

Mechanisms of Itch Evoked by β -Alanine

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β -alanine, a popular supplement for muscle building, induces itch and tingling after consumption, but the underlying molecular and neural mechanisms are obscure. Here we show that, in mice, β -alanine elicited itch-associated behavior that requires MrgprD, a G-protein-coupled receptor expressed by a subpopulation of primary sensory neurons. These neurons exclusively innervate the skin, respond to β -alanine, heat, and mechanical noxious stimuli but do not respond to histamine. In humans, intradermally injected β -alanine induced itch but neither wheal nor flare, suggesting that the itch was not mediated by histamine. Thus, the primary sensory neurons responsive to β -alanine are likely part of a histamine-independent itch neural circuit and a target for treating clinical itch that is unrelieved by anti-histamines.

Introduction

β -alanine is one of the very few naturally occurring β amino acids in humans. It is widely used as a supplement for muscle building because it is major component of the dipeptide carnosine (β -alanyl-L-histidine) and can boost the synthesis of intramuscular carnosine, which is an effective buffer to prevent muscle cells from becoming acidic, thereby decreasing fatigue during exercise (Baguet et al., 2010). A common sensory side effect of β -alanine is skin paresthesias including itch and tingling (Décombaz et al., 2012), but the underlying molecular mechanisms remain elusive.

β -alanine is widely distributed not only in muscle but in the nervous system and the skin (Crush, 1970; Tiedje et al., 2010). β -alanine binds and activates MrgprD receptors in humans and rodents (Shinohara et al., 2004). MrgprD, a G-protein-coupled receptor (GPCR), is highly expressed in a subset of small-diameter dorsal root ganglion (DRG) neurons implicated in mechanosensation (Dong et al., 2001; Shinohara et al., 2004; Cavanaugh et al., 2009). Strikingly, MrgprD⁺ axons exclusively innervate the epidermis of hairy and glabrous skin (Zylka et al., 2005), raising the untested possibility they may be involved in the skin-specific sensation of itch.

GPCRs play essential roles in mediating itch signals by directly detecting pruritic compounds in the skin (Liu et al., 2009, 2011).

Therefore, it is interesting to test whether β -alanine-induced itch is mediated by MrgprD. Here we show that a local intradermal injection of β -alanine directly induced itch in humans, confirming that β -alanine induces itch through a peripheral, cutaneous mechanism. We then show that β -alanine given as an oral supplement or injected intradermally elicited itch-associated behavior that was severely reduced in MrgprD^{-/-} mice. Furthermore, β -alanine directly excited DRG neurons via MrgprD. Lastly, by electrophysiologically recording from cutaneous sensory neurons, *in vivo*, we found that β -alanine activated a subset of MrgprD-expressing C-fiber mechanosensitive neurons, namely those responsive to heat. Because these neurons did not respond to histamine, they are candidates for mediating the itch and associated sensations elicited by histamine-independent pruritic stimuli, including β -alanine, heat, and mechanical stimuli.

Materials and Methods

Psychophysical study in humans

Eleven healthy subjects (aged 23–45 years; eight females; three males) were tested with protocols approved by the Yale University Human Investigation Committee.

The volar forearm was intradermally injected with 10 μ l of β - or L-alanine (Sigma Aldrich) in amounts of 22.5, 45, 90, or 180 μ g dissolved in a vehicle of sterile extracellular fluid (Sikand et al., 2011). The subjects used the generalized labeled magnitude scale to rate the perceived intensity of any itch, pricking/stinging, and burning evoked by the stimulus (Sikand et al., 2011).

Behavioral studies in mice

The behavioral testing and analyses were performed with an experimenter blind to experimental condition using protocols approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine. The mice (MrgprD knock-out) (Zylka et al., 2005; Rau et al., 2009) were 2–3 month-old males (20–30 g) that had been backcrossed to C57BL/6 mice for at least 10 generations.

Oral consumption of β -alanine. The previously acclimated mouse, held in the experimenter's hand, was fed, by pipette, either β -alanine (40 mg/ml) in a vehicle of 5% sucrose in water or the vehicle alone, and then placed in a test chamber. Behavior was video recorded for 40 min. From

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analyses of video recordings, the number of bouts of scratching of the cheek (vs the back) with the hindpaw and the number of wipes of the cheek with the forepaw (Shimada and LaMotte, 2008) were counted as these were the dominant behaviors.

