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Mechanisms of Pruritogen-Induced Activation of Itch Nerves in Isolated Mouse Skin

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Running Title: Characterization of itch causing nerves

Key Points Summary

Chloroquine (CQ) stimulates itch nerves and causes intense scratching in mice by activating the G-Protein Coupled Receptor (GPCR) MrgprA3. It is not known how stimulation of MrgprA3 (or other GPCRs) leads to activation of the itch nerve terminals in the skin, but previous studies have found that TRPA1 gene deletion blocks CQ-induced scratching.

In the present study we used a novel dorsal skin-nerve preparation to evaluate mechanisms underlying CQ- and histamine-induced action potential discharge in itch nerve terminals.

We found that CQ activation of the nerves requires the beta3 isoform of phospholipase C, however, TRPA1 or other TRP channel are not required. Evidence is provided for a role for calcium-activated chloride channels such as TMEM16a in GPCR-activation of itch nerve terminals.

The mechanism by which TRP channels participate in pruritogen-induced scratching may involve sites of action other than the primary afferent terminals.

Abstract:

Chloroquine (CQ) and histamine are pruritogens commonly used to study itch in the mouse. A novel skin-nerve preparation was used to evaluate chloroquine (CQ)- and histamine- induced activation of afferent nerves in the dorsal thoracic skin of the mouse. All CQ sensitive nerves were C-fibers, and were also sensitive to histamine. The response to CQ, but not histamine, was largely absent in mrgpr cluster Δ -/- mice supporting the hypothesis that CQ evokes itch largely via stimulation of MrgprA3

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receptors. The CQ-induced action potential discharge was largely absent in phospholipase Cβ3 knockout animals. The CQ and histamine responses were not influenced by removal of TRPA1, TRPV1, TRPC3 or TRPC6, nor by the TRP channel blocker Ruthenium Red. The bouts of scratching in response to CQ was not different between wild type and TRPA1 deficient mice. A selective inhibitor of TMEM16A, *N*-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid (MONNA) inhibited CQ-induced action potential discharge at itch nerve terminals and bouts of scratching by about 50%. Although TRPA1 and TRPV1 channels may be involved in the scratching responses to intradermal pruitogens, this is unlikely due to an effect at the nerve terminals, where chloride channels may play a more important role.

Abbreviations

AITC – Allyl isothiocyanate
CQ- Chloroquine
Mrgpr – Mas-related G-protein coupled receptor
TMEM16a – Transmembrane member 16A
TRP – Transient receptor Potential

Introduction

Recent studies have identified a major itch causing nerve in mouse skin as one defined by the expression of MrgprA3, a GPCR that when selectively activated by an agonist, e.g. chloroquine (CQ), causes intense scratching behavior (Liu *et al.*, 2009). The MrgprA3 expressing neurons comprise only about 4-6% of DRG neurons. Liu et al revealed that these neurons are a subset of TRPV1 and histamine H1 receptor expressing neurons. Selectively deleting the MrgprA3 expressing neurons using Cre-mediated diptheria toxin receptor methods largely inhibited scratching in response to MrgprA3 agonists, and also inhibited scratching in response to other pruritogens including histamine, 5-HT, and those released by mast cell activation, as well as the scratching associated with contact dermatitis (Han *et al.*, 2013). This indicates that the small subset of MrgprA3 expressing, CQ-sensing DRG nerves represent a major nerve subtype responsible for acute itch in the mouse.

Much of the work on the neurobiology underlying acute itch in mice has relied on behavioral assays in which bouts of scratching are quantified following injection of a pruritic substance intradermally into the nape of the neck or cheek pouch (McNeil & Dong, 2012; Akiyama & Carstens, 2013; Bautista *et al.*, 2014; LaMotte *et al.*, 2014; Ringkamp & Meyer, 2014). Depending on the pruritogen, acute

bouts of scratching behavior have been inhibited by TRPA1 and TRPV1 inhibitors, and reduced or even abolished in mice that have TRPA1 or TRPV1 genetically deleted. Such studies have led to the conclusion that TRPA1, and in some cases TRPV1, is essential in the itch pathways initiated by GPCR agonists like CQ and histamine (Imamachi *et al.*, 2009; Wilson *et al.*, 2011; Wilson & Bautista, 2014).

The general inference from these results is that TRPA1 or TRPV1 contribute to itch by acting as the cation channel that is opened downstream from GPCR signal transduction leading to action potential discharge in the itch nerve terminals. This supports the strategy of developing peripherally acting TRPA1/TRPV1 blockers for the treatment of pathological itch. However, this hypothesis has not yet been rigorously addressed experimentally. It is possible that the TRP channels involved in the itch-scratch pathway play a role at the level of the spinal cord or in the brain in addition to, or rather than, at the primary itch nerve terminals. With respect to the brain it is worth noting that *trpa1* -/- mice, or mice receiving TRPA1 antagonists, have strong behavioral abnormalities such as decreased immobility in force swim tests, and in general behave similarly to mice on large doses of anxiolytics and antidepressants(de Moura *et al.*, 2014). Mice lacking TRPV1 express similar behavioral abnormalities(Aguiar *et al.*, 2014). It is possible that these alterations in behavior contribute to the decrease in scratching associated with acute administration of a pruritogen. Indeed, antidepressants have been reported to have antipruritic activity in humans (Yosipovitch & Bernhard, 2013).

In this investigation, experiments were designed in which mouse skin was isolated along with the attending spinal nerves and DRG. Extracellular recordings from cell bodies of single DRG nerve fibers that terminate in the skin were carried out to characterize the CQ and histamine sensitive nerve population in the dorsal thoracic skin, and to quantify the action potential discharge in response to these pruritogens. We conclude that CQ and histamine stimulate action potential discharge in the same subpopulation of cutaneous C-fiber terminals, and that these nerves comprise about 25% of the C-fiber population in the dorsal skin. Activation of these nerve terminals by CQ or histamine occurs independently of the presence TRPA1, TRPV1, TRPC3, TRPC6, or other Ruthenium Red-sensitive cation channels.

Methods

Animals

All experiments were carried out were approved by The Johns Hopkins Animal Care and Use Committee, and conform to the principles and regulations, as described in (Grundy, 2015). Male mice C57BL/6J, *trpa1*$^{-/-}$, *trpv1*$^{-/-}$, *trpa1/trpv1* $^{-/-}$, and *trpc* 3/trpc6$^{-/-}$ weighing 22-27g were used. The *trpa1*$^{-/-}$, *trpv1*$^{-/-}$ were obtained from Jackson's Laboratory. The *trpa1/trpv1* $^{-/-}$ and *mrgpr cluster* Δ $^{-/-}$ animals were obtained by mating these homozygous mice in Xingzhong Dong's Laboratory. The *trpc* 3$^{-/-}$/*trpc6*$^{-/-}$ were obtained from the laboratory of Dr. D. Kass (Johns Hopkins University) that were originally developed in the laboratory of Dr. L. Birnbaumer (National Institutes of Environmental Health). The *plcb3* $^{-/-}$ mice were developed in the laboratory of Dr. M. Simon and provided by Dr. K-W Yau (Johns Hopkins University).

Sensitization

The allergen sensitization with ovalbumin was performed as described before(Potenzieri *et al.*, 2012). Ovalbumin (75µg, Sigma) with aluminum hydroxide gel (2.6mg, Sigma) in sterile PBS (total volume 200µl) was injected intraperitoneally three times (days 1, 3 and 5) and the mice were used for experiments at least 7 days after the last injection.

Ex vivo DRG nerve-skin preparation

The mouse was injected with heparin (5 IU g^{-1} in saline, i.p.) and killed 15 minutes later by CO_2 inhalation and exsanguination. The hair from the right back and side was removed using an electric shaver. Right dorsal side skin (fig. 1A and B) was dissected out along with the blood vessels namely the right subscapular artery (a branch from the right axillary artery that is distributed to right mouse back skin) and its branches. The right DRG nerves and ganglia (T7, T8, T9 or T10) were separated carefully from the connective tissue. The skin was then cut to the size approximately 3 cm X 3 cm such that the branches of the subscapular artery were positioned in the center of the preparation. The subcutaneous tissue except for the nerves and blood vessels was dissected out to facilitate the diffusion of chemicals. If present, the vessels other than the subscapular artery were ligated to prevent a rapid exit of chemicals infused into subscapular artery. The skin was gently stretched and pinned dermis side up in a Sylgard-lined perspex tissue chamber. The DRG ganglion (most often T9 or T8, occasionally T7 or T10) with the nerve was pulled through a small hole to adjacent Sylgard-

lined recording chamber and the hole was sealed with Vaseline. The tissue and recording chamber were separately superfused with Krebs solution composed of: NaCl, 118mM; KCl, 5.4mM; NaH_2PO_4, 1.0mM; $MgSO_4$, 1.2mM; $CaCl_2$, 1.9mM; $NaHCO_3$, 25.0mM; and dextrose, 11.1mM, and gassed with 95% O_2 – 5% CO_2, PH 7.4 containing indomethacin (3µM), at a flow rate of 4 ml min^{-1} (temperature 32-34 ^{o}C) and 3 ml min^{-1} (36-37^{o}C), respectively. It is possible that mechanically probing the isolated skin can lead to prostanoids production. Indomethacin was included to reduced the potential complicating factor of endogenous cyclooxygenase products sensitizing C-fibers over time (Emery *et al.*, 2016). For the intrarterial delivery of chemicals a 10mm long steel tip of 33 # gauge needle attached to PE-10 tubing (OD 0.61mm, ID 0.28mm) was inserted into the right subscapular artery, the blood in the skin preparation was flushed with buffer and the needle was secured with surgical silk.

