Activity-dependent silencing reveals functionally distinct itch-generating sensory neurons

David P Roberson[1,2], Sagi Gudes[3,4,8], Jared M Sprague[1,5,8], Haley A W Patoski[1], Victoria K Robson[1], Felix Blasl[3], Bo Duan[2,6], Seog Bae Oh[1,7], Bruce P Bean[2], Qiufu Ma[2,6], Alexander M Binshtok[3,4] & Clifford J Woolf[1,2]

The peripheral terminals of primary sensory neurons detect histamine and non-histamine itch-provoking ligands through molecularly distinct transduction mechanisms. It remains unclear, however, whether these distinct pruritogens activate the same or different afferent fibers. Using a strategy of reversibly silencing specific subsets of murine pruritogen-sensitive sensory axons by targeted delivery of a charged sodium-channel blocker, we found that functional blockade of histamine itch did not affect the itch evoked by chloroquine or SLIGRL-NH2, and vice versa. Notably, blocking itch-generating fibers did not reduce pain-associated behavior. However, silencing TRPV1+ or TRPA1+ neurons allowed allyl isothiocyanate or capsaicin, respectively, to evoke itch, implying that certain peripheral afferents may normally indirectly inhibit algogens from eliciting itch. These findings support the presence of functionally distinct sets of itch-generating neurons and suggest that targeted silencing of activated sensory fibers may represent a clinically useful anti-pruritic therapeutic approach for histaminergic and non-histaminergic pruritus.

Itch is a complex unpleasant cutaneous sensation that in some respects resembles pain, yet is different in terms of its intrinsic sensory quality and the urge to scratch. Histamine-mediated itch, as in patients with urticaria, can be effectively treated using histamine receptor antagonists[1]. However, itch accompanying most chronic pruritic diseases, including atopic dermatitis (eczema)[2], allergic itch[3] and dry skin itch[4], is not predominantly mediated by histamine[5]. The G protein–coupled receptors responsive to specific itch-generating ligands are distinct, although, at a cellular level, there is overlapping responsiveness of trigeminal and dorsal root ganglia (DRG) neurons to itch-producing pruritogens and pain-producing algogens[6–9]. Histamine-sensitive H1 receptors (H1Rs) generate histamine itch and are expressed by TRPV1+ phospholipase-β3 (PLCβ3)+ fibers[10,11]. Itch evoked by chloroquine is mediated by Mas-related G protein–coupled receptor (Mrgpr) A3 (refs. 6,12), whereas MrgprC11 is sensitized in dry skin itch[9,13] and activated by pruritogens released from mast cells during allergic itch[3]. Notably, co-activation of TRPV1 and H1R is required to produce histamine itch[14], whereas MrgprA3- or MrgprC11-mediated itch requires co-activation of TRPA1 (ref. 12), even though each of these TRP channels are canonical nociceptor transducers. *In vitro* calcium-imaging experiments have shown that neurons expressing MrgprA3 also respond to histamine, which has been interpreted as indicating a single neuronal path for histaminergic and MrgprA3-dependent itch[6]. Supporting this, ablation of neurons expressing MrgprA3 reduces the scratching evoked by histamine, chloroquine,

dry skin and allergic inflammation[15]. However, others report separate neural pathways mediating histamine and certain types of non-histamine itch[16,17]. Furthermore, although primary sensory neurons of juvenile mice respond to multiple itch mediators, this nonspecificity decreases with age[18]. Thus, it remains controversial whether there are separate afferents in the adult that mediate histamine itch and MrgprA3-dependent non-histamine itch. This distinction is clinically important, as therapies targeting histaminergic itch fibers might be ineffective for treating non-histaminergic itch if the neurons mediating the two itches are functionally distinct in the adult.

To study whether histaminergic and non-histaminergic itch are functionally distinct, we adapted a method originally designed for achieving a pain-specific peripheral nerve block[19,20] to selectively silence the peripheral terminals of different subsets of pruritogen- and algogen-responsive primary afferents in an activity-dependent manner. To do this, we targeted the charged, membrane-impermeable lidocaine derivative *N*-ethyl-lidocaine (QX-314, a sodium channel blocker) through large pore ion channels activated specifically by different algogens and pruritogens.

RESULTS
Targeted Na+ current block of pruritogen-activated neurons
Activation of TRPV1 channels permits entry of QX-314 selectively into DRG and trigeminal ganglion nociceptors through the TRPV1 pore to produce a selective block of sodium currents only in
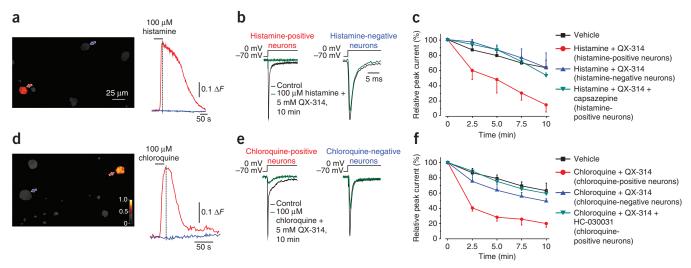
Figure 1 Application of pruritogens leads to a QX-314–mediated blockade of sodium currents selectively in pruritogen-sensitive trigeminal ganglion neurons. (**a**) Photomicrography (left) and representative traces (right) of changes in intracellular calcium concentration recorded from cultured trigeminal ganglia neurons at left. Red arrow indicates a small trigeminal neuron that responded (red trace) to histamine (60 s, 100 μM). Blue arrow indicates a cell that did not respond (blue trace) to application of histamine. Dotted line indicates the time point of the photomicrography frame. ΔF indicates the changes in absorbance ratio at 340 nm and 380 nm (340/380). (**b**) Representative, normalized traces of sodium currents recorded from histamine-positive (left) and histamine-negative (right) neurons before (black) and 10 min after (green) application of 100 μM histamine and 5 mM QX-314. (**c**) Time course of the changes in the amplitude of peak sodium current. The specific TRPV1 antagonist capsazepine (20 μM) abolished the histamine- and QX-314–mediated decrease in sodium current. Data are presented as mean ± s.e.m. of peak sodium current relative to control ($n = 3$ cells for each group; for statistical analysis, see **Supplementary Table 2**). (**d**) Photomicrography (left) and representative traces (right, dotted line indicates the time point of the photomicrography frame) of changes in intracellular calcium concentration following application of chloroquine (60 s, 100 μM). Color scale represents value of absorbance ratio at 340 and 380 nm (340/380). (**e**) Representative, normalized traces of sodium current recorded from chloroquine-positive (left) and chloroquine-negative (right) neurons before (black) and 10 min after (green) application of 100 μM chloroquine and 5 mM QX-314. (**f**) Time course of changes in amplitude of peak sodium current. The specific TRPA1 antagonist HC-030031 (100 μM) abolished chloroquine- and QX-314–mediated decrease in sodium current. Data are presented as mean ± s.e.m. of peak sodium current relative to control ($n = 3$ cells for each group; for statistical analysis, see **Supplementary Table 4**).

TRPV1-expressing nociceptors[19–24]. We examined whether histamine-mediated activation of TRPV1 channels[11,14] would allow sufficient QX-314 uptake to suppress sodium channel currents selectively in histamine-responsive trigeminal ganglion neurons. In trigeminal neuron cultures from adult male CD-1 mice, we recorded sodium currents using whole-cell voltage clamp from small (<25 μm diameter) neurons that showed an increase in intracellular calcium concentration following a 60-s bath application of 100 μM histamine (**Fig. 1a**). In these cells, a subsequent 2.5-min application of 100 μM histamine, together with 5 mM QX-314, significantly and progressively decreased sodium current amplitude, with a nearly complete block occurring after 10 min ($P < 0.05$, $n = 3$ cells; **Fig. 1b,c** and **Supplementary Tables 1** and **2**). This decrease was prevented by the TRPV1 channel blocker capsazepine (20 μM; **Fig. 1c**). Sodium currents recorded from trigeminal neurons that did not respond to histamine were not affected by co-application of histamine and QX-314 (**Fig. 1b,c**), indicating that extracellularly applied QX-314 by itself at this dose has no activity. Together with previous studies demonstrating that histamine produces downstream activation of TRPV1 channels[14], our data indicate that QX-314 enters histamine-responsive trigeminal neurons when they are activated by histamine, likely through TRPV1 channels.