Intradermal injection of β -alanine. In the mouse acclimatized to the test chamber, either β -alanine (50, 20, or 10 mM) or histamine dihydrochloride (27 mM) in 10 μ l of vehicle, or 10 μ l of vehicle (saline) alone, was injected intradermally into the cheek and behavioral responses video was recorded for 15 min. The video recordings were subsequently viewed to extract the number of bouts of scratching and the number of wipes directed toward the site of injection (Shimada and LaMotte, 2008).

Calcium imaging

Dorsal root ganglia from all spinal levels of 3-week-old mice were dissociated cultured for 18–20 h as previously described (Liu et al., 2009). There was no NGF or GDNF in the culture media for a calcium-imaging experiment; GDNF (20 ng/ml) was added to the media for whole-cell recordings. Neurons were loaded with Fura 2-acetomethoxyl ester (Invitrogen) for 30 min in the dark at room temperature. After washing, cells were imaged at 340 and 380 nm excitation to detect intracellular free calcium. We applied chloroquine (1 mM), histamine (100 μ M), and β -alanine (1 mM) to DRG neurons. To compare the response to histamine or β -alanine between WT and MrgprD-deficient DRG neurons, calcium-imaging assays were performed by an experimenter blind to genotype.

Whole-cell current-clamp recordings of cultured DRG neurons

Current-clamp recordings were performed as previously described (Liu et al., 2009). Briefly, action potential measurements were performed with an Axon 700B amplifier and the pCLAMP 9.2 software package (Molecular Devices). Electrodes were pulled (Model pp-830; Narishige) from borosilicate glass (WPI). Neurons were perfused with β -alanine for 10 s and washed for 3 min. All experiments were performed at room temperature (\sim 25°C).

In vivo electrophysiological recording

Nine adult male MrgprD-GFP knock-in (expressing GFP in MrgprD-expressing neurons) (Zylka et al., 2005), each weighing 23–29 g, were used. The use and handling of animals were approved by the Institutional Animal Care and Use Committee of the Yale University School of Medicine.

Primary sensory neurons innervating the skin on the hindlimb of the mouse were electrophysiologically recorded *in vivo* as described previously (Ma et al., 2010, 2012). Mice were anesthetized with isoflurane inhalation, artificially ventilated, and their core maintained at 36–38°C. After a laminectomy at the levels of L2–L6, the L3 or L4 DRG was exposed, separated from surrounding tissue, fixed to a plate, and superfused with warm, oxygenated, artificial cerebrospinal solution within a pool formed by a ring to which the skin was sewn. After removal of the epineurium, the cells on the surface of the ganglion were viewed using an upright microscope at 400 \times magnification either via reflection microscopy or epifluorescence microscopy (to identify the presence of GFP indicative of MrgprD expression).

Extracellular recording was performed using a micropipette electrode (Ma et al., 2010, 2012). The sensory submodality of a neuron was classified by its responses to mechanical, thermal, and chemical stimulation of its peripheral receptive field. These stimuli included innocuous stroking with a cotton-tipped probe, a gentle pinch using the experimenter's fingers and von Frey filaments with a fixed tip diameter (100 or 200 μ m) but delivering different bending forces (for noxious mechanical stimuli), a temperature-controlled chip-resistor heating probe, or ice (to deliver noxious thermal stimuli) (Ma et al., 2012). Neurons identified by conduction velocity as having C-fibers were classified as CM if responsive (exhibited multiple action potentials) to noxious mechanical stimuli alone or as CMH if responsive to mechanical and heat noxious stimuli (51°C, 5 s). Next, 5 μ l of vehicle (0.9% NaCl), histamine (1 μ g/ μ l in vehicle), or β -alanine (50 mM in vehicle) was injected intradermally into the receptive field using a microsyringe with a 31 gauge needle (sequence of chemical delivery varied). The chemically evoked discharges typically

lasted 2–10 min, after which at least 5 min elapsed before the next injection.