Extracellular recording of DRG fibers in the skin

Extracellular recordings were performed by using an aluminosilicate glass (O.D.: 1.0 mm, I.D.: 0.64mm) electrode (pulled with a Flaming-Brown micropipette puller, Sutter Instrument Company, Novade, CA, USA). The electrode was filled with 3M sodium chloride and placed into an electrode holder connected directly to a headstage (A-M Systems, Everett, WA, USA). A return electrode of silver-silver chloride wire and an earthed silver-silver chloride pellet were placed in the perfusion fluid of the recording chamber. The electrode resistance was approximately 2 M Ω. The recording signal was amplified (Microelectrode AC amplifier 1800, A-M Systems) and filtered (low cut off, 0.3 kHz; high cut off, 1 kHz) and the resultant activity was displayed on an oscilloscope (TDS 3054B, Tektronix, Beaverton, OR, USA) and Apple computer. The data were stored and analyzed by computer using the software TheNerveOfIt (PHOCIS, Baltimore, MD, USA). The action potential discharge in response to a stimulus was quantified for a 5 minute period, by this time the response has generally ceased.

The skin was searched with a small concentric stimulating electrode. When the electrical stimulus evoked an action potential, the response to mechanical and chemical stimuli was quantified. Essentially every fiber stimulated with the non-selective electrical stimulus was also mechanically sensitive. A mechanosensitive receptive field of a DRG fiber was identified when a

punctate mechanical stimulus (von Frey hair 4.0g) applied to the dermis surface of skin evoked a burst of action potentials. The fiber was only studied if the mechanically-induced action potential discharge waveform could be clearly discriminated from background noise and other nerve activity. In rare instances two fibers with distinct action potential waveforms were recorded simultaneously. Conduction velocity was determined from electrical stimulation of the nerve trunk and/or mechanosensitive receptive field by dividing the approximate length of the nerve fiber by conduction time. The chemicals were applied via infusion into the subscapular artery (as described above) at a rate of 40 µl s^{-1} in a total volume of 200µl. For every nerve studied, the vehicle was applied to ensure there was no artifactual response due to any change in mechanical force resulting from the injection. In >30 experiments we confirmed the accessibility of intraterially injected chemicals to mechanosensitive receptive filed by infusing Monastral Blue solution at the end of the experiment; in each case Monastral Blue was found saturating the receptive field (fig. 1C).

Antagonists were superfused over the tissue for at least 20 min prior to the application of CQ (or relevant stimulus) that was delivered in a solution that also contained the antagonist. To further ensure the antagonist were reaching the nerve terminals, it was also added intra-arterially every 10 min as described above until the stimulus was applied.

With experience this preparation required ~ 2 hours to set up. The preparation was suitable for robust extracellular recordings of action potential in single C-fibers for at least the ensuing 6 hours.

Patch clamp recording

Neurons were dissociated from DRG (T7-T10) isolated from MrgprA3-tdTomato mice, cultured in medium and studied within 24 hours. Amphotericin B-perforated whole-cell patch clamp technique was used to measure the membrane current, membrane potential and AP discharge on tdTomato-positive DRG neurons (~5% of total) isolated from MrgprA3-tdTomato mice. The latter were generated by mating the mice expressing MrgprA3-Cre with mice expressing Rosa26-loxP-STOP-loxP-tdTomato . Pilot experiment showed that all tdTomato-positive cells exhibit a robust AITC-induced inward current at -60 mV (n=18). In this study, only neurons with stable resting membrane potential were used. For voltage clamp experiments, the cells were held at -60 mV. The pipette solution contained (mM): KCl 40, K-gluconate 105 and HEPES 10, pH 7.2. Amphotericin B (30 µg/100 µl) was added to the pipette solution before experiments. The bath solution contained (mM): NaCl 135, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.5, HEPES 10 and glucose 10, pH 7.35. Chloroquine (1 mM) was dissolved in

bath solution and applied to bath for 1-2 min. HC 030031 stock (30 mM) was prepared in DMSO, and applied to bath at 30 µM 5-10 min before application of chloroquine. All recordings were performed at room temperature. The recorded membrane potential was corrected offline for the junction potential (-13 mV).

Calcium Assay

A conventional Fura-2 based calcium assay was used to evaluate the efficacy of the trpa1 deletion in DRG neurons isolated from *trpa1* -/- mice.

Behavior study

The extent of itch to a pruitogen was inferred from the number of bout of scratching as previously described (Liu *et al.*, 2009). Mice were put in a chamber such that they aluminum foil enwrapped plexiglass chamber. In front of the chamber was a video camera focused on a mirror beneath the chamber. The mice were unaware of the camera. Vehicle, or CQ were subcutaneously injected into the nape of the neck after acclimatization and scratching behavior was quantified for 30 min. We evaluated wild-type with *trpa1 -/-* animals in a paired experimental design. The two chambers were positioned far enough part so the mice could not detect each other, in an unused laboratory in the absence of distractions. The data subsequently quantified off-line.

Drugs and chemicals

Chloroquine, histamine and Ruthenium Red were dissolved in distilled water (stock solution 10mM). Capsaicin was dissolved in ethanol (stock solution 10mM). AITC, HC-030031, GSK2332255B, *N*-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid (MONNA) and gallein were dissolved in DMSO (stock solutions 1M, 100mM, 10mM, 100mM and 30mM respectively). All stock solutions were stored at -20 ^{o}C and the drugs were diluted in Krebs solution to their final concentrations on the day of use. Ovalbumin for extracellular studies was directly dissolved in Krebs solution to 1mg ml^{-1}. All drugs were purchased from Sigma-Aldrich except for GSK255B obtained from GSK and gallein was purchased from Tocris Bioscience.

Data analysis

The data are presented as the mean ± SEM. The peak frequency of the response to stimuli is

defined as the largest number of action potentials in any 1s bin. The total number of action potentials was determined as the number of spikes occurring during two minutes after the first spike, with the exception of OVA where the spikes were counted for 10 minutes. By these time periods the action potential discharge had returned back to baseline.

The scratching behavior was analyzed after the video was downloaded onto a computer and quantified as the number of scratching bouts during the 30 minutes post injection of CQ or vehicle. Student`s paired and non-paired tests were used as appropriate. The Fisher exact test was used to compare the percentage of drug-responsive fibers between the transgenic and wild-type mice. P values of <0.05 were considered significant.

The effect of CQ on evoking action potentials was quantified as peak frequency (Hz) and total number of spikes elicited. We carried out 228 experiments and learned that 26% of the nerve fibers responded to CQ.

The number of positive fibers evaluated was based on the co-efficient of variation of the response in wild-type animals. The coefficient of variation was 7.2 % for AP discharge Hz, so to obtained meaningful statistical analysis we carried out sufficient number of experiments until > 7 positive itch fibers were evaluated. For pharmacological analyses the data was evaluated in paired fashion (e.g. the response of the same nerve before and after treatment). The hypothesis here is that if the ion channel blocked is the key channel for the generator potential, the drug should essentially abolish the response to CQ. Because there is no desensitization to CQ response we were able to prove a lack of effect on CQ-induced responses after ~5 experiments.

Results:

Intrarterial injections in a skin-nerve preparation.

An electrical stimulus was used to search for single afferent nerve receptive fields in the isolated skin. We did not find many examples of “silent nociceptors” in this region of the skin. For example, in the last 100 consecutive experiments, all but 9 fibers could be activated by a mechanical force applied to the mechanical receptive field (generally $<2.5mm^2$) with a von Frey fiber (4.0g). Moreover, the mechanical probing was cursory to reduce damage to the preparation, so it is possible that we failed to probe precisely the receptive field of the nominally mechanically-

insensitive fibers. Chemical stimuli were superfused directly to the field of the afferent nerve under study. When compared to C-fibers in our airway-nerve preparations, where capsaicin produces immediate robust action potential discharge in a concentration range of 10nM -1 μM (Kollarik M, 2004), we noted that activation of C-fibers in the skin with capsaicin required large agonist concentrations (10 μM), and the response inevitably had a marked delay (>400 sec). We surmised that there is likely a substantive barrier in the underside of this region of the skin for rapid diffusion of superfused chemicals to the receptive field of the nerve. This barrier could be overcome with persistent application of a chemical, but because the rate at which a chemical stimulus reaches the terminal can influence the rate of the generator potential, and thus action potential initiation, we sought out a method to bypass this barrier. By dissecting the right subscapsular artery and its branches we were able to administer chemicals intra-arterially (i.a.). Monastral blue dye was routinely used to evaluate the vascular supply to the receptive field. When the dye was injected i.a. we noted immediate saturation of the receptive field (fig. 1C). When capsaicin was administered in this fashion it immediately (within 5 sec, n=76) activated the nerves even at a 10-fold lower concentration than needed when the drug was superfused (e.g. fig. 1D). Administration of vehicle i.a. was always included as a control and failed to evoke action potential discharge. We therefore evaluated the response of nerves to histamine, CQ, BAM8-22, capsaicin and AITC delivered intra-arterially to the terminals. The nature of the diffusion barrier was left unstudied but one might suspect the layer of cutaneous maximus muscle that allows for the skin to twitch may be involved(Pan *et al.*, 2012). For histological description of the potential diffusion barriers of the dorsal skin of a rodent see (Wells *et al.*, 2010).

