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Endogenous Inflammatory Mediators Produced by Injury Activate TRPV1 and TRPA1 Nociceptors to Induce Sexually Dimorphic Cold Pain That Is Dependent on TRPM8 and GFRα3

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The detection of environmental temperatures is critical for survival, yet inappropriate responses to thermal stimuli can have a negative impact on overall health. The physiological effect of cold is distinct among somatosensory modalities in that it is soothing and analgesic, but also agonizing in the context of tissue damage. Inflammatory mediators produced during injury activate nociceptors to release neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, inducing neurogenic inflammation, which further exasperates pain. Many inflammatory mediators induce sensitization to heat and mechanical stimuli but, conversely, inhibit cold responsiveness, and the identity of molecules inducing cold pain peripherally is enigmatic, as are the cellular and molecular mechanisms altering cold sensitivity. Here, we asked whether inflammatory mediators that induce neurogenic inflammation via the nociceptive ion channels TRPV1 (vanilloid subfamily of transient receptor potential channel) and TRPA1 (transient receptor potential ankyrin 1) lead to cold pain in mice. Specifically, we tested cold sensitivity in mice after intraplantar injection of lysophosphatidic acid or 4-hydroxy-2-nonenal, finding that each induces cold pain that is dependent on the cold-gated channel transient receptor potential melastatin 8 (TRPM8). Inhibition of CGRP, substance P, or toll-like receptor 4 (TLR4) signaling attenuates this phenotype, and each neuropeptide produces TRPM8-dependent cold pain directly. Further, the inhibition of CGRP or TLR4 signaling alleviates cold allodynia differentially by sex. Last, cold pain induced by both inflammatory mediators and neuropeptides requires TRPM8, as well as the neurotrophin artemin and its receptor GDNF receptor α3 (GFRα3). These results are consistent with artemin-induced cold allodynia requiring TRPM8, demonstrating that neurogenic inflammation alters cold sensitivity via localized artemin release that induces cold pain via GFRα3 and TRPM8.

Key words: **cold; inflammation; pain; TRPA1; TRPM8; TRPV1**

Significance Statement

The cellular and molecular mechanisms that generate pain are complex with a diverse array of pain-producing molecules generated during injury that act to sensitize peripheral sensory neurons, thereby inducing pain. Here we identify a specific neuroinflammatory pathway involving the ion channel TRPM8 (transient receptor potential cation channel subfamily M member 8) and the neurotrophin receptor GFRα3 (GDNF receptor α3) that leads to cold pain, providing select targets for potential therapies for this pain modality.

Introduction

Sensations such as touch and pain are initiated by activation of modality-specific sensory receptors on primary afferent nerve endings that respond to mechanical, chemical, or thermal stimuli (Basbaum et al., 2009). After injury, the sensitivity of these receptors is enhanced, thereby lowering thresholds such that pain intensity is increased (hyperalgesia) or stimuli that would normally be innocuous become painful (allodynia). Molecularly, damage to tissues and cells produces a variety of biologically active inflammatory mediators that either directly

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stimulate pain receptors or enhance their activity indirectly via an assortment of different signal transduction cascades (Woodhams et al., 2017; Ueda, 2021). In addition, robust stimulation of nociceptors induces the antidromic release of neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, leading to neurogenic inflammation and further aggravation of the inflammatory and pain states (Woolf and Ma, 2007; Basbaum et al., 2009). Thus, a key factor in the development of novel therapeutic options for acute and chronic pain is a better understanding of the molecules and pathways that lead to peripheral sensitization with injury or disease.

Intriguingly, unlike their effects on heat and mechanical sensitivity, many inflammatory mediators released at the site of injury, such as bradykinin, prostaglandins, and histamine, do not increase cold sensitivity, and may in fact inhibit normal cold responses (Linte et al., 2007; Zhang et al., 2012; Zhang, 2019). This effect involves receptor-mediated inhibition of transient receptor potential melastatin 8 (TRPM8; McKemy et al., 2002), the principal sensor of environmental cold in mammals that is required for appropriate responses to acute cold and pathologic cold pain, thereby diminishing the potential analgesic effects of cooling (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Knowlton et al., 2013). Nonetheless, like other somatosensory modalities, cold is a source of serious discomfort and pain (McKemy, 2018), and a better understanding of the molecules and signaling pathways that lead to cold allodynia is critical to any potential treatments.

The ion channels transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) are expressed in a heterogeneous subpopulation of primary sensory neurons that consists of peptidergic and nonpeptidergic C-fiber and Aδ-fiber nociceptors (Caterina et al., 1997; Story et al., 2003; Jordt et al., 2004). Stimulation of these channels results in neuropeptide release that elicits plasma protein extravasation and vasodilatation, collectively referred to as neurogenic inflammation (Woolf and Ma, 2007; Nassini et al., 2014; Matsuda et al., 2019). While TRPV1 is considered a noxious heat receptor (Caterina et al., 2000), TRPA1 was initially identified as a sensor of noxious cold (Story et al., 2003), although this has been under considerable debate in the field after TRPA1 was shown to be a receptor for environmental irritants (Jordt et al., 2004; Caspani et al., 2009). Nonetheless, genetic and pharmacological studies *in vivo* support a prominent role for TRPA1 in cold pain after injury, consistent with its established function as a key mediator of chronic inflammation (Bautista et al., 2013).

Here, we report that endogenous inflammatory mediators that stimulate neurogenic inflammation via activation of TRPA1 and TRPV1 lead to cold allodynia in a TRPM8-dependent manner. This cold pain phenotype is attenuated by block of both CGRP and substance P signaling, and we find that these neuropeptides can also directly provoke cold allodynia. Intriguingly, CGRP induces allodynia in female mice, but not in male mice, and antagonizing CGRP receptors only blocks neurogenic inflammation-induced cold sensation in females, whereas TLR4 inhibition prevents cold allodynia in males. Regardless of which cold pain inducer is used, we find that all require the availability of the neurotrophin artemin and its cognate receptor GDNF receptor α3 (GFRα3; Baloh et al., 1998a, b; Honma et al., 2002). Thus, our study reveals new mediators of cold pain that work via divergent signaling pathways, but all converge on artemin signaling via GFRα3 leading to increased cold sensitivity via TRPM8.

Table 1. Agents, manufacturers, concentrations, and vehicles

Agents	Manufacturer	Catalog #	Concentration	Vehicle
4HNE	Cayman Chemical	32100	120 nmol/20 µl	Ethanol
LPA	Sigma-Aldrich	L7260	4 µg/20 µl	PBS
$CGRP_{8-37}$	Tocris Bioscience	1169	5 nmol/20 µl	0.9% saline
TAK242	Sigma-Aldrich	614316	0.2 µg/20 µl	0.9% saline
α-CGRP rat	Sigma-Aldrich	C0292	5 µg/20 µl	0.9% saline
Artemin	R&D Systems	1085-AR-025/CF	0.2 µg/20 µl	HBSS
NADA	Sigma-Aldrich	A8848	10 nmol/20 µl	Ethanol
Substance P	Sigma-Aldrich	05–23-0600	20 nmol/35 µl	0.9% saline
SR140333	Tocris Bioscience	4012	90 nmol/20 µl	Ethanol
PBMC	Focus Biomolecules	10–1413	100 µg/20 µl	50% DMSO/saline

Materials and Methods

Animals. All experiments were approved by the University of Southern California Institutional Animal Care and Use Committee and in compliance with National Institutes of Health guidelines. Mice 8 to 14 weeks of age were used in all experiments, and equal numbers of male and female mice were used unless otherwise specified. All mice were housed in a temperature-controlled environment on a 12 h light/dark cycle with *ad libitum* access to food and water. All experiments were performed during the light cycle from 9:00 A.M. to 5:00 P.M. Wild-type mice (strain #000664), TRPM8-null mice ($Trpm8^{-/-}$; strain #008198), and TRPA1-null mice ($Trpa1^{-/-}$; strain #006401) were purchased from The Jackson Laboratory and were on the C57BL/6 background. GFRα3-null mice ($Gfrα3^{-/-}$; Honma et al., 2002; Lippoldt et al., 2016) were maintained in-house, and heterozygous mice were bred to obtain wild-type littermate controls ($Gfrα3^{+/+}$).

Pharmacological agents. All agents were purchased from the vendors listed in Table 1 and diluted to the desired concentration before being administered via an intraplantar injection in their respective vehicle (veh) solutions. All injections were 20 µl except for substance P (35 µl). The anti-artemin monoclonal antibody (mAb) and control IgG isotype antibodies were purchased from R&D Systems, reconstituted in sterile PBS solution, and injected intraperitoneally at a concentration of 10 mg/kg (Lippoldt et al., 2016).

