A dual role for peripheral GDNF signaling in nociception and cardiovascular reflexes in the mouse

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Group III/IV muscle afferents transduce nociceptive signals and modulate exercise pressor reflexes (EPRs). However, the mechanisms governing afferent responsiveness to dually modulate these processes are not well characterized. We and others have shown that ischemic injury can induce both nociception-related behaviors and exacerbated EPRs in the same mice. This correlated with primary muscle afferent sensitization and increased expression of glial cell line-derived neurotrophic factor (GDNF) in injured muscle and increased expression of GDNF family receptor α1 (GFRα1) in dorsal root ganglia (DRG). Here, we report that increased GDNF/GFRα1 signaling to sensory neurons from ischemia/reperfusion-affected muscle directly modulated nociceptive-like behaviors and increased exercise-mediated reflexes and group III/IV muscle afferent sensitization. This appeared to have taken effect through increased cyclic adenosine monophosphate (cAMP) response element binding (CREB)/CREB binding protein-mediated expression of the purinergic receptor P2X5 in the DRGs. Muscle GDNF signaling to neurons may, therefore, play an important dual role in nociception and sympathetic reflexes and could provide a therapeutic target for treating complications from ischemic injuries.

sensory neurons | neurotrophic factors | muscle pain | exercise-mediated reflexes

Group III/IV primary muscle afferents are subpopulations of thinly myelinated (III) and unmyelinated (IV) fibers with a variety of sensory capabilities that include mechanical sensation, thermal sensation, and chemosensation (1–3). Of the multiple potential functions of group III/IV muscle afferents, 2 have been clearly described. These neurons can serve as sensory transducers of noxious and nonnoxious peripheral stimuli from the muscles (2, 4). They also function as the sensory arm for the exercise pressor reflex (EPR), the cardiovascular response to muscle contraction that includes increased blood pressure and heart rate (HR) as well as increased ventilation (5–7). Each of these biological processes is influenced by the different sensory modalities of group III/IV afferents (8–13).

A common method to study peripheral sensory neurons in both nociception and sympathetic reflexes is to use a skeletal muscle ischemic injury model (14–18). After ischemic injuries, group III/IV muscle afferents display peripheral sensitization, including increased responsiveness to mechanical and chemical stimuli. Ischemia also increases responses to muscle contractions of ∼50% of group IV and ∼12% of group III muscle afferents (15). This correlates with changes in nociceptive behaviors, EPRs, and gene expression in the affected muscles and dorsal root ganglia (DRG) (14, 19).

Chemoreception and mechanical responsiveness of muscle afferents have been attributed to expression of a combination of acid-sensing ion channels (ASICs) and purinergic P2X receptors (8, 20–24). Each of these channels has been associated with altered nociception and EPR modulation after ischemic injury (9, 17, 25, 26). P2X and ASIC activity has been linked to fatigue sensations and ischemic pain (8, 20). These channels have also been associated with the responsiveness of DRG neurons to chemical stimulation (8, 10, 20). P2X5 has further been shown to modulate the pH sensitivity of ASIC3 (20), which may additionally shape afferent and behavioral responsiveness. The role of these channels in normal mechanical responses of nociceptors is not as clear, but ASIC3 and P2X receptors have been linked to mechanical hypersensitivity in different injury models (17, 21, 24, 27).

We previously reported a role for the cytokine interleukin-1β (IL-1β) modulating ASIC3 expression in mechanically and chemically sensitive afferents to regulate peripheral sensitization after ischemia with reperfusion (I/R) injury (17). This suggested that factors expressed in the injured muscles may have a substantial influence on afferent function and subsequent behavior. We and others also recently reported that growth factors (GFs) may be additional signaling molecules involved in modulating muscle injury-related hypersensitivity (28–31). Interestingly, of the numerous GFs tested, only glial cell line-derived neurotrophic factor (GDNF) was found to be up-regulated in the muscle 1 d after I/R. Its receptor, GDNF family receptor α1 (GFRα1), was also specifically induced in the I/R-affected DRGs (16). Previous studies have suggested that GDNF can sensitize peripheral nociceptors (32, 33) and produce muscle mechanical hypersensitivity (34). However, intramuscular injection of anti-GDNF antibodies can prevent mechanical hypersensitivity in a rat model of delayed-onset muscle soreness (35). GDNF can also regulate sensory neurons expressing ASIC3 and P2X receptors (32, 36).

Clinically, muscle I/R injuries are common characteristics of conditions such as peripheral vascular disease (PVD) and sickle cell anemia. A hallmark of these diseases is chronic musculoskeletal pain that is often observed alongside an exacerbation of the patient's EPR (37, 38), which can lead to exercise intolerance or further complicate underlying cardiovascular conditions

Significance

This work shows that increased muscle-derived glial cell line-derived neurotrophic factor (GDNF) acting through its receptor GDNF family receptor α1 on primary group III/IV muscle afferents modulates both nociception and the cardiovascular response to exercise. Importantly, unilateral ischemic muscle injuries not only produce pain-related behaviors in the affected limb, but they also affect sympathetic reflexes systemically. This dual function of primary muscle afferents under ischemic conditions potentially explains why patients with disorders such as sickle cell anemia or peripheral vascular disease concurrently report pain and altered exercise pressor reflexes. Results further highlight the potential use of localized anti-GDNF antibodies as a therapeutic option for these clinical conditions.

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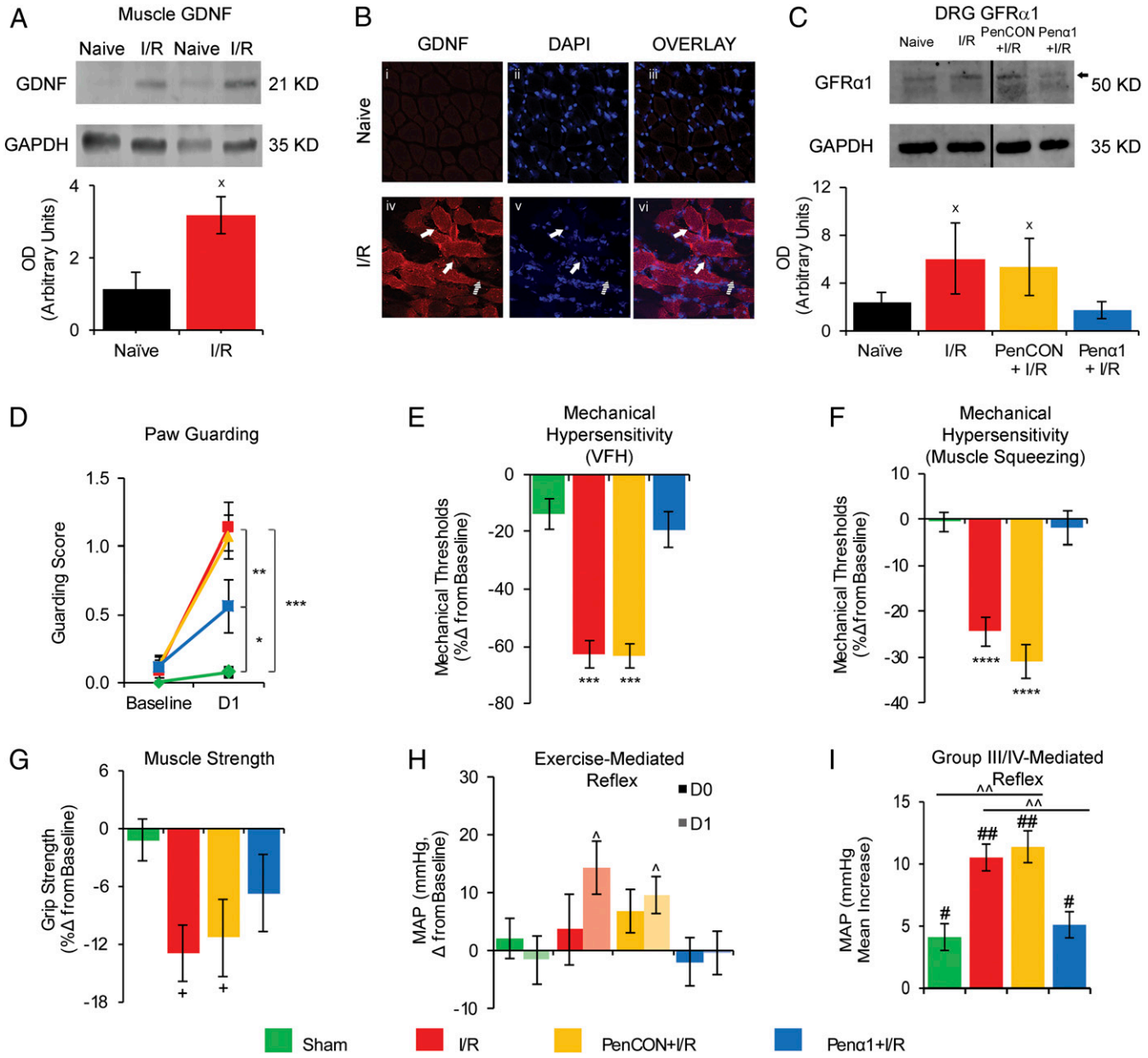