We then examined whether we could block sodium currents in chloroquine-responsive trigeminal neurons by co-application of chloroquine and QX-314. TRPA1 channels are activated by chloroquine in these cells[12]. We and others have shown that QX-314 and other cationic organic compounds with a similar molecular weight permeate the TRPA1 pore[23,25]. In trigeminal neurons that showed a robust increase in intracellular calcium after brief chloroquine application (100 μM, 60 s; **Fig. 1d**), subsequent co-application of 100 μM chloroquine and 5 mM QX-314 significantly decreased sodium currents

($P < 0.001$, $n = 3$ cells; **Fig. 1e,f** and **Supplementary Tables 3** and **4**). This effect was significantly reduced by pre-incubation with the TRPA1 channel blocker HC-030031 (100 μM, $P < 0.001$, $n = 3$ cells; **Fig. 1f**). In cells that did not respond to chloroquine, QX-314 and chloroquine had no effect on sodium current (**Fig. 1e,f**). Thus, pruritogen-mediated activation of TRP channels (histamine for TRPV1 and chloroquine for TRPA1) permitted sufficient entry of QX-314 selectively into particular pruritogen-sensitive trigeminal neurons to block sodium currents only in these neurons (**Supplementary Fig. 1**).

Selective silencing of pruriceptors *in vivo*
To determine whether we could selectively silence pruriceptors *in vivo*, we used intradermal cheek injections of pruritogens and QX-314, and then characterized pruriceptor function by quantifying ensuing behavioral responses related to itch (hindlimb scratching of cheek) or pain (forelimb wiping of cheek)[26]. Intradermal injection of histamine (100 μg per 20 μl) produced scratching (64.3 ± 7.5 bouts, $n = 6$ mice) that fully resolved in 30 min. There was no significant change in scratching (56.0 ± 8.3 bouts, $n = 6$ mice, $P > 0.05$) when histamine was injected with 1% QX-314. On the basis of the delayed time course of nociceptor block *in vivo* in response to a combination of capsaicin and QX-314 (ref. 19), and considering the gradual development of full sodium current block *in vitro* after QX-314 and histamine co-administration (**Fig. 1c**), we hypothesized that the short duration of histamine-evoked scratching behavior (~25 min) was too brief to detect the slow-onset blocking effects generated by co-administration of histamine with QX-314. To test this, we devised a behavioral model using two sequential intradermal injections, 30 min apart, of a pruritogen into the same intradermal cheek injection site. Identical pruritogen doses were used in each injection. To ensure that both injections
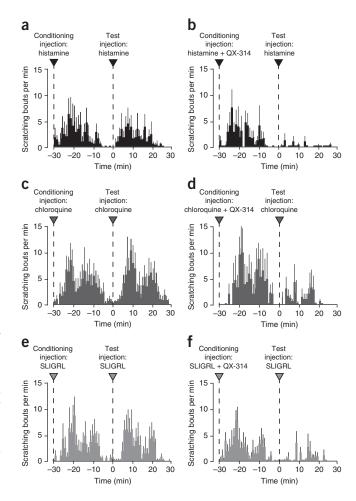
Figure 2 Co-administration of QX-314 and pruritogens inhibits subsequent pruritogen-evoked scratching. (**a**,**c**,**e**) Sequential pruritogen cheek injections at 30-min inter-stimulus intervals evoked similar levels of cheek scratching (itch) bouts. (**b**,**d**,**f**) Pruritogen-evoked scratching was inhibited 30-min after conditioning injection of pruritogen and 1% QX-314. (**a**) Conditioning injection: histamine (100 µg per 20 µl), total scratching bouts in 30 min (TSB) = 64.3 ± 7.5 bouts. Test injection: histamine (100 µg per 10 µl), TSB = 49.2 ± 9.7, $P > 0.05$, $n = 6$ mice, degrees of freedom (df) = 10. (**b**) Conditioning injection: histamine (100 µg per 20 µl) + 1% QX-314, TSB = 56.0 ± 8.3. Test injection: histamine (100 µg per 10 µl), TSB = 7.5 ± 3.8, $P < 0.001$, $n = 6$ mice, df = 10. (**c**) Conditioning injection: chloroquine (50 µg per 20 µl), TSB min = 103.0 ± 19.1. Test injection: chloroquine (50 µg per 10 µl), TSB = 109.0 ± 20.8, $P > 0.05$, $n = 7$ mice, df = 12. (**d**) Conditioning injection: chloroquine (50 µg per 20 µl) + 1% QX-314, TSB = 110.0 ± 13.7. Test injection: chloroquine (50 µg per 10 µl), TSB = 31.8 ± 13.3, $P < 0.01$, $n = 6$ mice, df = 10. (**e**) Conditioning injection: SLIGRL (50 µg per 20 µl), TSB = 80.8 ± 9.6. Test injection: SLIGRL (50 µg per 10 µl), TSB = 71.4 ± 12.0, $P > 0.05$, $n = 5$ mice, df = 8. (**f**) Conditioning injection: SLIGRL (50 µg per 20 µl) + 1% QX-314, TSB = 71.2 ± 16.1. Test injection: SLIGRL (50 µg per 10 µl), TSB = 17.7 ± 8.0, $P < 0.05$, $n = 6$ mice, df = 10. Data are presented as mean $\pm$ s.e.m. of total scratching bouts per minute for 30 min after conditioning injection and test injection. P values represent comparison of value of mean total scratching bouts in 30 min evoked by test injection to those evoked by conditioning injection.

were distributed in the same cutaneous area, however, we delivered the first dose (conditioning injection) in 20 µl of vehicle and the second dose (test injection) in 10 µl (**Supplementary Fig. 2**). Only behavior evoked by the test (second) injection was compared with behavior evoked by other identical test injections, with the experimental variable being the identity of the conditioning (first) injection given 30 min earlier.

When histamine alone was given for the conditioning injection (100 µg per 20 µl) and again 30 min later for the test injection (100 µg per 10 µl) at the same site, the amount of scratching evoked by the two injections was not significantly different ($P > 0.05$; **Fig. 2a**). However, histamine-evoked scratching was effectively abolished 30 min after a conditioning injection of both histamine and QX-314 (**Fig. 2b**), but not when it was preceded by an injection of QX-314 alone (**Supplementary Fig. 3a**). We conclude that histamine-mediated activation of large pore channels, such as TRPV1 (ref. 14), permitted uptake of QX-314 and produced a slow (<30 min) onset electrical silencing of the histamine-responsive sensory fibers, which then blocked the response to subsequent injection of histamine (**Fig. 2b**).

On the basis of our *in vitro* results (**Fig. 1d–f**), we hypothesized that intradermal administration of chloroquine with QX-314 could block chloroquine itch by permitting selective uptake of QX-314 through chloroquine-mediated activation of TRPA1 (ref. 12). Co-injection of QX-314 together with chloroquine (50 µg per 20 µl) inhibited scratching produced by subsequent chloroquine (50 µg per 10 µl) test injection, whereas injection of chloroquine alone or QX-314 alone did not (**Fig. 2c,d** and **Supplementary Fig. 3b**). We conclude that large-pore channels downstream of chloroquine-evoked MrgprA3 activation, likely TRPA1 (ref. 12), permit selective uptake of QX-314 and subsequent electrical silencing of chloroquine-sensitive afferent fibers.

