\times$ condition $\times$ exercise) were used to analyze behavioral data. One-way ANOVA was used to compare total post-injury distance traveled between groups that had been normalized to the average daily distance from all observed non-injured mice. The Holm-Sidak multiple comparisons correction was applied to all behavioral data requiring post-hoc analysis. Gene expression data obtained from real-time quantitative PCR was analyzed using one-way ANOVA with Holm-Sidak or Kruskal-Wallis one-way ANOVA on ranks with Dunn's test where appropriate. Differences in phenotype frequency between groups were compared with either χ^2 analysis (with Yates correction when applicable) or Fisher's exact test. Firing frequencies, thresholds to different stimuli, and cell counts were compared via Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. For all experiments, critical significance level was defined at $p < 0.05$.

3. Results

3.1. Voluntary exercise prior to ischemia and reperfusion injury (I/R) to the muscle prevents injury-induced muscle IL1 β upregulation and subsequent pain-related behaviors

We have previously reported that IL1 β signaling in neurons is crucial for the development of myalgia-like behaviors after ischemia and reperfusion injury (I/R) [61], and others have shown in a neuropathic pain model that the analgesic properties of moderate exercise are correlated with alterations in peripheral IL1 β levels [21]. To compare the role of cytokine and growth factor signaling within the context of exercise or I/R, real-time quantitative PCR was performed on forelimb muscle tissue of mice that had been housed individually in cages with running wheels for two days prior to injury and compared with naïve and injured mice that were not exposed to running wheels (see Fig. 1A). The mRNA expression of IL-4, 6, 10, and 1 β , artemin, nerve growth factor (NGF), glial derived neurotrophic factor (GDNF), and tumor necrosis factor α (TNF α ; Table 1) were all assessed. IL-4, -6, and -10 expression was not detectable across conditions, precluding these genes from analysis. IL1 β mRNA was found to be upregulated within the non-exercised I/R mice compared to both non-exercised Naïve animals and those with 2d voluntary exercise exposure ($n=3-5$, $p = 0.024$), whereas Naïve and 2d exercise exposed animals did not differ ($p=1.00$, Kruskal-Wallis one-way ANOVA on ranks). Following I/R, GDNF was also found to increase over naïve or 2d exercise levels in the muscle ($p = 0.041$), whereas TNF α showed an I/R-induced decrease ($p = 0.044$). Relative expression of NGF was no different between groups ($p=0.551$), but

artemin was found to be significantly upregulated following either I/R or exercise when compared to naïve animals (I/R: $p=0.023$; 2d exercise: $p=0.039$).

We then wanted to determine if this short course of voluntary exercise could prevent injury I/R-induced IL1 β upregulation in the muscles and subsequent pain-related behaviors since it was one of the factors specifically enhanced by I/R and inhibited by exercise in injured animals. Here, cohorts of naïve or I/R animals were housed individually in cages with running wheels for two days prior to injury and compared with groups of naïve or I/R mice that were not exposed to running wheels (Fig. 1A). The Naïve/Sham+Exercise and I/R +Exercise groups did not differ in distance traveled during this pretreatment period (Naïve/Sham: 4172 ± 490 , I/R: 4101 ± 668 meters/day, $p>0.05$). Similar to enhancements in IL1 β mRNA, the IL1 β protein content of forepaw muscles isolated from either of these 2d voluntary exercise exposed groups did not differ from that of non-exercised Naïve animals one day following injury (D1); however, the IL1 β in non-exercised I/R animals was significantly elevated compared to all other groups (Fig. 1B, $n=3-4$, $p<0.01$).