Fig. 1. Increased GDNF in muscle and up-regulation of GFRα1 in DRGs modulate pain-related behaviors and increased MAP after I/R. (*A*) GDNF is increased in muscle after I/R compared with naïve animals ($n = 4$ per group) as assessed by WB. OD, optical density. (*B*) Representative image of GDNF distribution within myofibers in naïve and I/R-injured forepaw muscle (magnification: 20×). DAPI marks nuclei. Solid arrows indicate GDNF+ myofibers. Dashed arrows indicate GDNF− myofiber. (*C*) WB of GFRα1 in DRGs showed increased expression after I/R and in PenCON+I/R. Penα1 prevented this up-regulation ($n = 3$ per group). (*D*) Paw guarding is increased 1 d after I/R and PenCON+I/R. This is partially prevented in Penα1+I/R animals. Mechanical withdrawal thresholds to VFH stimulation (*E*) and muscle squeezing (*F*) are decreased after I/R and PenCON+I/R. GFRα1 knockdown blocked these effects. (*G*) Grip strength is decreased 1 d after I/R and PenCON+I/R. This was partially reversed in Penα1+I/R animals. (*H*) Only injured mice (I/R and PenCON+I/R) displayed a significant increase in MAP after forced exercise (light-colored columns). The increase in MAP was not observed in Penα1+I/R mice. (*D–H*) $n = 12$ per group. D0, pre-I/R; D1, post-I/R. (*I*) When MAP is measured directly in anesthetized mice, all groups showed significant increases in MAP after electrical stimulation of the nerves, but I/R and PenCON+I/R groups showed a larger increase in MAP compared with sham and Penα1+I/R groups ($n = 4$ per group). One-way ANOVA, HSD post hoc (*A* and *C*) or 2-way repeated measures ANOVA, HSD post hoc (*D–I*). $^{x}P < 0.05$ vs. naïve; $^{*}P < 0.05$ vs. sham; $^{**}P < 0.01$ vs. Penα1+I/R; $^{***}P < 0.001$ vs. sham and Penα1+I/R; $^{****}P < 0.0001$ vs. sham and Penα1+I/R; $^{+}P < 0.01$ vs. sham; $^{\wedge}P < 0.05$ vs. preexercise MAP; $^{\wedge\wedge}P < 0.05$ vs. sham or Penα1+I/R; $^{\#}P < 0.05$ vs. baseline; $^{\#\#}P < 0.001$ vs. baseline.

(14, 38–40). While there is a plethora of knowledge regarding EPRs and the peripheral mechanisms of nociception from the skin (32, 41, 42), there is a significant disparity in our understanding of afferent mechanisms of ischemic myalgia. Furthermore, whether peripheral mechanisms of muscle nociception share common pathways with mechanisms of EPR modulation is relatively unexplored (14, 43). Here, we hypothesized that the GDNF/GFRα1 pathway sensitizes primary muscle afferents after I/R and regulates increased cardiovascular responses to exercise and pain-related behaviors.

Results

I/R Up-Regulates GDNF in the Muscle and Various Gene Expression in the DRGs That Correlate with Peripheral Hypersensitivity. Previously, we did not observe a significant increase in the expression of many GFs in the I/R-affected muscle, such as nerve growth factor

(NGF), brain-derived neurotrophic factor (BDNF), and Artemin. However, GDNF was significantly up-regulated (16). To characterize the role of GDNF signaling after I/R, we first quantified the amount of GDNF protein in muscle tissue by western blot (WB). Analysis revealed an increased amount of GDNF in the I/R-injured muscles compared with naïve mice 24 h after injury (Fig. 1*A*). Immunohistochemical labeling revealed expression of GDNF around and within myofibers (Fig. 1*B*).

While GDNF showed increased expression in I/R-injured muscle, its receptor, GFRα1, was up-regulated in the I/R-affected DRGs (Fig. 1*C*). To test the effects of GFRα1 up-regulation in DRG neurons, we used our previously described nerve-specific small interfering RNA (siRNA)-mediated knockdown strategy to block injury-related gene expression (14, 17, 42, 44, 45). We verified the effectiveness of the siRNA knockdown strategy via real-time PCR and WB. To control for nerve injections, a separate group of I/R-injured mice was injected with nontargeting siRNAs (PenCON+I/R). The PenCON+I/R mice showed increased messenger RNA (mRNA) expression of GFRα1 (438 ± 32%; $P < 0.05$ vs. naive) as did the I/R mice without siRNA injections (236 ± 17%; $P < 0.05$ vs. naive), while the Penα1+I/R mice (0 ± 19%; $P > 0.05$ vs. naïve; 1-way ANOVA with Holm–Sidak (HSD) post hoc) showed expression levels similar to naïve animals. Comparable results were also obtained at the protein level (Fig. 1*C*).

We then performed a variety of behavioral tests in I/R-injured animals with nerve-targeted GFRα1 knockdown. In these experiments, sham surgery controls were used for comparisons. I/R-injured and PenCON-injected animals with I/R showed increased paw guarding compared with sham-injured animals at 1 d. I/R-injured mice with GFRα1 knockdown (Penα1+I/R), however, showed significantly reduced paw guarding (Fig. 1*D*). Withdrawal thresholds to von Frey hair (VFH) stimulation of the plantar surface of the forepaw were also significantly decreased in the I/R and PenCON+I/R groups compared with sham-injured animals. This mechanical hypersensitivity was completely prevented by Penα1 injection (Fig. 1*E*). Similar results regarding mechanical hypersensitivity were also obtained in groups that underwent hind paw muscle squeezing (Fig. 1*F*). Grip strength was decreased after I/R alone and in the PenCON+I/R group. This decrease in grip strength was partially prevented by GFRα1 targeting siRNAs (Fig. 1*G*). Finally, we tested the cardiovascular response to exercise before and following injury to measure blood pressure and HR after a low-intensity exercise. This running protocol has been previously determined to be sufficient to induce an exercise-mediated cardiovascular reflex response but not strong enough that it can alter pain-related hypersensitivity (14). I/R-injured and PenCON+I/R mice showed a significant increase in the mean arterial pressure (MAP) 1 d after injury when compared with their preexercise baseline. This significant increase in MAP was completely absent in sham-injured or Penα1+I/R mice (Fig. 1*H*). We did not detect any change in the HR after exercise either before or 1 d after I/R.