SLIGRL-NH_2 (SLIGRL) acts at MrgprC11 receptors to produce itch[9]. MrgprC11-mediated itch also requires TRPA1 activation to generate scratching behavior[12]. Thus, we explored whether SLIGRL-mediated activation of TRPA1 channels is sufficient to introduce QX-314 and inhibit subsequent SLIGRL-evoked itch. SLIGRL-evoked (50 µg) scratching bouts were inhibited 30 min after injection of both
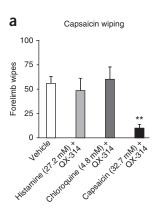
QX-314 and SLIGRL, but not after injections of either SLIGRL or QX-314 alone (**Fig. 2e,f** and **Supplementary Fig. 3c**). These findings suggest that histaminergic and non-histaminergic pruritogen-mediated entry of QX-314 into pruriceptors is sufficient to block activity of itch-generating fibers, as measured by a reduction in scratching behavior on subsequent injection of the same pruritogen.
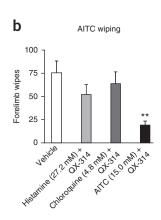
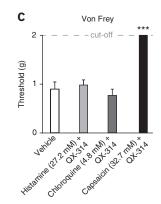## Pruriceptors are dispensable for non-itch sensations

It is unclear whether fibers that mediate histamine itch contribute to normal chemical, thermal and mechanical pain sensitivity. To explore this, we assessed pain-related behavioral responses to noxious chemical, thermal and mechanical stimulation after co-injection of QX-314 and either chloroquine (50 µg), histamine (100 µg), capsaicin (0.1%) or allyl isothiocyanate (AITC, 0.15%; **Fig. 3**). Injection of capsaicin in the cheek evoked robust forelimb wiping (indicative of TRPV1-evoked pain) 30 min after vehicle (0.9% NaCl) injection (**Fig. 3a**). Administration of both QX-314 and capsaicin abolished subsequent capsaicin-evoked wiping, as expected (**Fig. 3a**). However, capsaicin-evoked wiping was unchanged 30 min after injection of QX-314 and either histamine or chloroquine (**Fig. 3a**), even though such injections blocked scratching generated by these pruritogens (**Figs. 2** and **4**). Injections of histamine, chloroquine or QX-314 alone did not reduce subsequent capsaicin-evoked wiping (**Supplementary Fig. 4a**).

Test injection of AITC (0.15%) evoked robust forelimb wiping (indicative of TRPA1-evoked pain) 30 min after vehicle (0.9% NaCl) injection (**Fig. 3b**). AITC-evoked wiping was blocked after a conditioning injection of both QX-314 and AITC (0.15%), but not after
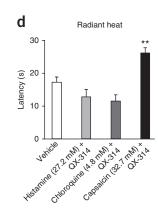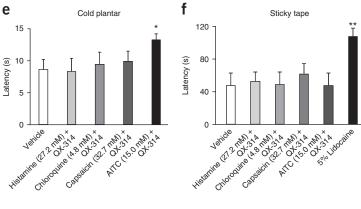
Figure 3 Silencing pruritogen-responsive neurons does not alter behavioral responses to non-itch stimuli. (**a**,**b**) Forelimb wipes (pain-related behavior) evoked by capsaicin (0.1%, 10 µl, **a**) and AITC (0.15%, 10 µl, **b**) 30 min after cheek conditioning injections of vehicle or 1% QX-314 together with pruritogens (histamine, chloroquine) or algogens (capsaicin, AITC). (**c**–**e**) Hindpaw withdrawal to punctate mechanical (von Frey) stimulus (**c**), a radiant heat (52 °C) stimulus (**d**) or focally applied cold stimulus (**e**) (see Online Methods) 30 min after intraplantar conditioning injections of vehicle or 1% QX-314 together with pruritogens or algogens. (**f**) Latency to bite, lick or attempt removal of an adhesive dot on the plantar surface of the hindpaw 30 min after intraplantar conditioning injections of vehicle or 1% QX-314 together with pruritogens or algogens was compared with a group receiving saline or intraplantar injection of 5% lidocaine (after 5 min). P values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Error bars represent s.e.m. ($n = 5$–7 mice for all groups).

conditioning injections of QX-314 and either chloroquine (50 µg) or histamine (100 µg), or by prior administration of QX-314, chloroquine or histamine alone (**Fig. 3b** and **Supplementary Fig. 4b**).

We next asked whether blocking histamine- or chloroquine-responsive fibers affects mechanical or thermal pain sensitivity. Intraplantar co-injection of QX-314 (20 µl) and either histamine (100 µg) or chloroquine (50 µg) had no effect on mechanical sensitivity to von Frey filaments, whereas co-application of capsaicin (0.1%) and QX-314 abolished mechanical pain responses (**Fig. 3c**). Likewise, intraplantar injection of QX-314 (20 µl) and either histamine (100 µg) or chloroquine (50 µg) did not alter responses to noxious heat (52 °C). Noxious thermal sensitivity was, however, abolished when tested after co-administration of capsaicin (0.1%) and QX-314 (**Fig. 3d**).

To determine whether histamine and chloroquine itch-generating fibers are dispensable for sensitivity to cold or tactile stimuli, we carried out a focal cold plantar assay[27] and an adhesive dot plantar tactile assay[28] 30 min after intraplantar injections of pruritogens and algogens together with QX-314 (**Fig. 3e,f**). Cold sensitivity was unaltered 30 min after injections of QX-314 and either histamine (100 µg), chloroquine (50 µg) or capsaicin (0.1%). However, cold sensitivity was significantly reduced after co-injection of AITC (0.15%) and QX-314 ($P < 0.05$; **Fig. 3e**). We next used latency to attend to an adhesive dot placed on the plantar paw surface as a measure of tactile sensitivity[28]. The latency to attend was unchanged 30 min after injections of QX-314 and either histamine (100 µg), chloroquine (50 µg), capsaicin (0.1%) or AITC (0.15%), whereas intraplantar injection of lidocaine (5%) significantly increased latency ($P < 0.01$; **Fig. 3f**).

We propose that, although histamine and chloroquine activate TRPV1- and TRPA1-expressing pruriceptors, respectively, these

fibers are not essential for acute thermal, mechanical and chemical pain sensitivity, which likely involve different or additional subsets of afferents to generate pain-related behavior. Likewise, we conclude that chloroquine- and histamine-sensitive itch fibers are not essential for normal tactile sensitivity.

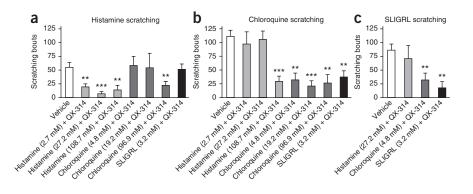### Distinct fibers mediate histamine and non-histamine itch

We next used the silencing strategy to reveal whether different pruritogens activate the same or unique peripheral afferent pathways by administering test injections (10 µl) of histamine (100 µg), chloroquine (50 µg) or SLIGRL (50 µg) 30 min after conditioning injections (20 µl) of histamine (100 µg), chloroquine (50 µg) or SLIGRL (50 µg) with or without QX-314. We hypothesized that if histaminergic and non-histaminergic itch are transmitted by different subsets of afferent fibers, application of both histamine and QX-314 will block histaminergic itch, whereas chloroquine or SLIGRL itch will remain intact, and vice versa.

We first examined whether histamine-evoked scratching is affected by the targeted silencing of chloroquine-activated pruriceptors. Histamine-evoked scratching was unchanged 30 min after a conditioning injection of both chloroquine and QX-314 (**Fig. 4a**), even though this treatment blocked subsequent chloroquine itch (**Figs. 2b** and **4b**). Similarly, conditioning injection of SLIGRL and QX-314 (which reverses later SLIGRL-evoked scratching; **Figs. 2c** and **4c**) did not reduce subsequent histamine (100 µg per 10 µl) itch (**Fig. 4a**).

