Inflammatory and neuropathic cold allodynia are selectively mediated by the neurotrophic factor receptor GFRα3

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Tissue injury prompts the release of a number of proalgesic molecules that induce acute and chronic pain by sensitizing pain-sensing neurons (nociceptors) to heat and mechanical stimuli. In contrast, many proalgesics have no effect on cold sensitivity or can inhibit cold-sensitive neurons and diminish cooling-mediated pain relief (analgesia). Nonetheless, cold pain (allodynia) is prevalent in many inflammatory and neuropathic pain settings, with little known of the mechanisms promoting pain vs. those dampening analgesia. Here, we show that cold allodynia induced by inflammation, nerve injury, and chemotherapeutics is abolished in mice lacking the neurotrophic factor receptor glial cell line-derived neurotrophic factor family of receptors-α3 (GFRα3). Furthermore, established cold allodynia is blocked in animals treated with neutralizing antibodies against the GFRα3 ligand, artemin. In contrast, heat and mechanical pain are unchanged, and results show that, in striking contrast to the redundant mechanisms sensitizing other modalities after an insult, cold allodynia is mediated exclusively by a single molecular pathway, suggesting that artemin–GFRα3 signaling can be targeted to selectively treat cold pain.

Gfrα3 | artemin | cold | pain | allodynia

When pain continues past its usefulness as a warning of potential tissue damage, it becomes a debilitating condition for which few viable treatments are currently available. The result can be an exacerbation of pain in response to both innocuous (allodynia) and noxious (hyperalgesia) stimuli (1). For example, pain felt with normally pleasant mild cooling (cold allodynia) occurs in many pathological conditions, such as fibromyalgia, multiple sclerosis, stroke, and chemotherapeutic-induced polyneuropathy, but what underlies this specific form of pain at the cellular or molecular level is largely unknown (2–5). Pain-sensing afferent neurons (nociceptors) are sensitized during injury or disease, in part, by a vast array of proalgesic compounds termed the "inflammatory soup" (e.g., neurotrophic factors, protons, bradykinin, prostaglandins, and ATP) (1). These substances are released locally at the site of injury by infiltrating immune cells, such as macrophages, neutrophils, and T cells, as well as resident cells, including keratinocytes and mast cells (6), and either directly activate sensory receptors or sensitize them to subsequent stimuli (7). Moreover, prolonged inflammation can lead to central sensitization (in the spinal cord and brain) and bring about long-lasting chronic pain that persists after acute inflammation has resolved. Thus, a better understanding of the molecules involved in neuroinflammation may lead to therapeutic options for acute and chronic pain.

Of the range of proalgesics known to promote pain, only nerve growth factor (NGF) and the glial cell line-derived neurotrophic factor family ligand (GFL) artemin have been shown to lead to cold hypersensitivity (8–11). Both are major components of the inflammatory soup and produce nociceptor sensitization and pain through their cognate cell surface receptors. NGF, the classical proalgesic neurotrophic factor, leads to thermal and mechanical sensitization directly through its receptor tyrosine kinase TrkA expressed on nociceptors and indirectly through the activation of peripheral cells (12). Glial cell line-derived neurotrophic factor family of receptors-α (GFRαs) are typically coupled to the receptor tyrosine kinase Ret (1). However, GFRαs are more widely expressed than Ret, and Ret-independent GFL-induced neuronal sensitization has been reported, suggesting that these receptors may signal through additional transmembrane proteins (13–16).

Here, we show that cold allodynia induced by inflammation, nerve injury, or chemotherapeutics is completely abolished in mice null for GFRα3 (*Gfrα3*$^{-/-}$). In contrast, heat and mechanical hyperalgesia are unaltered in *Gfrα3*$^{-/-}$ mice, indicating that GFRα3 has a limited role in pain associated with these sensory modalities but predominates the potentiation of cold sensitivity after injury. This specificity strongly suggests that therapeutic interventions into cold allodynia should focus on artemin–GFRα3 signaling. Indeed, we find that pathological cold pain alone is ameliorated in animals treated with artemin-neutralizing antibodies. These results show that cold allodynia is mediated exclusively by artemin–GFRα3 signaling and that blocking this pathway is a viable treatment option for cold pain.

Results

Previously, we showed that intraplantar hind paw injections of artemin or NGF induce a robust and transient TRPM8-dependent cold allodynia (8). The NGF/TrkA signaling pathways and their requirement in sensory neuron development and sensitization are well-established (1), but how GFRα receptors induce sensory neuron sensitization is poorly understood. Therefore, to determine how artemin leads to cold pain, we first examined acute sensitivity of mice lacking the artemin receptor GFRα3 (*Gfrα3*$^{-/-}$) to thermal or mechanical stimuli, which to the best of our knowledge, has not been reported for these animals (17, 18). Using the cold plantar (19), von Frey (mechanical), and Hargreaves (radiant heat) assays, we compared thermal and mechanically evoked behaviors of WT and *Gfrα3*$^{-/-}$ mouse littermates, finding no differences between the two genotypes (Fig.