To corroborate that observed changes in MAP after exercise in injured animals were not due to increased pain during exercise, we performed intraarterial measurements of blood pressure in lightly anesthetized animals. To stimulate the forepaw muscles, an electrical stimulus was applied to the median and ulnar nerves. Interestingly, a stimulus of 3 times twitch threshold required to induce a sustained muscle contraction for 30 s was not effective at inducing any detectable change in MAP. In addition, neither a tail pinch nor delivery of a noxious mechanical stimulus to the right forepaw (>400 g) induced any variation in MAP. In contrast, a 1-mA nerve stimulus capable of activating group III and IV muscle afferents was effective at inducing a significant increase in MAP in all conditions. However, both I/R and PenCON+I/R conditions showed an increase in MAP significantly larger than sham-injured animals. This exacerbated response was dampened in the Penα1+I/R group (Fig. 1*I*). Results collectively indicate that afferent GFRα1 up-regulation in the DRG is important in dually modulating nociception and exercise-mediated reflexes after I/R.

To test whether GFRα1 up-regulation played a role in afferent sensitization after I/R, we performed electrophysiological recordings in an ex vivo muscle, nerve, DRG, spinal cord preparation. As previously reported (16, 17), we found that group III/IV muscle afferents from I/R and PenCON+I/R mice have decreased mechanical thresholds compared with naïve animals. GFRα1 knockdown prevented observed mechanical sensitization in sensory neurons (Fig. 2 *A* and *B*). As shown in our previous work (16, 17), naïve afferents in this report were found to respond almost exclusively to either 15 mM lactate and 1 μM adenosine triphosphate (ATP), pH 7.0 ("low" metabolite mixtures) or 50 mM lactate and 5 μM ATP, pH

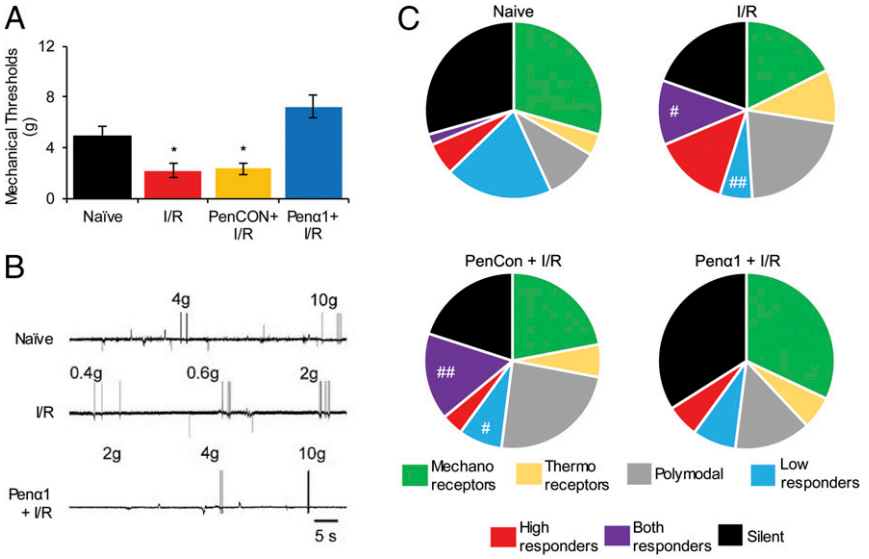

Fig. 2. I/R induces mechanical hypersensitivity in group III/IV muscle afferents and a change in prevalence of chemosensitive fibers. (*A*) Group III/IV muscle afferents showed decreased mechanical thresholds 1 d after I/R or PenCON+I/R compared with naïve. Penα1 injection prevented I/R-induced mechanical sensitization (naïve: $n = 26$, I/R: $n = 21$, PenCON+I/R: $n = 26$, Penα1+I/R: $n = 20$). (*B*) Representative traces of afferent mechanical responses from select groups. (*C*) In I/R and PenCON+I/R groups, there was a significant decrease in the total number of "low responders" and a significant increase in the number of "both responders." These changes were not observed in Penα1+I/R animals (naïve: $n = 51$, I/R: $n = 51$, PenCON+I/R: $n = 50$, Penα1+I/R: $n = 50$. One-way ANOVA, HSD post hoc (*B*) and χ^2 (*C*)). $^{*}P < 0.05$ vs. naïve and Penα1+I/R; $^{\#}P < 0.05$ vs. naïve; $^{\#\#}P < 0.01$ vs. naïve.

Table 1. Select DRG gene expression 1 d after I/R

Gene: DRGs	I/R, %
ASIC1	364 ± 15*
ASIC3	110 ± 10*
P2X3	114 ± 10*
P2X4	194 ± 10*
P2X5	70 ± 10*
GFRα1	236 ± 16*
TrkA	−23 ± 1
TrKB	−22 ± 1
TrkC	−19 ± 2
GFRα2	−5 ± 1

Values indicate percentage change vs. naïve. Mean ± SEM.
*$P < 0.05$ vs. naïve, 1-way ANOVA.

6.6 ("high" metabolite mixtures) but not both (Fig. 2*C*). I/R and PenCON+I/R groups showed an increase in the total number of afferents that responded to stimulation of the muscles with both low and high metabolite mixtures. This phenotypic change was prevented by selective GFRα1 knockdown. Surprisingly, the number of afferents recorded in Penα1+I/R mice that showed any response to metabolites tended to be lower than that observed in naïve animals (Fig. 2*C*). This suggests that GFRα1 up-regulation not only plays a role in afferent mechanosensation after I/R but is also important for chemosensory functions of group III/IV afferents.

The increased expression of GFRα1 in the affected DRG was accompanied by a significant up-regulation of various genes encoding receptors involved in sensory transduction. Similar to previous reports (16, 17), we found that ASIC1, ASIC3, and purinergic receptors P2X3, P2X4, and P2X5 were significantly up-regulated 1 d after I/R. Other receptors from the GFR family, including GFRα2 and GFRα3, were not up-regulated after I/R. The tyrosine receptor kinase (trk) family of receptors (trkA, trkB, and trkC) was also not up-regulated in the DRGs after I/R (Table 1).

We, therefore, assessed the effects of GFRα1 knockdown on up-regulated receptor expression in the DRGs after I/R. We did not find any significant difference in the expression levels between I/R and PenCON+I/R mice and thus, grouped the data for simplicity of presentation (I/R control). Penα1+I/R animals showed a significant decrease in the expression level of ASIC3 but not ASIC1 compared with I/R control animals. However, knockdown did not completely revert levels of ASIC3 to those observed in uninjured mice. Interestingly, the only purinergic channel with increased expression that was significantly blocked by selective GFRα1 knockdown after I/R was P2X5. The I/R-induced changes in P2X3 or P2X4 were not reversed by Penα1 injection (Table 2).

These latter results were corroborated by total cell counts in the DRGs where I/R and PenCON+I/R animals showed a significant increase in the total number of individual cells positive for either GFRα1 or P2X5 and the total number of neurons coexpressing GFRα1 and P2X5 (Fig. 3). Both of these increases in total number of immunopositive cells were prevented by selective knockdown of GFRα1, suggesting a direct relationship between GFRα1 and P2X5 expression after injury.

To gain better insight on whether the phenotypic alterations in chemosensitive muscle afferents induced by I/R corresponded with the expression of GFRα1 and P2X5, after electrophysiological characterization of identified muscle afferents we filled chemosensitive neurons with neurobiotin and performed immunohistochemistry in the DRG containing the labeled neuron. As shown in Fig. 3*E* and Table 3, after I/R, ∼90% (9 of 10 GFRα1+, 9 of 10 P2X5+) of the neurons that became responsive to both metabolite mixtures expressed either P2X5 or GFRα1, and 80% of these expressed both receptors (8 of 10 GFRα1+/P2X5+). In the low-responder subpopulation, only 25 to 30% of cells were positive for both receptors (1 of 4 in naïve, 1 of 3 in I/R control, and 0 of 1 in Penα1+I/R) (Table 3). These observations suggest that, while the coexpression of GFRα1 and P2X5 is not a requirement for the normal chemosensitive function of metaboreceptors, there may be a strong link between the coexpression of both GFRα1 and P2X5 and the phenotypic switch observed in chemosensitive primary muscle afferents after I/R.