CQ vs Capsaicin sensitivity

All fibers that responded to CQ conducted action potentials in the C-fiber range. A total of 228 C-fibers were studied from 138 mice. We have previously reported that about 4% of neurons in the DRG *per se* are CQ-sensitive (Liu *et al.*, 2009). This population is, as expected, enriched when evaluating C-fiber nerves specifically in the the skin. The CQ-sensitive C-fibers comprised 26% of the C-fiber population. The average CV of CQ-sensitive fibers was 0.75 ±0.01 m/sec. This was slightly but statistically ($P<0.05$) slower than the CV of CQ-insensitive C-fibers 0.83±0.01 m/sec (fig. 2). The response to CQ was generally a burst of action potential that at its peak averaged 12 ± 1 Hz. There was no desensitization between consecutive treatments of CQ on a given fiber when administered 30 minutes apart ($P>0.1$ between response of first and second application).

Capsaicin sensitivity is often used as a marker of pain evoking C-fibers. Among the CQ-sensitive C-fibers the vast majority (73.5%) also responded to capsaicin. The capsaicin response, as quantified by the peak discharge Hz (peak number of spikes in any 1 sec bin), was similar to that observed with CQ averaging 15 ± 2 Hz. Capsaicin also stimulated CQ-insensitive C-fibers. We found that the capsaicin response was stronger in the CQ-sensitive population than in the CQ-insensitive population of C-fibers, based on peak frequency of action potential discharge. The maximum capsaicin-induced discharge in CQ-insensitive C-fibers averaged 6 ± 1 Hz, n=41, which was significantly less than the 15 ± 2 Hz evoked in CQ-sensitive C-fibers ($P<0.05$).

All CQ-sensitive C- fibers (7 of 7) responded to the TRPA1 agonist AITC, however this required large concentrations ($\geq$ 300 μM), and the peak Hz of the response was significantly weaker than that evoked by CQ or capsaicin averaging only 4 ± 0.5 Hz (fig. 3).

Characterization of the CQ and histamine sensitive nerves in skin

Histamine (100 μM) did not evoke as strong of a burst of action potentials as CQ averaging only 4 ±0.8 Hz at its peak ($P <0.05$ compared to CQ), but the response was more persistent (fig 3 and representative tracing shown in fig. 8A). Every histamine-sensitive fiber studied also responded to CQ and only 4 of 23 CQ-sensitive fibers were histamine-insensitive. In these few histamine-insensitive fibers, CQ may have been acting independently of MrgprA3 (see below). Among 17 CQ-insensitive C-fibers none responded to histamine. In other words, histamine and CQ sensitivity essentially defined the same C-fiber subpopulation in the region of the skin we studied.

Allergen Response

We evaluated the activation of 42 skin C-fibers by the allergen, ovalbumin (OVA) in OVA-sensitized mice (OVA has no effect on C-fibers in non-sensitized mice). OVA consistently evoked action potential discharge in 12 of 12 CQ-sensitive C-fibers averaging 6 ± 2 Hz and 100 ± 19 action potentials over baseline (fig. 3). In contrast, OVA modestly if at all stimulated action potentials in 30 CQ-insensitive C-fibers (1.6 ± 0.2 Hz total spike of 23 ± 3; $P <0.01$ compared to CQ-sensitive fibers, data not shown).

Mrgpr

It has previously been noted that CQ stimulates DRG neurons via MrgprA3 (Liu *et al.*, 2009). We evaluated 10 histamine-responsive C-fibers in *mrgpr cluster* $\Delta^{-/-}$ mice. The response to histamine was not different than wildtype ($P>0.1$), but the response to CQ was substantially reduced ($P<0.01$)

(fig. 4A and B). Unlike studies in wildtype animals where all histamine-sensitive nerves responded strongly to CQ, only one of the 10 histamine-sensitive nerves studied in *mrgpr cluster* $\Delta^{-/-}$ mice responded strongly (243 action potentials) to CQ, two additional fibers responded with a small increase of action potential discharge over baseline (<30 action potentials, e.g. fig. 4A).

MrgprC11

DRG neurons expressing MrgprA3 largely overlaps with those expressing MrgprC11 (Zylka *et al.*, 2003). Moreover in a calcium assay we previously noted that 3.6% of neurons in the DRG that responded to the MrgprC11 stimulant BAM8-22, also responded to CQ (Liu *et al.*, 2009). We evaluated the overlap in responsiveness between CQ and BAM8-22- sensitive C-fibers in the mouse skin. In preliminary dose-response studies we found that BAM8-22 stimulated C-fibers with a threshold concentration of 0.1 μM and a maximum effect at 10 μM. We evaluated 12 C-fibers that were activated by CQ and 100% were also stimulated by BAM8-22 with burst of action potentials of similar intensity to CQ (fig.5). We evaluated 13 fibers that were CQ-insensitive and found only 1 that was weakly activated by BAM8-22. Therefore, as predicted, the CQ responsive and BAM8-22 responsive C-fibers in the skin represent the same population of nerves.

Phospholipase β3

The Mrgprs are GPCRs that are often linked to Gq. Gq-coupled receptors stimulate phospholipase C (PLC). However studies at the cell soma have indicated that CQ activates the neurons independently of PLC (20). An RNAseq analysis of MrgprA3 expressing neurons revealed that the dominant PLC isoform expressed is PLCβ3 isoform (Usoskin *et al.*, 2015). We therefore evaluated the effect of CQ in PLCβ3 knock out animals. We evaluated 26 C-fibers in 8 *plcβ3*$^{-/-}$ mice. There was no action potential discharge in 23 of these C-fibers. In the 3 that responded the total number of action potentials elicited were only 5, 10, and 5 (fig. 6A and B). As a positive control, capsaicin stimulated C-fibers from *plcβ3*$^{-/-}$ mice (15 ± 5.1 Hz); the number of action potentials evoked by capsaicin was not statistically different than wild type mice (fig. 6). Gallein, an inhibitor of G-protein βγ signaling had no effect on CQ-induced action potential discharge (fig. 10).

Lack of TRP channel involvement in CQ-induced action potential discharge

Previous studies have shown that CQ-induced scratching is strongly dependent on TRPA1(Wilson *et al.*, 2011). We evaluated 15 cutaneous C-fibers from *trpa1*$^{-/-}$ mice. As with C-fibers in wild type mice

about 27% (4/15) responded to CQ and histamine. The peak response and total number of action potentials were not different than that observed in wild type. OVA stimulated action potential discharge in 3 of 3 CQ-sensitive C-fibers in skin of *trpa1-/-* mice in a fashion similar to wildtype, averaging 5± 1Hz. AITC (300 μM) failed to activate C-fibers in the *trpa1*$^{-/-}$ mice whereas, as mentioned above, this concentration of AITC activated C-fibers studied in wildtype mice. These data are summarized in fig. 7 A. Consistent with these observations, we found no difference in the bouts of scratching in response to intradermal CQ between 18 pairs of wild type vs *trpa1-/-* mice (fig 7B).

To further substantiate the completeness of the *trpa1* deletion we quantified the effect of the TRPA1 agonist cinnamaldehyde (250 μM) and capsaicin (150nM) in elevating calcium in neurons isolated from the DRG. Capsaicin increased calcium in 27% of the neurons (51/190) whereas cinnamaldehyde failed to increase calcium in any neurons from *trpa1*$^{-/-}$ mice (data not shown).

A more thorough investigation was carried out in 20 *trpa1*$^{-/-}$*/trpv1*$^{-/-}$ double knockout mice. The proportion of C-fibers that responded to CQ (8 of 40) was again not different than wild type (P>0.5). Neither the peak Hz of discharge nor total number of action potentials evoked was different than that found in wild type animals (fig. 8 A, B, C). Likewise, the response to histamine was not different in CQ-sensitive C-fibers from wild type and *trpa1*$^{-/-}$*/trpv1*$^{-/-}$ animals (fig. 8 A, B, C)). Capsaicin was added at the end of all experiments and none of the fibers evaluated in the *trpa1*$^{-/-}$*/trpv1*$^{-/-}$ animals responded with action potential discharge (fig. 8 B and C).

We next evaluated the response to CQ in C-fibers isolated from *trpc3*$^{-/-}$*/trpc6*$^{-/-}$mice. Among the 20 C-fibers studied 30% responded to CQ and the peak discharge frequency and total number of action potentials evoked were not different than wild type (fig. 9A).