Significance

There are few effective treatments for chronic cold pain induced by tissue damage, nerve injury, or chemotherapeutic polyneuropathies. Here, we show that the specific artemin receptor, glial cell line-derived neurotrophic factor family of receptors-α3, is absolutely required for injury-induced cold pain, and we show results of a specific transduction pathway that can be targeted selectively to treat cold pain.

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S1 *A–C*) ($P > 0.05$). These data show that acute nociceptive behaviors are not altered in GFRα3-deficient mice.

Among the four distinct GFL α-receptor subtypes (GFRα), artemin has been reported to be highly selective for GFRα3 (20) but has also been suggested to cross-react with other GFL receptors (21). Therefore, to determine if artemin's effects on cold sensitivity are GFRα3-specific, we examined cold sensitivity after intraplantar artemin injections in both WT and *Gfrα3*$^{-/-}$ mice. In the WTs, the latency to a paw withdrawal from a radiant cold stimulus using the cold plantar assay was significantly decreased at 1 and 3 h after artemin injection (Fig. S1*D*) ($P < 0.001$ at 1 h vs. basal or vehicle-injected; $P < 0.01$ at 3 h). However, consistent with this ligand's selectivity for GFRα3 (20), hind paw injections of artemin failed to alter cold sensitivity in *Gfrα3*$^{-/-}$ mice (Fig. S1*E*) ($P > 0.05$). Similar results were observed in *Gfrα3*$^{-/-}$ mice using the evaporative cooling assay (Fig. S1*F*), showing that artemin-induced cold hypersensitivity is GFRα3-dependent.

Next, to test the role of GFRα3 in pathological cold pain, we examined adult WT and *Gfrα3*$^{-/-}$ littermates in classical models of inflammation, nerve injury, and chemotherapeutic-induced neuropathic pain (22, 23). WT mice show robust cold allodynia 2 d after unilateral injections of the inflammatory agent complete Freund's adjuvant (CFA) (Fig. 1*A*) ($P < 0.01$, pre- vs. post-CFA or ipsilateral vs. contralateral), which we and others have previously reported (22–24). In contrast, *Gfrα3*$^{-/-}$ mice show no differences in their hind paw lift latencies between the ipsilateral (inflamed) and the contralateral (control) sides, and there were no differences in their sensitivity compared with the basal, pre-inflamed state (Fig. 1*A*) ($P > 0.05$). To determine the general nature of this inability of *Gfrα3*$^{-/-}$ mice to mount a cold allodynic response after injury, we also examined animals with neuropathic pain caused by chronic constriction injury (CCI) of the sciatic nerve (25). As with inflammation, cold allodynia was observed in WT animals (Fig. 1*B*) ($P < 0.01$, preinjury vs. 7 d postinjury; $P < 0.001$, ipsilateral vs. contralateral), but cold sensitivity was remarkably unchanged in *Gfrα3*$^{-/-}$ mice (ipsilateral vs. contralateral; preinjury vs. 7 d postinjury; $P > 0.05$). Lastly, one of the major side effects of platin-based chemotherapeutics is cold pain (26), a phenotype that can be modeled in mice given a single systemic injection of oxaliplatin (22, 23). As with the previous pain models, the cold allodynia observed in WT mice ($P < 0.001$, basal vs. 7 d postinjection) was completely absent in mice null for GFRα3 (Fig. 1*C*) ($P > 0.05$, pre- vs. postinjection and *Gfrα3*$^{-/-}$ postinjection vs. WT mice preinjection). We observed similar results in all three pathological pain models when cold sensitivity was determined by evaporative cooling (Fig. S2).

We asked how specific the role of GFRα3 signaling is for cold pain vs. other pain modalities. To address this question, we examined mechanical- and radiant heat-evoked responses in WT and *Gfrα3*$^{-/-}$ mice in the three pain models tested previously. In striking contrast to cold-evoked behaviors, we observed robust mechanical hyperalgesia in both WT and *Gfrα3*$^{-/-}$ mice in the context of inflammation (Fig. 2*A*) ($P < 0.001$, pre- vs. post-CFA or ipsilateral vs. contralateral), with nerve injury (Fig. 2*B*) ($P < 0.01$, pre- vs. postinjury; $P < 0.001$, ipsilateral vs. contralateral), and with oxaliplatin-induced polyneuropathy (Fig. S3) ($P < 0.001$, basal vs. postinjection). Next, we examined heat hyperalgesia in both the CFA inflammatory and CCI neuropathic pain models (oxaliplatin does not induce heat hyperalgesia). As with mechanical pain, we observed strong heat hyperalgesia in both WT and Gfrα3$^{-/-}$ mice with inflammation (Fig. 2*C*) ($P < 0.05$ and $P < 0.01$, pre- vs. post-CFA for WT and *Gfrα3*$^{-/-}$ mice, respectively; $P < 0.001$, ipsilateral vs. contralateral) or irritation of the sciatic nerve (Fig. 2*D*) ($P < 0.01$, pre- vs. postinjury; $P < 0.001$, ipsilateral vs. contralateral). Furthermore, there was no difference between the levels of mechanical and heat hyperalgesia between the two genotypes ($P > 0.05$), showing that GFRα3 is not absolutely required for heat and mechanical pain, such as it is for cold. These remarkable results show that, unlike the redundant nature of heat or mechanical sensitization, which is mediated by several algogenic receptors (1), injury-evoked cold allodynia, both inflammatory and neuropathic, requires the artemin receptor GFRα3.