P2X5 Plays a Role in Pain-Related Behaviors and Increased Exercise-Mediated Reflexes after I/R. qPCR results suggested that GFRα1 regulated the development of muscle pain-related behaviors and exacerbated exercise-mediated reflexes after I/R through modulation of the expression of ASIC3 and/or P2X5. Since we previously have shown that ASIC3 is crucial for I/R-related peripheral sensitization (17), here we assessed the role of P2X5. Using similar strategies to those described for GFRα1 knockdown, we confirmed that injection of Penetratin-linked P2X5 targeting siRNAs into the median and ulnar nerves of mice with I/R was able to completely block the I/R-induced up-regulation of this channel at the mRNA (I/R: 70 ± 10%; $P < 0.01$ vs. naïve; PenCON+I/R: 70 ± 20%; $P < 0.01$ vs. naïve; PenX5: −65 ± 36%; $P < 0.001$ vs. naïve; 1-way ANOVA with HSD post hoc) and protein levels (Fig. 4*A*). Behavioral testing revealed that animals injected with P2X5-targeting siRNAs (PenX5+I/R) had lower guarding scores than both I/R- and PenCON+I/R-treated animals, although this did not reach sham levels (Fig. 4*B*). Reduced mechanical withdrawal thresholds to VFH stimulation (Fig. 4*C*) were partially reversed by P2X5 knockdown after I/R. However, the reductions in withdrawal thresholds to forepaw muscle squeezing (Fig. 4*D*) were not significantly inhibited in the PenX5+I/R group. Grip strength deficits were partially rescued from the effect of I/R by PenX5 injection (Fig. 4*E*). Interestingly, PenX5 injection completely prevented the exacerbation of exercise-mediated reflexes after I/R, similar to that observed with Penα1 injection. Results suggest that P2X5 (in possible conjunction with other receptors, like ASIC3 [17]) plays an important role in pain-related behaviors and enhanced exercise-mediated reflexes after I/R.

Cyclic Adenosine Monophosphate Response Element Binding (CREB) Binding Protein Inhibition Prevents I/R-Induced Overexpression of P2X5 and Development of Ischemic Myalgia-Like Behaviors. Results suggested that enhanced GDNF/GFRα1 signaling increased P2X5 (and ASIC3) expression in muscle afferents to dually modulate nociception and exercise-mediated reflexes after I/R. However, we did not know how GDNF signaling influenced transcription. We, therefore, performed reverse transcription (RT) array analysis of DRGs from mice with I/R to perform an unbiased screen of several transcription factors simultaneously. Surprisingly, no transcription factors were up-regulated at the mRNA level in the DRGs 1 d after I/R using this approach (*SI Appendix*, Table S1). We, therefore, retrogradely labeled afferents from the forepaw muscles using fluorogold, dissociated the DRGs in vitro, and treated them with GDNF. Unlike our previous reports analyzing IL-1β–treated muscle afferents (17), GDNF was not found to alter the numbers of cells containing activated c-Jun–N-terminal

Table 2. Effects of GFRα1 knockdown on I/R-related gene expression in DRGs

Gene: DRGs	I/R control, %	Penα1+I/R, %
ASIC1	511 ± 11*	520 ± 17*
ASIC3	112 ± 9*	48 ± 9%†
P2X3	100 ± 14*	108 ± 4*
P2X4	386 ± 17*	232 ± 10*
P2X5	69 ± 19‡	18 ± 10
GFRα1	438 ± 31*	0 ± 19

Values indicate percentage change vs. naïve. Mean ± SEM. $n = 6$ per group; combined I/R control, $n = 12$.
*$P < 0.001$ vs. naïve; 1-way ANOVA with HSD post hoc test.
†$P < 0.01$ vs. naïve and $P < 0.001$ vs. I/R control; 1-way ANOVA with HSD post hoc test.
‡$P < 0.01$ vs. naïve; 1-way ANOVA with HSD post hoc test.

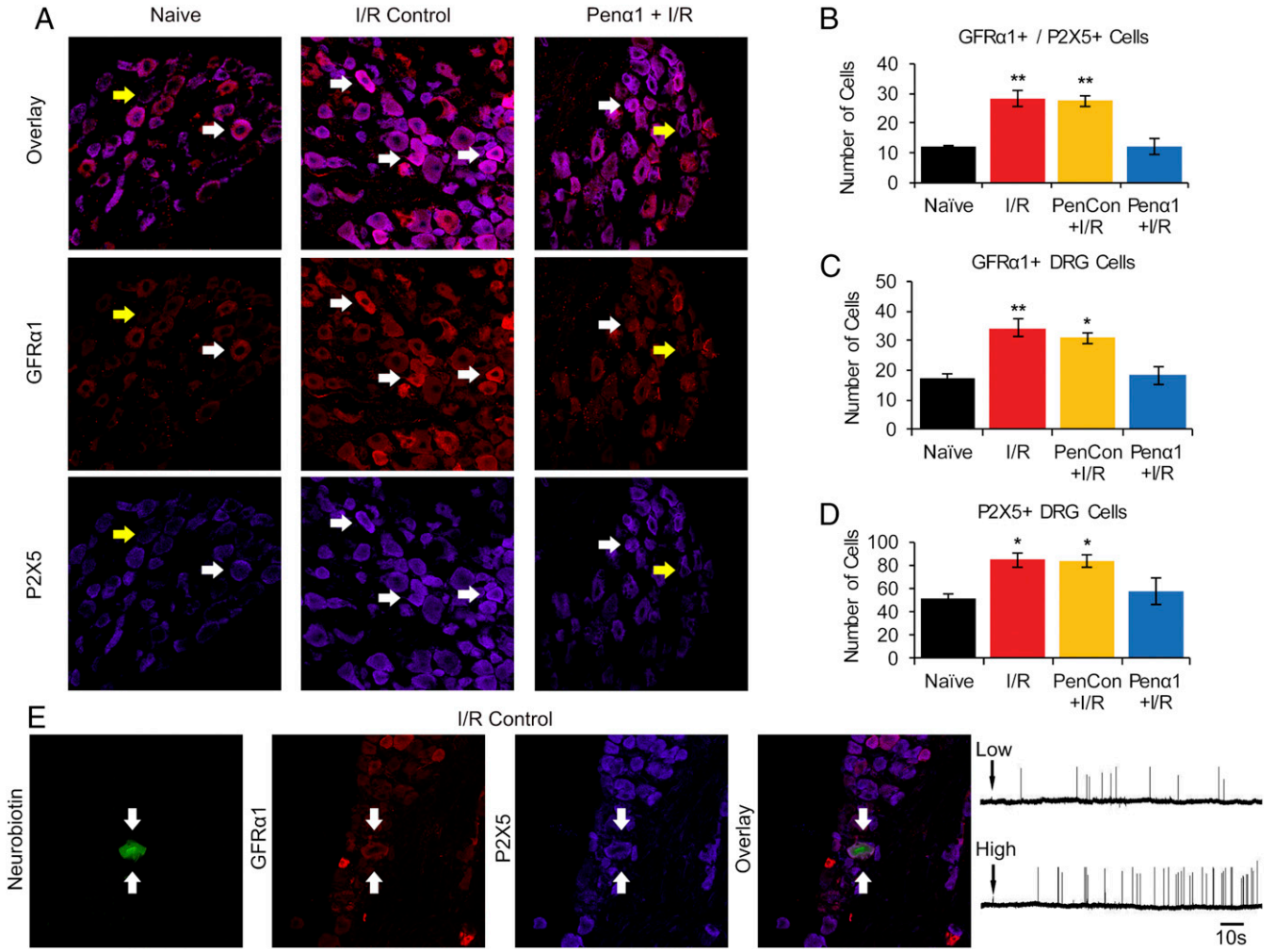

Fig. 3. I/R increases the number of cells positive for GFRα1 and P2X5. (*A*) Representative images of DRGs immunostained for GFRα1 and P2X5. Yellow arrows indicate GFRα1- cells. White arrows indicate GFRα1+ cells. (*B–D*) After I/R, there was a significant increase in the total number of cells immunopositive for both GFRα1 and P2X5 (*B*) as well as total cells positive for either GFRα1 (*C*) or P2X5 (*D*). The increase in immunopositive cells was prevented in Penα1+I/R ($n = 3$ per group). (Magnification: 20×.) One-way ANOVA, HSD post hoc. *$P < 0.05$ vs. naïve and Penα1+I/R; **$P < 0.01$ vs. naïve and Penα1+I/R. (*E*) Representative functionally identified muscle afferent obtained during ex vivo recording intracellularly filled with neurobiotin (green) was found to be immunopositive for GFRα1 (red) and P2X5 (blue) and was responsive to both low and high metabolite mixtures.

kinase. GDNF also did not increase the numbers of cells with phosphorylated mitogen-activated protein kinase (MAPK) p38 (*SI Appendix*, Fig. S1).