Results

Intradermal injection of β -alanine evokes itch in humans

It is not known whether β -alanine, consumed orally in humans, elicits itch by acting directly (or through a metabolite) on the nerve endings of pruriceptive neurons or at some other site along the pathways mediating itch. Here, in tests in humans, intradermally injected β -alanine (90 μ g or 100 mM) elicited itch in all subjects, who also reported lesser sensations of pricking/stinging and burning (Fig. 1A). The sensations peaked within the first few minutes and declined slowly over time. The same dose of L-alanine, the proteinogenic α -amino acid that cannot bind or activate MrgprD (Shinohara et al., 2004), produced a weak, transient itch in only five of 11 subjects (Fig. 1A). The mean area under the curve (AUC), peak magnitude, and duration of itch were each significantly greater for β -alanine compared with L-alanine ($p < 0.05$, paired Student's *t* tests). In comparison with β -alanine, L-alanine also evoked significantly lesser sensations of pricking and burning (as measured by peak magnitude, duration, and/or AUC; $p < 0.05$). A significant effect of dose was obtained for AUC ($p = 0.012$), peak magnitude ($p = 0.04$), and duration ($p = 0.04$) of itch only for β -alanine (one-way repeated-measures ANOVA; Fig. 1B). But the highest magnitude of itch was observed with 90 μ g (100 mM). That is, an effect of dose was obtained on all the itch parameters. But there was no effect of dose on any parameters measured for pricking/stinging and burning produced by the four tested doses. These studies demonstrate that β -alanine can act directly and peripherally to elicit the sensation of itch. Importantly, no flare or wheal was induced by β -alanine injection in human skin, suggesting that β -alanine-induced itch is mediated by a histamine-independent neural pathway.

MrgprD mediates β -alanine-induced itch

To determine whether the β -alanine-induced itch response is mediated by MrgprD, we compared the scratching behavior between wild-type (WT) and MrgprD^{-/-} mice. Cheek injections of a series of concentrations of β -alanine elicited dose-dependent site-directed bouts of scratching in WT mice (Fig. 2A). The mice exhibited few if any pain-associated behaviors denoted by site-directed wiping of the injection site with the forepaw, suggesting β -alanine mainly induces itch and not pain in mice. However, it cannot be ruled out that nonpainful nociceptive sensations, such as the pricking or burning reported by humans in response to β -alanine, might also be present in mice but of insufficient magnitude to elicit site-directed wiping. Almost no scratching was detected in MrgprD^{-/-} mice (WT vs KO, 41.1 \pm 6.5 vs 1.8 \pm 0.9 bouts, $n = 6$ vs 5, $p = 0.002$; Fig. 2A). MrgprD^{-/-} mice did not have any scratching deficits in response to cheek injection of histamine (50 μ g/10 μ l) compared with WT controls (WT vs KO, 26 \pm 3.2 vs 30.3 \pm 11.1 bouts, $n = 11$ vs 12, $p = 0.72$), indicating that MrgprD specifically mediates β -alanine-induced itch. As a negative control, L-alanine failed to induce a scratching response (Fig. 2A). These results suggest that β -alanine directly induces itch sensation in an MrgprD-dependent manner.

Since oral consumption of β -alanine evokes itch sensation in humans, we asked whether feeding mice with β -alanine can induce itch-associated behavior. To establish an animal model for β -alanine-evoked side effects, we fed WT mice sucrose water supplemented with β -alanine (40 mg/kg), a dose equivalent to the lower limit of the dose that people normally take (Harris et al., 2006). The latency of the onset of the scratching (occurring pri-

marily on the cheek and nape of the neck) in each mouse was ~ 10 min (617 ± 188 s), which is similar to that reported by humans (~ 20 min) when taking β -alanine orally (Harris et al., 2006). *MrgprD*^{-/-} mice exhibited significantly less scratching than WT controls (28.2 ± 6.0 vs 10.4 ± 3.3 bouts, $n = 5$, $p = 0.032$; Fig. 2B). The level of scratching found in the mutant mice was similar to that of both genotypes after drinking sucrose water (9.75 ± 4.21 bouts). Together, these data demonstrate that oral administration of β -alanine induces itch behavior in mice and that this effect is *MrgprD*-dependent.