Experimentally induced overexpression of artemin in peripheral tissues has been shown to lead to heat and mechanical

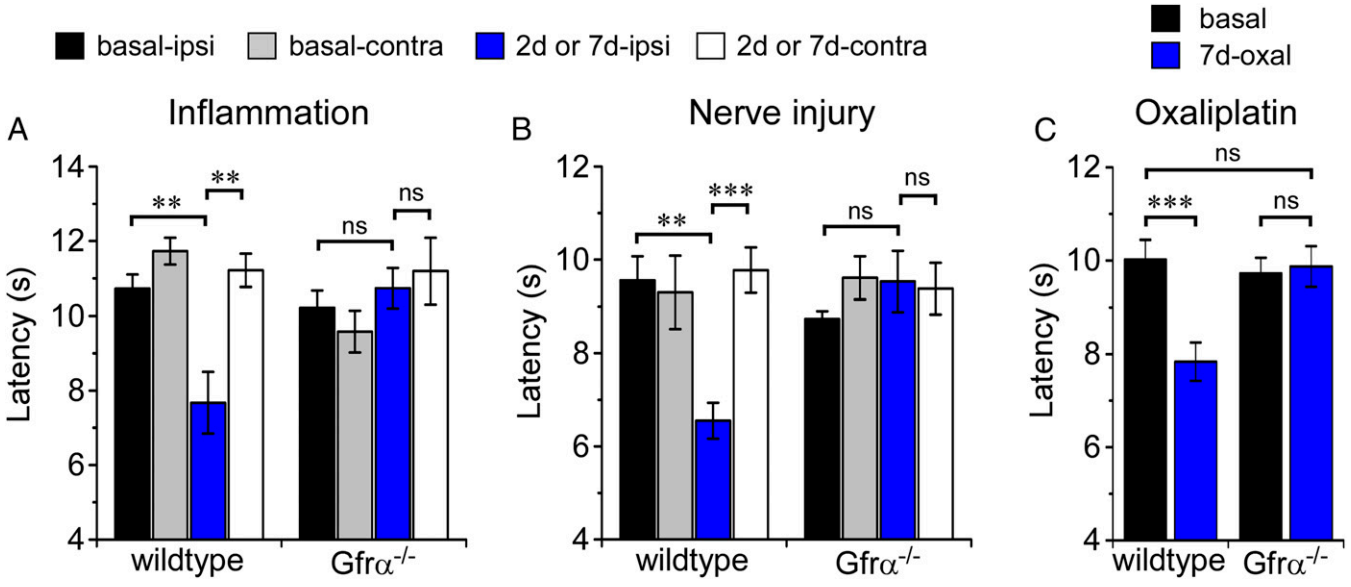

Fig. 1. GFRα3 is required for cold allodynia induced by inflammation, nerve injury, and chemotherapy polyneuropathy. (*A*) Decreased cold-evoked withdrawal latencies in WT but not *Gfrα3*$^{-/-}$ mice 2 d after an intraplantar injection of CFA. Post-CFA latencies for *Gfrα3*$^{-/-}$ mice were not statistically different ($P > 0.05$) than basal. $^{**}P < 0.01$ ($n = 7$–9). (*B*) Cold allodynia observed in the ipsilateral hind paw in WT mice after CCI was absent in *Gfrα3*$^{-/-}$ mice with postinjury withdrawal latencies identical to preinjury times ($P > 0.05$; $n = 6$–7). $^{**}P < 0.01$; $^{***}P < 0.001$. (*C*) Oxaliplatin-induced decreases in withdrawal latencies to cold observed in WT controls were absent in *Gfrα3*$^{-/-}$ mice, with response times the same as preinjection times for both genotypes ($P > 0.05$; $n = 11$–12). contr, Contralateral; ipsi, ipsilateral; ns, not significant; oxal, oxaliplatin. $^{***}P < 0.001$.

