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Capsaicin-responsive corneal afferents do not contain TRPV1 at their central terminals in trigeminal nucleus caudalis in rats

Deborah M. Hegarty^a, Sam M. Hermes^a, Tally M. Largent-Milnes^a, and Sue A. Aicher^{a,#}

^aDepartment of Physiology and Pharmacology, Oregon Health & Science University, Mail code: L334, 3181 Sam Jackson Park Road, Portland, OR 97239-3098, United States

Abstract

We examined the substrates for ocular nociception in adult male Sprague-Dawley rats. Capsaicin application to the ocular surface in awake rats evoked nocifensive responses and suppressed spontaneous grooming responses. Thus, peripheral capsaicin was able to activate the central pathways encoding ocular nociception. Our capsaicin stimulus evoked c-Fos expression in a select population of neurons within rostral trigeminal nucleus caudalis in anesthetized rats. These activated neurons also received direct contacts from corneal afferent fibers traced with cholera toxin B from the corneal surface. However, the central terminals of the corneal afferents that contacted capsaicin-activated trigeminal neurons did not contain TRPV1. To determine if TRPV1 expression had been altered by capsaicin stimulation, we examined TRPV1 content of corneal afferents in animals that did not receive capsaicin stimulation. These studies confirmed that while TRPV1 was present in 30% of CTb-labeled corneal afferent neurons within the trigeminal ganglion, TRPV1 was only detected in 2% of the central terminals of these corneal afferents within the trigeminal nucleus caudalis. Other TRP channels were also present in low proportions of central corneal afferent terminals in unstimulated animals (TRPM8, 2%; TRPA1, 10%). These findings indicate that a pathway from the cornea to rostral trigeminal nucleus caudalis is involved in corneal nociceptive transmission, but that central TRP channel expression is unrelated to the type of stimulus transduced by the peripheral nociceptive endings.

Keywords

Confocal microscopy; immunocytochemistry; cholera toxin B subunit; TRP

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#Corresponding author: Department of Physiology and Pharmacology, Oregon Health & Science University, Mail code: L334, 3181 Sam Jackson Park Road, Portland, OR 97239-3098, United States, Tel: 503-418-2550; Fax: 503-494-4352, aichers@ohsu.edu, hegartyd@ohsu.edu (D. M. Hegarty); hermess@ohsu.edu (S. M. Hermes); milnes@ohsu.edu (T. M. Largent-Milnes)

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CONTRIBUTIONS

All authors participated in the acquisition, analysis and interpretation of the data and in the writing and revising of this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

1. INTRODUCTION

The cornea is uniquely suited for orofacial pain studies as it is the most densely innervated tissue in the body and is innervated exclusively by thinly myelinated A-delta and unmyelinated C-fibers (Belmonte et al., 2004; De Felipe et al., 1999; Marfurt and Del Toro, 1987). Painful stimulation of the cornea is transduced by these afferents and transmitted via the ophthalmic branch of the trigeminal nerve to neurons in the trigeminal nucleus caudalis (Vc). Corneal afferent terminals send their highest density projections to the caudal and rostral transition areas of Vc with cervical spinal cord and trigeminal nucleus interpolaris (Vi), respectively (Aicher et al., 2013; Belmonte et al., 2004; Hegarty et al., 2010; Marfurt and Del Toro, 1987). The peripheral ends of the corneal afferents contain members of the transient receptor potential (TRP) channel family that are thought to transduce mechanical, thermal, cold and chemical stimuli (Guo et al., 1999; Hiura and Nakagawa, 2012; Murata and Masuko, 2006; Nagata et al., 2005; Nakagawa et al., 2009; Parra et al., 2010). The most well-characterized of these TRP channels is the transient receptor potential vanilloid 1 (TRPV1) channel, previously known as vanilloid receptor 1 (VR1) (Caterina et al., 1997; Caterina et al., 2000). The TRPV1 channel is activated by noxious heat, spider toxins, low pH and capsaicin (Caterina et al., 2000; Patapoutian et al., 2009). Capsaicin is the active ingredient of hot chili peppers and has been established as a noxious stimulus when applied to orofacial structures in rodents (Caterina et al., 2000; Klein et al., 2011; Neubert et al., 2008; Shimada and LaMotte, 2008). As demonstrated in TRPV1 knockout mice, the TRPV1 channel is the exclusive transducer of capsaicin stimulation (Caterina et al., 2000).