In these same exercise and injury conditions, we also assayed three ischemic myalgia-like behaviors that we have previously shown to be enhanced at D1 post-I/R [61,62]: ongoing/spontaneous paw guarding [77], mechanical hypersensitivity, and muscle weakness. Similar to our previous reports, mice with I/R show increased guarding behaviors in the affected forelimb that persist to day three following injury (D3) when compared to all other groups ($p<0.001$) as well as to pre-injury baseline (BL) measurements ($p<0.001$; Fig. 1C). Mechanical thresholds at D1 and D3 (Fig. 1D), along with grip strength at D1 (Fig. 1E), were also significantly decreased in the I/R group ($p<0.05$ compared to BL and all other groups); whereas mice with 2d prior voluntary exercise exposure showed no I/R-induced changes in any behavioral assay when compared to either BL ($p=0.22$) or Naïve group measurements ($p=0.32$). An additional group of animals underwent a differing exercise paradigm of short duration forced treadmill running employed immediately after I/R. This mild forced exercise regimen, however, was not effective in mitigating I/R-induced increases in muscle IL1 β ($n=3-4$; $p<0.01$ vs naïve) or pain-related behaviors ($n=5-6$; $p<0.016$) at D1. At D3, forced exercise after injury appeared to prolong I/R-induced mechanical hypersensitivity compared to Naïve controls (Fig. 2) and all other I/R conditions, regardless of exercise exposure (Fig. S1). Forced exercise in mice with I/R was not found to alter grip strength at D3.

3.2. IL-1 receptor antagonist attenuates pain-related behaviors caused by I/R

Because prior exposure to exercise was found to prevent the I/R-induced increases in both pain-related behaviors and IL1 β protein in the muscles at D1 following I/R, we wanted to investigate if targeting IL1 β directly could similarly ameliorate these I/R-evoked behavioral changes. Here we compared the effects of IL1RA and vehicle (H₂O) treatment on guarding behavior, mechanical hypersensitivity, and grip strength at D1 following I/R when compared to BL measurements (Fig. 3A). Both groups were similar in BL guarding measurements ($p=0.864$; Fig. 3B), but Vehicle+I/R animals showed a dramatic increase in guarding behaviors on D1 compared to either their BL or to IL1RA+I/R animals on D1 ($p<0.001$). IL1RA+I/R animals did not statistically differ in average guarding scores at BL and D1

($p=0.08$; two-way RM ANOVA with Holm-Sidak, time $\times$ condition interaction $p<0.001$). When examining mechanical hypersensitivity (Fig. 3C), we found that both IL1RA+I/R and Vehicle+I/R animals showed a significant decrease in paw withdrawal threshold when comparing D1 to BL ($p<0.001$). Vehicle+I/R animals, however, were significantly more hypersensitive than IL1RA treated mice on D1 ($p<0.001$). Additionally, grip strength was significantly decreased in both groups (Fig. 3D), but D1 measurements did not differ between IL1RA or Vehicle treatment ($p>0.05$).

3.3. IL-1 receptor antagonist prevents I/R-induced alterations in muscle afferents

We wanted to further investigate the effects of IL1RA treatment at the primary afferent level, so we utilized our *ex vivo* muscle preparation to compare the effects of IL1RA or Vehicle on afferent plasticity 1 day following I/R (Fig. 4A). To better understand the relationship between injury and treatment, these data were also compared to previously acquired naïve data pooled from our recent studies [61,62]. We first compared phenotype distribution across conditions to determine the numbers of cells responsive to mechanical, thermal, and chemical stimulation (Fig. 4B). There were no significant changes in the numbers of thermally responsive cells, nor in the response properties of cells to either hot or cold stimulation, between groups (Table S3). While Vehicle+I/R mice displayed a significant increase in the number of cells responsive to mechanical stimulation when compared to naïve ($p=0.028$) neither group differed from IL1RA+I/R mice (24/54, $p=0.178$). However, the decreased mechanical thresholds seen in the Vehicle+I/R condition ($p=0.037$ vs. naïves) were blocked by IL1RA treatment ($p=0.056$ vs. naïves; $p<0.001$ vs. Vehicle+I/R; Figs. 4C, D). IL1RA+I/R mice showed decreased firing to mechanical stimulation relative to naïve mice [Naïve (20 cells): 52.34 ± 9.58 , IL1RA+I/R (22 cells): 25.96 ± 10.38 Hz, $p=0.033$], but this parameter was unaffected in Vehicle+I/R animals [Vehicle+I/R (19 cells): 49.81 ± 23.66 Hz, $p>0.05$].