β -alanine directly excites DRG neurons in an *MrgprD*-dependent manner

We next examined whether β -alanine directly activated *MrgprD*⁺ DRG neurons and whether the lack of β -alanine-induced scratching in *MrgprD*^{-/-} mice is due to a deficit in the response of mutant DRG neurons to β -alanine. Indeed, 1 mM β -alanine triggered an intracellular calcium increase in $\sim 12\%$ of total DRG neurons from WT mice, whereas it failed to activate any DRG neurons from *MrgprD*^{-/-} mice (Fig. 3A), suggesting that *MrgprD* is required for the response of DRG neurons to β -alanine. There was no effect of the KO on the responses to histamine (Fig. 3A). To confirm that β -alanine-sensitive neurons express *MrgprD*, we used *MrgprD*-GFP knock-in (*MrgprD*^{GFP/+} mice that have one copy of WT *MrgprD* and one copy of *MrgprD* deleted by GFP knockin) in which *MrgprD*-expressing neurons are labeled with GFP (Zylka et al., 2005). We found β -alanine only activated GFP⁺ *MrgprD*-expressing neurons and not neurons that did not express GFP. In addition, only 43.3% of GFP⁺ neurons showed intracellular calcium rises in response to β -alanine, suggesting that downstream signaling components are not completely coexpressed with *MrgprD*. We also examined whether β -alanine induced action potentials in dissociated DRG neurons using whole-cell patch recording. In WT, β -alanine-sensitive neurons fired multiple action potentials upon β -alanine treatment and could repeatedly be activated by β -alanine at intervals after wash-out of 3–5 min without significant tachyphylaxis (Fig. 3C). In contrast, no *MrgprD*-deficient neurons (i.e., GFP⁺ DRG neurons from *MrgprD*^{GFP/GFP} mice; i.e., two copies of *MrgprD* deleted by GFP knockin) fired action potentials upon similar treatment (Fig. 3C). These data suggest that β -alanine-induced itch sensation is likely caused by direct activation of *MrgprD* that leads to firing of DRG neurons.