While TRPV1 channels located at the periphery are known to transduce capsaicin stimulation, it is unclear what role TRPV1 channels on the central afferent terminals may play (Kim et al., 2014; Largent-Milnes et al., 2014; Patapoutian et al., 2009; Patwardhan et al., 2009). It is often presumed that primary afferent neurons that contain TRPV1 at peripheral sites also contain the same transducer at central sites. In the present study, we examined whether the central terminals of corneal afferents making direct contacts with capsaicin-activated neurons contain TRPV1. We also assessed the content of TRP channels in unstimulated animals to verify that TRP content was not altered by noxious stimuli.

Previous studies from this laboratory have successfully used cholera toxin B (CTb) to trace corneal afferents to caudal and rostral Vc (Aicher et al., 2013; Aicher et al., 2014; Hegarty et al., 2010). We have demonstrated that a substantial percentage of CTb-labeled corneal afferents contain vesicular glutamate transporter 1 (VGluT1) (33%) or VGluT2 (28%), with fewer afferents containing calcitonin gene-related peptide (CGRP) (15%) and very few afferents containing substance P (SP) (3%) (Hegarty et al., 2010). We also found that the glutamatergic and peptidergic neurochemical composition differs between caudal and rostral Vc corneal afferents (Hegarty et al., 2010). In later studies we found that the corneal afferents to rostral Vc preferentially contact parabrachial-projecting neurons (Aicher et al., 2013; Aicher et al., 2014) which are responsive to multi-modal corneal stimulation (Aicher et al., 2014). In the current study, we sought anatomical and functional confirmation that neurons in caudal and rostral Vc receiving direct contacts from corneal afferents are activated by noxious corneal stimulation with capsaicin. We used immunocytochemical detection of c-Fos protein to determine the extent of capsaicin-induced activation of second-

order neurons in Vc, since c-Fos has been used previously as a marker of neuronal activation after noxious stimulation of the cornea (Chang et al., 2010; Martinez and Belmonte, 1996; Meng and Bereiter, 1996; Strassman and Vos, 1993).

2. MATERIALS AND METHODS

2.1. Experimental animals

All protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University and all experiments adhered to the guidelines of the National Institutes of Health and the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP) and conform to the European Union Directive 2010/63/EU for animal experimentation. Male Sprague-Dawley rats (n = 24, 230 - 450 g; Charles River Laboratories, Wilmington, MA) were housed in pairs on a 12/12 light/dark cycle and were given access to food and water *ad libitum*. The animals that were included in the behavioral study were not used in the corneal tract tracing and immunocytochemical studies described below.

2.2. Behavioral assessments after ocular capsaicin application

Unanesthetized animals were lightly restrained by one experimenter while a second experimenter pipetted 10 μ l of 0.1% capsaicin (Sigma-Aldrich, St. Louis, MO; n = 3) or its vehicle (1% Tween-80 and 1% ethanol in saline; n = 3) onto the left ocular surface. Immediately following capsaicin or vehicle application, the animal was placed into a plexiglass chamber for 15 minutes and the number of eye blinks, eye wipes, and grooming bouts were counted. Eye blinks were defined as blinks occurring exclusively in the eye that received capsaicin or vehicle application. Eye wipes were counted if the animal used the forelimb ipsilateral to the eye that received capsaicin or vehicle to wipe the eye. Spontaneous bouts of grooming were performed with both forepaws and were counted if the bout followed a typical pattern of cleaning the forepaws, followed by both sides of the face, stomach, back and hind limbs. Eye wipes were distinct from bouts of grooming. After behavioral assessment, the animal was removed from the chamber and returned to the home cage.