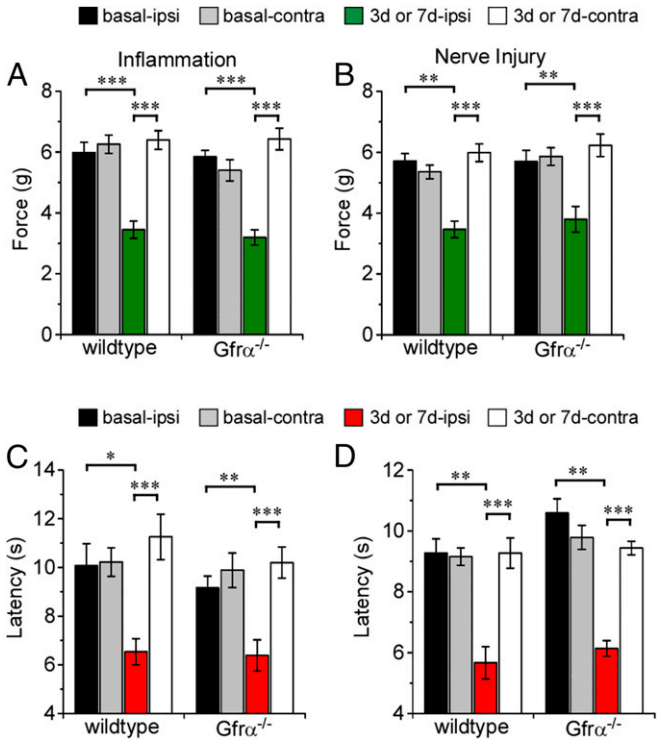

Fig. 2. Heat and mechanical hyperalgesia are not dependent on GFRα3. Both WT and *Gfrα3*$^{-/-}$ mice exhibit reduced threshold forces inducing a paw withdrawal (*A*) 3 d after unilateral CFA injection or (*B*) 7 d after CCI surgery (ipsilateral vs. contralateral; n = 6–7). Similarly, heat hyperalgesia was observed (*C*) 3 d after the induction of inflammation or (*D*) 7 d after nerve injury (ipsilateral vs. contralateral; n = 7–8). contr, Contralateral; ipsi, ipsilateral. *P < 0.05; **P < 0.01; ***P < 0.001.

hyperalgesia as well as altered expression of molecules involved in sensory transduction (27, 28). However, these studies involved either genetically induced artemin overexpression in the periphery (27) or multiple plantar injections of exogenous artemin (28), making it unclear if artemin–GFRα3 signaling influences afferent expression phenotypes under physiological conditions. Thus, to determine if the lack of a cold allodynic phenotype in *Gfrα3*$^{-/-}$ mice is a result of alterations in sensory afferent development caused by the absence of GFRα3, we used quantitative PCR to determine expression of array markers involved in cold thermosensation (29). In adult dorsal root ganglia (DRG) (Fig. S4*A*) and trigeminal neurons (Fig. S4*B*), we observed no differences (P > 0.05) in transcript expression of either known thermosensory receptors (*Trpm8*, *Trpa1*, or *Trpv1*) or channels implicated in excitability of thermosensory afferents (*Nav1.8*, *Nav1.6*, *Task3*, *Traak*, and *Trek1*) in WT and *Gfrα3*$^{-/-}$ mice (30).

Next, using immunohistochemistry, we examined the protein expression phenotype of *Gfrα3*$^{-/-}$ mice, first establishing that immunoreactivity for GFRα3 was absent in adult L4–L6 DRG from these animals (Fig. S5). TRPM8 is the principle cold thermoreceptor in mammals, and we have shown that it is required for artemin-mediated cold allodynia (8, 31). GFRα3 is expressed in one-half of TRPM8^{+} DRG neurons (8), but consistent with our transcript expression analysis, we observed no difference in the number of TRPM8-positive neurons between WT and *Gfrα3*$^{-/-}$ mice (Fig. S5*F*) (P > 0.05). GFRα3 is found exclusively in TRPV1-positive afferents (27, 32), but we observed no difference in TRPV1 expression in *Gfrα3*$^{-/-}$ mice (Fig. S5 *A* and *F*). Moreover, the numbers of neurons immunoreactive to antibodies to calcitonin gene-related peptide, a marker of peptidergic nociceptors (Fig. S5 *B* and *F*), and bound to the nonpeptidergic neuronal marker isolectin IB4 (Fig. S5 *C* and *F*) were similar. There was also no difference in fiber-type distribution, because the numbers of neurons labeled for the A-fiber marker NF200 (Fig. S5 *D* and *F*) and the C-fiber marker peripherin (Fig. S5 *E* and *F*) were similar between genotypes. These results show that the development of DRG neurons is not influenced by GFRα3 signaling, results consistent with prior analyses of these mice as well as those lacking artemin expression (17, 18).