Cold plantar assay. The cold plantar assay was used to assess the sensitivity to cold stimulation as previously described (Brenner et al., 2014; Ongun et al., 2018; Yamaki et al., 2021). Mice were habituated in Plexiglas chambers for 2 h on a 6-mm-thick glass surface maintained at 30°C. A compressed dry ice pellet was applied to the surface of the glass directly under the hindpaw to be tested, and hindpaw withdrawal latencies were recorded. A 30 s cutoff time was used between each measurement to avoid potential tissue damage. Three trials were taken and averaged for each hindpaw, and baseline (BL) measurements were recorded before each experiment. In time course experiments, mice were returned to home cages after measurements at 4 h to access food and water and returned to their original chambers for 30 min for habituation before next measurements.

Hargreaves assay. The Hargreaves assay was used to assess the sensitivity to heat stimulation (Yamaki et al., 2021). Mice were habituated in Plexiglas chambers (IITC Life Science) for 2 h on a glass surface on which temperature was maintained at 32°C. A radiant light source was used as a heat stimulus and was focused on the plantar surface of hindpaw. Hindpaw withdrawal latency was recorded with a cutoff time of 20 s between each measurement to avoid potential tissue damage. Three trials were taken and averaged for each hindpaw. Baseline measurements were recorded before treatment. Mice were returned to the original chamber after treatment, and new measurements were taken after 30 or 60 min.

Statistical analysis. The experimenters were blinded to either genotype of the mice receiving treatments or substances injected, and a Power analysis was performed to determine an appropriate sample size. All data were presented as the mean ± SEM, and all statistical analysis was performed using GraphPad Prism 9 software, as described in the figure legends.

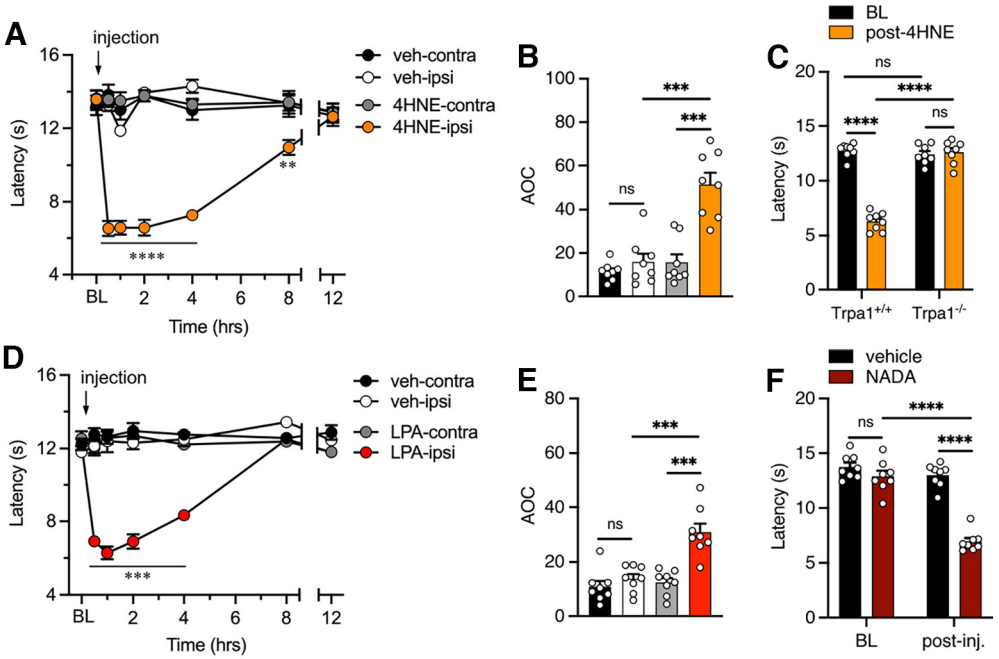

Figure 1. Endogenous inflammatory mediators induce cold allodynia. ***A***, Cold allodynia in mice receiving an intraplantar injection of 4HNE starts at 30 min postinjection and lasts up to 8 h as evidenced by a significantly lower withdrawal latency compared with BL measurements or to vehicle-injected mice ($n = 8$ for each condition, two-way ANOVA with a Dunnett's multiple-comparisons test for BL vs each time point; and Bonferroni's multiple-comparisons test for vehicle vs 4HNE at each time point; $^{****}p < 0.0001$, $^{**}p < 0.01$). ***B***, Area over the curve (AOC) was measured for each animal over time and averaged between groups, demonstrating that 4HNE produced robust cold allodynia in wild-type mice (paired two-tailed *t* test for ipsi vs contra; unpaired *t* test with Welch's correction for vehicle vs 4HNE; $^{ns}p > 0.05$, $^{***}p < 0.001$). ***C***, TRPA1-null (*Trpa1*$^{-/-}$) mice displayed normal cold sensitivity after injection with 4HNE, whereas wild-type (*Trpa1*$^{+/+}$) littermates showed robust cold allodynia ($n = 8$ for each genotype, paired two-tailed *t* test for BL vs post-4HNE; unpaired *t* test with Welch's correction for post-4HNE between genotypes; $^{ns}p > 0.05$, $^{****}p < 0.0001$). Cold sensitivity was similar between genotypes before injection (unpaired *t* test with Welch's correction, $^{ns}p > 0.05$). ***D***, Intraplantar LPA also produced robust cold allodynia for at least 4 h postinjection compared with baseline or vehicle ($n = 8$ for each condition, two-way ANOVA with a Dunnett's multiple-comparisons test for BL comparisons and Bonferroni's test for vehicle, $^{***}p < 0.001$). ***E***, LPA-induced cold allodynia was also significantly different in mice given vehicle in the contralateral hindpaw (paired two-tailed *t* test for ipsi vs contra; unpaired *t* test with Welch's correction for vehicle vs LPA; $^{ns}p > 0.05$, $^{***}p < 0.001$). ***F***, Intraplantar NADA induced cold allodynia ($n = 8$, unpaired *t* test with Welch's correction for vehicle vs NADA; paired two-tailed *t* test for BL vs post-NADA; $^{ns}p > 0.05$, $^{****}p < 0.0001$).

Results

Endogenous inflammatory mediators induce cold allodynia

The mechanisms whereby inflammatory mediators, produced by tissue injury or other physical insults, lead to cold pain are poorly understood. To address this gap in our knowledge, we tested the effects of established endogenous proalgesic molecules, with distinct receptor mechanisms, and their ability to induce cold sensitivity in mice. First, we tested 4-hydroxy-2-nonenal (4HNE), a reactive aldehyde produced when reactive oxygen species peroxidize membrane phospholipids and a known endogenous agonist of the irritant receptor TRPA1 (Benedetti et al., 1980; Trevisani et al., 2007). Using the Cold Plantar assay, a measure of the sensitivity of an animal to an innocuous cold stimulus (Brenner et al., 2014; Ongun et al., 2018), we measured baseline latencies for a hindpaw lift on cold stimulation in an equal number of male and female C57/BL6 wild-type mice, then performed a unilateral intraplantar hindpaw injection of 4HNE (120 nmol) or vehicle, with cold sensitivity reassessed over a 12 h period. 4HNE produced a reduction in withdrawal latencies that was significantly different from baseline by 30min post-injection, an effect that remained for up to 8 h (Fig. 1*A*). The area over the curve (AOC) for each time course showed that 4HNE induced significant cold allodynia compared with vehicle-treated animals or the uninjected contralateral hindpaw (Fig. 1*B*). To determine the receptor dependency of 4HNE on cold allodynia, we repeated these assays in TRPA1-null mice (*Trpa1*$^{-/-}$; Kwan et al., 2006; Trevisani et al., 2007) finding that the 4HNE-induced reduction in the withdrawal latency observed in wild-type littermates (*Trpa1*$^{+/+}$) was absent in *Trpa1*$^{-/-}$ mice (Fig. 1*C*).