When combined, the proportion of chemically sensitive afferents was no different across conditions (Naïve: 40/91, Vehicle+I/R: 17/31 IL1RA+I/R: 18/40; $p>0.05$); however, we found that the population of afferents responding to only low metabolites, only high metabolites, or both low and high metabolites was condition-dependent. Vehicle+I/R animals were similar to previous results from I/R conditions [61,62] in that they exhibited a smaller population of afferents responsive to low metabolites only, and a greater population of afferents responsive to both low and high metabolite solutions (Low Responders/Both Responders: Naïve: 18/7; Vehicle+I/R: 4/8, $p<0.05$; Fig. 4E). While IL1RA-treated animals had greater numbers of cells responsive to high metabolites only (11/18), this proportion was not found to be statistically significant compared to naïve (15/40, $p=0.165$) or Vehicle+I/R (5/31 $p=1.0$). IL1RA+I/R animals also showed a partial recovery from the enhanced firing to low metabolites seen in the Vehicle+I/R group ($p=0.033$ vs. naïves), as they were no different from either prior naïve data ($p=0.629$) or the Vehicle+I/R condition ($p=1.0$; Fig. 4F). Firing to high metabolite solutions was no different between groups (Naïve: 2.87 ± 0.8 Hz, Vehicle+I/R: 5.33 ± 1.4 Hz, IL1RA+I/R: 6.14 ± 2.2 Hz; $p=0.611$, Kruskal-Wallis one-way ANOVA on ranks, $n=8-13$).

We also performed cell counts on the affected C7, C8, and T1 DRGs from naïve, Vehicle +I/R, and IL1RA+I/R mice to quantify the expression of specific pain-related channels (Fig. 5A) previously thought to play a role in I/R related responses in the DRGs [61]. We found that ASIC3 expression was increased in the DRGs of Vehicle+I/R mice (64.11 ± 14.23 cells/field of view, $p=0.018$), but not IL1RA+I/R mice (13.75 ± 2.74 , $p=0.788$), relative to naïves (8.83 ± 2.35 , Fig. 5B). The increase in P2X3 caused by I/R was not alleviated by IL1RA treatment (Naïve: 14.92 ± 4.19 ; Vehicle+I/R: 44.11 ± 7.71 , $p=0.007$ vs. naïve; IL1RA+I/R: 31 ± 3.11 , $p=0.039$ vs. naïve; Fig. 5C). Co-expression of P2X3 and ASIC3 did not differ between IL1RA+I/R animals and naïves (Naïve: 2.67 ± 0.78 , IL1RA+I/R: 3.5 ± 1.22 , $p=1.0$), whereas Vehicle+I/R animals showed significantly more cells co-expressing ASIC3 and P2X3 than found in either of the other groups (26.0 ± 6.17 ; $p>0.05$; Fig. 5D).

3.4. IL1RA treatment in mice with acute running wheel exposure ameliorates I/R-induced pain-related behaviors

To better understand the relationship between I/R injury, IL1 β , and exercise, we also characterized the I/R-induced behavioral changes in IL1RA or vehicle-treated mice that were acutely exposed to a voluntary running wheel (Fig. 6A), and compared them with non-injured vehicle or IL1RA-injected controls. In this acute exercise exposure paradigm, mice were habituated to the wheel cages for 15–20 minutes between BL behavior and occlusion surgery, and their activity was monitored for two days following I/R. We first assessed paw guarding, mechanical hypersensitivity and grip strength in these other cohorts to confirm I/R related pain-like behaviors in mice acutely exposed to a running wheel. Similar to the previous experiment (Fig. 3), I/R-induced changes in pain related behaviors were greatly reduced by IL1RA treatment. Guarding behaviors (Fig. 6B) were significantly increased on D1 in Vehicle+I/R animals, but this was moderately reduced by IL1RA treatment, so that all groups significantly differed at this time point ($p<0.001$). However, IL1RA+I/R animals were no different from controls on D3, whereas guarding scores in Vehicle+I/R mice remained elevated relative to their BL ($p=0.006$) and to controls at D3 ($p=0.009$). IL1RA treatment blocked the substantial D1 decreases in mechanical paw withdrawal threshold (Fig. 6C) and grip strength (Fig. 6D) seen in Vehicle+I/R animals. However, mechanical hypersensitivity was not fully restored to control levels at D3 in IL1RA+I/R mice ($p=0.017$).