Our results suggest that cold allodynia is specifically mediated by artemin signaling through its receptor GFRα3, which likely functions upstream of molecules involved in cold transduction, highlighting what seems to be a highly specific pathway leading to cold pain. Because of this extraordinary specificity, we hypothesized that in vivo artemin neutralization in mouse models of inflammatory and neuropathic pain could selectively block cold allodynia, even that which is localized at or near a site of injury, whereas heat and mechanical pain would remain intact. Artemin-neutralizing antibodies are known to effectively inhibit binding of artemin with GFRα3 in vivo and serve as a potential pharmacological mechanism to ameliorate or prevent the effects of artemin exposure (33–35). Therefore, we tested whether a systemic injection of an established artemin-neutralizing mAb could reverse inflammatory and neuropathic pain. Remarkably, both inflammatory cold allodynia (Fig. 3*A*) and oxaliplatin-induced (Fig. 3*B*) cold allodynia were ameliorated in WT mice 4 h after intradermal injection (10 mg/kg) of the antiartemin antibody MAB1085 (P > 0.05, ipsilateral vs. contralateral), whereas mice injected with an isotype control antibody remained sensitized to cold (Fig. 3 *A* and *B*) (P < 0.01, ipsilateral vs. contralateral or pre- vs. postinjection). We observed a similar reduction in cold allodynia in mice tested with the evaporative cooling assay (Fig. S6).

Moreover, in agreement with our genetic analysis, treatment with MAB1085 had no effect on mechanical (Fig. 3 *C* and *D*) (P < 0.001) or heat (Fig. 3*E*) (P < 0.001) hyperalgesia observed in the ipsilateral vs. contralateral hind paws, and the level of hyperalgesia was not different between control and MAB1085-treated mice (P > 0.05). These data show that artemin-neutralizing antibodies can reverse multiple types of injury-induced cold pain in an effective and highly specific manner.

To date, unlike heat and mechanical hyperalgesia, only artemin and to a lesser extent, NGF have been found to induce cold hypersensitivity in mice when administered by intraplantar injections (8, 9, 11). NGF signals through the tyrosine kinase TrkA and is a major mediator of heat hyperalgesia through sensitization of the heat-gated capsaicin receptor TRPV1 (36, 37). Based on the extensive literature on NGF/TrkA signaling, we expected that NGF-induced cold sensitization was mediated by TrkA through cellular signal transduction cascades that sensitize molecules involved in cold transduction. However, to our surprise, we found that cold allodynia observed in WT mice 1 h after intraplantar NGF injection (Fig. 4*A*) (P < 0.01 at 1 h vs. basal and vehicle-injected) was absent in *Gfrα3*$^{-/-}$ mice injected with NGF (Fig. 4*B*) (P > 0.05 at 1 and 3 h postinjection; P > 0.05, NGF vs. vehicle at all times tested), results similar to those observed after artemin injection. To ensure that this absence of cold allodynia was not because of a general reduction in NGF sensitization in these mice, we tested heat hyperalgesia, finding that, consistent with our analyses of inflammatory and neuropathic pain, heat hyperalgesia remained intact in *Gfrα3*$^{-/-}$ mice (Fig. 4*D*) (P < 0.001 at 1 h postinjection and NGF vs. vehicle; P < 0.01 at 3 h postinjection and NGF vs. vehicle), similar to that observed in WT animals (Fig. 4*C*). Thus, these results show that NGF-induced cold allodynia requires GFRα3, suggesting for the first time, to our knowledge, that cellular mechanisms leading to cold hypersensitivity converge on GFRα3.

How then does NGF prompt GFRα3-dependent cold allodynia? NGF does not directly interact with or stimulate GFRα receptors (38). However, in addition to direct sensitization of nociceptors, NGF also acts indirectly through activation of various peripheral cell types and the subsequent release of a host of