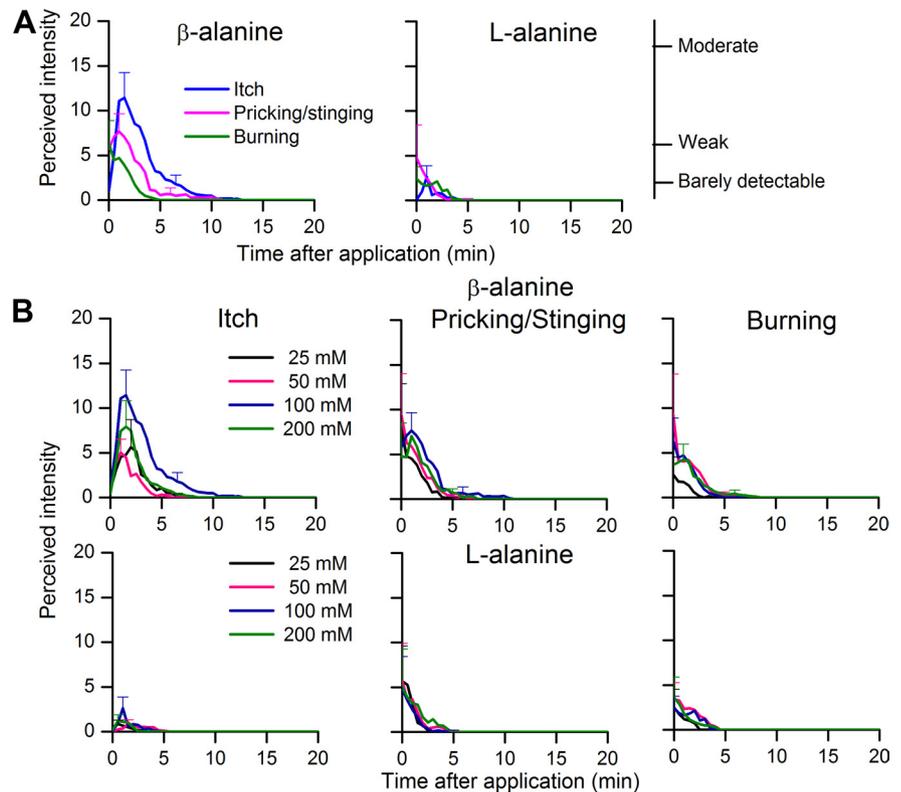


Figure 1. Intradermal injection of β -alanine evokes itch in humans. **A**, Time course of the mean perceived intensity of itch and nociceptive sensations evoked by intradermal injection of $90 \mu\text{g}$ (100 mM) of β -alanine or L-alanine. The mean ratings of itch, pricking/stinging, and burning obtained from 11 subjects are plotted for successive intervals of 30 s after injection. Beginning with the peak rating of each sensory quality, the SEM is plotted every 5 min (error bars). The positions of three verbal descriptors (vertical scale on the right) are shown in correspondence with the ratings of mean perceived intensity (y -axes). **B**, Time course of the mean perceived intensity of itch and nociceptive sensations evoked by different doses of β -alanine and L-alanine in the same subjects.

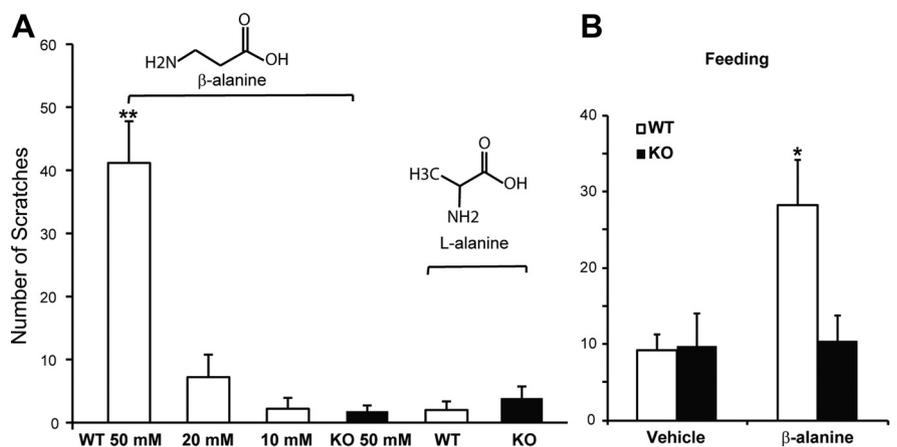


Figure 2. *MrgprD* is required for β -alanine-induced scratching in mice. **A**, Cheek injection of β -alanine induced site-directed scratching in WT but not *MrgprD*^{-/-} mice. β -alanine induced significant scratching in WT mice during the first 15 min after injection in a dose-dependent manner (50 mM , $n = 6$; 20 mM , $n = 5$; 10 mM , $n = 5$). In contrast, few scratching bouts were detected in *MrgprD*^{-/-} mice after β -alanine cheek injection ($n = 5$). L-alanine, the proteinogenic α -amino acid, did not induce a significant itch response (WT, $n = 6$; KO, $n = 8$). **B**, Orally administering β -alanine induced itch in WT but not *MrgprD*^{-/-} (KO) mice. The total number of scratching bouts during the 40 min after β -alanine feeding (40 mg/kg body weight) was significantly higher in WT mice than in *MrgprD*^{-/-} mice ($n = 5$ per genotype; *WT vs KO). All error bars represent SEM. * $p < 0.05$, ** $p < 0.01$; Student's t test.

β -alanine sensitivity defines a unique subset of histamine-insensitive DRG neurons

To further characterize the population of β -alanine-sensitive neurons, we tested their responsiveness to other itch-inducing molecules in cultured DRG using calcium imaging. All