Next, we sought to determine whether the sensitization induced by 4HNE was a general effect by asking whether molecularly and functionally distinct endogenous inflammatory mediators also induce cold allodynia. Specifically, we focused on lysophosphatidic acid (LPA), a phospholipid produced by activated platelets and microglia on tissue damage (Ma et al., 2010a; Nieto-Posadas et al., 2011). LPA is an agonist of the capsaicin receptor TRPV1 (Huang et al., 2002; Nieto-Posadas et al., 2011), which, like TRPA1, when activated, induces neurogenic inflammation and peripheral sensitization (Basbaum et al., 2009). Intriguingly, we found that an intraplantar injection of LPA (4 μg) also produced cold allodynia that lasted for several hours (Fig. 1*D*) and was significantly different compared with vehicle and the contralateral paw (Fig. 1*E*). To confirm that this effect is not molecule specific, we also tested the endogenous TRPV1 agonist *N*-arachidonoyldopamine (NADA), a putative endocannabinoid that induces robust heat hyperalgesia via activation of TRPV1 (Huang et al., 2002), observing that NADA produced similar effects on cold sensitivity as does 4HNE and LPA in wild-type mice (Fig. 1*F*). Thus, inflammatory mediators produced by tissue insults lead to cold hypersensitivity, and, to the best of our knowledge, the effects of these molecules on cold pain, and their associated cellular mechanisms, have not been determined.

While TRPA1 is proposed to be a cold sensor (Story et al., 2003), this has been controversial (Sinica and Vlachova, 2021), and, as we have previously reported (Yamaki et al., 2021), we observed no differences in baseline responses to cold between the two genotypes (Fig. 1C), suggesting that TRPA1 does not mediate cold responses in the cold plantar assay. Furthermore,

TRPV1 is a noxious heat sensor and has not been linked to acute cold sensing, suggesting that the effects of LPA or NADA are downstream of TRPV1 channel activation. Thus, it is unlikely that cold allodynia induced by these endogenous molecules is mediated directly by either TRPV1 or TRPA1. The menthol receptor TRPM8 is the predominant cold sensor in mammals, and we asked whether cold allodynia induced by 4HNE and LPA is dependent on TRPM8 by repeating these assays in TRPM8-null mice (*Trpm8*$^{-/-}$; Bautista et al., 2007; Knowlton et al., 2010). To test 4HNE and LPA, both wild-type and *Trpm8*$^{-/-}$ mice were first tested for cold sensitivity before a single intraplantar injection of either 4HNE (Fig. 2*A*) or LPA (Fig. 2*B*), as above, then cold withdrawal latencies were retested 30 min (for 4HNE) or 60 min (for LPA) later when we observed no differences in cold acuity in *Trpm8*$^{-/-}$ mice preinjection versus postinjection. To confirm that the absence of cold allodynia in *Trpm8*$^{-/-}$ was not because of off-target effects that can be associated with knock-out animals, we asked whether coinjection of the TRPM8-specific antagonist 1-phenylethyl-4-(benzyloxy)-3-methoxybenzyl (2-aminoethyl)carbamate (PBMC; Knowlton et al., 2011) with either 4HNE (Fig. 2*C*) or LPA (Fig. 2*D*) prevented cold allodynia. In both cases, pharmacological inhibition of TRPM8 prevented cold allodynia induced by either mediator of neurogenic inflammation.

Last, to ensure that this phenotype was not because of an overall deficit in sensitization in TRPM8-null mice, we repeated these experiments and measured heat hyperalgesia with the Hargreaves assay (Hargreaves et al., 1988), a behavioral paradigm like the cold plantar assay. As predicted, *Trpm8*$^{-/-}$ mice displayed no differences in heat sensitivity compared with wild-type mice at baseline, whereas 4HNE (Fig. 2*E*) or LPA (Fig. 2*F*) induced heat hyperalgesia in TRPM8-null mice that was indistinguishable from that observed in wild-type mice. These results suggest that cold sensitization produced by these inflammatory mediators is downstream of their receptor channels and converge on sensitizing TRPM8.

Substance P and CGRP mediate cold allodynia produced by inflammatory mediators. As stated above, 4HNE and LPA are potent agonists for TRPA1 and TRPV1, respectively, nociceptor-expressed nonselective cation channels whose activation leads to neurogenic inflammation and peripheral sensitization to somatosensory stimuli via antidromic release of proinflammatory peptides, such as substance P and CGRP (Basbaum et al., 2009). Thus, to determine how these agents lead to cold pain, we first asked whether the cold allodynia they produce was mediated by substance P signaling. To test this, we tested whether the inhibition of the neurokinin 1 receptor (NK1R) by the peripherally acting, nonpeptide antagonist SR140333 (Oury-Donat et al., 1994) altered cold allodynia induced by intraplantar 4HNE or LPA. First, we performed an intraplantar injection of SR140333 (90 nmol; Teodoro et al., 2013) in an equal number of male and female wild-type mice and tested whether basal cold sensitivity was altered 30 min postinjection, observing no effect on basal cold sensitivity with NK1R antagonism. Next, these mice were injected with 4HNE (Fig. 3*A*) and we tested cold responses 30 min postinjection, observing a significantly reduced level of cold allodynia compared with mice pretreated with vehicle. However, SR140333 did not completely prevent cold allodynia as there was a slight but significant reduction in the withdrawal latencies compared with baseline and after injection of SR140333. We repeated this assay with LPA, finding that pretreatment with SR140333 reduced LPA-induced cold allodynia compared with those pretreated with vehicle (Fig. 3*B*).

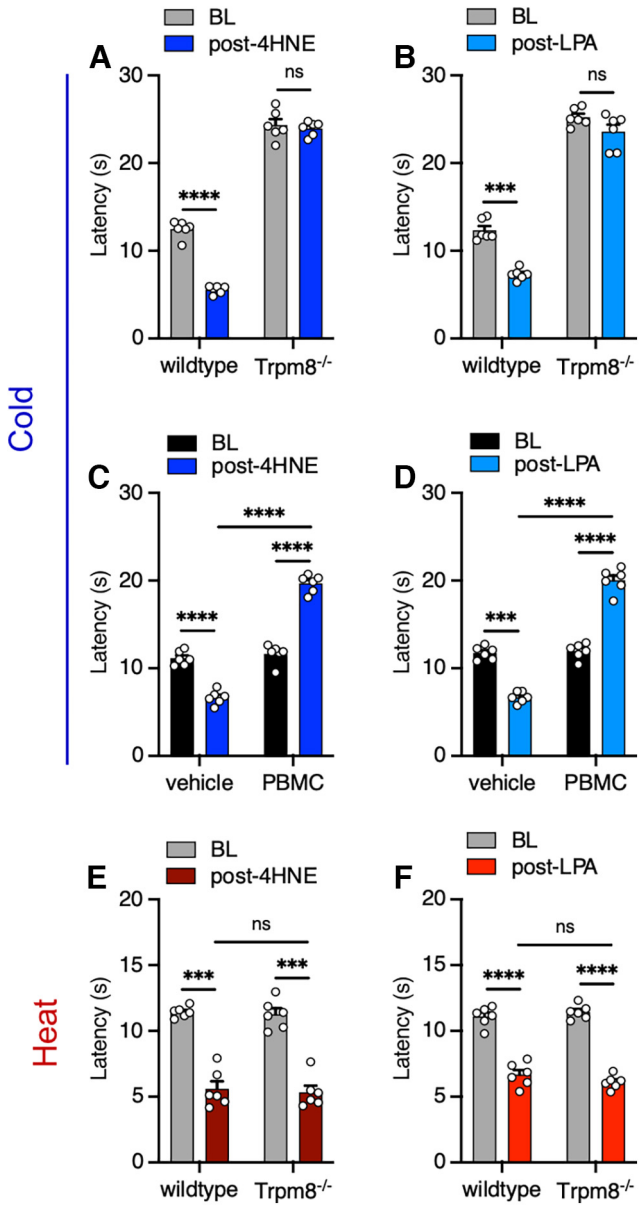

Figure 2. Endogenous inflammatory mediator-induced cold allodynia requires TRPM8. ***A***, ***B***, An intraplantar injection of either 4HNE (***A***) or LPA (***B***) induced cold allodynia in wild-type mice but not *Trpm8*$^{-/-}$ mice ($n = 6$, BL vs postinjection paired two-tailed t test, $^{ns}p > 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$). ***C***, ***D***, Coinjection of the TRPM8 antagonist PBMC inhibited cold allodynia by 4HNE (***C***) and LPA (***D***; $n = 6$ each, BL vs postinjection paired two-tailed t test; vehicle vs PBMC unpaired t test with Welch's correction, $^{***}p < 0.001$, $^{****}p < 0.0001$). ***E***, ***F***, Conversely, both genotypes exhibited heat hyperalgesia after injection of either 4HNE (***E***) or LPA (***F***), with no differences between wild-type and *Trpm8*$^{-/-}$ mice either preinjection or postinjection ($n = 6$, BL vs postinjection paired two-tailed t test; wild-type vs *Trpm8*$^{-/-}$ postinjection unpaired t test with Welch's correction, $^{ns}p > 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Unlike 4HNE, there was no difference between the measurements taken after SR140333 injection and those of antagonist plus LPA, whereas there was again a subtle yet significant difference between the latter and baseline responses (Fig. 3*B*). Next, we asked whether substance P can directly induce cold allodynia in mice and whether this was also TRPM8 dependent. Wild-type female and male mice (Fig. 3*C*) were tested for basal cold sensitivity then given either an intraplantar injection of vehicle or substance P (20 nmol) then retested 30 min later when we observed robust cold allodynia, a phenotype that