Since our previous reports suggested that MAPKs, such as extracellular signal-related protein kinases 1/2 (ERK1/2), were not up-regulated or activated by I/R in the DRGs (21), we performed additional WB analysis from I/R-injured animals to assess other putative transcription factors that may be activated (phosphorylated) at the protein level by increased GDNF/GFRα1 signaling. Although no changes in pERK5 were detected, we found that cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein was phosphorylated after I/R in the DRGs. However, CREB phosphorylation in the Penα1 condition, although seemingly lower than I/R, was not statistically different from I/R or naive (Fig. 5*A*). Since CREB has been linked to receptor tyrosine kinase signaling (46, 47) and these molecules execute their functions through transcription factor complexes (48–50), we decided to assess the CREB binding protein (CBP) in mice with I/R. Not only did I/R up-regulate CBP along with phosphorylation of CREB, but knockdown of GFRα1 in the DRGs after I/R prevented CBP up-regulation (Fig. 5*A*).

We, therefore, used a pharmacological approach to assess whether disruption of the CBP/CREB transcription factor complex could blunt I/R-related hypersensitivity and altered exercise-mediated reflexes. Treatment of mice with the CBP antagonist xx-650-23 not only inhibited the ischemic myalgia-like behaviors after I/R (Fig. 5 *B–F*), but it also reduced the levels of P2X5 in the DRGs (Fig. 5*G*). Results indicated that I/R-related behaviors are modulated by increases in GDNF/GFRα1 signaling through the regulation of P2X5 induction in the DRGs. The effects of GDNF/GFRα1 on P2X5 expression seem to be due, at least in part, to modulation of CREB/CBP-mediated transcription.

Targeting GDNF Directly in Injured Muscles Prevents Pain-Related Behaviors and Exacerbated Exercise-Mediated Reflexes after I/R. To test whether targeting increased levels of muscle GDNF directly could prevent ischemic myalgia-like behaviors, we then injected anti-GDNF antibodies, vehicle, or immunoglobulin G (IgG) into the affected forepaw immediately after sham or I/R and assessed pain-related behaviors and exercise-mediated reflexes 1 d after injury. Mice injected with vehicle or IgG were not found to be different from each other. However, as indicated for GFRα1, P2X5, and CBP inhibition, GDNF antibody injection into the muscles significantly inhibited I/R-induced paw guarding, mechanical

Table 3. Expression of GFRα1 and P2X5 in chemosensitive group III/IV muscle afferents

Receptor type and condition	GFRα1+	P2X5+	GFRα1+/P2X5+
Low responders			
Naïve	2/4	1/4	1/4
I/R control	1/3	3/3	1/3
I/R	0/1	1/1	0/1
PenCON+I/R	1/2	2/2	1/2
Penα1+I/R	0/1	0/1	0/1
High responders			
Naïve	1/1	1/1	1/1
I/R control	3/4	3/4	3/4
I/R	2/2	2/2	2/2
PenCON+I/R	1/2	1/2	1/2
Penα1+I/R	0/0	0/0	0/0
Both responders			
Naïve	1/1	1/1	1/1
I/R control	9/10	9/10	8/10
I/R	3/3	2/3	2/3
PenCON+I/R	6/7	7/7	6/7
Penα1+I/R	0/0	0/0	0/0

hypersensitivity, and grip strength and altered exercise-mediated reflexes compared with control antibody-injected mice with I/R (Fig. 6).

Discussion

Here, we describe a mechanism of muscle afferent sensitization after I/R injuries that regulated pain-related behaviors and exacerbated cardiovascular responses to exercise. Peripheral sensitization after I/R was modulated by increased GDNF from injured muscles acting on up-regulated GFRα1 in DRG neurons, which (among other possible players) modulated CREB/CBP-dependent transcription of P2X5 (Fig. 7).

Multiple models of musculoskeletal pain have suggested a prominent role for GDNF signaling (33–35). Various studies have shown that chemosensitive group III/IV muscle afferents are the sensory arm of the EPR and that ischemic injuries are capable of increasing their response to metabolite stimulation and muscle contraction (5, 9, 15, 51). In addition, group III/IV muscle afferents also function as nociceptors and can be sensitized by ischemic injury. Interestingly, of all combinations of GFs and their receptors up-regulated in the muscles and DRGs, only GDNF and GFRα1 were collectively induced after I/R (16) (Table 1). Selective knockdown of GFRα1 in muscle afferents partially prevented injury-related paw guarding and completely blocked the I/R-induced decrease in mechanical withdrawal thresholds to VFH stimulation and to muscle squeezing (Fig. 1).

However, GFRα1 knockdown was only partially effective in preventing the decreased grip strength observed after injury, suggesting that there are other mechanisms involved in altering specific muscle-related tasks after I/R, such as cytokine-mediated regulation of ASICs (17). Previous work showed that preventing the I/R-induced up-regulation of Interleukin 1 receptor, type 1 (IL1r1) in DRGs completely prevented ASIC3 induction and in turn, fully prevented the development of all pain-related behaviors after injury (17, 18). Studies using ASIC3 knockout mice report a lack of mechanical hyperalgesia after muscle inflammation that can be restored by expressing ASIC3 in the neurons innervating the affected tissue (22, 23). However, in this report, selective knockdown of GFRα1 fully blocked the I/R induced up-regulation of the purinergic receptor P2X5 but only partially that of ASIC3. Together, this provides a plausible explanation as to why Penα1 injection only partially blunted I/R-induced paw guarding and injury-reduced grip strength. Thus, pain-related