We then asked whether chloroquine itch is affected following silencing of histamine- or SLIGRL-sensitive itch fibers by injecting chloroquine 30 min after administration of QX-314 and either histamine or SLIGRL. Chloroquine-evoked itch was blocked after

Figure 4 Distinct primary afferents mediate histaminergic itch and non-histaminergic itch. Pruritogen-evoked cheek scratching (itch) bouts 30 min after different conditioning injections, indicated at the x axes, are shown. (a–c) Intradermal test injection of histamine (100 μg per 10 μl) alone (a), chloroquine (CQ, 50 μg per 10 μl) alone (b) or SLIGRL (50 μg per 10 μl) alone (c) 30 min after conditioning injection of vehicle or pruritogens together with 1% QX-314. P values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; $**P < 0.01$, $***P < 0.001$). Error bars represent s.e.m. ($n = 5$–7 mice for all groups).

co-injection of SLIGRL and QX-314, but not by co-administration of histamine and QX-314 (**Fig. 4b**). Likewise, SLIGRL-evoked itch was unaffected 30 min after injection of histamine with QX-314, but was significantly reduced after injection of chloroquine and QX-314 ($P < 0.01$; **Fig. 4c**).

It appears that a common afferent population mediates chloroquine and SLIGRL itch, but this population is functionally distinct from the neurons responsible for histamine itch. These findings contrast, however, with prior *in vitro* data showing that all chloroquine-responsive DRG neurons respond to histamine[6]. A possible explanation for this discrepancy is that peripheral terminals of trigeminal neurons differ from DRG neuron cell bodies in terms of their responsiveness to multiple pruritogens. To explore this, we repeated the same silencing strategy on the back below the neck. Treatment with both histamine and QX-314 did not reduce subsequent chloroquine-evoked scratching when compared with treatment with vehicle, whereas intradermal injection of both chloroquine and QX-314 inhibited subsequent chloroquine-evoked scratching (**Supplementary Fig. 5**), indicating that facial and somatic itch appear to be similar in terms of the functional independence of histamine and non-histamine itch.

Another possibility is that different pruriceptor subsets exist with high or low sensitivity to either histamine or non-histamine pruritogens. To investigate this, we varied the dose of pruritogen given with 1% QX-314 (20 μl) for conditioning injections. Application of a lower dose of histamine (10 μg) together with QX-314 inhibited subsequent histamine-evoked scratching without reversing chloroquine (50 μg) scratching (**Fig. 4a,b**), which was essentially the same as the observed effects following a higher dose of histamine (100 μg) with QX-314. However, increasing the concentration of histamine to 108.7 mM (400 μg per 20 μl) with QX-314 for conditioning injections blocked scratching evoked by both histamine and chloroquine (**Fig. 4a,b**), indicating that an overlap manifests only at very high doses of histamine. Higher doses of chloroquine (200 or 400 μg) and QX-314 blocked later chloroquine itch behavior, but not scratching evoked by histamine. However, using 96.9 mM chloroquine (1 mg per 20 μl) together with QX-314 for conditioning injections blocked subsequent histamine- and chloroquine-evoked scratching (**Fig. 4a,b**). These data suggest that histamine and chloroquine generally activate functionally distinct pruriceptor populations, but these fibers can respond to multiple pruritogens, either directly or indirectly, when they are presented at very high, presumably non-physiological[29,30], concentrations (histamine, 107.8 mM; chloroquine, 96.9 mM).

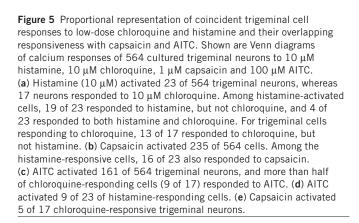**Histamine and chloroquine act on different sets of neurons**

The responsiveness of DRG neurons to multiple pruritogens decreases with age[18]. In juvenile mice, 60–100% of all chloroquine-responsive DRG cells also respond to histamine[6,18], whereas only half of

chloroquine-responsive DRG neurons respond to histamine in adolescent mice[18]. We asked whether distinct chloroquine- and histamine-sensitive neuronal populations could be revealed in adult (2–4 months old) mice by their sensitivity to pruritogens *in vitro*. MrgprA3 receptors are activated by 10 μM chloroquine and maximally activated by 1 mM chloroquine in HEK293 cells[6]. Murine DRG neurons respond to histamine at doses as low as 10 μM[14]. We examined coincident calcium responses of 564 cultured trigeminal neurons to 10 μM histamine and 10 μM chloroquine. Histamine activated 4.1% (23 of 564) of trigeminal neurons, whereas 3.0% (17 of 564) responded to chloroquine (**Fig. 5a**). A majority of chloroquine- and histamine-responding trigeminal neurons responded to only one pruritogen. Among chloroquine-activated cells, 76.5% (13 of 17) responded to chloroquine, but not to histamine, whereas the remaining 23.5% (4 of 17) responded to both 10 μM histamine and 10 μM chloroquine. Likewise, trigeminal neurons responding to histamine were largely unresponsive to chloroquine: 82.6% (19 of 23) responded to histamine, but not chloroquine, and 17.4% (4 of 23) responded to both histamine and chloroquine.

To determine whether responsiveness of adult trigeminal neurons to chloroquine or histamine is dependent on pruritogen dose, we further explored coincident calcium responses to 100 μM histamine and 100 μM chloroquine. Most trigeminal neurons that responded to either 100 μM histamine (4.8% of all cells) or 100 μM chloroquine (4.3% of all cells) did not respond to both (0.9% of all cells) (**Supplementary Fig. 6a**). Moreover, we also found that the populations of fibers responding to chloroquine and histamine in DRG neurons from adult mice were, as in the trigeminal ganglia, largely distinct (**Supplementary Fig. 6b**). These data indicate that primary afferent populations responding to histamine and chloroquine are largely distinct in adult mice.

The requisite expression of TRPV1 for histaminergic itch[14] and TRPA1 for chloroquine-evoked itch[12] raises the question of whether relative expression patterns of TRPA1 and TRPV1 differ among chloroquine- and histamine-sensitive neuronal populations. To answer this, we exposed cultured trigeminal neurons to 100 μM AITC and 1 μM capsaicin and measured the coincident responses of neurons to both agents. Consistent with the described receptor expression patterns for neurons that mediate histamine itch (for example, H1R and TRPV1)[10,14] and chloroquine itch (for example, MrgprA3 and TRPA1)[6,12], we found that a majority (16 of 23, 69.6%) of histamine-responsive cells responded to capsaicin (**Fig. 5b**) and most (9 of 17, 52.9%) of the chloroquine-activated neurons were sensitive to AITC (**Fig. 5c**). The inverse relationships were also true; most (14 of 23 neurons, 60.8%) histamine-responsive trigeminal cells did not respond to AITC (**Fig. 5d**), and capsaicin failed to activate a majority (12 of 17, 70.6%) of chloroquine-activated neurons (**Fig. 5e**).

5

Figure 5 Proportional representation of coincident trigeminal cell responses to low-dose chloroquine and histamine and their overlapping responsiveness with capsaicin and AITC. Shown are Venn diagrams of calcium responses of 564 cultured trigeminal neurons to 10 µM histamine, 10 µM chloroquine, 1 µM capsaicin and 100 µM AITC. (**a**) Histamine (10 µM) activated 23 of 564 trigeminal neurons, whereas 17 neurons responded to 10 µM chloroquine. Among histamine-activated cells, 19 of 23 responded to histamine, but not chloroquine, and 4 of 23 responded to both histamine and chloroquine. For trigeminal cells responding to chloroquine, 13 of 17 responded to chloroquine, but not histamine. (**b**) Capsaicin activated 235 of 564 cells. Among the histamine-responsive cells, 16 of 23 also responded to capsaicin. (**c**) AITC activated 161 of 564 trigeminal neurons, and more than half of chloroquine-responding cells (9 of 17) responded to AITC. (**d**) AITC activated 9 of 23 of histamine-responding cells. (**e**) Capsaicin activated 5 of 17 chloroquine-responsive trigeminal neurons.
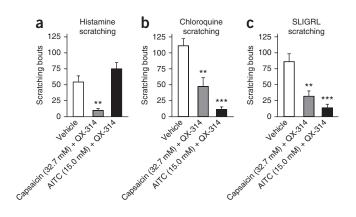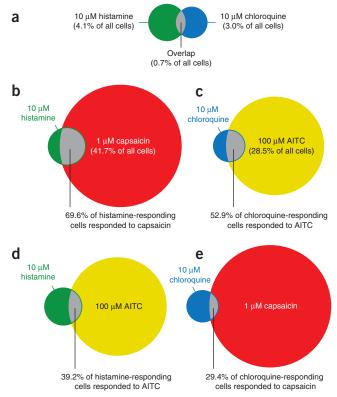