We then tracked their voluntary activity for two days following I/R surgery, since this parameter is another clinically-relevant measure often decreased by ischemic myalgia [48,81]. Distance traveled on the wheel was significantly decreased in Vehicle+I/R mice on D1 (Fig. 6E; $p<0.05$ vs other groups), but this deficit was ablated with IL1RA treatment. Furthermore, IL1RA-treated I/R animals also showed a persistent increase in average velocity that surpassed naïve and injection control mice (Fig. 6F; $p<0.05$).

Since we had measured voluntary activity in mice who received 2d voluntary exercise exposure prior to I/R (shown in Fig. 1), we compared the total distance traveled following I/R (normalized to the average 2d distance traveled by naïve mice to control for habituation to wheel exposure; Fig 6G) between these complimentary data sets. Whereas Vehicle+I/R animals with acute exposure to voluntary running wheels covered significantly less distance than naïves in the two days following injury, the animals who had 2d prior voluntary

exercise exposure were protected from the I/R-induced activity decrease (Vehicle+I/R $p = 0.014$ vs all other conditions). Altogether, this suggests that the I/R-associated decrease in voluntary activity is not only ablated by IL1RA treatment, but also by exercise pretreatment.

3.5 Reward Motivation is Unchanged Following IL1RA and/or I/R

Lastly, to better understand the effect of motivation in our model of ischemic injury, we utilized a saccharin preference assay [23]. Sucrose is typically used in this task of anhedonic-like behavior; however, in order to avoid any potential confounding effects of sucrose on activity level and/or pain, an equally preferred saccharin solution was used [66,73]. For all groups, BL fluid intake and saccharin preference did not differ ($p = 0.57$; data not shown), and neither IL1RA administration nor I/R injury affected saccharin preference (Drug $p=0.918$, Injury $p=0.878$, Drug x Injury $p=0.619$; Fig. 7A) or total fluid intake (Drug $p=0.509$, Injury $p=0.677$, Drug x Injury $p=0.433$; Fig. 7B) on D1.

4. Discussion

Transient ischemia and reperfusion injury (I/R) of the forelimb, evokes a robust ischemic myalgia-like behavioral phenotype in affected mice [61,62]. IL1 β /IL1r1 signaling regulates the injury-induced sensitization of primary group III and IV muscle afferents. Specifically, I/R-evoked increases in IL1 β within the muscle tissue act at the upregulated IL1r1 in affected DRGs to stimulate *de novo* ASIC3 expression, leading to enhanced chemical and mechanical sensitivity in individual primary muscle afferents, and increased pain-related behaviors *in vivo* [61,62]. To better understand the role of IL1 β signaling in I/R-induced sensitization, we investigated additional methods of blocking peripheral IL1 β action, including voluntary exercise, in this study. Here we show that prior exposure to 2d of voluntary exercise completely blocks I/R induced increases in both muscle IL1 β and pain-related behaviors, in agreement with multiple recent studies showing positive effects of exercise in reducing the pain-related behaviors in various injury models [9,21,39,57]. We also test the effects of a potential pharmacological therapy for ischemic myalgia (IL1RA) on the response properties of *individual* muscle afferents and pain-like behaviors post I/R.

4.1 IL1 β Inhibition by Exercise and IL1RA

IL1 β , a pro-inflammatory mediator that has been linked to the development of muscle hyperalgesia [4,6,7,16,17], is also important to the generation of an ischemic myalgia-like phenotype in our mouse model of I/R [61,62]. Here, we confirmed robust increases of IL1 β in the affected muscle 1d after I/R in both non-exercised animals (Fig. 1) and those submitted to mild, short duration, forced treadmill running immediately following reperfusion surgery (Fig. 2). Our previous findings showed that this mild forced running protocol, whether given prior to or during a prolonged occlusive injury, neither attenuated nor enhanced injury-evoked ischemic myalgia-like behaviors [58]. We therefore elected to administer this regimen following the I/R injury here. Regardless, animals who had undergone forced treadmill running were not protected from I/R-induced increases in pain-related behaviors. Rather, these animals show increased guarding behaviors in the affected forelimb and decreased mechanical threshold and grip strength, similar to non-exercised mice (Figs. 2, S1). Interestingly, whereas a mild forced exercise intervention did little to

mitigate the effects of I/R, and may even prolong the effects of injury (Fig. S1), prior exposure to 2d of voluntary wheel running protected against I/R-induced IL1 β enhancement (Figs. 1, 2; Table 1). Furthermore, I/R+2d Prior Exercise mice also failed to develop any I/R-associated pain-related behaviors or show a decrement in voluntary activity following injury (Figs. 1, 6), even though I/R animals acutely exposed to the same running wheels in our recent study [61] and in Fig. 6 still displayed enhanced ischemic myalgia-like behaviors.