We addressed the hypothesis of redundancy among the TRP channels such that any one channel is sufficient to evoke action potential discharge. The response of the potent TRPC3/TRPC6 antagonist GSK2332255B (Seo *et al.*, 2014) was evaluated on the CQ response in C-fibers isolated from *trpa1* $^{-/-}$ */trpv1* $^{-/-}$animals. The response in the presence of GSK2332255B averaged 84 ±13% of the initial response studied in the absence of GSK2332255B (P>0.5) (fig. 9B and C).

Consistent with the finding with *trpa1*$^{-/-}$ mice, in wild type mice we found that the CQ-induced response was not significantly inhibited by the TRPA1 antagonist HC030031 (30 μM) (fig. 10). HC030031 did however inhibit the response to AITC (300 μM). In the presence HC0031, AITC evoked 0.8 ± 0.8 Hz stimulation (n=5) compared to 4 ± 0.4 Hz (n=7) in untreated tissue, P<0.05. We evaluated the effect of Ruthenium Red (30 μM), a substance that nonselectively blocks many TRP

channels and other cation channels. The response to CQ was not inhibited following 20 min treatment with Ruthenium Red (fig. 10 A and B).

Patch clamp studies on cell bodies

Previous studies have indicated that the electrophysiological effects of CQ on the cell soma require TRPA1. Based on the data presented above this would suggest a distinction between the mechanisms occurring at the cell soma vis-a-vis terminals. To directly address this hypothesis we evaluated the response of the MrgprA3-expressing neurons to CQ using MrgprA3-tdTomato mice. At a holding potential of -60 mV, 100 µM CQ elicited an inward current (0.46 ± 0.19 pA/pF, n=6). At 1mM CQ this current was increased to 3.0 ± 1.6 pA/pF (n=13). In current-clamp mode, 1mM CQ caused a membrane depolarization by 8.8± 0.7 mV (n=9). In 7 of 9 neurons this was sufficient to evoked action potential discharge ranging from 3 to 61 APs (averaging 24 ± 8). We next evaluated the effect of the TRPA1 antagonist HC-030031 on the responses at the cell bodies. We found that the CQ-evoked current was reduced by 52.5 ± 10.5% (n=6) upon second stimulation with 1 mM CQ (10 min interval between two CQ applications). We therefore evaluated the effects of HC 030031 in a non-paired fashion. In neurons pretreated with HC 030031, the CQ-induced membrane depolarization was the same as control averaging 9.6 ± 1.2 mV (n=9). In 6 of 9 HC treated neurons, CQ evoked action potential discharge that was not different from control (ranging from 2 to 57 and averaging 22 ± 7). These data are illustrated in fig. 11. Although trpa1 does not appear to be required for CQ-induced membrane depolarization, the MrgprA3-expressing neurons were however responsive to AITC. In fact, when studied in voltage clamp mode, the inward current evoked by AITC (100 µM) averaged 22.3 ± 2.8 PA/pF which was ~ 7-fold larger than the 3.0 ± 1.6 pA/pF evoked by 1mM CQ ($P<0.01$) (data not shown).

M-Current

Blocking of neuronal M-type potassium channels (KCNQ/Kv7) provide for a mechanism by which GPCR/ PLC can evoke a membrane depolarization and an increase in excitability of neurons (Hernandez *et al.*, 2008; Linley *et al.*, 2008). We found that the effect of CQ was not mimicked by an effective Kv7 blocker XE991, nor was the Peak Hz or total number of action potentials evoked by CQ altered in the presence Kv7 blockade (fig. 10).

TMEM16A

In cell bodies of neurons isolated from the DRG and vagal sensory ganglia, bradykinin evokes a depolarizing current by activating calcium-activated chloride channels (Oh & Weinreich, 2004; Lee *et al.*, 2005; Liu *et al.*, 2010). At the level of C-fiber terminals in the isolated trachea, the bradykinin-induced action potential discharge is inhibited by nonselective chloride channel blockers. TMEM16a is a calcium activated chloride channels that is expressed in MrgA3 expressing DRG neurons. Using an siRNA strategy to knockdown TMEM16a expression in small diameter DRG neurons decreases bradykinin-induced depolarizing currents in the rat Liu *et al.*, 2010. Recently, MONNA has been described as a potent and selective inhibitor of TMEM16A(Oh *et al.*, 2013b). We found that MONNA (10 μM) inhibited CQ-induced action potential discharge from itch nerves in the skin by approximately 50% (fig. 12A). Consistent with the electrophysiological studies was the finding that MONNA inhibited the number of scratching bouts by about 50% (fig. 12B).

Discussion

The results show that the CQ-induced activation of itch fibers terminating in mouse skin depends on Mrgpr GPCRs and the stimulation of PLCβ3 isozymes. The data also show that the ion channels down stream from GPCR actvation with CQ or histamine that are required for membrane depolarization and action potential discharge at the peripheral terminals of itch nerves are unlikely to include TRP channels, but may involve, at least partially, calcium activated chloride channels.

We found little evidence for a CQ-sensitive, histamine-insensitive nerve population in the skin. Likewise, we found little evidence for a histamine-sensitive, CQ-insensitive nerve population. These findings are consistent with the hypothesis that either CQ or histamine can be used as a marker of the MrgprA3 expressing itch fibers in this region of the skin. Our observations that CQ sensitive fibers are histamine-and capsaicin-sensitive C-fibers is in agreement with nerve recording from the mouse skin *in vivo* (Han *et al.*, 2013). The conclusion that CQ-sensitive nerves in the skin are the same population as histamine sensitive fibers is also consistent with the observation that selective deletion of MrgprA3 expressing neurons not only reduces the scratching in response to CQ, but also to histamine(Han *et al.*, 2013). This conclusion is at odds with findings in the cheek where histamine- and CQ-sensitive nerves appear to represent distinct populations (Roberson *et al.*, 2013). This may represent a difference between itch neurons in the trigeminal ganglia vs DRG. We used concentrations of histamine and CQ (100μM) that based on other studies are expected to be saturating for histamine receptors and MrgprA3. It remains possible that there may be a nerve subtype in the mouse skin that responds only to larger concentrations of histamine or CQ.

Our previous studies showed that among the Mrgpr receptors, CQ selectively stimulated MrgprA3(Liu *et al.*, 2009). The results with the C-fibers in the *mrgpr cluster* $\Delta^{-/-}$ animals are consistent with this conclusion. It has previously been reported that MrgprA3 and MrgprC11 are expressed in the same overlapping population of DRG neurons (Zylka *et al.*, 2003). In 12 experiments where the C-fiber responded strongly to CQ, the nerve also responded strongly to the MrgprC11 agonist BAM8-22, whereas CQ-insensitive neurons were also insensitive to BAM8-22. This provides additional support that C-fibers activated by CQ are those that express MrgprA3. BAM8-22 is a pruritogen in human volunteers presumably due to its effect on MrgrX1, the human functional ortholog for MrgprC11 (Dong *et al.*, 1990), although BAM8-22 may also have Mrgpr-independent sensitizing on C-fiber afferent nerves (Hager *et al.*, 2008). It is worth noting that in one histamine-sensitive fiber, CQ caused a robust discharge of action potentials in absence of MrgprA3. An additional two other fibers also responded to CQ, with a subtle but noticeable increase in action potential discharge over baseline. Caution is therefore required when using CQ as a *specific* MrgprA3 agonist. This is also the case for behavioral assays; CQ causes significant bouts of scratching in *mrgpr cluster* $\Delta^{-/-}$ animals (Liu *et al.*, 2009) and in mice in which MrgA3 expressing neurons are deleted(Han *et al.*, 2013), although significantly less than in wild type animals.

Since the strong activation by CQ appears to depend on Mrgpr activation it is likely that GPCR-Gq signaling is involved(Han *et al.*, 2002). A consequence of GPCR-Gq activation is the simulation of PLC. There are six families of PLC that comprise thirteen isozymes. An RNAseq analysis of MrgprA3 expressing mouse DRG neurons revealed that the isoform expressed is almost exclusively limited to PLCβ3(Usoskin et al., 2015). CQ caused no (or very minimal) action potential discharge in C-fibers terminating in the skin of PLCβ3 deficient mice; a finding consistent with a GPCR-Gq-PLC dependent nerve terminal activation.

The ionic mechanisms downstream of PLCβ3 activation that underlie action potential discharge are not known. A surprising finding of the present study was the lack of influence of TRPA1 or TRPV1 in the activation of the C-fiber terminals by CQ or histamine. Other studies have found that stimulation of a depolarizing current in DRG cell bodies, or elevations in intracellular calcium by CQ in nerve cell bodies dissociated from DRG is strictly dependent on TRPA1. Moreover, the scratching response to intradermal injections of CQ were virtually absent in some studies with *trpa1*$^{-/-}$ mice(Wilson *et al.*, 2011). The present results show that TRPA1 is not required for the action potential response to CQ at the C-fiber nerve terminals. Our patch clamp analysis also revealed no electrophysiological evidence for an important role of TRPA1 in the CQ-induced membrane depolarization and action potential discharge in the cell soma.