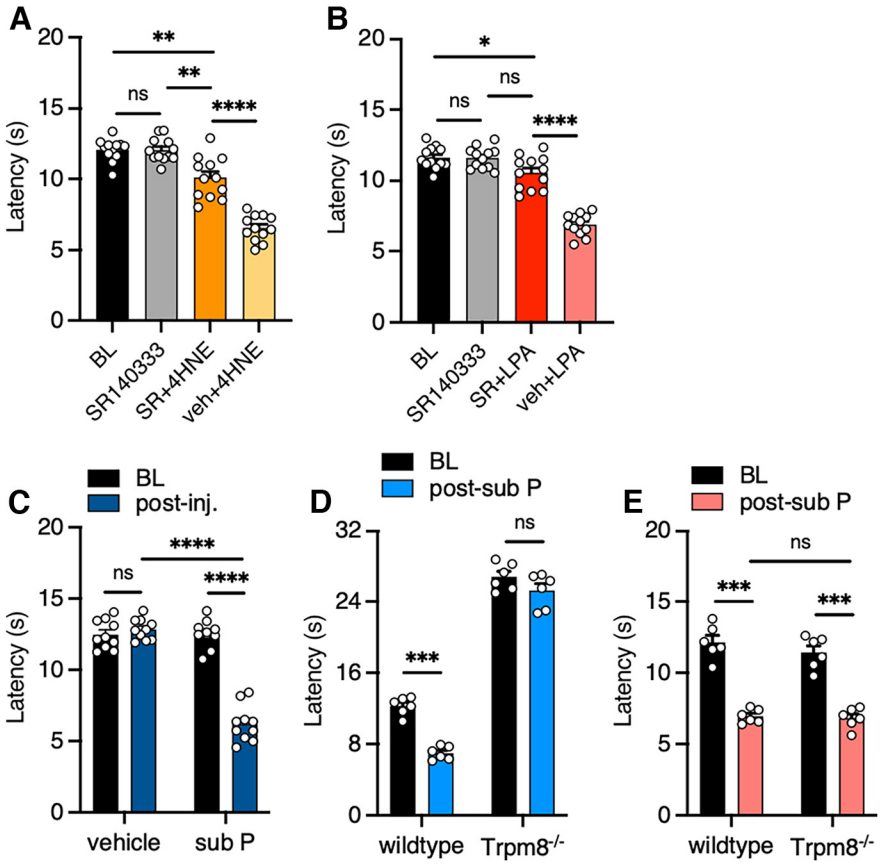

Figure 3. Substance P leads to TRPM8-dependent cold allodynia. ***A***, ***B***, Male and female wild-type mice exhibited reduced cold withdrawal latencies after intraplantar injections of 4HNE (***A***) or LPA (***B***) when pretreated with SR140333 ($n = 12$ mice each condition, one-way ANOVA with a Tukey's multiple-comparisons test for BL, SR140333, and SR140333(SR) + 4HNE or SR140333(SR) + LPA; unpaired t test with Welch's correction for SR140333(SR) + 4HNE or SR140333(SR) + LPA vs veh + 4HNE or veh + LPA; $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$). ***C***, Intraplantar substance P induced cold allodynia in male and female wild-type mice ($n = 10$, paired two-tailed t test for BL vs postinjection; unpaired t test with Welch's correction for vehicle vs substance P; $^{ns}p > 0.05$, $^{****}p < 0.0001$). ***D***, ***E***, Substance P does not induce cold allodynia in *Trpm8*$^{-/-}$ mice (***D***) but does produce heat hyperalgesia (***E***) in TRPM8-null mice ($n = 6$ for each, paired two-tailed t test BL vs post-substance P; unpaired t test with Welch's correction wild-type vs *Trpm8*$^{-/-}$; $^{ns}p > 0.05$, $^{***}p < 0.001$).

was dependent on TRPM8 as it was absent in male and female *Trpm8*$^{-/-}$ mice (Fig. 3*D*). Furthermore, this TRPM8 dependence is cold specific as *Trpm8*$^{-/-}$ mice of both sexes exhibited heat hyperalgesia with intraplantar substance P (Fig. 3*E*).

Next, we asked whether CGRP might also play a role in TRPM8-dependent cold pain. To this end, 30 min after baseline cold sensitivity testing, an equal number of male and female wild-type mice received an intraplantar injection of the CGRP receptor antagonist $CGRP_{8-37}$ (5 nmol), then were retested 30 min postinjection at which time we observed no differences in cold sensitivity with CGRP receptor antagonism. We then injected 4HNE as above and observed a modest reduction in the withdrawal latency compared with baseline or after $CGRP_{8-37}$ treatment (Fig. 4*A*). However, compared with animals injected with vehicle before 4HNE (veh + 4HNE), mice treated with $CGRP_{8-37}$ before 4HNE exhibited significantly less cold allodynia. We similarly tested the effect of CGRP antagonism on LPA-induced cold hypersensitivity, finding that $CGRP_{8-37}$ inhibited cold allodynia induced by this agent compared with baseline and vehicle + LPA-treated mice (Fig. 4*B*). With either agonist, we observed that the latencies in mice treated with the CGRP receptor antagonist displayed substantial variability, prompting us to take a closer look at the data where we detected a clear sexual dimorphism. Specifically, when we increased the number of animals for each sex, we found that $CGRP_{8-37}$ essentially prevented 4HNE-induced cold allodynia in female mice (Fig. 4*C*). We did observe a subtle, yet significant difference in female mice given $CGRP_{8-37}$ and LPA compared with baseline ($p = 0.021$), but these latencies were not significantly different compared with those taken after $CGRP_{8-37}$ and were significantly different from mice given vehicle before LPA (Fig. 4*D*). Conversely, male mice were unaffected by pretreatment with $CGRP_{8-37}$ before 4HNE (Fig. 4*E*) or LPA (Fig. 4*F*), demonstrating that antagonizing CGRP receptors inhibit neurogenic cold allodynia in a sex-dimorphic manner.

Next, we reasoned that if CGRP receptor antagonism altered cold allodynia, CGRP itself should be cold sensitizing, as well as show sex differences. To directly determine whether peripheral CGRP can induce cold allodynia, we performed unilateral hindpaw injections of α-CGRP (5 µg) or vehicle in both male and female wild-type mice and tested cold sensitivity 30 min postinjection. Consistent with the CGRP receptor antagonist results, α-CGRP produced cold allodynia in female (Fig. 5*A*) but not male mice (Fig. 5*B*). Next, we asked whether CGRP-induced cold allodynia required TRPM8, finding that the neuropeptide had no effect on cold sensitivity in female *Trpm8*$^{-/-}$ mice (Fig. 5*C*). Last, to determine whether this sexual dimorphism was specific for cold sensing, we repeated the injections of CGRP and assessed heat sensitivity using the Hargreaves's assay, finding that withdrawal latencies were also reduced in female (Fig. 5*D*) but not in male wild-type mice (Fig. 5*E*), and that CGRP did induce heat hyperalgesia in female TRPM8-null mice (Fig. 5*F*). Thus, peripheral CGRP induces cold allodynia via TRPM8 in a sexually dimorphic manner, consistent with prior reports of female-specific effects of CGRP in peripheral tissues (Paige et al., 2022). Of note, compared with CGRP antagonism, we observed a mildly incomplete inhibition of cold allodynia with NK1R receptor antagonism (Fig. 3), which would not be unexpected as CGRP is likely also altering sensitivity under these conditions. Further, effects of peripheral substance P are not known to show sex differences, and we did not observe any dimorphism when we analyzed our data by sex ($p > 0.05$, multiple unpaired t tests with Welch's correction for male vs female mice at each condition, Fig. 3*A*,*B*).