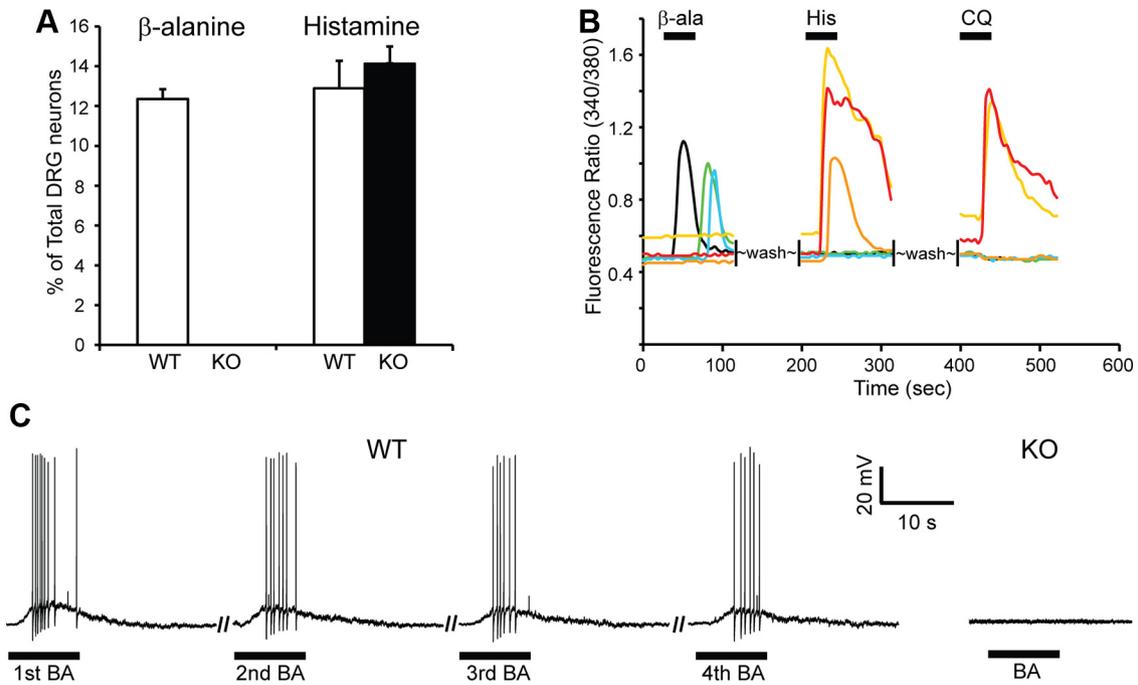


Figure 3. The response of DRG neurons to β -alanine is *MrgprD*-dependent. **A**, Approximately 12% of WT DRG neurons responded to β -alanine (β -ala; 1 mM) with increased $[Ca^{2+}]_i$, whereas *MrgprD*^{-/-} DRG neurons did not respond (number of neurons analyzed: WT, *n* = 282; KO, *n* = 212, *n* = 3 mice per genotype). The response to histamine was not impaired in *MrgprD*^{-/-} neurons. The percentage of *MrgprD*^{-/-} neurons responding to histamine (100 μ M) was similar to that of WT neurons (>300 neurons analyzed, 3–4 mice per genotype). Error bars represent SEM. **B**, Representative traces of DRG neurons from *MrgprD*^{GFP/+} mice in calcium imaging assays. β -alanine only activated GFP⁺ neurons as monitored by increased $[Ca^{2+}]_i$ with calcium imaging. β -alanine-sensitive neurons (black, green, and blue traces; *n* = 91) did not respond to histamine (His; 100 μ M) or CQ (1 mM), and histamine- and CQ-sensitive neurons (yellow, red, and orange traces) did not respond to β -alanine (>200 neurons from 3 mice). **C**, β -alanine (BA; 1 mM) induced action potentials in DRG neurons. In WT DRG, β -alanine-sensitive neurons (as determined by calcium imaging, *n* = 8) fired action potentials upon repeated β -alanine treatment. In contrast, none of the neurons tested from *MrgprD*^{-/-} mice showed any response to the drug (*n* = 7).