Roles of TRP channels in histamine and non-histamine itch

The different expression patterns of TRPV1 and TRPA1 among histamine- and chloroquine-sensitive neurons *in vitro* (**Fig. 5**) suggest that targeted silencing of TRPV1 or TRPA1 fibers may differently affect histamine itch and chloroquine itch. To explore this, we administered 20-µl conditioning injections of capsaicin (0.1%) or AITC (0.15%), with or without QX-314, and then administered 10-µl test injections of the pruritogens histamine (100 µg), chloroquine (50 µg) or SLIGRL (50 µg) 30 min later at the same site. Injection of capsaicin alone did not significantly reduce histamine-, chloroquine- or SLIGRL-evoked scratching ($P > 0.05$; **Supplementary Fig. 3**). However, when the conditioning injection of capsaicin was administered together with QX-314, the scratching evoked by subsequent histamine injection was abolished (**Fig. 6a**). Administration of capsaicin with QX-314 also significantly reduced, but did not eliminate, scratching produced by subsequent injections of chloroquine or SLIGRL ($P < 0.01$; **Fig. 6b,c**), even though only a third of chloroquine neurons were capsaicin sensitive (**Fig. 5e**), implying that the TRPV1⁺ subset of chloroquine-sensitive neurons may have a particularly prominent role in eliciting behavioral itch responses.

When QX-314 was administered together with AITC for the conditioning injection, subsequent SLIGRL-evoked scratching was significantly reduced ($P < 0.001$; **Fig. 6c**). Likewise, chloroquine-evoked scratching was virtually abolished 30 min after injection of QX-314 together with AITC (**Fig. 6b**). In contrast, injection of QX-314 with AITC did not reduce subsequent histamine-evoked scratching (**Fig. 6a**). In summary, chloroquine- and SLIGRL-evoked scratching is effectively blocked when TRPA1⁺ (AITC responsive) fibers are electrically silenced. Histamine-evoked scratching, on the contrary, is largely abolished when TRPV1⁺ fibers are electrically silenced, but

is unaffected when TRPA1⁺ (AITC responsive) fibers are blocked. Histamine itch is predominantly associated with TRPV1⁺ pruriceptor fibers, and non-histamine itch is associated with TRPA1⁺ fibers.

TRPV1⁺/TRPA1⁺ afferents are involved in itch inhibition

Cheek injection of capsaicin normally evokes only a wiping (pain) response without any substantial scratching (itch)[26,31]. We found, however, that cheek injection of capsaicin (0.1%, 10 µl) produced significant ($P < 0.05$) scratching (itch) as well as wiping (pain) when TRPA1-responsive nociceptors were first silenced by conditioning co-injection of AITC (0.15%, 20 µl) and 1% QX-314 (**Fig. 7a**). Injection of capsaicin (0.1%, 10 µl) 30 min after 20 µl injection of AITC (0.15%), capsaicin (0.1%), QX-314 or vehicle (0.9% NaCl) alone produced only wiping (**Supplementary Fig. 4a**). Capsaicin-evoked scratching following co-injection of AITC (0.15%) with QX-314 was prevented when the conditioning combination included histamine (100 µg) (**Supplementary Fig. 3d**). These data suggest that a subset of TRPA1⁺ sensory neurons normally mask capsaicin-evoked itch and that silencing these neurons allows capsaicin to generate itch-related behavior through uninhibited activation of TRPV1⁺ histaminergic itch fibers.

When we repeated the experiments using AITC (0.15%, 10 µl) as the test algogen (**Fig. 7b**), we again observed that algogen-mediated electrical silencing, this time by co-injection of capsaicin (0.1%) and

Figure 6 Selective silencing of nociceptor populations differentially inhibits histamine itch and non-histamine itch. (**a–c**) Cheek scratching (itch) following intradermal test injection of histamine (100 µg per 10 µl) alone ($n = 6$ mice, **a**), chloroquine (CQ, 50 µg per 10 µl) alone ($n = 6–7$ mice, **b**) and SLIGRL (50 µg per 10 µl, $n = 5–6$ mice, **c**) 30 min after a conditioning injection of vehicle (0.9% NaCl, 20 µl) or 1% QX-314 together with capsaicin or AITC. P values represent comparison to vehicle (white column) value (not significant, $P > 0.05$; **$P < 0.01$, ***$P < 0.001$). Error bars represent s.e.m.
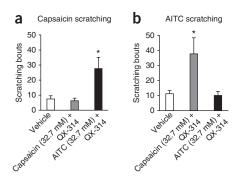
Figure 7 TRPV1⁺/TRPA1⁺ fibers suppress itch. (**a**) Hindlimb scratching (itch) evoked by capsaicin (0.1%, 10 µl) 30 min after intradermal injection of vehicle (0.9% NaCl, 20 µl), capsaicin (0.1%) and 1% QX-314 (20 µl), or AITC (0.15%) and 1% QX-314 (20 µl). Note the significant increase in capsaicin-mediated scratching following application of AITC with QX-314. (**b**) Intradermal cheek injection of AITC (0.15%, 10 µl) generated little hindlimb scratching between 30 and 60 min after injection of vehicle (0.9% NaCl, 20 µl), and this was not changed following a conditioning injection of AITC and 1% QX-314. However, AITC-evoked scratching increased following intradermal cheek injection of capsaicin (0.1%) and 1% QX-314 (20 µl). P values represent comparison to vehicle value (not significant, $P > 0.05$; *$P < 0.05$). Error bars represent s.e.m. ($n = 6$ mice for all groups).

QX-314, resulted in both wiping and scratching following test injection of AITC (**Fig. 7b**). The AITC-evoked scratching was largely reversed when chloroquine- and capsaicin-responsive fibers were both silenced by a conditioning injection of chloroquine (50 µg), capsaicin (0.1%) and QX-314 (**Supplementary Fig. 3e**). These findings suggest that a subset of TRPV1-expressing sensory neurons normally mask itch following AITC activation of chloroquine-sensitive itch fibers.

Thus, silencing TRPA1⁺ fibers and silencing TRPV1⁺ fibers allow capsaicin and AITC, respectively, to evoke scratching (itch) responses. Our interpretation is that there is a subset of peripheral TRPV1⁺ and TRPA1⁺ neurons that, when activated, normally inhibit or mask itch via central inhibitory interneurons[32] and whose silencing then allows either capsaicin or AITC to abnormally evoke itch.

DISCUSSION

We selectively inhibited pain- and itch-related behaviors by targeting the membrane-impermeant sodium channel blocker QX-314 into peripheral axon terminals of distinct populations of trigeminal pruriceptors or nociceptors. The specific population silenced was determined by the pattern of activation of particular large pore channels by different pruritogens or algogens; TRPV1 for capsaicin and histamine, TRPA1 for AITC, chloroquine and SLIGRL. Using electrophysiological recordings, we found that activation of these large pore channels by histamine and chloroquine enabled sufficient permeation of QX-314 into trigeminal neurons to block sodium currents, as shown previously for capsaicin[19], and that this effect was specific; only pruritogen-activated neurons were blocked. The fact that specific TRP-channel blockers (TRPV1 for histamine and TRPA1 for chloroquine) can prevent permeation of QX-314 indicates that its effect is mediated by TRP channels. This enabled us to exploit the silencing of different afferents to tease out their functional sensitivity to defined stimuli. Our approach differs from interventions that only block a particular receptor (for example, H1R and H4R)[33] or channel (for example, TRPA1)[34] in that it targets action potential generation

and conduction of the activated axon, and differs from genetic targeted ablation of different sensory neuronal subtypes[35] in that it is temporary, with no known compensatory changes.