Reviews of exercise therapy for the treatment of chronic pain have suggested that the timing and intensity, and perhaps the voluntary, rather than compulsory nature [57], of an activity regimen strongly influences its beneficence [1,3,10,27,38,44,51]. This may correlate with the differing effects of the various exercise treatments used in this study. Although 2d of wheel running prior to I/R completely block the injury's painful effects, if an animal begins running immediately before the occlusion through the duration of the study, D1 injury effects will remain just as severe as if they had not run at all (Figs. 1, 2, 3, 6, S1).

Interestingly, the presence of this behavioral phenotype coincides with increased IL1 β expression within the affected muscle, which animals with 2d prior exercise do not display. This may not be surprising as physical activity has been increasingly suggested to inoculate against pro-inflammatory cytokine effects [54,55], and within the context of I/R, regulation of disrupted IL1 β signaling may contribute to exercise's anti-nociceptive effects.

Exercise is known to dramatically increase interleukin-1 receptor antagonist, IL1RA, within the circulation, and enhances muscle IL1 β expression to a lesser extent [7,26,53]. Because IL1RA and IL1 β have similar binding affinities to IL1r1, the larger increase in IL1RA likely prevents IL1 β action [13]. In an effort to untangle the associations between IL1 β , exercise, and I/R, we pharmacologically inhibited IL1 β action at IL1r1 using a recombinant form of mouse IL1RA. This is similar to a commercially used recombinant human IL1RA employed to combat immune-mediated joint degradation for the treatment of rheumatoid arthritis (RA). Use of this drug has also been shown to relieve RA-associated pain [22,36]. IL1RA treatment in rodents decreases pain-related behaviors evoked in models of intense swimming exercise [6], inflammatory pain [4], bone cancer [4], and intramuscular acid injection [69]. We found that IL1RA treatment in I/R blocks increased paw guarding and partially restores mechanical thresholds (Fig. 3). Exogenous IL1RA administration, however, may also decrease the ability of injured tissues to heal [36,50], which may account for the drug's lack of effect on I/R-evoked grip strength reduction.

Due to the ability of IL1RA to reduce the ischemic myalgia-like behavioral phenotype provoked by I/R, we explored functional effects on individual group III and IV muscle afferents with our *ex vivo* muscle electrophysiology preparation [31,58,61,62]. The Vehicle +I/R condition yielded comparable results to our previous studies, namely, lower thresholds to mechanical stimulation and skewed proportions of metabolite responders, when compared with IL1RA-treated animals. There were significantly more mechanically sensitive afferents in the Vehicle+I/R condition (Fig. 4) than either the IL1RA+I/R condition or naïve comparison data. Systemic IL1RA administration elicited similar results to our recent study of nerve injection of IL1r1-targeting siRNA during I/R [61]. Furthermore, both treatments are effective in blocking enhanced ASIC3, but not P2X3, expression induced by I/R (Fig. 5). This suggests that either method is sufficient to inhibit IL1 β action at IL1r1 in the peripheral

afferents to prevent I/R-induced *de novo* ASIC3 expression that modulates mechanical and chemical responses in individual primary muscle afferents, leading to increased pain-related behaviors (Figs. 1–5). It is important to note that we did not assess the effects of 2d voluntary exercise on afferents. A full confirmation of its effects on nociceptor function in relation to IL1RA treatment in the future are necessary.