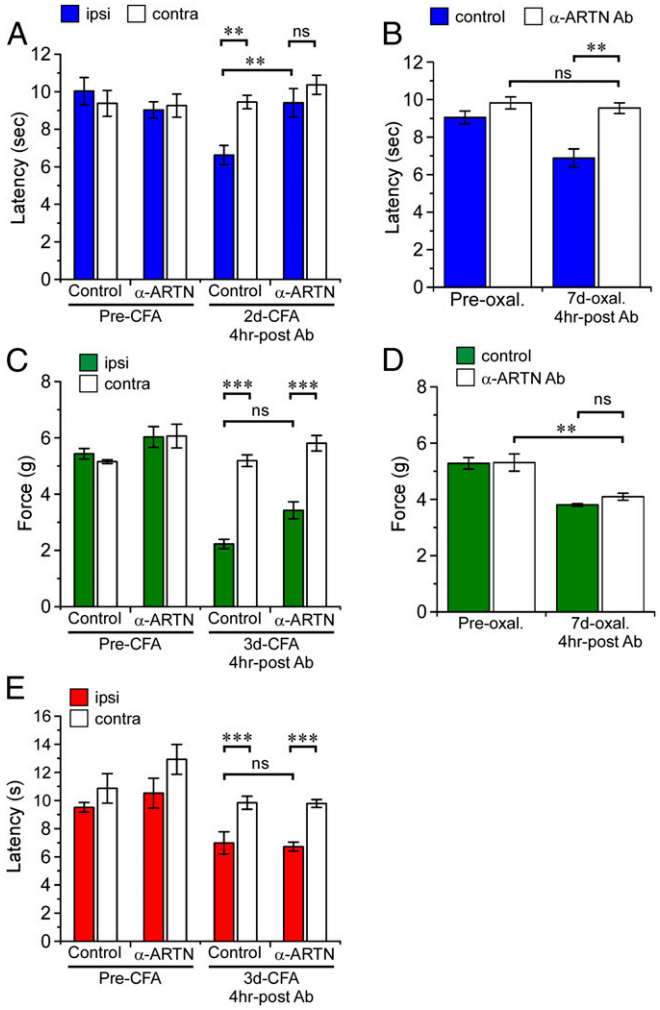

Fig. 3. Artemin neutralization selectively attenuates cold hypersensitivity. (*A*) Inflammatory cold allodynia was attenuated in WT mice 4 h after s.c. injection of an artemin-neutralizing antibody ($P > 0.05$, ipsilateral vs. contralateral; $n = 6$–7) compared with in control mice. $^{**}P < 0.01$. (*B*) Chemotherapeutic-induced cold pain was attenuated after antibody injection ($P > 0.05$, preoxaliplatin vs. postantibody) and significantly different from controls. $^{**}P < 0.01$. (*C*) Conversely, inflammatory mechanical hyperalgesia was unaffected ($P > 0.05$, ipsilateral control vs. ipsilateral antibody; $P < 0.001$, ipsilateral vs. contralateral for both treatments; $n = 5$–8). $^{***}P < 0.001$. (*D*) Chemotherapeutic-induced mechanical hyperalgesia was unaffected ($P > 0.05$, postantibody vs. control; $P < 0.01$, preoxaliplatin vs. postantibody; $n = 5$–6). $^{**}P < 0.01$. (*E*) Inflammatory thermal hyperalgesia was unaffected ($P > 0.05$, ipsilateral control vs. ipsilateral antibody; $P < 0.001$, ipsilateral vs. contralateral for both treatments; $n = 6$). ARTN, artemin; contr, contralateral; ipsi, ipsilateral; ns, not significant; oxal, oxaliplatin. $^{***}P < 0.001$.

inflammatory mediators, which in turn, sensitize sensory afferents (12, 39–41). Several inflammatory conditions stimulate artemin release from a number of peripheral cell types, including keratinocytes, fibroblasts, and immune cells (28, 42, 43), and we hypothesized that NGF-induced cold allodynia was mediated by NGF indirectly promoting artemin release. To test this hypothesis, we again used artemin-neutralizing antibodies and found that NGF-evoked cold allodynia observed in control mice (Fig. 5*A*) ($P < 0.001$, NGF vs. vehicle, ipsilateral vs. contralateral), measured 1 h after intraplantar NGF injections, was blocked when MAB1085 was administered systemically 1 h before NGF treatment (Fig. 5*A*) ($P > 0.05$, NGF vs. vehicle, ipsilateral vs. contralateral). This absence of NGF-evoked sensitization was again modality-specific, because MAB1085 had no effect on heat hyperalgesia compared with controls (Fig. 5*B*) ($P < 0.001$, NGF vs. vehicle, ipsilateral vs. contralateral for both conditions). Thus, these results show that NGF-induced cold allodynia is mediated by artemin signaling through its cognate cellular receptor GFRα3, further validating the necessity and specificity of this signaling pathway on pathological cold pain.

Discussion

To our knowledge, this study is the first rigorous report of nociception in mice lacking GFRα3, and our results are consistent with prior studies that found no discernable phenotype in peripheral sensory ganglia in both artemin and GFRα3-null mice (17, 18). The lack of any salient somatosensory abnormalities in naïve *Gfrα3*$^{-/-}$ mice suggests that the receptor has no substantial role in sensory nervous system development, despite the fact that it is expressed in ∼20% of adult DRG neurons.

Nonetheless, we now show the necessity of GFRα3 in pathological cold pain induced by an important inflammatory mediator (NGF) and inflammation itself and in two distinct forms of neuropathic pain. What is a remarkable and seminal result of our study is that injury-induced cold allodynia of multiple etiologies is totally dependent on GFRα3, unlike the redundant nature of heat and mechanical pain that signal through a diverse repertoire of cell surface receptors (1). NGF, protons, bradykinin, and histamine, to name a few, are all capable of potentiating TRPV1 responses (7, 29, 44, 45) and responsible for the development of heat hyperalgesia. However, only artemin and NGF induce cold allodynia in a manner similar to that found after injury (8). Here,