Inhibition of TLR4 prevents cold allodynia produced by inflammatory mediators in male mice

To further address these sex differences associated with cold allodynia, we sought to determine whether other pain-signaling pathways known to produce male-specific sexual dimorphism also participate in cold allodynia after stimulation by 4HNE or LPA. The toll-like receptor 4 (TLR4) contributes to microglial-

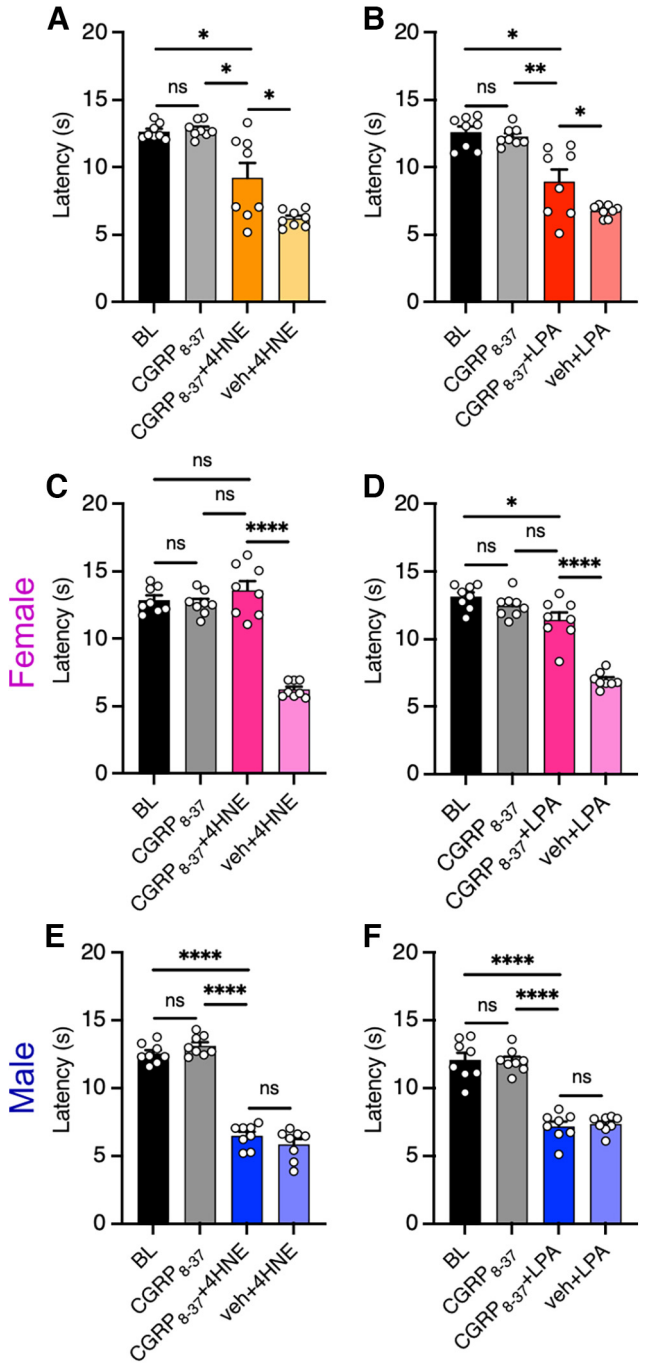

Figure 4. Cold allodynia produced by endogenous inflammatory mediators requires CGRP signaling in female mice. ***A***, Intraplantar injection of $CGRP_{8-37}$ in male and female mice did not alter basal cold sensitivity but slightly decreased cold allodynia induced by a subsequent injection of 4HNE ($n = 8$ mice, one-way ANOVA with a Tukey's multiple-comparisons test for BL, $CGRP_{8-37}$, and $CGRP_{8-37}$ + 4HNE; unpaired *t* test with Welch's correction for $CGRP_{8-37}$ + 4HNE vs veh + 4HNE; $^{ns}p > 0.05$, $^{*}p < 0.05$). ***B***, Similarly, $CGRP_{8-37}$ reduced cold allodynia induced by LPA ($n = 8$ mice, one-way ANOVA with a Tukey's multiple-comparisons test for BL, $CGRP_{8-37}$, and $CGRP_{8-37}$ + LPA; unpaired *t* test with Welch's correction for $CGRP_{8-37}$ + LPA vs veh + LPA; $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$). ***C***, ***D***, Pretreatment with $CGRP_{8-37}$ essentially prevented cold allodynia induced by either 4HNE (***C***) or LPA (***D***) in female mice. ***E***, ***F***, Conversely, CGRP receptor antagonism had no effect on 4HNE-induced (***E***) or LPA-induced (***F***) cold allodynia in male mice ($n = 8$ mice for each condition, one-way ANOVA with a Tukey's multiple comparison test for BL, $CGRP_{8-37}$, and $CGRP_{8-37}$ + 4HNE or $CGRP_{8-37}$ + LPA; unpaired *t* test with Welch's correction for $CGRP_{8-37}$ $CGRP_{8-37}$ + 4HNE or $CGRP_{8-37}$ + LPA vs veh + 4HNE or veh + LPA; $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{****}p < 0.0001$). Data in ***A*** and ***B*** are included in the sex-specific comparisons in ***C–F***.

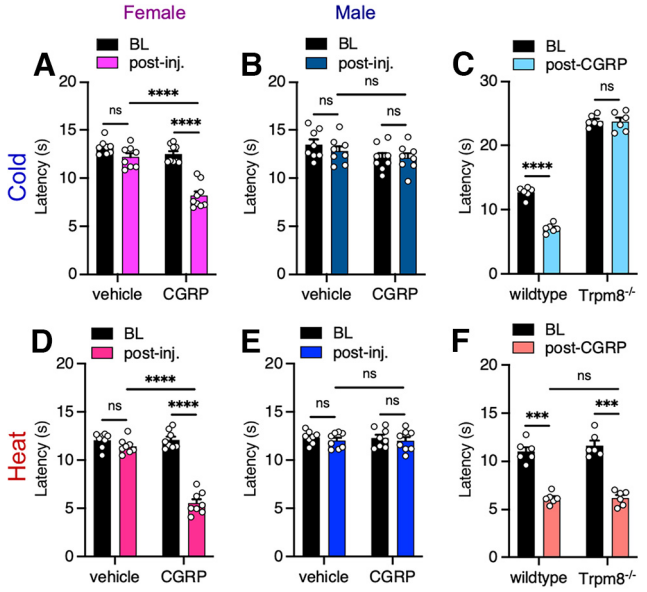

Figure 5. Intraplantar CGRP induces sexually dimorphic cold allodynia. ***A***, Female mice given an intraplantar injection of α-CGRP exhibited robust cold allodynia compared with pre-injection or vehicle-injected mice ($n = 8$, paired two-tailed *t* test preinjection vs postinjection, unpaired *t* test with Welch's correction vehicle vs α-CGRP, $^{ns}p > 0.05$, $^{****}p < 0.0001$). ***B***, Conversely, α-CGRP did not alter cold sensitivity in male mice ($n = 8$ for each condition; $^{ns}p > 0.05$). ***C***, α-CGRP-induced cold allodynia was absent in *Trpm8*$^{-/-}$ mice ($n = 6$ for each genotype, paired two-tailed *t* test BL vs post-CGRP; $^{ns}p > 0.05$, $^{****}p < 0.0001$). ***D***, ***E***, α-CGRP also induced a similar dimorphic effect of heat sensitivity in female (***D***) and male (***E***) mice ($n = 8$, paired two-tailed *t* test preinjection vs postinjection; unpaired *t* test with Welch's correction vehicle vs α-CGRP; $^{ns}p > 0.05$, $^{****}p < 0.0001$). ***F***, α-CGRP-induced heat hyperalgesia was similar in wild-type and *Trpm8*$^{-/-}$ mice ($n = 6$ for each genotype, paired two-tailed *t* test BL vs post-CGRP; unpaired *t* test with Welch's correction between genotypes; $^{ns}p > 0.05$, $^{***}p < 0.001$).

dependent early pain in males, but to a lesser extent in females (Sorge et al., 2011; Woller et al., 2016; Huck et al., 2021), and we asked whether inhibiting TLR4 altered cold sensitivity. TAK242 is a small molecule that selectively binds to an intracellular domain of TLR4 and prevents signaling by inhibiting the recruitment of signaling adapter molecules (Matsunaga et al., 2011). Using this antagonist, wild-type female and male mice were tested for baseline cold sensitivity and then given an intraplantar injection of TAK242 (0.2 µg), with neither sex exhibiting any alterations in cold sensitivity when retested 30 min postinjection of TAK242. These animals were then given an injection of 4HNE or LPA, as above, with cold sensitivity assessed 30 or 60 min later, respectively. Consistent with the male-specific role of TLR4 in pain, we observed no attenuation of 4HNE-induced (Fig. 6*A*) or LPA-induced (Fig. 6*B*) cold allodynia in female mice pretreated with TAK242, whereas this sensitization was completely absent in male mice similarly treated with either 4HNE (Fig. 6*C*) or LPA (Fig. 6*D*). Thus, cold allodynia shows intriguing sexual dimorphism with peripheral CGRP and TLR4 signaling required for female and male cold pain, respectively.