Additionally, it is possible that exogenous IL1RA might act synergistically with exercise to promote recovery from I/R (Fig. 6). This combined exposure completely blocked the decreases in mechanical threshold, voluntary activity, and grip strength, and significantly attenuated the increased guarding behaviors observed following I/R, thus providing an improvement over treatment with IL1RA alone. Finally, due to the potential of anhedonia to decrease voluntary activity in our model, we also investigated how reward seeking may be affected by IL1RA and/or I/R. For this, we assessed consumption of a saccharin solution, another natural reward, compared to that of drinking water, in a separate cohort of non-activity exposed animals (Fig. 7). A failure to develop a bias toward the saccharin solution can be indicative of anhedonia/depression, but despite an I/R-induced decrease in wheel running, we did not see any differences in saccharin preference in any of our groups, suggesting that the decreased activity levels following I/R are more related to ability than to motivation. However, as alterations in reward pathways may be integral to exercise effects in other conditions, this potential interaction should be considered when using voluntary activity as a means of assessing pain-like behaviors.

4.2 Clinical Relevance

Muscle pain is a widely experienced clinical problem that can arise from a number of conditions. Exercise is a commonly recommended therapy; however, effective pain management is often etiology-dependent [10,51]. In diseases such as fibromyalgia [15,37], complex regional pain syndrome (CRPS) [11,28], peripheral vascular disease [1,42], and chronic fatigue syndrome (CFS) [2,43], myalgia may originate from peripheral perfusion deficits, leading to ischemic-like conditions within affected muscles. Recent evidence is emerging, however, that exercise programs that are effective for analgesia in some patient populations may in fact be hyperalgesic in others [43,71]. In particular, this may be exacerbating underlying conditions and thus precipitating the prevalence in noncompliance seen in people afflicted with ischemic myalgia-related disorders [18,27,72].

In a recent study of patients with CFS, IL1RA was ineffective in relieving muscle pain symptoms [60]. Species differences and difficulty in generating an appropriate animal model of CFS may account for this discrepancy. On the other hand, the clinical trial was performed in women only and basic research on IL1RA administration in muscle pain models, including the current study, has been performed in males. We have now confirmed IL1 β is integral for the induction of an ischemic myalgia-like phenotype following I/R in male mice; however, there has been a recent bevy of research suggesting that the development of hyperalgesia requires sex-dependent mechanisms of immune response [67,68]. Since IL1r1 inhibition blocks I/R induced changes in individual muscle afferents and pain-related behaviors, perhaps these sex differences in IL1RA treatment response are manifested at the level of the primary afferents, implying that the role of IL1 β in injury-induced sensitization

may be applicable only to males. It will also be important to investigate the effects of exercise on other factors such as GDNF (Table 1) in the future to help determine this. Regardless, because multiple I/R-related conditions, disproportionately affect women [12,59,60], mechanisms of ischemic myalgia generation and maintenance in females should be addressed in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Two days of prior exposure to voluntary wheel running blocks ischemia and reperfusion injury (I/R)-induced changes in muscle IL1 β and pain-related behaviors
 (A) Singly-housed mice were either exposed to an in-cage running wheel or kept in traditional housing for two days prior to occlusion and reperfusion or sham surgeries. Behavioral assays were performed immediately prior to injury surgeries (baseline, BL), at one day following occlusion surgeries (D1), and at three days following surgery (D3). (B) Western blot of protein isolated from the affected forepaw muscles of similarly treated cohorts at D1 (n=3–4) shows that voluntary exercise-exposed animals are protected from I/R-related increases in IL1 β . (C) Spontaneous/ongoing pain-like behavior induced by I/R is ablated in voluntary exercise exposed animals (significant interaction condition $\times$ time $p < 0.001$). Decreases in paw withdrawal threshold to mechanical stimulation (D; significant main effect of condition $p < 0.001$) and in grip strength (E; significant interaction condition $\times$ time $p = 0.43$) are also prevented with 2d prior voluntary activity. Data are represented as mean $\pm$ SEM and were analyzed via two-way ANOVA or two-way RM ANOVA with Holm-Sidak *post hoc* tests. ** $p < 0.001$ vs BL and other groups, * $p < 0.05$ vs. BL and other groups.