Using this selective silencing strategy, we found that the fibers that mediate histamine and non-histamine itch are functionally separable. Moreover, we observed that activation of these itch-generating fibers was not required for eliciting normal responses to acute mechanical and thermal stimuli. Our *in vitro* data confirm the presence of adult sensory neurons that respond only to histamine or only to chloroquine.

Separate afferent lines have been described for histamine and cowhage itch[16,36] and a distinct non-histamine itch pathway activated by β-alanine[17]. Our data support separate functional pathways for histamine itch and itch mediated by the MrgprA3 and MrgprC11 ligands chloroquine and SLIGRL, respectively. The separation of these afferents on the basis of the silencing approach was defined for a broad range of pruritogen concentrations for targeting QX-314 into afferent terminals through activated TRP channels. At extremely high concentrations (>90 mM) of pruritogen *in vivo*, however, an overlap did occur between histamine and chloroquine populations. This cross-activation between the two populations could be secondary to release of endogenous mediators from keratinocytes, mast cells or other non-neuronal cells activated secondary to high-dose pruritogen administration, or it could reflect a very limited sensitivity of the peripheral terminals of histaminergic pruriceptors to chloroquine and SLIGRL and of the non-histaminergic terminals to histamine. Given that the high concentrations of the opposing pruritogen required to co-activate the separate histamine or non-histamine responsive set of afferents are unlikely to be found in most natural conditions[29,30], we consider it probable that the two sets are functionally distinct and normally act independently.

Our calcium imaging data also confirm the existence of distinct trigeminal and DRG neuron populations that respond to either chloroquine or histamine at a range of doses in adult mice. Prior *in vitro* experiments have shown that MrgprA3 lineage DRG neurons in juvenile (4 week old) mice respond both to chloroquine (1 mM) and histamine (50 µM)[6]. Similarly, ablation of MrgprA3⁺ neurons in 5-week-old mice substantially attenuates both chloroquine- and histamine-evoked scratching behavior[15]. However, a recent study found that the proportion of DRG neurons responding to both histamine and chloroquine was markedly less in 7–9-week-old mice compared with 3–4-week-old mice[18]. We interpret these collective data as suggesting that, in adult (2–4 month old) mice, Mrgpra3⁺ neurons are likely composed of pruriceptors with a differential sensitivity to, but not absolute selectivity for, chloroquine, and that these afferents normally contribute primarily to non-histaminergic itch. Microneurographic studies in humans have identified a distinct set of histamine-insensitive fibers that are activated during cowhage-evoked itch[16]. The finding that cowhage spicules activate MrgprA3⁺ neurons suggests that a common pathway may mediate chloroquine and cowhage itch[15]. However, cowhage spicules act nondiscriminatively on many nociceptor subtypes and may instead trigger itch nonspecifically through focal activation of superficial nociceptor terminals[37].

H1R and MrgprA3 receptors rely on downstream activation of TRPV1 or TRPA1 channels, respectively, to generate itch behavior[11,12,14], which is somewhat counterintuitive, as these TRP channels are also activated by algogens (capsaicin and AITC) that normally produce pain. This raises questions as to whether there are different subsets of TRPV1⁺ or TRPA1⁺ neurons involved in processing pain or itch and why pain normally predominates. Our data indicate that histamine itch is mediated by TRPV1⁺ fibers that do not express appreciable levels of TRPA1, as histamine itch is inhibited by silencing

capsaicin-activated fibers, but not by silencing AITC-activated fibers. This correlates with the coincidence of capsaicin and histamine responsiveness in most histamine-responsive trigeminal neurons. In contrast, chloroquine and SLIGRL itch appears to be mediated mainly by TRPA1^{+} fibers. However, using capsaicin-induced silencing and trigeminal neuron calcium imaging, we found that a subset of these fibers also co-expressed TRPV1. TRPV1 is therefore promiscuously expressed in nociceptors and both histaminergic and non-histaminergic pruriceptors.

Electrical silencing of either histamine-sensitive or chloroquine-sensitive primary afferents blocks itch, but does not alter a wide range of pain-associated behaviors, suggesting that, although these primary afferent fibers are required for itch, as are gastrin-releasing peptide receptor–expressing neurons in the dorsal spinal cord[35,38], they are not necessary for eliciting acute thermal or mechanical pain, as observed for the MrgprA3^{+} population[15]. Our data do not rule out the possibility, however, that activation of some histamine-sensitive or chloroquine-sensitive fibers might be sufficient to produce pain. For example, histamine can evoke pain in rodents and humans[39,40], particularly in bradykinin-sensitized nociceptors[41].

We also found a group of peripheral neurons that expressed both TRPA1 and TRPV1 and appeared to be involved in a functional inhibition or masking of itch, as silencing either TRPA1^{+} fibers or TRPV1^{+} fibers allowed capsaicin or AITC to evoke itch, rather than pain. Consistent with this, when nociceptive sensitivity is reduced by abolishing vesicular glutamate transporter type 2–dependent synaptic glutamate release in nociceptors, intradermal capsaicin injection is then able to generate scratching[42]. Thus, we propose that the algogen-evoked itch that followed electrical silencing of contra-algogen-responsive fibers may represent a pain-to-itch synesthesia produced only when TRPV1^{+} histaminergic pruriceptors or TRPA1^{+} non-histaminergic pruriceptors are activated and a normally itch-inhibiting subset of TRPV1^{+}/TRPA1^{+} nociceptors is silenced (**Supplementary Fig. 7**). In the absence of such silencing, the combination of activation of nociceptive pathways and inhibition of itch by TRPV1 or TRPA1 expressing nociceptors will lead to pain dominating as a sensation.

Our findings suggest that primary afferent itch-generating neurons encode functionally distinct histamine and chloroquine itch pathways (**Supplementary Fig. 7**). In addition to revealing modality specificity and functional specialization of somatosensory afferents, our findings could also help to direct development of new treatments for itch. Administration of QX-314 may be an effective treatment for pruritus caused by either histamine or non-histamine pruritogens if they are associated with sufficient activation of TRPV1 or TRPA1. Alternatively, given that large-pore channels are present in both histamine- and chloroquine-sensitive pruriceptors, broadly targeting QX-314 into these fibers via co-activation of both TRPV1 and TRPA1 channels, for example, by using a non-pungent TRPV1/TRPA1 co-activator[23,24,43], may have therapeutic promise for preventing or blocking both histamine-evoked itch and histamine-independent itch, albeit at the expense of also producing analgesia.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

AUTHOR CONTRIBUTIONS
D.P.R. conceived, designed and conducted the behavioral experiments, analyzed behavioral data, and wrote the manuscript. S.G. performed the combined calcium-imaging and electrophysiology experiments. J.M.S. carried out the calcium-imaging experiments. H.A.W.P. and V.K.R. conducted behavioral experiments and analyzed behavioral data. F.B. contributed to the combined calcium-imaging and electrophysiology experiments. B.D. contributed to behavioral experiments and interpretation of behavioral data. S.B.O. gathered behavioral pilot data and gave manuscript advice. B.P.B. conceived the silencing strategy, gave technical and conceptual advice, and edited the manuscript. Q.M. provided critical analysis and interpretation of behavioral data, designed behavioral experiments, and contributed to critical revision of the manuscript. A.M.B. conceived the silencing strategy, conceived and designed behavioral experiments and the combined calcium-imaging and electrophysiology experiments, gathered behavioral pilot data, supervised the project, and wrote the manuscript. C.J.W. conceived the silencing strategy, supervised the project and wrote the manuscript.