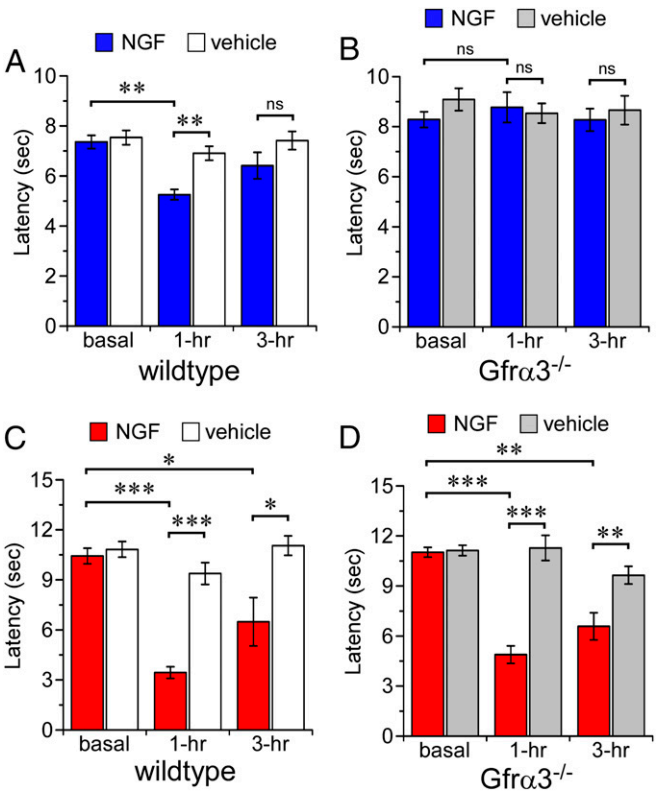

Fig. 4. NGF-induced cold allodynia is GFRα3-dependent. (*A*) WT mice exhibit cold allodynia 1 h but not 3 h after intraplantar NGF injections ($P > 0.05$; $n = 9$–11), whereas (*B*) *Gfrα3*$^{-/-}$ mice showed no change in cold sensitivity compared with vehicle-injected mice in the cold plantar assay ($P > 0.05$; $n = 9$–11). $^{**}P < 0.01$. Both (*C*) WT and (*D*) *Gfrα3*$^{-/-}$ mice displayed robust heat hyperalgesia 1 and 3 h after NGF administration ($n = 6$). ns, Not significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

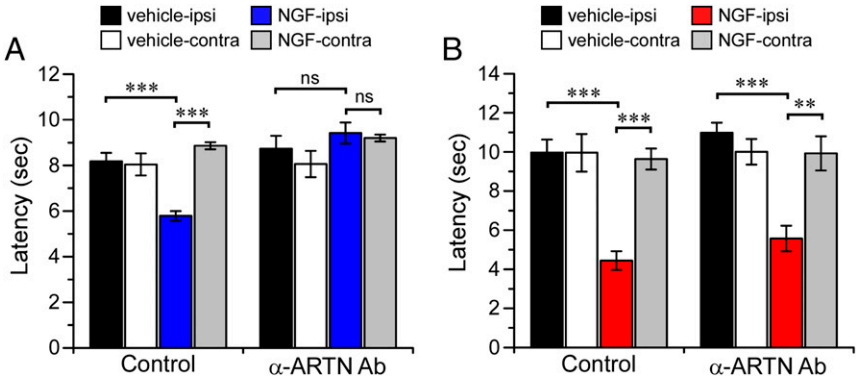

Fig. 5. Artemin neutralization blocks NGF-induced cold allodynia. (*A*) NGF-induced cold allodynia was attenuated in WT mice by artemin neutralization ($P > 0.05$, pre- vs. post-NGF and vs. vehicle-injected mice; $n = 4$) 1 h before intraplantar NGF injection. Control mice show robust cold allodynia after NGF injection (pre- vs. post-NGF and vs. vehicle-injected; $n = 4$). ***$P > 0.001$. (*B*) NGF-induced heat hyperalgesia was unaffected by antibody treatment and similar to controls (pre- vs. post-NGF and vs. vehicle-injected; $n = 4$). **$P < 0.01$; ***$P > 0.001$. ARTN, artemin; contr, contralateral; ipsi, ipsilateral; ns, not significant.

we show that both proalgesics promote cold pain through GFRα3 and, surprisingly, that NGF-induced cold allodynia occurs through a mechanism that involves artemin, because it is ameliorated with artemin neutralization. The latter result is consistent, however, with an indirect action of NGF on nociceptors, in which NGF activates immune cells to release a host of inflammatory mediators, including artemin (12, 39).