Figure 2. Forced running immediately after reperfusion does not prevent increases in injury-associated pain-related behaviors or muscle IL1 β

(A) Cohorts of age-matched Naïve and I/R mice underwent a moderate forced treadmill running protocol immediately following reperfusion surgery. (B) As measured by Western blot, the upregulation of muscle IL1 β protein expression in forced run I/R vs Naïve animals was comparable to that seen in non-exercised animals (see Fig. 1B). (C) Paw guarding behaviors were significantly increased at D1 following forced running in animals with I/R (interaction effect of condition x time $p < 0.001$). Similarly, forced running did not affect I/R-induced reductions in mechanical threshold (D; interaction effect of condition x time $p = 0.003$) or grip strength (E; interaction effect of condition x time $p = 0.011$), but also did not affect these behaviors in uninjured animals. Data from the non-exercised groups from Fig. 1 have also been included for comparison to forced run conditions. All data are represented as mean $\pm$ SEM. Behavioral data were analyzed via three-way ANOVA (condition x time x exercise) with Holm-Sidak, and Western blot data were analyzed using one-way ANOVA with Holm-Sidak. ** $p < 0.001$ vs BL and uninjured groups, * $p < 0.05$ vs. BL and uninjured groups.

Figure 3. Treatment with the interleukin-1 receptor antagonist (IL1RA) attenuates increases in spontaneous pain-like behaviors and evoked mechanical hypersensitivity, but does not rescue grip strength impairment, associated with I/R

(A) Immediately before occlusion and following reperfusion surgery, mice were intraperitoneally injected with 3 mg/kg IL1RA or vehicle (H₂O). Pain-related behavioral assays were performed at BL and D1. (B) IL1RA ablates I/R-evoked increases in ongoing paw guarding (significant interaction condition x time $p < 0.001$), and improves decreases in paw withdrawal threshold to mechanical stimulation (C; significant interaction condition x time $p = 0.010$), but does not fully restore thresholds to BL levels (IL1RA+I/R BL vs D1 $p < 0.05$). (D) Grip strength was similarly decreased on D1 from BL in both groups. Data are represented as mean $\pm$ SEM and were analyzed via two-way RM ANOVA with Holm-Sidak *post hoc* tests. ** $p < 0.001$ vs BL and other groups, * $p < 0.05$ vs. BL.

Figure 4. Effects of IL1RA treatment on I/R-induced alterations on Group III and IV primary muscle afferents as assessed by ex vivo electrophysiology

(A). Compared to previous pooled data from uninjured mice, IL1RA+I/R mice were no different in total number of mechanosensitive or chemosensitive cells, but Vehicle+I/R mice had significantly more mechanically sensitive cells (B). (C) The decrease in mechanical threshold following I/R seen in vehicle-treated animals (vs naïve comparison $p=0.037$) is absent in IL1RA+I/R animals (vs naïve comparison $p=0.056$, vs Vehicle+I/R $p<0.001$; representative traces during mechanical stimulation of the muscles, D). (E) Although the total number of metabolite responders was unaffected by I/R or IL1RA, proportions of cells sensitive to Low, High, or Both metabolite solutions were significantly altered from naïve and IL1RA+I/R proportions in vehicle-treated animals ($p=0.045$ and 0.007 , respectively), whereas naïve and IL1RA+I/R metabolite proportions did not differ ($p>0.05$). I/R also causes an increase in IF to low metabolites that may be partially rescued with IL1RA treatment (F; Vehicle+I/R vs naïve $p=0.033$, IL1RA $p>0.05$ vs other groups). Differences in phenotype distribution were analyzed with χ^2 , and response properties were analyzed with Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Phenotype data are presented as percentage of cells responsive to a certain stimulus out of the total tested for that stimulus within that condition, and response properties are represented as mean $\pm$ SEM, wherein these reference values from pooled naïve data are reflected as solid and dashed lines, respectively. * indicates $p<0.05$ compared to naïve; # indicates $p>0.05$ compared to naïve and $p<0.05$ vs Vehicle+I/R, ^ indicates $p<0.05$ vs naïve but not IL1RA+I/R.