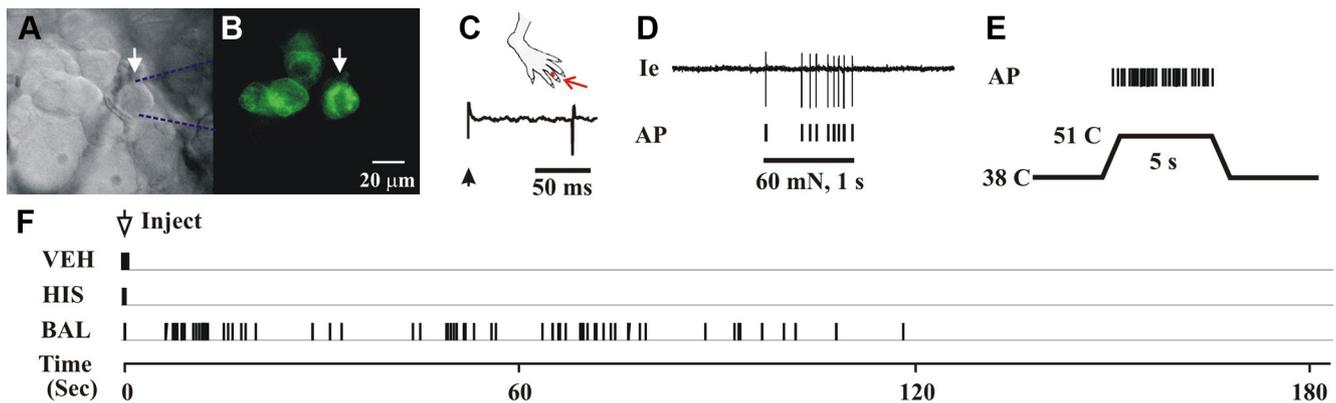


Figure 4. β -alanine-sensitive neurons are C-mechanoheat nociceptive and do not respond to histamine. **A**, Bright-field image of a neuronal recording (arrow) with an extracellular electrode (outlined with dashed blue lines). **B**, Fluorescent microscopy revealed the expression of GFP (i.e., *MrgprD*⁺) in this neuron. **C**, Location of the cutaneous receptive field (red dot) of this neuron on the hairy skin of the hindpaw and conduction velocity (0.64 m/s, lower trace) obtained with electrical stimulation (arrow) of the receptive field. **D**, Responses of this neuron to a 60 mN force via a 200 μ m diameter probe applied to the receptive field (1 s) with original extracellular recording trace (Ie) and action potentials (AP) indicated below. **E**, Response to heat stimulation (38–51°C, 5 s) indicates that this is a CMH nociceptive neuron. **F**, Responses of the above CMH neuron to the intradermal injection of vehicle (VEH), histamine (HIS), and β -alanine (BAL).

β -alanine-sensitive neurons failed to respond to histamine (Fig. 3B), suggesting that β -alanine sensitivity may define a histamine-independent itch-sensing neuronal population in the DRG. Consistently, the percentages of total WT and *MrgprD*^{-/-} DRG neurons responding to histamine were similar (Fig. 3A). Furthermore, β -alanine-responding neurons were not sensitive to chloroquine (CQ; Fig. 3B), a drug used to treat and prevent malaria but with serious pruritic side effects (Mnyika and Kihamia, 1991). We previously found that *MrgprA3* functions as a major receptor for CQ and mediates CQ-induced itch (Liu et al., 2009). CQ-sensitive DRG neurons also respond to histamine (Liu et al.,

2009). *In situ* hybridization experiments indicated that *MrgprA3* and *MrgprD* are expressed by different populations of DRG neurons (Dong et al., 2001; Zylka et al., 2005), which is consistent with the current finding that β -alanine-sensitive neurons respond to neither CQ nor histamine.

We next studied *MrgprD*⁺ neurons in a more physiologically relevant setting with an *in vivo* electrophysiological recording method (Ma et al., 2010). The advantage of this method is that it allows us to first positively identify neurons of interest (in this case, GFP⁺ neurons in *MrgprD*^{GFP/+} mice; Fig. 4A,B) and then study their peripheral receptive field