It is possible that TRPA1 may be selectively involved at lower levels of GPCR stimulations, and its role was surmounted by the 100 µM concentration of CQ used in the present study. This is unlikely however, as our previous concentration-response analysis has noted that 100 µm is not a supramaximal concentration for MrgprA3 activation (Liu *et al.*, 2009). We also saw a lack of TRPA1 involvement in the action potential discharge evoked by endogenous "physiological concentrations" of pruritogens released as a consequence of allergen (OVA) activated mast cells. The mechanism of antigen activation of the CQ-sensitive nerves was not studied, but in this context it is worth noting that upon immunological activation mast cells can release mediators that stimulate MrgprC11 (Lee *et al.*, 2008).

Our results considered along with the findings of others that TRPA1 is involved in CQ-induced scratching would indicate that the location of the TRPA1 channels involved in the scratch reflex in healthy mice may be somewhere other than the nerve terminals, most likely in the central nervous system(Akiyama & Carstens, 2014). Mice in which *trpa1* is genetically deleted, or are treated with TRPA1 antagonists have strongly altered behavior responses to stressors, similar to mice treated with large doses of benzodiazepines or antidepressant drugs (de Moura *et al.*, 2014). Consistent with our findings with respect to itch nerve terminal activation, we failed to find a difference in the CQ-induced bouts of scratching between wild type and *trpa1 -/-* mice. Therefore, the effect of TRPA1 on scratching may depend on subtleties of experimental design, a conclusion more in keeping with a central site of action rather than a dependency on TRPA1 for action potential generation at the itch nerve terminals.

Agonists of GPCRs, notably Gq-coupled receptors, often lead to TRPV1 activation(Veldhuis *et al.*, 2015). The CQ-sensitive terminals are more responsive to TRPV1 stimulation than TRPA1, therefore we thought it likely that TRPV1 may be involved in the CQ-induced action potential discharge. This hypothesis was not supported by the data showing that the percentage of neurons responding and the magnitude of the action potential discharge induced by either CQ or histamine was not different between wild-type and *trpa1*$^{-/-}$*/trpv1*$^{-/-}$ double knockout animals, nor inhibited by ruthenium red.

Than et al. noted that CQ stimulates calcium increases in a subset of DRG neurons independently of TRPA1 or TRPV1. In these neurons, the calcium rise was inhibited by TRPC3 blockade (Than *et al.*, 2013). The MrgprA3 expressing neurons in mouse DRG express TRPC3 and TRPC6 mRNA(Usoskin *et al.*, 2015). Our observation that the action potential discharge in the terminals was the same between wild type mice and *trpc3*$^{-/-}$ */ trpc6*$^{-/-}$ mice indicates that these channels are not critical for

the action potential responses. The lack of inhibitory effect of removing TRPA1, TRPV1, TRPC3 or TRPC6 is not likely due to strong redundancy among these channels, because the potent TRPC3/TRPC6 inhibitor GSK2332255B (Seo *et al.*, 2014) had little effect on CQ-induced responses even when evaluated in the skin from *trpa1*$^{-/-}$*/trpv1* $^{-/-}$ animals.

Gq-GPCR activation of PLC can lead to neuronal membrane depolarization by blocking the so-called M-current carried by KCNQ/Kv7 channels (Hernandez *et al.*, 2008; Linley *et al.*, 2008). An effective M-current blocker did not mimic the effect of CQ or histamine, nor did it alter the action potential discharge evoked by CQ. Moreover, the MrgprA3 expressing subpopulation of neurons in the DRG express relatively little KCNQ mRNA(Usoskin *et al.*, 2015).

It is known that adult primary sensory nerves concentrate intracellular Cl such that the equilibrium potential for Cl^- (E_{Cl})is more positive than the resting membrane potential (Rohrbough & Spitzer, 1996). Previous studies at the soma of sensory neurons, and nerve terminals of vagal C-fibers, revealed that a component of bradykinin B2 receptor mediated nerve stimulation is inhibited by chloride channel blockers (Oh & Weinreich, 2004; Lee *et al.*, 2005), and in the case of small diameter DRG neurons by specifically inhibiting TMEM16a expression (Liu *et al.*, 2010). The MrgprA3 expressing nerves express the calcium-activated chloride channels including TMEM16A (Aon-1) (Usoskin *et al.*, 2015), and this channel has been liked to GPCR/PLC-dependent stimulation (Stolz *et al.*, 2015). Recently MONNA has been characterized as a selective TMEM16A (Oh *et al.*, 2013b). The finding that this antagonist inhibited CQ-induced C-fiber stimulation and bouts of scratching by 50% supports the hypothesis that chloride efflux through TMEM16A channels contributes the CQ-induced generator potential in itch nerves; as has been previously hypothesized for bradykinin-induced activation of DRG neurons in the rat (Liu *et al.*, 2010). . This conclusion is based on a single antagonist, and TMEM16A blockers including MONNA may have relevant nonselective effects (Boedtkjer *et al.*, 2015). Therefore, this hypothesis needs to be cautiously considered until independent verification is obtained.

The data presented here argue against a strategy targeting TRP channels *at the level of the itch nerve terminals* to control GPCR-dependent itch. It should be kept in mind however, that all studies were carried out in healthy animals and the pruritogens were limited to an evaluation of histamine and CQ. Inhibition of TRPA1 inhibits bile acid-induced scratching in mice, and stimulation of the TGR5 receptor with bile acid can lead to TRPA1 activation in cell systems(Lieu *et al.*, 2014). It is possible that TRPA1 may play more of a role in itch associated with inflamed skin. TRPA1 is expressed in multiple cell types, and inflammation associated with dermatitis is associated with elevation of TRPA1 expression. Blocking TRPA1 inhibits scratching associated with dry skin (Wilson *et al.*, 2013),

atopic(Oh *et al.*, 2013a) and contact dermatitis(Liu *et al.*, 2013). This however, was associated with inhibition of the inflammatory response and production of pruritogens, and may not necessarily have been due to direct regulation of action potential generation at the nerve terminals(Liu *et al.*, 2013; Wilson *et al.*, 2013).

Acknowledgement

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fig1

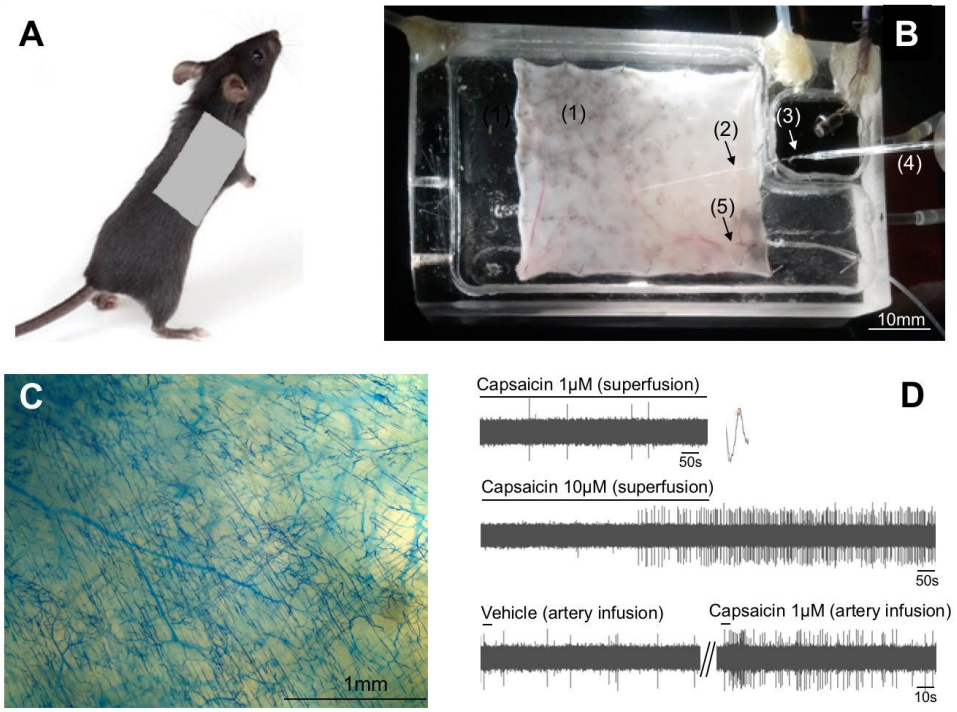

Figure 1. Extracellular recording of single fiber nerve fiber activity in isolated DRG-skin preparation. (A) The location of skin used for preparation is shown on the right back side of the mouse. (B) The DRG nerve-skin preparation. The skin (1) is pinned dermis (corium) side up in the tissue chamber, the T9 thoracic nerve (2) passes through a hole into the recording chamber and the T9 DRG (3) at the distal end of is pinned. Extracellular recording electrode (4) has the tip positioned in the DRG. The tissue and the recording chamber are separately superfused with warmed Krebs solution. Mechanical stimuli (von Frey hairs) are delivered to the dermis (corium) side of the skin. The drugs are delivered via cannulated right subscapular artery (5) connected to PE tubing. (C) Filling of the microvasculature following the infusion of Monastral Blue into the subscapular artery shows extensive distribution of the dye in the skin achieved by this route of drug delivery. (D) An example showing the difference of DRG nerve fiber activation

evoked by capsaicin delivered in superfusing fluid (upper two traces) and infused into subscapular artery (lower trace). The time to onset of action potential discharge was notably shorter and the magnitude of the response was higher when capsaicin was infused into subscapular artery. Note the large difference in the time scale between upper two traces and lower trace.