Cold allodynia produced by endogenous proalgesics requires the neurotrophin artemin and its receptor GFRα3

When taken together, our results suggest that neurogenic inflammation leads to the release of proalgesic neuropeptides that lead to TRPM8-dependent cold pain. However, how these pathways alter TRPM8 cold signaling is unknown. Previously, we have shown that the neurotrophin artemin sensitizes mice to cold in a

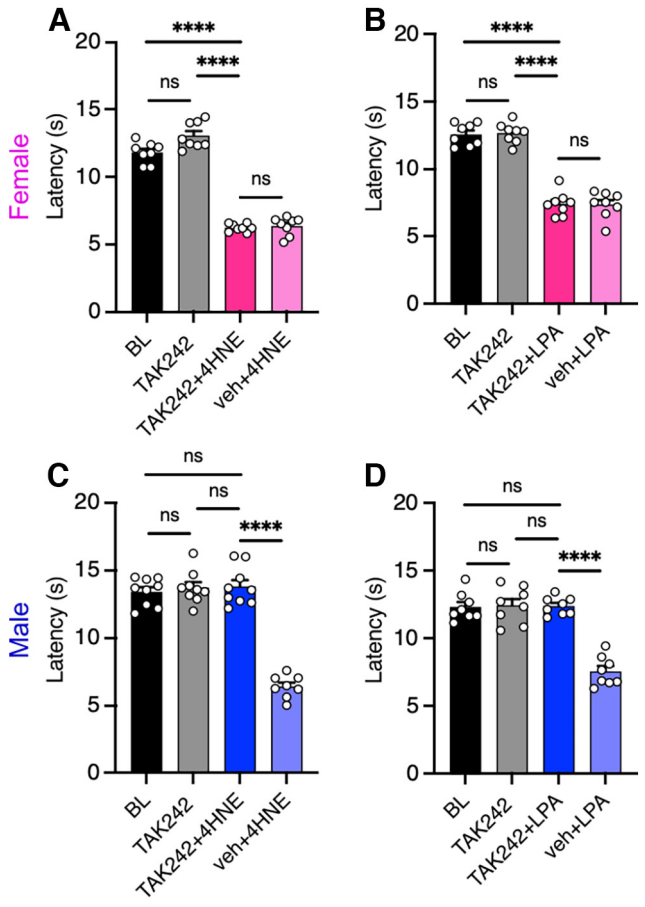

Figure 6. Inhibition of TLR4 prevents cold allodynia produced by endogenous inflammatory mediators in male mice. ***A***, ***B***, An intraplantar injection of the TLR4 antagonist TAK242 in female mice did not alter basal cold sensitivity for 4HNE-induced (***A***) or LPA-induced (***B***) cold allodynia. ***C***, ***D***, Conversely, TAK242 prevented cold allodynia induced by 4HNE (***C***) or LPA (***D***) in male mice ($n = 8$, one-way ANOVA with a Tukey's multiple-comparisons test for BL, TAK242, and TAK242 + 4HNE/LPA; unpaired t test with Welch's correction for TAK242 + 4HNE/LPA vs veh + 4HNE/LPA; $^{ns}p > 0.05$, $^{****}p < 0.0001$).

manner that is TRPM8 dependent, and that its receptor GFRα3 is required for inflammatory or neuropathic cold allodynia (Lippoldt et al., 2013, 2016). Therefore, we reasoned that neurogenic cold allodynia was similarly dependent on this signaling pathway. To test this, we performed intraplantar injections of 4HNE, LPA, CGRP, or substance P in GFRα3-null mice (*Gfrα3*$^{-/-}$; Honma et al., 2002; Lippoldt et al., 2016) to determine whether this receptor was required for cold allodynia. Both *Gfrα3*$^{-/-}$ and *Gfrα3*$^{+/+}$ littermates were tested for basal cold sensitivity and, as we have reported previously (Lippoldt et al., 2016), the latencies to lift were similar in both genotypes, as was heat sensitivity (Fig. 7). However, the robust cold allodynia observed in *Gfrα3*$^{+/+}$ mice after intraplantar injection of either 4HNE (Fig. 7*A*), LPA (Fig. 7*B*), CGRP (Fig. 7*C*), or substance P (Fig. 7*D*) was absent in *Gfrα3*$^{-/-}$ animals. Furthermore, while cold allodynia induced by these agents was dependent on GFRα3, heat hyperalgesia remained in *Gfrα3*$^{-/-}$ mice injected with 4HNE (Fig. 7*E*), LPA (Fig. 7*F*), CGRP (Fig. 7*G*), or substance P (Fig. 7*H*).

Neutralization of artemin in the periphery selectively resolves both inflammatory and neuropathic cold allodynia (Lippoldt et al., 2016; Jeong et al., 2022). Thus, we asked whether this is also true for cold allodynia induced by neuroinflammatory agents injected into the hindpaw. Naive mice were first tested for BL cold sensitivity then 4HNE (Fig. 8*A*), LPA (Fig. 8*B*), CGRP (Fig. 8*C*), or substance P (Fig. 8*D*) were injected then these mice were retested as above, with each agent producing the expected decrease in withdrawal latencies. Immediately after testing, these mice were then given an intraperitoneal injection of either an anti-artemin antibody MAB1085 (10 mg/kg) or an equal amount of an isotype control antibody (IgG), with cold sensitivity tested 60 min later. For all mice injected with the isotype control, cold allodynia was still present at the time of testing as there was a significant difference in the withdrawal latencies compared with baseline. Furthermore, in the animals treated with LPA (Fig. 8*B*) or CGRP (Fig. 8*C*), there were no differences in responses before and after treatment with the control mAb. We did observe a slight yet significant lengthening of the latencies in 4HNE-injected ($p = 0.025$) and substance P-injected ($p = 0.001$) mice given the control mAb (Fig. 8*A*,*D*), but there was still a robust difference compared with baseline. In contrast, we observed an essentially complete amelioration of cold allodynia in mice injected with the anti-artemin mAb (α-ARTN) to all four proalgesics with no significant differences in these withdrawal latencies compared with baseline measurements. In addition, there was a significant difference in cold sensitivity in mice treated with the control versus the anti-artemin mAb in all cases. These results indicate that, like both inflammatory and neuropathic cold allodynia, cold sensitization induced by endogenous inflammatory mediators and proalgesic neuropeptides is mediated exclusively by artemin acting on its receptor GFRα3 (Lippoldt et al., 2013, 2016).

Artemin induces cold allodynia downstream of either CGRP, substance P, or TLR4 signaling

Our data have lent to a potential model whereby activation of nociceptive afferents expressing TRPV1 or TRPA1 triggers CGRP, substance P, and TLR4 signaling pathways, which, in turn, prompt the activation of GFRα3, presumably through artemin, and subsequent sensitization of TRPM8 cells that mediate cold sensing. This predicts that these signaling pathways are upstream of artemin and, as artemin itself induces TRPM8-dependent cold allodynia (Lippoldt et al., 2013, 2016), we asked whether cold allodynia induced by an intraplantar artemin injection could be altered by inhibition of these pathways. To this end, wild-type mice were treated with $CGRP_{8-37}$ (Fig. 9*A*), SR140333 (Fig. 9*B*), or TAK242 (Fig. 9*C*), or their vehicles, as above, then were given an intraplantar injection of artemin 30 min later. For all three antagonists, mice treated with artemin showed robust cold allodynia that was not significantly different from mice given their respective vehicles. Thus, the inhibition of these signaling pathways does not alter artemin-induced cold allodynia, suggesting that artemin is downstream of the products of tissue injury and neurogenic inflammation.