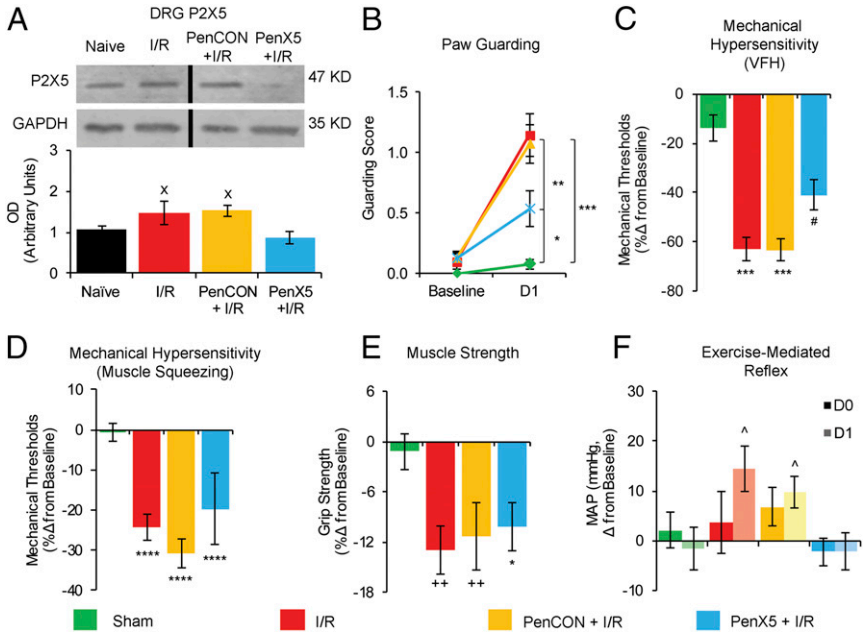

Fig. 4. Knockdown of P2X5 in muscle afferents inhibits pain-related behaviors and exacerbated exercise-mediated reflexes after I/R. (*A*) P2X5 is increased in DRGs 1 d after I/R or PenCON+I/R, and this is prevented by PenX5 injection in mice with I/R ($n = 3$ per group). OD, optical density. (*B*) Paw guarding is increased 1 d after I/R and PenCON+I/R compared with shams, and this is partially prevented in PenX5+I/R animals. (*C*) Mechanical withdrawal thresholds to VFH stimulation are decreased after I/R and PenCON+I/R, and this is partially rescued in the PenX5+I/R group. (*D*) Mechanical withdrawal thresholds to muscle squeezing are decreased after I/R, PenCON+I/R, and PenX5+I/R. (*E*) PenX5 injection in mice with I/R partially inhibited I/R-related grip strength deficits observed in I/R alone or PenCON+I/R groups. (*F*) A significant increase in MAP after exercise was observed in injured mice (I/R and PenCON+I/R), but this was not observed in the PenX5+I/R group. (*D* and *E*) $n = 12$ per group. Data for naïve, sham controls, I/R, and PenCON+I/R are similar to those presented in Fig. 1. One-way ANOVA, HSD post hoc (*A*) or 2-way repeated measures ANOVA, HSD post hoc (*B–F*). D0, pre-I/R; D1, post-I/R. $^{*}P < 0.05$ vs. sham; $^{**}P < 0.01$ vs. PenX5+I/R; $^{***}P < 0.001$ vs. sham and PenX5+I/R; $^{****}P < 0.0001$ vs. sham; $^{\#}P > 0.05$ vs. sham, I/R, and PenCON+I/R; $^{x}P < 0.05$ vs. naïve; $^{++}P < 0.01$ vs. sham; $^{\wedge}P < 0.05$ vs. preexercise MAP.

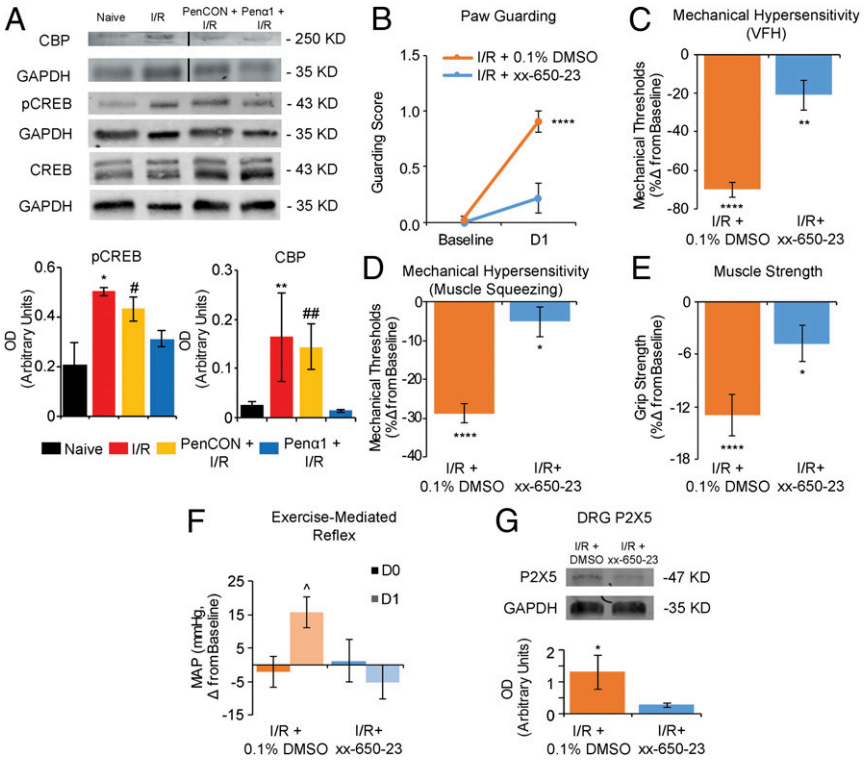

Fig. 5. Disruption of the CBP/CREB transcription factor complex regulates I/R-related pain-like behaviors, exercise-mediated reflexes, and P2X5 expression. (*A*) pCREB and CBP are increased after I/R compared with naïve as assessed with WB. Penα1 injection partially reduced the I/R-provoked up-regulation of pCREB and fully inhibited the up-regulation of CBP in DRGs. $n = 4$ to 7 per group. $^{*}P < 0.05$ vs. naïve but $P < 0.13$ vs. Penα1; $^{**}P < 0.05$ vs. naïve and Penα1; $^{\#}P < 0.08$ vs. naïve but $P < 0.4$ vs. I/R; $^{\#\#}P < 0.14$ vs. naïve and Penα1 but $P < 0.9$ vs. I/R. OD, optical density. (*B*) Paw guarding is increased 1 d after I/R in vehicle-treated (0.1% DMSO) animals, and this is prevented by treatment with xx-650-23. Mechanical withdrawal thresholds to VFH stimulation (*C*) and muscle squeezing (*D*) are significantly decreased after I/R+0.1% DMSO and I/R+xx-650-23. However, both of these behavioral effects were significantly blunted in xx-650-23–treated animals. (*E*) Decreased grip strength observed 1 d after I/R+0.1% DMSO was partially rescued by xx-650-23. (*F*) Exacerbated exercise-induced MAP after I/R 0.1% DMSO was blocked by xx-650-23. (*B–F*) $n = 10$ per group. $^{*}P < 0.05$ vs. baseline; $^{**}P < 0.01$ vs. baseline; $^{****}P < 0.0001$ vs. baseline; $^{\wedge}P < 0.05$ vs. preexercise MAP. (*G*) xx-650-23 significantly reduced the levels of P2X5 in the DRGs of mice with I/R compared with I/R+0.1% DMSO-treated animals. (*G*) $n = 3$ per group. One-way ANOVA, HSD post hoc (*A* and *G*) or 2-way repeated measures ANOVA, HSD post hoc (*B–F*). $^{*}P < 0.05$ vs. I/R+xx-650-23.

behaviors and altered exercise-mediated reflexes from I/R are due to activation of several signaling pathways that likely includes both cytokine and GF signaling mechanisms.

GFRα1 signaling appeared to play a role in the development of primary afferent mechanical hypersensitivity after I/R as GFRα1 knockdown blocked I/R-induced reductions in group III/IV muscle afferent thresholds (Fig. 2). We also observed a phenotypic switch from observing mostly 2 mutually exclusive subpopulations of chemosensitive fibers, metaboreceptors (low metabolite responders) or metabonociceptors (high metabolite responders), to a subpopulation that became responsive to both low and high metabolite concentrations. This finding confirms our previous reports of a phenotypic switch after I/R (16, 17), but in contrast with this earlier study, selective knockdown of GFRα1 after I/R did not revert the phenotypic switch. Instead, it vastly decreased the numbers of detectable chemosensitive neurons. Although we did not assess cell death in these studies, results suggest that GFRα1 signaling, especially after I/R, plays an important role in the maintenance of peripheral chemosensitivity.

Results could be explained by the downstream effects on P2X5 (Figs. 3–5), a key player in the chemosensitive function of muscle afferents (8, 20). P2X5 has been shown to modulate the pH sensitivity of ASIC3 in vitro (20). Previous work also showed that exposure to a combination of metabolite stimuli is necessary to effectively activate chemosensitive afferents (1, 8). Thus, it is reasonable to suggest that disrupting one of the components of the chemosensitive apparatus could render the primary neurons incapable of responding to a chemical stimulus (depending on the concentrations or combinations of metabolites present) (8). This could also explain why Penα1+I/R mice and PenX5+I/R mice do not exhibit increased exercise-mediated reflexes after injury as chemosensitivity of group III/IV muscle afferents is a key component of the cardiovascular response to exercise (6, 9, 12, 25, 52, 53). Nevertheless, regulation of P2X5 expression via CREB/CBP-dependent transcription (Fig. 6) may be a key means through which GFRα1 modulated muscle afferent sensitization after I/R.