Fig 2

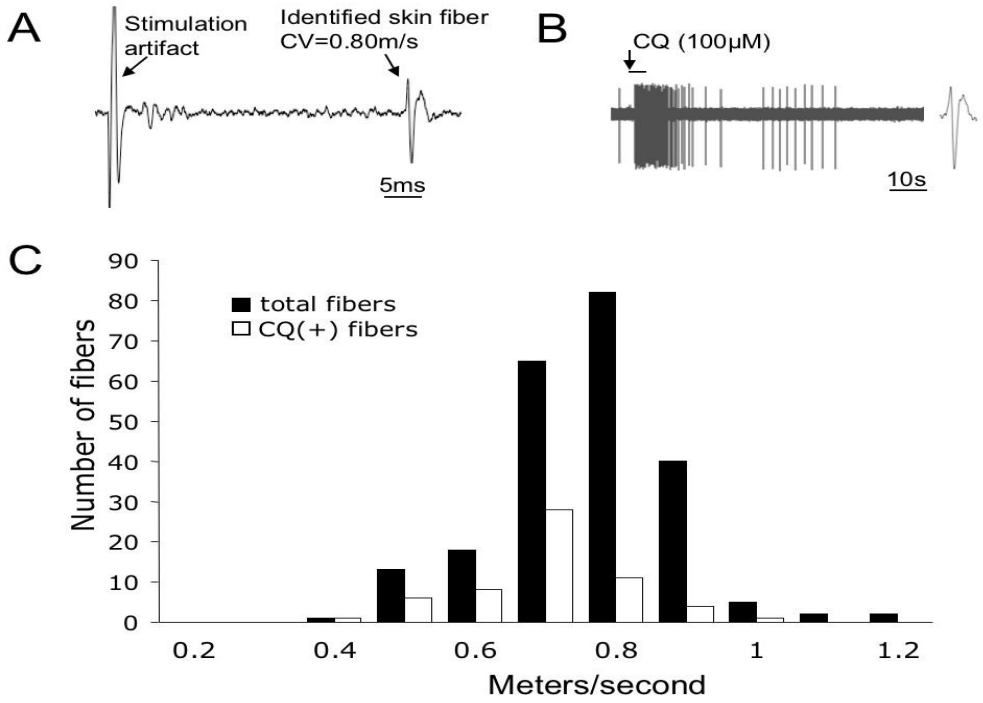

Figure 2. The conduction velocity distribution of CQ-sensitive skin DRG C-fibers. (A) Representative trace showing the electrical stimulation of the mechanosensitive receptive field. Conduction velocity was determined by dividing the approximate length of the nerve fiber by conduction time. (B) Representative trace showing the action potential discharge evoked in response to CQ delivered via artery infusion in the same fiber. Note identical action potential waveform in (A) and (B). (C) The conduction velocity distribution of CQ-positive skin DRG C-fibers (n=59) and the total skin DRG C-fiber population (n=228).

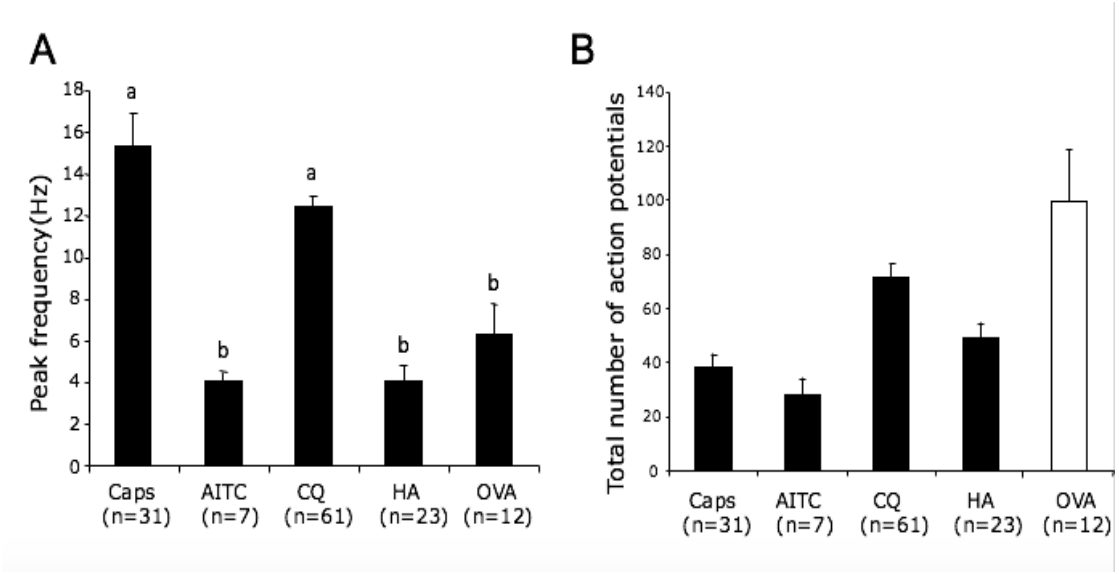

Figure 3. Quantification of the response to capsaicin, AITC, CQ, HA(histamine) and OVA in the skin DRG CQ-positive C-fibers in wild type mouse. (A) The peak frequency and (B) the total number of action potentials. Capsaicin(1μM), AITC(300μM), CQ(100μM) and HA(100μM) were delivered by artery infusion and the response was recorded for 2 minutes. Among the CQ-positive C-fibers (n=61) 31 of 40 fibers tested responded to capsaicin, all tested fibers responded to AITC (n=7) and 23 of 25 responded to HA. Ovalbumin (OVA) was evaluated in sensitized mice. Ovalbumin (1mg/ml) was delivered by both arterial infusion and superfusion and the response was recorded for 30 minutes. Among the CQ-positive C-fibers 12 of 13 responded to OVA. The bars represent mean ± SEM of (n) number of individual fibers studied (1 experiment per animal for OVA and capsaicin, 1-2 experiments per animal for other stimuli). An ANOVA followed by a Tukey Kramer revealed a statistically greater peak Hz response for Caps and CQ (denoted with an a) compared to the other stimuli (denoted with a b).

Fig 4

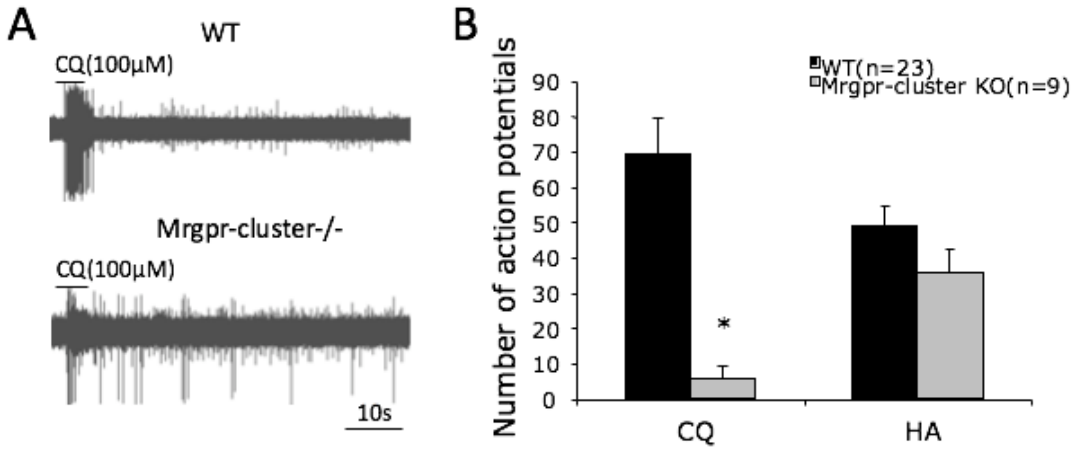

Figure 4 CQ and histamine (HA) stimulation in HA-sensitive cutaneous C-fiber in wildtype (WT) compared to Mrgpr-cluster Δ-/- mice. (A) Representative traces of the response to CQ in wild type and Mrgpr-cluster Δ-/- mice. (B) The mean ± SEM number of action potentials evoked by CQ and HA in HA-sensitive C-fibers wild type (n=23) and mrgpr-cluster Δ-/- mice (n=9). One of the histamine–sensitive C-fiber studied in mrgpr-cluster Δ-/- mice responded with a burst of 159 action potentials after a delay post- addition of CQ (and was excluded) 7 fibers did not respond and 2 responded with a low level of activation ($P<0.05$, including all 10 fibers). The bars represent mean ± SEM of (n) number of individual fibers studied; an astrick (*) denotes significantly different than wildtype ($P<0.05$).