properties in living animals. A total of 19 GFP⁺ DRG neurons from MrgprD^{GFP/+} mice were recorded. All neurons were cutaneous C-nociceptive units (conduction velocity range: 0.50–0.75 m/s), including nine CMH (responding to both mechanical and heat stimuli) and 10 CM (responding only to noxious mechanical stimulation) neurons (Fig. 4C–E). Two of the CMH neurons also responded to cold stimulation (ice, 20 s). All neurons were initially silent (not spontaneously active) before testing. Injection of vehicle never evoked any discharges beyond insertion of the needle. Five of the nine CMH neurons were tested with β -alanine, two were tested with histamine only, and the remaining two were tested with both chemicals. All 10 CM neurons were tested with β -alanine, of which seven were also tested with histamine. Each chemical was injected intradermally into the receptive field in the hindpaw. β -alanine evoked discharges in all seven CMH neurons tested but not in any of the CM neurons (Fig. 4F). In contrast, the four CMH and seven CM neurons tested did not show any response to histamine (Fig. 4F). Histamine injection was able to activate other CMH neurons that did not express GFP (data not shown). Both *in vitro* DRG calcium imaging and *in vivo* DRG recording data demonstrate that MrgprD-expressing neurons were of two types: those that were activated by β -alanine and by heat and those that were not. Neither type was activated by histamine.

Discussion

Our findings indicate that skin sensations evoked by β -alanine are mediated by MrgprD expressed in cutaneous sensory neurons. Although another study showed that MrgprD enhanced the excitability of small-diameter DRG neurons (Rau et al., 2009), its function in somatosensation has not been directly demonstrated. In this study, we provide the first evidence that β -alanine can directly trigger action potentials in both *in vitro* cultures and *in vivo* DRG neuron recordings. Furthermore, our mouse behavioral and human psychophysical data suggest that a major role of MrgprD in somatosensation is itch. These studies provide a molecular explanation for β -alanine-induced itch and demonstrate that β -alanine-induced itch-mediating neural activity originates in the peripheral and not the central nervous system.

β -alanine sensitivity defines a unique population of itch-mediating neurons and further demonstrates the functional diversity of primary sensory neurons in DRG. MrgprD-expressing neurons comprise the majority of nonpeptidergic, small-diameter neurons in the DRG. Dissociated DRG neuron recordings, skin nerve recordings, and neuronal ablation studies suggest MrgprD⁺ neurons are unmyelinated (C-fiber), mechanosensitive, nociceptive neurons (Dussor et al., 2008; Cavanaugh et al., 2009; Rau et al., 2009). Behaviorally, mice lacking MrgprD⁺ neurons exhibit reduced mechanical but not thermal pain, suggesting MrgprD⁺ neurons are mainly involved in mechanosensation *in vivo* (Cavanaugh et al., 2009). Here we reveal the unexpected heterogeneity and functional diversity of MrgprD⁺ neurons. Only a subset of MrgprD-expressing neurons is involved in β -alanine-evoked itch. Interestingly, this subset has physiological properties distinct from those of β -alanine-insensitive but MrgprD-positive neurons. *In vivo* DRG recordings showed that β -alanine-sensitive neurons account for ~40% of total MrgprD-expressing neurons and respond to heat stimuli. In contrast, the β -alanine-insensitive but MrgprD-positive neurons are insensitive to heat. In the future, it would be interesting to in-

vestigate the molecular basis of the distinct physiological properties of MrgprD-expressing neurons. Furthermore, β -alanine sensitivity implies a functional diversity of MrgprD-expressing neurons and raises the question of whether a fraction of MrgprD-expressing neurons are dedicated to the transduction of pruritic chemicals and mediate itch whereas others mediate mechanically evoked pain. Our data are consistent with the presence of two different types of itch-mediating neurons, one responsive to β -alanine but not to histamine and another responsive to histamine but not MrgprD⁺ (Liu et al., 2009). The MrgprD molecular marker coupled with the response to β -alanine provide a means of identifying the same itch-mediating neurons *in vitro* and *in vivo*.

Our studies also developed a novel, physiologically relevant method to study itch. Most if not all current animal models of acute itch entail skin injection of itch-inducing compounds. However, most drug-, supplement-, and food-induced itch are triggered by oral consumption. Oral administration of β -alanine provides a valuable way to study itch, especially for investigating itch side effects caused by many orally taken medicines (Reich et al., 2009). Because only healthy volunteers were tested, it remains to be determined whether the levels of β -alanine or some other endogenous chemical mediator may be capable of activating MrgprD-expressing neurons and contribute to itch under pathological conditions. Together, the identification of MrgprD as an itch-mediating receptor should open new avenues for the exploration of pruriceptive neuronal function and circuitry as well as the development of novel antipruritic drugs.

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