The signal transduction mechanisms that lead to cold sensitization after artemin activation of GFRα3 remain unclear. We recently reported that artemin- and NGF-evoked cold allodynia was dependent on TRPM8 channels, but artemin and GFRα3 have not been shown to directly sensitize TRPM8 channels in vitro. The molecular processes whereby NGF/TrkA activation leads to heat hyperalgesia are well-documented (7), but to date, the molecular nature of GFL signaling on nociception has yet to be elucidated. For example, we and others have shown that acute exposure of GFLs, including artemin, in vivo leads to heat hyperalgesia (8, 42, 45). Similarly, GFLs potentiate capsaicin responses in dissociated DRG neurons recorded by Ca^{2+} imaging, showing sensitization of $TRPV1^{+}$ cells (45). However, specific changes in TRPV1 channel activity have not been reported, and the preponderance of data suggests that artemin-induced heat hyperalgesia is caused by increased TRPV1 expression (27, 28, 46, 47). Moreover, unlike the established TRPM8 dependence of artemin- and NGF-evoked cold allodynia (8) or the necessity of TRPV1 for NGF-induced heat hyperalgesia (36), the molecule determinants of GFL-evoked alterations in nociception in vivo are unknown (48).

What then underlies artemin- and GFRα3-dependent cold pain? As a glycosyl-phosphatidylinositol (GPI)-linked extraceullular receptor, GFRα3 must bind to a transmembrane protein to transduce a signal, which in many systems, is the tyrosine kinase Ret (49). However, recent evidence has uncovered GFRα actions that are Ret-independent, and although the exact transduction mechanisms underlying the GFL signaling in the absence of Ret have yet to be elucidated, both the neural cell adhesion molecules and integrin-β1 are reported to act as coreceptors with GFRαs (50, 51). These receptor complexes signal intracellularly through similar molecular mechanisms, including protein kinases (MAPK, p38/JNK, PI3K, src family, PKA, and PKC) and phospholipases (PLCβ and PLCγ) (15, 49, 50), pathways also known to modulate heat sensitivity (52–56), suggesting a potentially similar mechanism of action.

Artemin- and NGF-induced sensitization of cold responses is TRPM8-dependent, and multiple studies have shown that TRPM8 plays a role in the development of cold allodynia (8, 22–24, 57). However, inflammatory cold allodynia is also diminished by both an TRPA1 antagonist and reduced TRPA1 transcript expression, and cold hypersensitivity can be induced by TRPA1 agonism in WT but not *Trpa1*$^{-/-}$ mice (58, 59). Thus, both TRPM8 and TRPA1 are involved in pathological cold pain, although it should be noted that artemin directly inhibits TRPA1 channels (60), but it is unknown how channel inhibition influences any potential changes to TRPA1 function downstream of GFRα3 activation.

Lastly, ion channels involved in neuronal excitability have been implicated in cold pain. For example, two-pore, nongated potassium channels contribute to cold pain as they are down-regulated after oxaliplatin treatment, thereby leading to enhanced neuronal excitability (61, 62). Moreover, antagonism of the voltage-gated sodium channel Nav1.6 attenuated neuropathic cold allodynia (63). Thus, the mechanisms that potentiate cold responses at the molecular and cellular levels are diverse, but our data strongly suggest that future studies into cold pain should center around the effect of GFRα3 activation on these pathways.

Finally, we provide evidence here that artemin and GFRα3 are potential therapeutic targets for conditions in which cold alloydnia is a symptom. Artemin-neutralizing antibodies can ameliorate both inflammatory and neuropathic cold pain, which has already been established in the animal, suggesting that interfering with artemin is a potential therapeutic strategy for this pain modality. Our results are consistent with other recent reports that suggest that artemin neutralization was found to inhibit noninflammatory heat hyperalgesia in the tongue and reduce bladder hyperalgesia (33–35). Moreover, this approach is analogous to therapeutic interventions to block pain with NGF-neutralizing antibodies that are currently ongoing (64, 65). The finding that antiartemin antibodies can block oxaliplatin-induced cold allodynia provides a particularly promising prospect for clinical application. When taken as a whole, these studies show that, unlike the broad range of mediators of mechanical and heat pain, the exacerbation of cold pain after injury is mediated exclusively by the GFL artemin and its receptor GFRα3, providing the first evidence, to our knowledge, of a proalgesic agent singularly required for cold sensitization. Thus, artemin and GFRα3 can be considered as valuable therapeutic targets because of their effectiveness and specificity to cold pain.

Materials and Methods

Behavioral Assays. Details are in *SI Materials and Methods*. All experiments were approved by the University of Southern California Institutional Animal Care and Use Committee and performed in accordance with the recommendations of the International Association for the Study of Pain and *Guide for the Care and Use of Laboratory Animals* by the NIH (66). Adult WT or GFRα3$^{-/-}$ mice (a gift from Brian Davis, University of Pittsburgh, Children's Hospital of Pittsburgh, Pittsburgh, PA) of both sexes were used in behavioral assays. Cold, heat, and mechanical sensitivity were assayed as described (19, 22).

Artemin and NGF Injections. Artemin and NGF injections to the hind paw were performed as described previously (8).

Pain Models. Inflammation was induced unilaterally by intraplantar injection of 20 μL CFA into the hind paw. Neuropathic pain was induced using the CCI model or chemotherapeutic oxaliplatin (22, 25).

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