Fig5

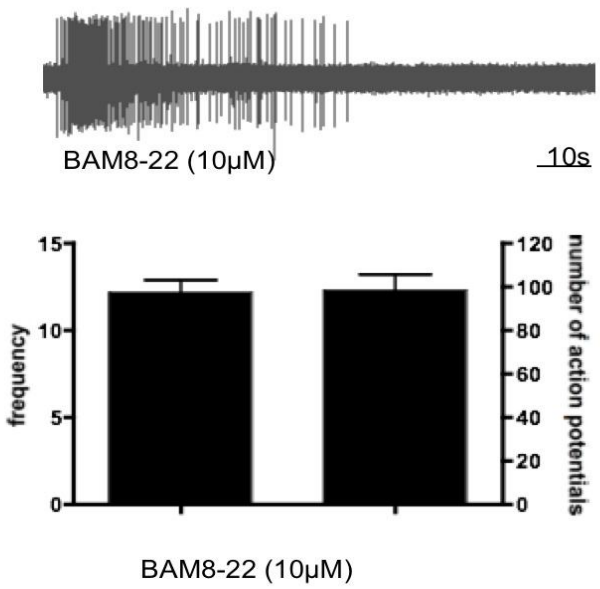

Figure 5 Activation of CQ-sensitive C-fibers by the MrgprC11 agonist BAM8-22. All (12 of 12 C-fibers that responded to CQ were also strongly activated by BAM8-22. The bar graphs are mean ± SEM of n= 12 C-fibers. 12 of 13 C-fibers that did not responded CQ,also failed to respond to BAM8-22 (not shown).

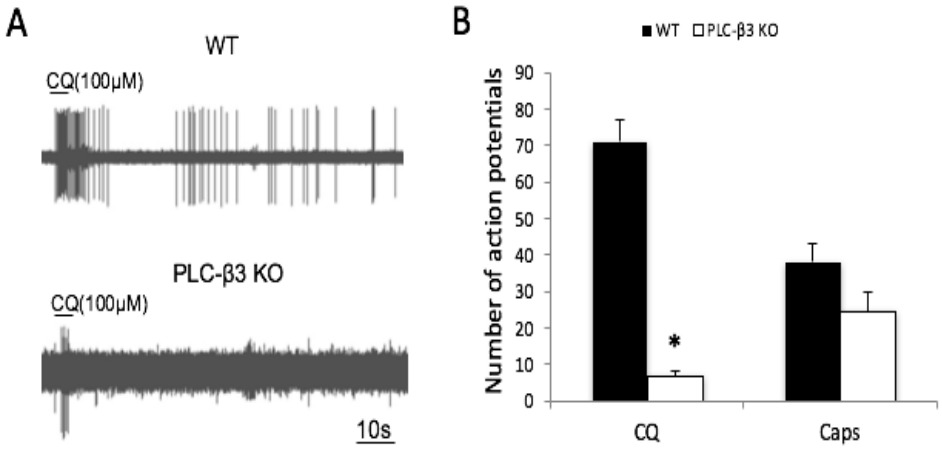

Figure 6 CQ stimulation in cutaneous C-fiber in wild type compared to PLCβ3 ko mice. (A) Representative traces of the response to CQ in wild type and PLCβ3 ko mice. (B) The number of action potentials evoked by CQ in wild type and PLCβ3 ko mice. The wild type data are the same as presented in figure 3. 26 C-fibers in 8 PLCβ3 ko were evaluated. Among these fibers 23 had no response and 3 had a minimal activation. The histogram reflects the mean ± SEM of 61 and the 3 CQ responding fibers in WT and PLCβ3 ko mice, respectively. As a positive control, the number of action potentials evoked by capsaicin was not statistically different than wild type mice.

fig7

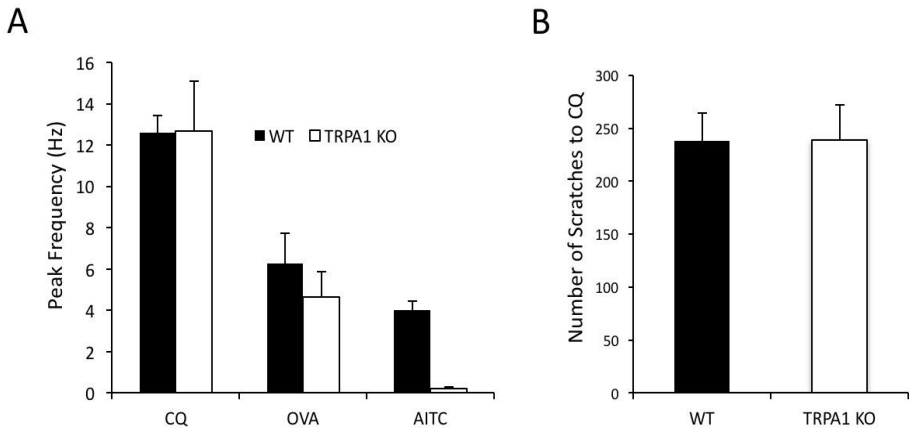

Figure 7. CQ, OVA, and AITC-induced stimulation of action potential discharge in C-fibers innervating the skin of wild type and *trpa1-/-* mice. (A)The bar graphs represent the mean ± SEM peak action potential discharge frequency observed in C-fibers innervating the skin of wildtype (black bars) and *trpa1 -/-* mice (white bars). CQ (100 μM) activated 4 of 15 C-fibers in *trpa1 -/-* mice which was not different than the % of CQ sensitive C-fibers in wild-type (P>0.1). The OVA (1mg/ml) and AITC (300 μM) data were derived from C-fibers that were previously determined to be CQ-sensitive. The respective n values for wild type and *trpa1 -/-* animals was, CQ (33 and 4); OVA (12 and 3); AITC (7 and 3). (B) Scratching bouts in wildtype (WT) compared to *trpa1 -/-* mice in response to CQ injection. Each day one wild type and one *trpa1 -/-* animal was evaluated. The bars represent the mean ± SEM number of bouts evoked over a 30-minute period in n= 18 pair of mice.

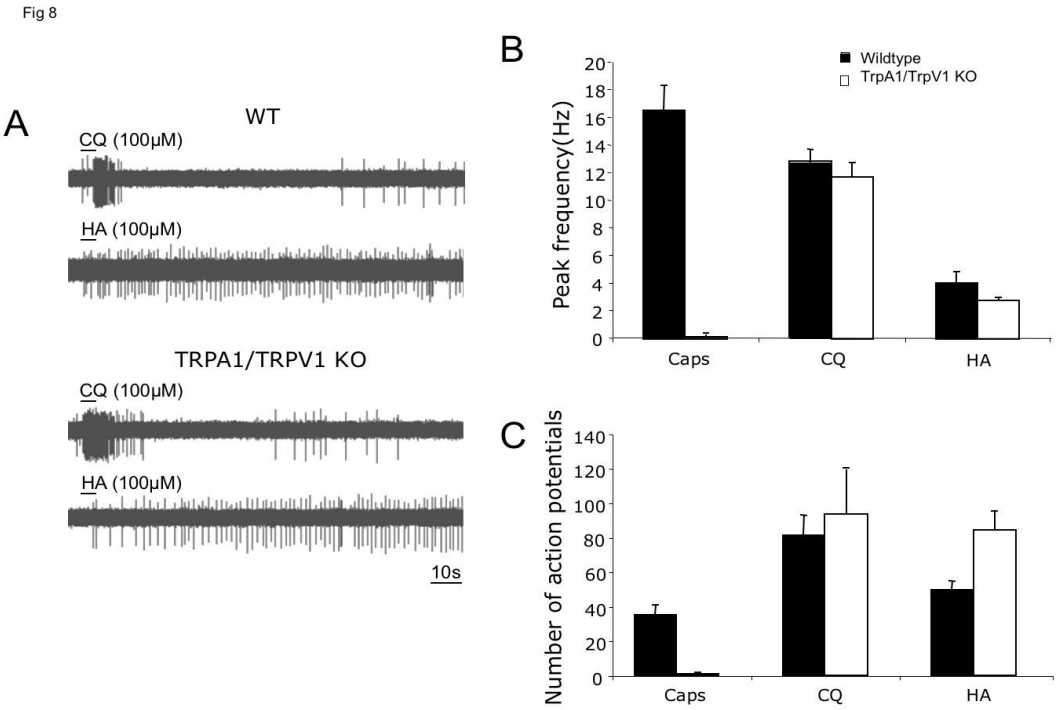

Figure 8. CQ, histamine, and capsaicin-induced stimulation of action potential discharge in C-fibers innervating the skin of wild type and *trpa1/trpv1 -/-* double KO mice.. (A) The pattern of response to CQ (the burst of action potentials with an onset within 10 seconds) was similar between the wild type and *trpa1/trpv1 -/-* mice. (B) The peak frequency and (C) the total number of action potentials evoked by capsaicin, CQ and HA in wild type (black bars) and *trpa1/trpv1 -/-* mice. The data on wild type mice C-fibers are taken from Fig. 3. In the *trpa1/trpv1 -/-* mice 8 of 40 C-fibers responded to CQ which was not significantly different than the % of CQ-sensitive fibers in wild type animals. Among the CQ-positive *trpa1/trpv1 -/-* C-fibers, 4 of 4 C-fibers responded to histamine and 0 of 4 C-fibers responded to capsaicin. The bars represent the mean ± SEM. The n values for responding nerves in wildtype as in Figure 3; the n values for responding C-fibers in the *trpa1/trpv1 -/-* skin are 4, 8 , and 4 for caps, CQ, and HA, respectively (typically one nerve studies per animal).