Discussion

Here, we show that neurogenic inflammation induced by TRPV1 or TRPA1 causes cold allodynia via CGRP receptors, NK1R, and TLR4. Further, our studies have uncovered an intriguing sex dimorphism in the mechanisms that induce cold hypersensitivity. Regardless of the signaling pathways involved or the sex differences underlying these phenotypes, what is remarkable is that all require the neurotrophin artemin, GFRα3, and TRPM8. These results are consistent with other pain models, including localized general inflammation, nerve injury, and chemotherapeutic polyneuropathy (Lippoldt et al., 2016), highlighting the

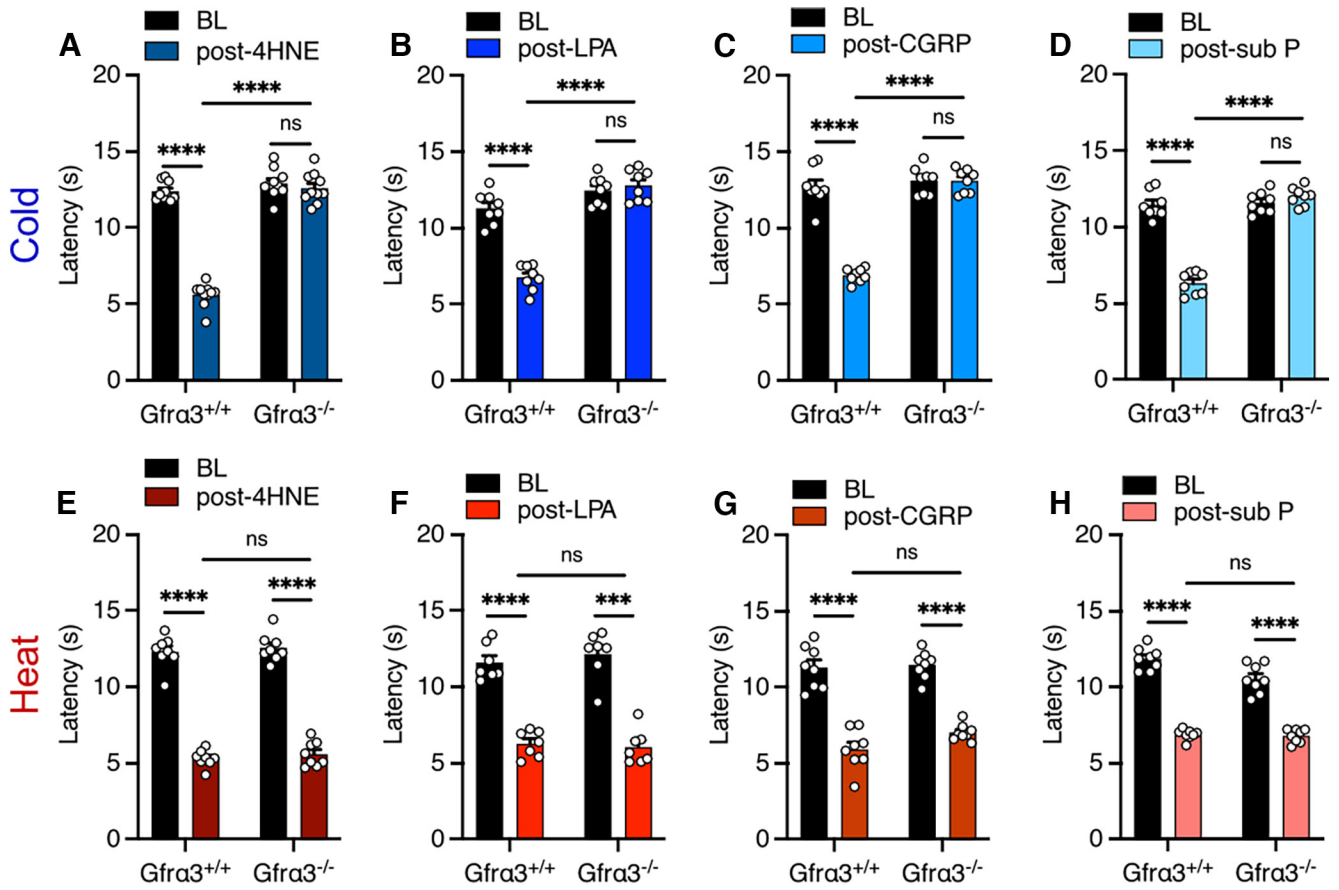

Figure 7. Cold allodynia produced by proalgesics requires GFRα3. ***A–D***, Cold allodynia observed in wild-type (*Gfrα3*$^{+/+}$) mice after injection of 4HNE (***A***), LPA (***B***), CGRP (***C***), or substance P (***D***) was absent in Gfrα3$^{-/-}$ mice. ***E–H***, Conversely, there was no difference in heat hyperalgesia between the two genotypes when injected with 4HNE (***E***), LPA (***F***), CGRP (***G***), or substance P (***H***; $n = 8$ each, paired two-tailed *t* test BL vs postinjection; unpaired *t* test with Welch's correction postinjection for *Gfrα3*$^{+/+}$ vs *Gfrα3*$^{-/-}$; $^{ns}p > 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

signaling specificity of cold compared with other modalities in which a plethora of molecules and sensory receptors produce painful sensitization after tissue insults (Basbaum et al., 2009). These findings also highlight specific molecular targets to therapeutically treat this pain modality without altering sensory processing of either heat or mechanical sensation. Indeed, our use of anti-artemin monoclonal antibodies to selectively block established cold pain serves as an exemplar of a possible intervention approach (Lippoldt et al., 2016; Yamaki et al., 2021; Jeong et al., 2022).

GFRα receptors are glycosylphosphatidylinositol-linked extracellular receptors that must work with a transmembrane coreceptor for intracellular signal transduction, classically the protooncogene Ret receptor tyrosine kinase (Durbec et al., 1996; Trupp et al., 1996). However, GFRαs are known to signal independent of Ret (Ibanez et al., 2020), which is intriguing as we find that Ret is absent from sensory neurons that coexpress TRPM8 and GFRα3 (Lippoldt et al., 2013, 2016). Thus, the signal transduction pathways that lead to artemin and GFRα3-mediated cold allodynia are unclear but require TRPM8 channels (Lippoldt et al., 2013, 2016; Yamaki et al., 2021). Further, cellular cold sensitivity is modulated by a small number of mechanisms that alter either TRPM8 function or cell excitability. For the latter, potassium conductances are known to be key regulators of neuronal cold sensitivity, with Shaker-like Kv1 and nongated two-pore channels serving as excitability brakes setting thermal thresholds of TRPM8 neurons (Kang et al., 2005; Madrid et al., 2009). Further, injury reduced the expression of potassium conductances on cold nociceptors expressing TRPM8, increasing cold sensitivity (Noël et al., 2009; Pereira et al., 2014; González et al., 2017). For TRPM8, cold sensitivity is dependent on phosphorylation, the association of the channel with the phospholipid PIP2 and Gαq proteins (Liu and Qin, 2005; Premkumar et al., 2005; Rohacs et al., 2005; Abe et al., 2006; Zhang et al., 2012; Zhang, 2019; Rivera et al., 2021), studies focusing on how these pathways inhibit TRPM8 channel function (McKemy, 2018). If these TRPM8 modulators, or altered K^+ conductances, underlie the basis for the effect of artemin and GFRα3 on cold sensitivity is unclear.

Our results also add to the increasing appreciation of the significant sex differences in pain (Mogil, 2020), with evidence that CGRP and TLR4 mediate cold pain in females and males, respectively. For CGRP, the most well characterized sex differences occur in migraine-related pain, which is more prominent in females compared with males (Ahmad and Rosendale, 2022). Intravenous CGRP triggers headaches in migraineurs (Lassen et al., 2002) and produces migraine-like pain only in female rodents when applied directly to the dura (Avona et al., 2019). In the case of nonmigraine pain, surprisingly little is known of the potential sex differences of CGRP, likely as most preclinical pain studies have used male mice almost exclusively (Mogil, 2020). However, a recent report found that intrathecally applied CGRP antagonists inhibited hyperalgesic priming in female mice only, and intrathecally applied CGRP caused a long-lasting mechanical hypersensitivity in females, effects that were transient in male mice (Paige et al., 2022).