An important consideration is that not all chemosensitive neurons are the same regarding GFRα1 or P2X5. Our neurochemical analysis of functionally identified chemosensitive neurons shows that metaboreceptors (low metabolite responders) may only partially rely on GFRα1 or P2X5 as only ~33 to 25% (Table 2) of these cells coexpressed both receptors. In contrast, metabonociceptors (high metabolite responders) often express both GFRα1 and P2X5 (Fig. 3 and Table 3). After I/R, the new subpopulation of afferents that responds to both metabolite concentrations almost always coexpresses GFRα1 and P2X5, reinforcing the idea that enhanced coexpression of both receptors is important in the observed phenotypic switch in chemosensitive neurons after I/R.

The behavioral experiments on P2X5-targeted knockdowns somewhat support this notion as the guarding scores on PenX5+I/R mice almost perfectly mimic those observed in the Penα1+I/R group. Yet, both mechanical hypersensitivity and muscle function were not rescued by PenX5, suggesting that this aspect of afferent sensitization may be explained by concurrent cytokine/ASIC signaling (17). Nevertheless, P2X5 knockdown in I/R-affected afferents was quite effective in preventing exacerbated exercise-mediated reflexes 1 d after injury, highlighting the importance P2X5 in modulating the chemosensitive function of muscle afferents. This

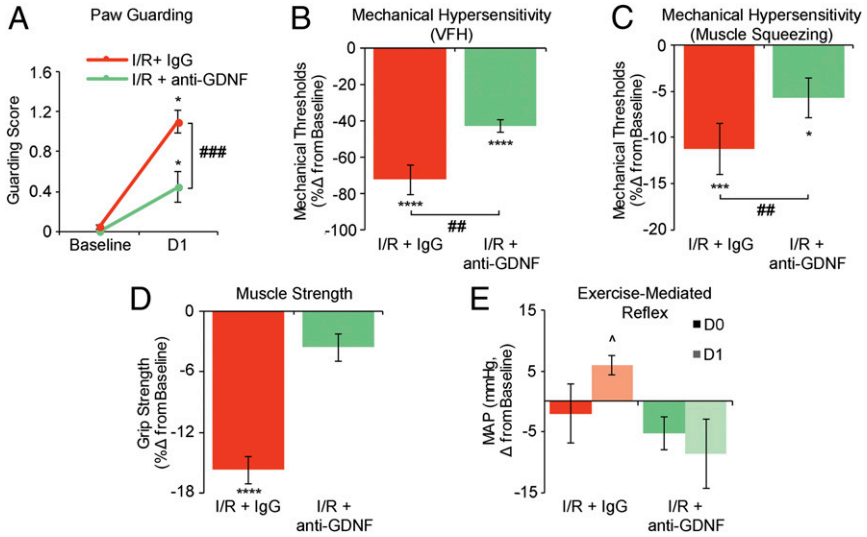

Fig. 6. Intramuscular injections of anti-GDNF antibody partially prevent pain-related behaviors and enhanced exercise-mediated reflexes after I/R. (*A*) Paw guarding is increased 1 d after I/R+IgG, and this is partially prevented by treatment with anti-GDNF at the time of injury. (*B*) Mechanical withdrawal thresholds to VFH stimulation are significantly decreased after both I/R+IgG and I/R+anti-GDNF. (*C*) Mechanical withdrawal thresholds to muscle squeezing are also decreased after I/R+IgG and I/R+anti-GDNF; however, anti-GDNF partially reversed I/R-related effects on muscle hypersensitivity. (*D*) Muscle strength was decreased 1 d after I/R+IgG, but anti-GDNF injection prevented the reduction in grip strength. (*E*) Changes in MAP after forced running were unaffected by IgG injection in mice with I/R, but anti-GDNF–treated animals did not show any changes in postexercise MAP. D0, pre-I/R; D1, post-I/R. (*A–E*) $n = 10$ per group. Two-way repeated measures ANOVA, HSD post hoc. $^{*}P < 0.05$ vs. baseline; $^{***}P < 0.001$ vs. baseline; $^{****}P < 0.0001$ vs. baseline; $^{\#\#}P < 0.01$ vs. I/R+anti-GDNF; $^{\#\#\#}P < 0.01$ vs. I/R+anti-GDNF; $^{\wedge}P < 0.05$ vs. preexercise MAP.

complements previous in vitro reports that showed that P2X5 can modulate ASIC3 pH sensitivity and could explain the increased behavioral and cardiovascular responses observed after injury here (20). Future studies are needed to assess afferent sensitization in mice with I/R and P2X5 knockdown.

The origin of the observed increase in muscle GDNF levels after I/R is still up for debate. Our results suggested that GDNF could come directly from the I/R-affected myofibers (Fig. 1). Previous studies support this notion in that skeletal myofibers themselves are capable of releasing GDNF (54, 55). The immunohistochemistry performed in injured muscles also shows a distinct "halo-like" pattern around the injured muscle cells that highly resembles previous reports, hinting that GDNF could originate in myocytes (55). While the effect of GFRα1 up-regulation in the DRG after I/R seems to be an important player in injury-related hypersensitivity, the cause of increased receptor expression was not elucidated.

The observed increase in MAP after I/R resembles previous reports in other models of ischemic insult (14, 15, 53, 56, 57). However, in this report, unilaterally targeting a neurotrophic factor or its receptor prevented the development of exacerbated exercise-mediated reflexes postinjury. While we were able to detect an exacerbated MAP after I/R, we did not observe significant changes in HR after exercise before or after injury. This could be due to a fast recuperation of HR after exercise compared with the observed MAP changes. Nonetheless, the fact that our strategies targeting GDNF/GFRα1 were effective at preventing the exacerbated exercise-mediated reflexes after I/R reveals how important this signaling pathway may be for the sensitization of muscle afferents and subsequent effects on cardiovascular reflexes or nociceptive-like responses.

One important consideration is that sensory neurons, which regulate both the nociceptive as well as the exercise-mediated reflex circuits, are affected by the increased muscle GDNF observed after I/R. Indeed, both biological phenomena seem to be heavily interconnected at least in the basic molecular mechanisms that are shared between them, such as P2X receptors and possibly, ASICs. This characteristic highlights the difficulty of studying one phenomenon independently from the other. However, our data clearly indicate that, in anesthetized animals that would not experience "pain," that group III/IV afferent stimulation is needed to observe a change in MAP (Fig. 1*I*).

Clinical Significance

Patients who experience pathologies characterized by musculoskeletal ischemic injury, such as sickle cell disease, PVD, or complex regional pain syndrome, often present both pain and altered autonomic vasomotor responses to tissue ischemia (37, 58–60). Our data suggest that muscle GDNF combined with GFRα1 up-regulation can sensitize muscle primary afferents after I/R. To determine whether targeting this pathway was a potential therapeutic for I/R-related hypersensitivity, we targeted GDNF at the source by injecting antibodies into the I/R-injured muscles, similar to strategies used in other disease models (35, 61, 62). Our results highlight the potential therapeutic value of locally blocking this pathway in ischemic injuries as our mice displayed reduced pain-related behaviors and exercise-mediated reflexes after I/R upon intramuscular injection of GDNF-targeting antibodies (Fig. 7). While these findings still need to be confirmed in human tissues, our results suggest that reducing GDNF signaling may be an option to treat patients who suffer musculoskeletal ischemic injuries and that this strategy may not only alleviate their pain but also, prevent cardiovascular complications associated with injury.

Methods

Animals. Experiments were conducted with young adult (3 to 8 wk) male Swiss Webster mice housed in a barrier facility that were maintained on a 12:12-h light–dark cycle with a temperature-controlled environment and given food and water ad libitum. All procedures were approved by the Cincinnati Children's Hospital Institutional Animal Care and Use Committee and adhered to NIH Standards of Animal Care and Use (63) under the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved practices.