1. Grundmann, S. & Stander, S. Chronic pruritus: clinics and treatment. *Ann. Dermatol.* **23**, 1–11 (2011).
2. Yosipovitch, G. & Papoiu, A.D. What causes itch in atopic dermatitis? *Curr. Allergy Asthma Rep.* **8**, 306–311 (2008).
3. Lee, M.G. *et al.* Agonists of the MAS-related gene (Mrgs) orphan receptors as novel mediators of mast cell-sensory nerve interactions. *J. Immunol.* **180**, 2251–2255 (2008).
4. Akiyama, T., Carstens, M.I. & Carstens, E. Enhanced scratching evoked by PAR-2 agonist and 5-HT but not histamine in a mouse model of chronic dry skin itch. *Pain* **151**, 378–383 (2010).
5. Papoiu, A.D., Tey, H.L., Coghill, R.C., Wang, H. & Yosipovitch, G. Cowhage-induced itch as an experimental model for pruritus. A comparative study with histamine-induced itch. *PLoS ONE* **6**, e17786 (2011).
6. Liu, Q. *et al.* Sensory neuron–specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. *Cell* **139**, 1353–1365 (2009).
7. Akiyama, T., Carstens, M.I. & Carstens, E. Facial injections of pruritogens and algogens excite partly overlapping populations of primary and second-order trigeminal neurons in mice. *J. Neurophysiol.* **104**, 2442–2450 (2010).
8. Patel, K.N. & Dong, X. Itch: cells, molecules and circuits. *ACS Chem. Neurosci.* **2**, 17–25 (2011).
9. Liu, Q. *et al.* The distinct roles of two GPCRs, MrgprC11 and PAR2, in ich and hyperalgesia. *Sci. Signal.* **4**, 45 (2011).
10. Han, S.-K., Mancino, V. & Simon, M.I. Phospholipase Cbeta 3 mediates the scratching response activated by the histamine H1 receptor on C-fiber nociceptive neurons. *Neuron* **52**, 691–703 (2006).
11. Imamachi, N. *et al.* TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proc. Natl. Acad. Sci. USA* **106**, 11330–11335 (2009).
12. Wilson, S.R. *et al.* TRPA1 is required for histamine-independent, Mas-related G protein–coupled receptor–mediated itch. *Nat. Neurosci.* **14**, 595–602 (2011).
13. Akiyama, T., Carstens, M.I. & Carstens, E. Enhanced responses of lumbar superficial dorsal horn neurons to intradermal PAR-2 agonist but not histamine in a mouse hindpaw dry skin itch model. *J. Neurophysiol.* **105**, 2811–2817 (2011).
14. Shim, W.-S. *et al.* TRPV1 mediates histamine-induced itching via the activation of phospholipase A2 and 12-lipoxygenase. *J. Neurosci.* **27**, 2331–2337 (2007).
15. Han, L. *et al.* A subpopulation of nociceptors specifically linked to itch. *Nat. Neurosci.* **16**, 174–182 (2013).
16. Namer, B. *et al.* Separate peripheral pathways for pruritus in man. *J. Neurophysiol.* **100**, 2062–2069 (2008).
17. Liu, Q. *et al.* Mechanisms of itch evoked by beta-alanine. *J. Neurosci.* **32**, 14532–14537 (2012).
18. Akiyama, T. *et al.* Cross-sensitization of histamine-independent itch in mouse primary sensory neurons. *Neuroscience* **226**, 305–312 (2012).
19. Binshtok, A.M., Bean, B.P. & Woolf, C.J. Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature* **449**, 607–610 (2007).
20. Brenneis, C. *et al.* Phenotyping the function of TRPV1-expressing sensory neurons by targeted axonal silencing. *J. Neurosci.* **33**, 315–326 (2013).
21. Kim, H.Y. *et al.* Selectively targeting pain in the trigeminal system. *Pain* **150**, 29–40 (2010).
22. Puopolo, M. *et al.* Permeation and block of TRPV1 channels by the cationic lidocaine derivative QX-314. *J. Neurophysiol.* **109**, 1704–1723 (2013).

23. Binshtok, A.M. *et al.* Coapplication of lidocaine and the permanently charged sodium channel blocker QX-314 produces a long-lasting nociceptive blockade in rodents. *Anesthesiology* **111**, 127–137 (2009).
24. Roberson, D.P., Binshtok, A.M., Blasl, F., Bean, B.P. & Woolf, C.J. Targeting of sodium channel blockers into nociceptors to produce long-duration analgesia: a systematic study and review. *Br. J. Pharmacol.* **164**, 48–58 (2011).
25. Chen, J. *et al.* Pore dilation occurs in TRPA1 but not in TRPM8 channels. *Mol. Pain* **5**, 3 (2009).
26. Shimada, S.G. & LaMotte, R.H. Behavioral differentiation between itch and pain in mouse. *Pain* **139**, 681–687 (2008).
27. Brenner, D.S., Golden, J.P. & Gereau, R.W. A novel behavioral assay for measuring cold sensation in mice. *PLoS ONE* **7**, e39765 (2012).
28. Ogle, M.E., Gu, X., Espinera, A.R. & Wei, L. Inhibition of prolyl hydroxylases by dimethyloxaloylglycine after stroke reduces ischemic brain injury and requires hypoxia inducible factor-1alpha. *Neurobiol. Dis.* **45**, 733–742 (2012).
29. Petersen, L.J. Quantitative measurement of extracellular histamine concentrations in intact human skin *in vivo* by the microdialysis technique: methodological aspects. *Allergy* **52**, 547–555 (1997).
30. Khalil, I.F. *et al.* Development of ELISA-based methods to measure the anti-malarial drug chloroquine in plasma and in pharmaceutical formulations. *Malar. J.* **10**, 249 (2011).
31. Akiyama, T., Carstens, M.I. & Carstens, E. Differential itch- and pain-related behavioral responses and micro-opoid modulation in mice. *Acta Derm. Venereol.* **90**, 575–581 (2010).
32. Ross, S.E. *et al.* Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. *Neuron* **65**, 886–898 (2010).
33. Ohsawa, Y. & Hirasawa, N. The antagonism of histamine H1 and H4 receptors ameliorates chronic allergic dermatitis via anti-pruritic and anti-inflammatory effects in NC/Nga mice. *Allergy* **67**, 1014–1022 (2012).
34. Liu, T. & Ji, R.R. Oxidative stress induces itch via activation of transient receptor potential subtype ankyrin 1 in mice. *Neurosci. Bull.* **28**, 145–154 (2012).
35. Sun, Y.-G. *et al.* Cellular basis of itch sensation. *Science* **325**, 1531–1534 (2009).
36. Schmelz, M. *et al.* Chemical response pattern of different classes of C-nociceptors to pruritogens and algogens. *J. Neurophysiol.* **89**, 2441–2448 (2003).
37. Namer, B. & Reeh, P. Scratching an itch. *Nat. Neurosci.* **16**, 117–118 (2013).
38. Sun, Y.G. & Chen, Z.F. A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. *Nature* **448**, 700–703 (2007).
39. Rudick, C.N., Bryce, P.J., Guichelaar, L.A., Berry, R.E. & Klumpp, D.J. Mast cell–derived histamine mediates cystitis pain. *PLoS ONE* **3**, e2096 (2008).
40. Sikand, P., Shimada, S.G., Green, B.G. & LaMotte, R.H. Similar itch and nociceptive sensations evoked by punctate cutaneous application of capsaicin, histamine and cowhage. *Pain* **144**, 66–75 (2009).
41. Koppert, W., Reeh, P.W. & Handwerker, H.O. Conditioning of histamine by bradykinin alters responses of rat nociceptor and human itch sensation. *Neurosci. Lett.* **152**, 117–120 (1993).
42. Liu, Y. *et al.* VGLUT2-dependent glutamate release from nociceptors is required to sense pain and suppress itch. *Neuron* **68**, 543–556 (2010).
43. Leffler, A. *et al.* The vanilloid receptor TRPV1 is activated and sensitized by local anesthetics in rodent sensory neurons. *J. Clin. Invest.* **118**, 763–776 (2008).