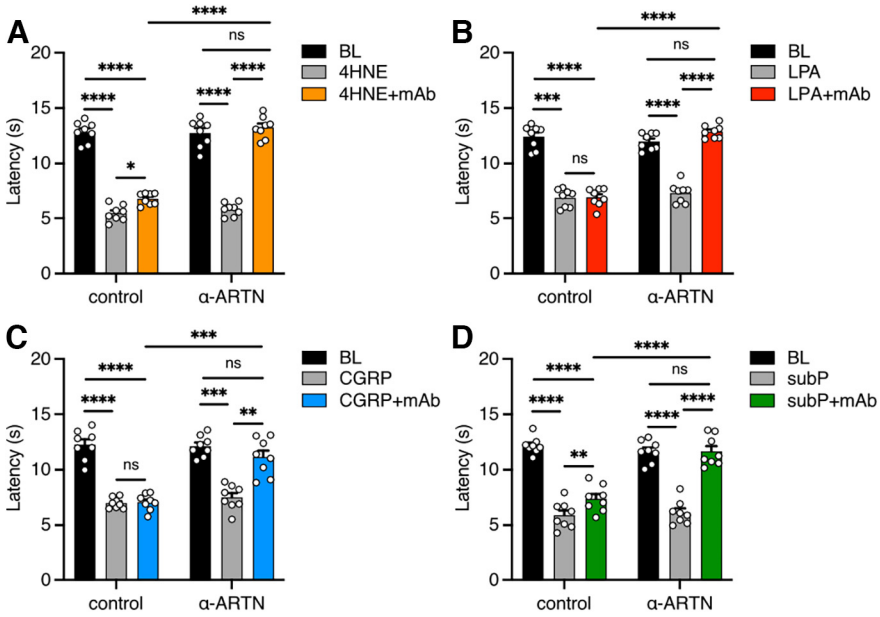

Figure 8. Artemin neutralization ameliorates cold allodynia induced by endogenous proalgesics. ***A–D***, Mice treated with control IgG show robust cold allodynia after injection of 4HNE (***A***), LPA (***B***), CGRP (***C***), or substance P (***D***), whereas this cold pain phenotype was ameliorated in mice given an anti-artemin mAb ($n = 8$ for each, one-way ANOVA with Tukey's multiple comparison tests for BL, proalgesic, and proalgesic + mAb, unpaired t test with Welch's correction for control vs anti-artemin mAb comparisons; $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

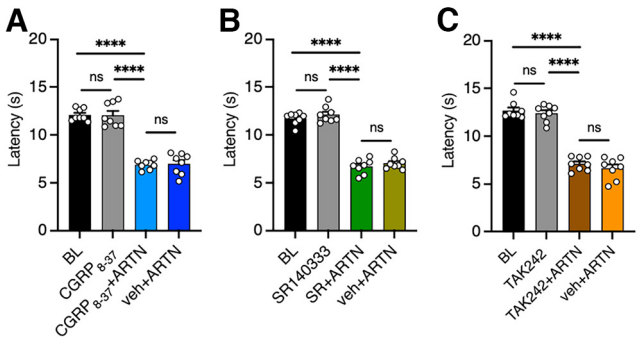

Figure 9. Artemin-induced cold allodynia is insensitive to inhibition of CGRP, substance *P*, or TLR4 signaling. ***A–C***, Wild-type mice pretreated with $CGRP_{8-37}$ (***A***), SR140333 (***B***), or TAK242 (***C***) show robust cold allodynia after an intraplantar injection of artemin ($^{ns}p > 0.05$, $^{****}p < 0.0001$; one-way ANOVA with Tukey's multiple-comparisons test for BL, antagonist, and antagonist + ARTN; unpaired t test with Welch's correction for agonist + ARTN vs veh + ARTN).

We show for the first time that peripherally (hindpaw) applied CGRP induced thermal hypersensitivity (cold and heat) in females only, demonstrating that sex differences occur in peripheral tissues. Conversely, antagonism of TLR4 prevented neurogenic cold allodynia in males but not in females, which is consistent with some preclinical studies reporting that inhibiting this receptor was only effective at improving pain in males (Sorge et al., 2011, 2015; Ramachandran et al., 2019), although other studies suggest the involvement of TLR4 in pain is more complicated (Woller et al., 2016; Huck et al., 2021; Szabo-Pardi et al., 2021). TLR4 is expressed in myeloid-lineage cells both peripherally and centrally, including macrophages, microglia, neurons, astrocytes, and endothelial cells (Ji et al., 2016). Thus, it is unclear where the actions of TLR4 lead to sexually dimorphic pain. For example, genetic deletion of TLR4 in $Nav1.8^{+}$ sensory neurons reduced nerve injury-induced mechanical hypersensitivity in female but not male mice (Szabo-Pardi et al., 2021). Further, conditional knockout of TLR4 in microglia reduced chronic allodynia more robustly in male mice in a tibial fracture model (Huck et al., 2021). The interaction between CGRP and TLR4 is complicated as CGRP modulates inflammatory responses induced by TLR4 activation, whereas TLR4 signaling leads to CGRP release and modulates the expression in TLR4-activated macrophages (Gomes et al., 2005; Ma et al., 2010b; Assas et al., 2014; Baliu-Piqué et al., 2014; Jia et al., 2021). The TLR4 antagonist used in this study inhibits mechanical allodynia in a postoperative pain model in male rats (females were not tested) when administered peripherally via an intraplantar injection (Xing et al., 2018). Here, we find similar results for neurogenic cold allodynia, suggesting that peripheral TLR4 contributes in a sex-dimorphic manner, but further studies are needed to determine whether there are also central effects on cold signaling. TLR4 is likely not coexpressed with TRPM8 neuronally since modulating TLR4 expression in sensory neurons does not induce cold behavioral changes (Szabo-Pardi et al., 2021), which is consistent with our hypothesis of GFRα3 involvement.

Previous studies have shown that substance P release contributes to thermal hyperalgesia (Renback et al., 1999; Massaad et al., 2004; Rogoz et al., 2014), as well as cold nociception in cornea (Li et al., 2019). We find that antagonizing CGRP fully reverses cold allodynia, whereas an NK1R antagonist provided incomplete inhibition. Interactions among CGRP, substance P, and other neuropeptides modulate the effects of substance P in plasma extravasation, and linkages between substance P and TLR4 are also known (Gomes et al., 2005; Rogoz et al., 2014; Schlereth et al., 2016; Huck et al., 2021). Nonetheless, our data are the first to show that intraplantar injection of substance P can induce cold allodynia in mice, regardless of sex. What is striking is that regardless of signaling pathways used herein, each ultimately converges on the release of artemin. Artemin and GFRα3 are known to play important roles in multiple forms of pain, including migraine, osteoarthritis-associated pain, and inflammatory bone pain (Shang et al., 2016, 2017; Nencini et al., 2019; Minnema et al., 2022). In addition, artemin can enhance CGRP release from nociceptors (Schmutzler et al., 2009), as well as regulate TRP channels expression (Elitt et al., 2008; Ikeda-Miyagawa et al., 2015). Why artemin has such a specific role in cold nociception, compared with redundant mechanisms in heat and force nociception, remains unknown and requires further analysis.

Last, one of the key findings of this study is it supports the premise that TRPA1 is not a molecular sensor of cold but serves an important role in amplifying cold pain associated with injury, as it does for other modalities (Bautista et al., 2013). Since their cloning, TRPM8 and TRPA1 have been considered the primary molecular detectors of cold in the peripheral nervous system, with the premise that, because of their temperature activation thresholds *in vitro*, TRPM8 mediated innocuous cool and TRPA1 noxious cold (McKemy et al., 2002; Story et al., 2003).

While the role of TRPM8 in cold is largely incontrovertible, TRPA1 as a molecular sensor of cold has been an intense topic of debate in the field after it was shown to be a receptor for pungent and inflammatory molecules (Bandell et al., 2004; Jordt et al., 2004; Trevisani et al., 2007). *In vivo*, the strongest evidence for TRPA1 mediating cold has come from studies showing that inflammatory or neuropathic cold pain is lessened in animals with reduced TRPA1 expression or treated with antagonists (Obata et al., 2005; da Costa et al., 2010; del Camino et al., 2010; Palkar et al., 2015). Further, in some cases cold sensitivity is increased when animals are treated peripherally with TRPA1 agonists, as we show here (del Camino et al., 2010; Honda et al., 2014). These convincing data demonstrated that TRPA1 is involved in cold pain in these pathologic settings leading to the reasonable supposition that it is a cold sensor *in vitro*. However, our findings provide a plausible explanation for the *in vitro* results described above in that the activation of TRPA1 and TRPA1 afferents does not directly send cold signals centrally but lead to neurogenic inflammation that promotes sensitization of TRPM8 cold signaling. This premise is further supported by the corollary findings that activation of TRPV1 leads to an identical cold phenotype, including directly inducing cold allodynia, the dependence on CGRP, substance P, and TLR4 signaling, and the dependence on artemin and GFRα3 for this cold pain phenotype.

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