I/R Injury and Interventions. I/R was induced as previously described (16). Except for the baseline behavioral time points, all assessments were performed 18 h after the reperfusion procedure. Specific targeting siRNAs were used to selectively knock down the expression of either the GDNF receptor, GFRα1, or the ATP receptor, P2X5 (Thermo), and compared with control, nontargeting siRNAs in identified groups. siRNAs were conjugated to Penetratin-1 (MP

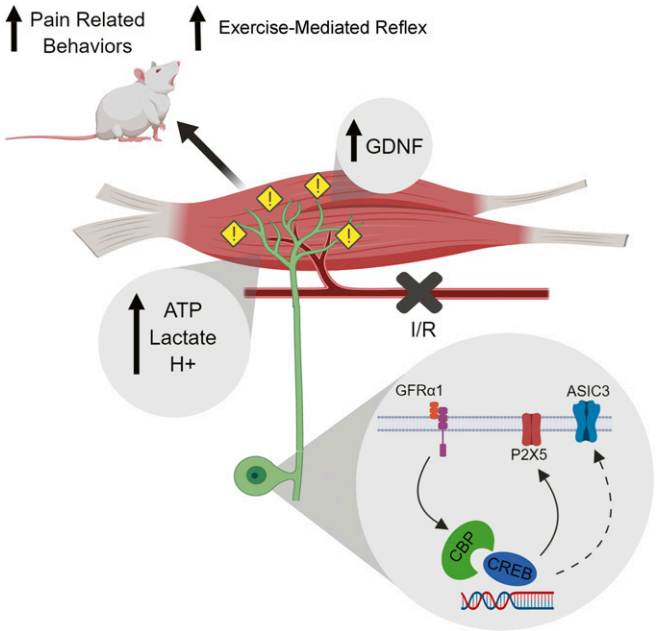

Fig. 7. A role for peripheral GDNF signaling in the development of ischemic myalgia. After I/R, injured muscles release a variety of metabolites including ATP, lactate, and protons that stimulate group III/IV muscle afferents. Simultaneously, GDNF is released in the injured tissues that induces a signaling cascade in sensory neurons through its receptor GFRα1 involving increased function of CREB/CBP, which in turn, modulates expression of ATP-sensitive channel P2X5 and to a lesser degree, ASIC3. This molecular change consequently modulates the appearance of pain-related behaviors and affects the exercise-mediated reflex after injury. Targeting the overexpression of P2X5 or GFRα1 in sensory neurons or the increased GDNF within the muscle appears to inhibit many of these ischemic myalgia-like phenomena.

Biomedicals) prior to injection into the median and ulnar nerves 2 d before I/R (14, 44, 45, 64). Other cohorts of mice (with or without I/R) were injected with 10 μg of anti-GDNF, antibody (Alomone), IgG (R&D), or vehicle (sterile water) in a volume of 5 μL into the right forepaw muscles. Antibody dose was determined from Murase et al. (35). Compound xx-650-23 was used to block the interaction between CREB and CBP; 20 mg/kg of xx-650-23 (Glixx) or an equivalent volume of vehicle (0.1% dimethyl sulfoxide [DMSO]) was injected intraperitoneally in <200 μL immediately after occlusion surgery under isoflurane.

Pain-Related Behaviors and Cardiovascular Assessments. Separate groups of sham, I/R, siRNA+I/R, local antibody injection+I/R, or antagonist+I/R were used for behavioral analysis ($n = 8$ to 12 per group). Testing of pain-related behaviors was performed as previously described by Ross et al. (16). Separate cohorts of mice were tested using a digital Randall–Sellito device (IITC) to assess withdrawal thresholds to muscle squeezing (65, 66).

The exercise-mediated reflex was determined using a low-intensity forced run protocol on a modular treadmill (Columbus Instruments) at 0° inclination based on previous work (14, 67, 68) (ramp speed: 9 m/min to 13 m/min; distance: 500 m). This protocol is well below the speed and distance previously reported to induce anaerobic metabolism in the muscle or tissue damage and has not been shown to alter pain-related hypersensitivity due to ischemic injury (14, 69, 70). Blood pressure and HR were then measured using a tail cuff system (Kent Scientific), and MAP reported.

To measure central cardiovascular responses, under isoflurane (3%), a 1.2F pressure catheter (Transonic) was inserted into the carotid artery and advanced toward the thoracic cavity. Anesthesia was reduced to 1.5% for recording. To trigger cardiovascular reflexes, the median and ulnar nerves were stimulated with bipolar electrodes (30 s, 20 Hz, 0.01-ms pulses) first at 3× twitch threshold (variable between animals but never more than 250 μA) and then, at 1 mA. After electrical stimulation, a mechanical stimulus using a Randall–Sellito device with blunt probe was delivered at ∼400 g and held for ∼5 s followed by tail pinch. HR, left ventricular pressure, and systolic and diastolic pressures were captured using chart software (ADInstruments; $n = 4$ per group).

Ex Vivo Recording Preparation. Ex vivo recording was performed according to Jankowski et al. (1) and Ross and coworkers (14). After dissection, the preparation was transferred to a recording chamber containing oxygenated artificial cerebrospinal fluid. The forepaw was pinned on an elevated platform, keeping the entire paw perfused in a chamber isolated from the DRGs (C7-T1) and the spinal cord. The bath was warmed to 32 °C. Intracellular single-unit recordings were made from the C7/C8/T1 DRGs. Peripheral receptive fields (RFs) in the muscles were localized by electrically stimulating the muscles with a concentric bipolar electrode. Only driven cells with RFs in the muscles then underwent mechanical, thermal, and chemical testing.

Immunohistochemistry. DRGs containing neurobiotin-filled cells from ex vivo recording were immersion fixed with 3% paraformaldehyde in 0.1 M phosphate buffer, embedded in optimal cutting temperature medium, sectioned (15 μm) on a cryostat, and processed for GFRα1 (R&D) or P2X5 (Alomone). Forepaw muscles were processed similarly for GDNF (Alomone). Appropriate secondary antibodies (Thermo) as well as FITC-Avidin (Vector) to label neurobiotin-filled cells were then used prior to mounting with Fluoromount G (EMS). Sections were imaged on a Nikon confocal microscope. Total cells containing GFRα1 and P2X5 were quantified using Neurolucida software in C7 or C8 DRGs ($n = 3$ per condition) using a slightly modified methodology as previously reported by Christianson et al. (71) and Jankowski et al. (42).

Western Blotting. Protein from C7-T1 DRGs ($n = 3$ per condition) was isolated, separated by polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes according to previous procedures (14). Membranes were processed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Pro-Sci), GFRα1 (R&D), P2X5 (Alomone), GDNF (Alomone), CREB (Abcam), pCREB (Abcam), or CBP (Abcam). After primary antibody incubation, membranes were washed and incubated with the appropriate infrared-conjugated secondaries (LiCor). Immunoreactive bands were imaged using an LICOR Odyssey laser scanner and analyzed using NIH ImageJ.

Real-Time PCR and Transcription Factor Arrays. DRGs (C7-T1) were collected ($n = 3$ per condition) and RNA isolated using Qiagen RNeasy kits according to the manufacturer's protocol. Appropriate complimentary DNA was generated and either RT^2 Profiler PCR Arrays (Mouse Signal Transduction Pathway Finder; Qiagen) or real-time PCR reactions were performed using a Step-One real-time PCR machine (Applied Biosystems). Cycle time (Ct) values for all targets were all normalized to a GAPDH internal control, and fold change was determined as $2^{\Delta\Delta Ct}$ (Applied Biosystems). Values were converted and reported as a percentage change, where 2-fold change = 100% change.

Statistics. All datasets were analyzed using Prism statistical software. Data were first assessed for normality and equal variance prior to performing the indicated statistical tests. Data are represented as mean ± SEM. Significance was defined with $P \leq 0.05$.

SI Appendix contains detailed methods. All data discussed in the paper will be made available on reasonable request.

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