ONLINE METHODS

Cell culture. Trigeminal neuron cultures were prepared from adult (2–3 month old) CD-1 mice[44]. In short, trigeminal neurons were removed and placed into Hank's balanced salt solution and 1% penicillin-streptomycin (vol/vol, Sigma), then digested in 5 mg ml^{-1} collagenase and 1 mg ml^{-1} Dispase II (Roche). Cells were triturated in the presence of DNAse I inhibitor (50U) and centrifuged through 10% BSA (vol/vol, Sigma). The cell pellet was resuspended in 1 ml Neurobasal (Sigma) containing B27 supplement (Invitrogen), penicillin and streptomycin (Sigma), 10 μM AraC. Cells were plated onto poly-D-lysine–coated (500 μg ml^{-1}) and laminin-coated (5 mg ml^{-1}) 35-mm tissue culture dishes (Becton Dickinson) at 8,000–9,000 per dish, at 37 °C, in 5% carbon dioxide.

Ratiometric calcium imaging. Cultured adult trigeminal neurons were loaded for 45–60 min with 1 μM fura-2 acetoxymethyl ester dye (stock in DMSO) in a bath solution composed of 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES and then rinsed for 45–60 min for de-esterification of intracellular acetoxymethyl esters. Neurons were perfused continuously at 2 ml min^{-1} and examined with an inverted microscope (Eclipse Ti) equipped with Epi-Fl attachment, perfect focus system (Nikon) and Exi Aqua monochromator (QImaging). Intracellular $[Ca^{2+}]_i$ was measured fluorometrically as an absorbance ratio at 340 and 380 nm ($\Delta F340/380$, 510 nm for emission, Lambda DG4, Sutter Instruments). Images were taken every 1 s, monitored online and analyzed offline using Nikon Elements AR Software (Nikon). Histamine (100 μM) and chloroquine (100 μM) were briefly bath applied (60 s) using a fast-step valve control perfusion system (Harvard Apparatus). In all responsive neurons, the changes in ratio (ΔF) following application of histamine and chloroquine were larger than $0.1\Delta F$ and were easily distinguishable from optic noise, which was less than $0.025\Delta F$. Whole-cell voltage-clamp recordings were then performed from the responsive cells (histamine positive or chloroquine positive) and non-responsive cells (histamine negative or chloroquine negative).

Electrophysiology. For electrophysiological recordings of transmembrane sodium currents, to decrease driving force for sodium, we replaced the solutions after completing calcium imaging measurements to thereafter contain 60 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM $CdCl_2$, 10 mM HEPES, 10 mM glucose, 5 mM 4-AP, 60 mM choline chloride and 15 mM TEA-Cl (pH 7.4). Pipette solution contained 110 mM CsCl, 25 mM CsOH, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA and 10 mM HEPES (pH = 7.4 with CsOH). Recordings were performed using a Multiclamp 700 B amplifier (Molecular Devices) at 22 ± 2 °C. Data were low-pass filtered at 1 kHz (−3 dB, 4 pole Bessel filter) and sampled at 10 kHz. Patch pipettes were pulled from thick-walled borosilicate glass capillaries (1.5-mm outer diameter, Sutter Instrument) on a Sutter Instrument P-1000 puller (Novato) and had a resistance of 2–5 MΩ. Pipette potential was zeroed before seal formation and membrane potential not corrected for the small liquid junction potential (−2.2 mV). Care was taken to maintain membrane access resistance as low as possible (usually 3–7 MΩ and always less than 10 MΩ). Capacity currents were cancelled and series resistance 80% compensated. Linear leakage currents were digitally subtracted on-line using a P/4 procedure. Command voltage protocols were generated on-line with a Digidata 1200 A/D interface (Molecular Devices). Data were digitized on-line using pCLAMP 10.2 (Molecular Devices). Data averaging and peak detection were made using pCLAMP 10.2 software (Molecular Devices). Data were fitted using QPlot.

For these experiments, we used data only from neurons in which input resistance and leak current did not substantially change during 10 min of drug application (20 min of recording). Because of the relatively low number of responsive cells and long and complex protocol of subsequent electrophysiological recording, we used data from three cells per group (seven groups).

Calcium imaging (alone) experiments. All dissections were performed on adult (2–4 month old) male CD-1 mice. The calcium-indicator dye Fura-2 a.m. was introduced at 2 μg ml^{-1} for 30 min at 22 ± 2 °C, washed and analyzed on a Nikon Eclipse Ti inverted microscope with exi-aqua CCD camera and NIS-elements AR 3.10 software. Each cell was given 3 min to recover from each pruritogen exposure and 8 min to recover between exposure to AITC and capsaicin. Each exposure lasted for 60 s, except for capsaicin, which was exposed for 10 s. Pruritogens were locally applied, with the perfusion opening placed approximately 150 μm from the field of view, and preliminary exposure to standard extracellular solution was employed before exposure to reagents made in SES. Pruritogen order was assigned at random (coin toss) for each plate, and no order effects were observed between differently ordered groups.

Behavioral studies. All animal procedures were approved by the Boston Children's Hospital Animal Care and Use Committee. Naive adult (2–4 month old) male CD-1 mice (Charles River Laboratories) housed in groups of five mice using a normal 12-h light/dark cycle were used. Mice were fully habituated to handling before all experimental procedures and were randomly assigned to experimental groups. The day before beginning itch experiments, mice were briefly anesthetized by inhalation of 1–2% isofluorane (vol/vol) and a ~1-cm^2 area of hair was shaved on the right cheek of each mouse. Capsaicin (Sigma-Aldrich) was freshly prepared by dilution in vehicle (20% ethanol (vol/vol), 5% Tween 20 (vol/vol), in saline, 10 ml). All other drug solutions were prepared fresh in normal saline (0.9% NaCl, wt/vol).

Itch assay. Mice received intradermal microinjections of pruritogen, algogen, QX-314, vehicle or a combination of pruritogen or algogen together with QX-314 intradermally in the cheek. To ensure proper intradermal injection, needle puncture with a 28-gauge needle was initiated bevel-up at 5° to the plane of taut skin until initial penetration, then inserted horizontally until the needle tip was 0.5 cm beyond the point of insertion before intradermal evacuation of syringe contents. Correct injection was confirmed by presence of a slightly domed bulla immediately following removal of needle (**Supplementary Fig. 2c**). For sequential injection experiments, the intradermal bullae of the conditioning (first) injection were outlined with fine-tip permanent marker to denote the extent of intradermal drug distribution, thereby providing visible drug distribution boundaries for subsequent injections (**Supplementary Fig. 2**). Mice not receiving proper drug injections were noted and excluded from the study before observation. Immediately after conditioning injection, mice were placed in in a custom-built itch observation apparatus and video recorded during the mouse dark cycle as previously described[26]. Scratches and/or wipes were subsequently quantified by blinded observers.

Von Frey assay. Mice were assessed manually using the up-down method to determine the median 50% (5 of 10) withdrawal threshold[45].

Radiant heat (Hargreaves) assay. Radiant heat withdrawal threshold was determined using the Plantar Analgesia Meter (IITC Life Science) according to previously described methods[46].

Focal cold plantar (Brenner) assay. Latency to remove hindpaw from focally applied cold stimulus was performed according to methods previously described[27] only using 3/8-inch-thick (4.78 mm, as measured by electronic caliper) Neoceram N-0 thermal-shock-resistant glass (Nippon Electric Glass).

Adhesive dot plantar tactile (sticky tape) assay. Latency to bite, lick or attempt removal of a 9-mm diameter circular adhesive Microtube Tough-Spots label (Diversified Biotech) was performed according to methods previously described[28], only using hindpaw instead of forepaw for application of stimulus.

Statistical analysis. Sample sizes for all experiments were chosen according to standard practice in the field. For electrophysiological experiments, the significance of the effect was calculated using two-way ANOVA non-parametric test followed by Bonferroni post-test. Comparison of group means for behavior studies was performed using Student's t test. All bar graphs are plotted as mean ± s.e.m. For behavioral studies, n represents the total number of mice used in each group.

44. Malin, S.A., Davis, B.M. & Molliver, D.C. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat. Protoc.* **2**, 152–160 (2007).
45. Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M. & Yaksh, T.L. Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* **53**, 55–63 (1994).
46. Hargreaves, K., Dubner, R., Brown, F., Flores, C. & Joris, J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**, 77–88 (1988).