Figure 5. DRG ASIC3 upregulation after I/R is prevented with IL1RA treatment

(A) Representative immunocytochemical staining of ASIC3 (blue) and P2X3 (red) protein in whole DRGs from naïve, Vehicle+I/R and IL1RA+I/R conditions. (B) The Vehicle+I/R condition contained significantly more cells immunopositive for ASIC3 than IL1RA+I/R animals ($p=0.018$), but P2X3 expression did not differ (C; $p>0.05$). (D) Co-expression of ASIC3 and P2X3 was significantly increased in Vehicle+I/R mice compared to IL1RA+I/R mice ($p<0.039$). Scale bar for all images, 60 μm . Experimental data are represented as mean $\pm$ SEM and were analyzed with Kruskal-Wallis one-way ANOVA on ranks with Dunn's test. Solid and dashed lines represent the mean and upper or lower extremes of the SEM, respectively, of pooled naïve comparison data. * $p < 0.05$ compared to other group(s).

Figure 6. Acute exercise with IL1RA treatment decreases pain-related behaviors and increases voluntary activity following I/R similar to 2d prior exercise exposure

Mice were briefly habituated to a running wheel, then intraperitoneally injected with 3 mg/kg IL1RA or vehicle (H₂O) before occlusion surgery, and placed back into running wheel housing for remainder of study. Following reperfusion surgery, they were again injected with IL1RA or vehicle (A). (B) IL1RA treatment with acute exercise exposure attenuates increased guarding score evoked by I/R, and ensures D3 recovery in treated mice (significant interaction condition x time $p < 0.001$). (C) Although IL1RA+I/R animals showed a mild decrease from BL in paw withdrawal threshold on D1, they did not differ from uninjured mice at any time point (significant interaction condition x time $p = 0.042$). (D) Grip strength in IL1RA+I/R animals also did not differ at any time point (significant interaction condition x time $p = 0.006$). IL1RA treatment protects from I/R-associated decrease in voluntary activity. Vehicle+I/R animals ran shorter distances on D1 compared to both IL1RA+I/R and uninjured mice (E; significant main effects of condition $p = 0.035$ and time $p < 0.001$), but average velocity only differed from IL1RA+I/R animals on D1 (F; significant main effects of condition $p = 0.047$ and time $p = 0.002$). (G) Following normalization to average naïve 2d distance, total 2d post-I/R distance traveled from animals in Fig. 1 was compared to that from Vehicle+I/R and IL1RA+I/R groups, showing that 2d prior exercise exposure and IL1RA treatment similarly recover voluntary activity following I/R (Vehicle+I/R $p = 0.014$ vs all other conditions). Data are represented as mean $\pm$ SEM and were analyzed via two-way RM ANOVA (B–F) or one-way ANOVA (G) with Holm-Sidak *post hoc* tests. $^{\wedge}p < 0.05$ vs other time points, $*p < 0.05$ vs other groups, $\#p < 0.05$ vs uninjured controls only, $^{\>}p = 0.034$ vs IL1RA+I/R only.

Figure 7. Reward-seeking behavior is unaffected by I/R and/or IL1RA

Saccharin preference was assayed in an additional cohort of mice with injury and/or injection treatment (n=5–6). During the week leading up to I/R surgery, mice of the various conditions were habituated to saccharin exposure during two overnight periods. Then, following reperfusion surgery mice were again provided with both water and saccharin solution. (A) Total liquid intake in these mice over the night following I/R surgery was no different between groups ($p>0.05$). (B) There was no clear effect of IL1RA or I/R on saccharin preference ($p>0.05$). Data are represented as mean $\pm$ SEM and were analyzed via two-way ANOVA (drug $\times$ injury).

Table 1

Changes in mRNA expression of various cytokines and growth factors with ischemia and reperfusion (I/R) injury or 2d prior voluntary running wheel exposure.

Gene	I/R	2d Prior Exercise
Artemin	240.1 ± 20 [^]	152.4 ± 22 [^]
GDNF	87.7 ± 3 [*]	7.7 ± 3
IL-1β	148.8 ± 5 [*]	- 13.3 ± 19
NGF	- 26.7 ± 8	- 2.6 ± 8
TNFα	- 66.0 ± 32 [*]	- 1.9 ± 17

Values are presented as percent change in expression relative to age-matched naïve mice ± SEM (n=3–5 per condition).

^{*}
p < 0.05 vs all other conditions,

[^]
p < 0.05 vs naïve only.