Fig 9

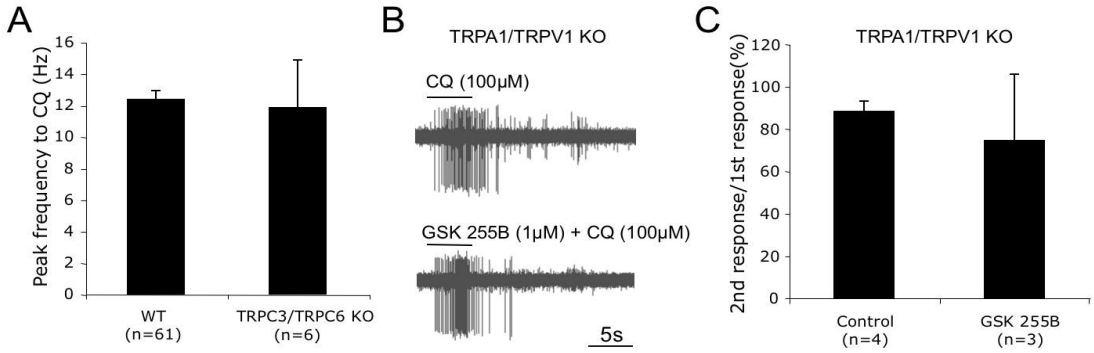

Figure 9. The response to CQ is not altered in *trpC3/6* double KO or by TRPC3/6 antagonist in *trpa1/trpv1 -/-* double KO mice. (A) Bar graphs showing the mean ± SEM of the peak frequency (Hz) evoked by CQ in wild type and TRPC3/C6 KO mice. (B) Representative traces of response to CQ in the absence and in the presence of the TRPC3/6 antagonist GSK255B in *trpa1/trpv1 -/-* mice. (C) The response to repeated administration of CQ in the absence and in the presence of the TRPC3/6 antagonist GSK255B in the *trpa1/trpv1 -/-* mice. The bar graphs depict the mean ± SEM of the number of action potentials evoked presented as the ratio of the second CQ response to the first CQ response .

Fig 10

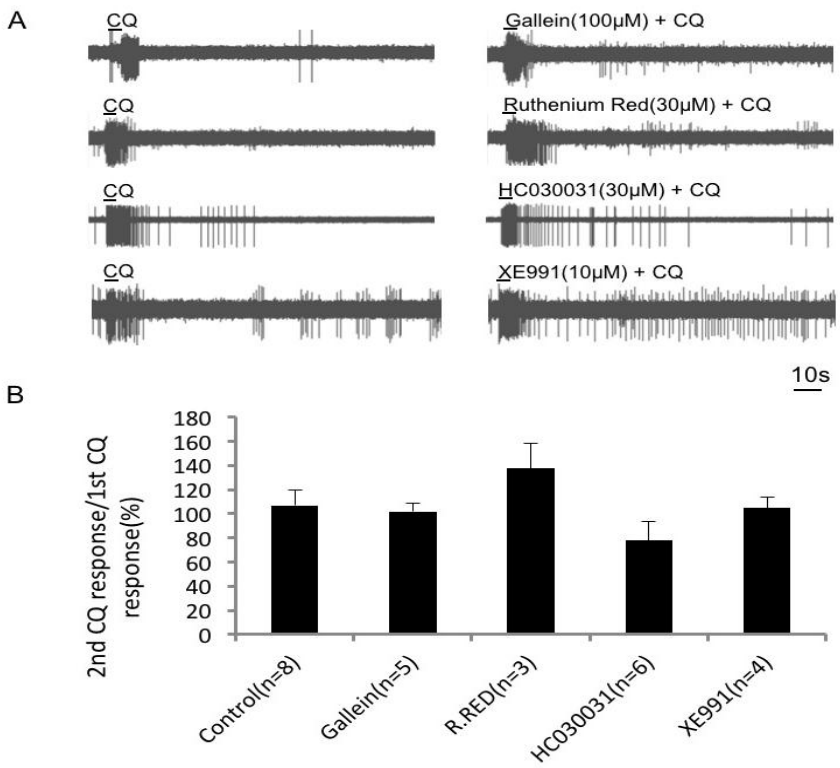

Figure 10. The response to CQ is not inhibited following 20 min treatment with gallein, Ruthenium Red, HC030031 and XE991 (A) Representative traces of the response to CQ in the absence and in the presence of gallein (100 μM), Ruthenium Red (30 μM), HC030031 (30 μM) and XE991 in WT mice. (B) The response to repeated administration of CQ in the absence and in the presence of the various inhibitors. The data are presented as mean ± SEM of the ratio of the second CQ response to the first CQ response (the response quantified as number of action potentials evoked). No drug significantly inhibited the CQ response (P>0.1)

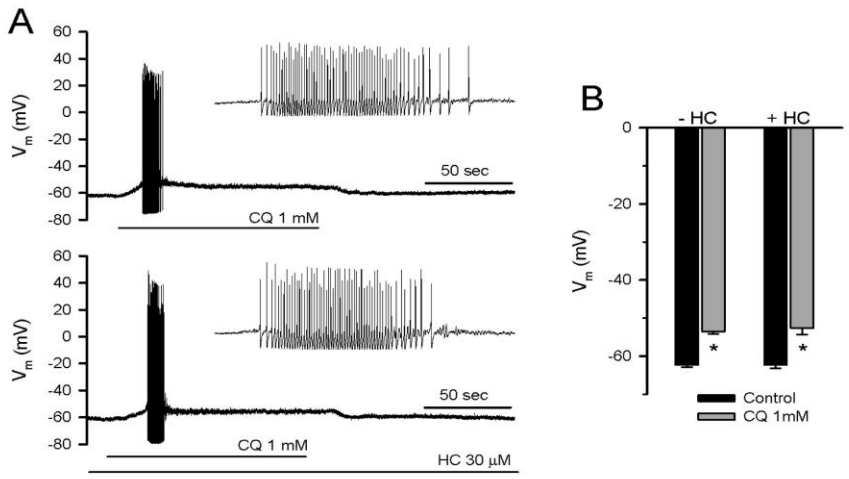

Figure 11. Whole-cell patch clamp recording of mrgprA3-td tomato expressing neurons. A. Studies in current-clamp mode showing the membrane depolarization and action potential discharge evoked by CQ (1 mM) in a control neuron (top) and a neuron pretreated for 5 min with HC 030031 (30 μM) (bottom). The number of action potentials was not influenced by the TRPA1 antagonist (mean data are presented in the text). B. The depolarizing effect of CQ (1mM) on the membrane potential in control neurons and neurons treated with 30 μM HC 030031 (* denotes P< 0.01 relative to control, bars represent the mean ± SEM of 9 experiments.

fig

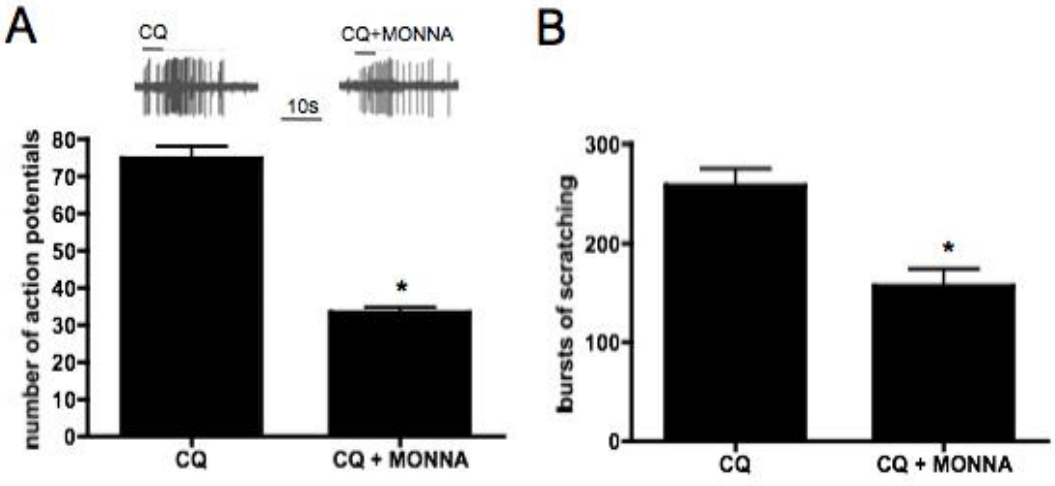

Figure 12. The effect of MONNA on CQ-induced action potential discharge and bouts of scratching. (A) the mean ± SEM of CQ in the absence and presence of MONNA (10 μM, 15 min), in 10 CQ-sensitive C-fibers (* denotes P <0.05). B. The mean ± SEM of the number of bouts of scratching over 30 min in the presence of CQ (10 mM) (n=7) or CQ + MONNA (1 mM), n=5 (* denotes P <0.01).

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Additional Information

• Competing Interests: There are no competing interests.

• Author Contributions

F.R. works in laboratories at the JHU Asthma Center. He was involved with the conception of the study, experimental design, data acquisition, and interpretation, as well as editing the paper for content.

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