2.3. Corneal afferent tract tracing and capsaicin application techniques for immunocytochemistry studies

2.3.1. Cholera toxin B subunit—(CTb; 1% solution in 0.1 M phosphate buffer (PB); List Biological Laboratories, Inc., Campbell, CA) was used as an anterograde tracer to identify central projections of corneal afferents into the ventrolateral dorsal horn of trigeminal nucleus caudalis (Vc) as previously described (Aicher et al., 2013; Hegarty et al., 2010). In order to achieve uptake of CTb into corneal afferents, the outer epithelial layer of the left cornea within a steel ring (7-gauge, Small Parts, Miramar, FL; affixed to the cornea with petroleum jelly) was abraded with a 1 minute application of 1-heptanol (99%; Alfa Aesar, Ward Hill, MA) followed by saline rinses (Aicher et al., 2013; De Felipe et al., 1999; Hegarty et al., 2010). This method was used because it produces a uniform abrasion of the outer epithelial layer that is restored by 48 hours after abrasion (De Felipe and Belmonte, 1999; Gallar et al., 1990). The heptanol method does not evoke protein expression of c-Fos

suggesting that removal of the outer epithelial layer does not produce overt injury to the cornea (De Felipe and Belmonte, 1999). After rinsing, the eye was dried again, the steel ring replaced around the abraded surface with petroleum jelly, and CTb (10-15 μ l) was placed inside the ring for 30 min before being rinsed off with saline. Rats received a subcutaneous injection of ketoprofen (2.5 mg/kg) to reduce discomfort and were observed for two hours in their home cage before being returned to the colony.

2.3.2. Capsaicin application—Seven days after CTb application, animals designated for capsaicin-induced c-Fos immunocytochemical experiments were anesthetized again with vaporized isoflurane in oxygen (5% induction, 2-3% maintenance). Animals were randomized to one of three treatment groups: 0.1% capsaicin (n = 3), vehicle control solution (n = 3) and anesthesia control (n = 3). Experimental animals received 10 μ l capsaicin applied to the same cornea that received CTb one week earlier. After 1 minute, the capsaicin was wicked away, and the eye was rinsed briefly with saline. Vehicle control animals received 10 μ l of 1% Tween-80 and 1% ethanol in saline on the left cornea for 1 minute. The vehicle solution was then wicked away and the eye was rinsed briefly with saline. The anesthesia control animals were anesthetized for a comparable amount of time as the experimental and vehicle control animals. The eye was not stimulated during this time. Animals recovered from anesthesia and were returned to their home cage. Two hours later, animals were perfused for immunocytochemical processing.

2.4. Immunocytochemistry and tissue preparation for microscopy

2.4.1. Transcardial perfusion—Rats were overdosed with pentobarbital sodium (150 mg/kg) and perfused transcardially as previously described (Aicher et al., 2013; Hegarty et al., 2010; Hermes et al., 2006) through the ascending aorta with one of two sequences of solutions, based on the best results for each of the antibodies being used for the analyses. The sequence for most animals was as follows: (1) 10 ml heparinized saline (1000 units/ml), and (2) 600ml 4% paraformaldehyde (in PB, pH 7.4). The sequence for animals used in the CTb and TRPA1 co-localization analysis was as follows: (1) 10 ml heparinized saline (1000 units/ml), (2) 50 ml 3.8% acrolein in 2% paraformaldehyde, and (3) 200 ml 2% paraformaldehyde (in 0.1 M PB, pH 7.4). The brainstem was then removed and incubated in the final fixative for 30 minutes, and then placed in 0.1 M PB. For all cases, the brainstem was sectioned coronally (40 μ m) on a vibrating microtome and processed for appropriate immunocytochemical procedures. To distinguish the left and right sides, a small puncture was placed through the contralateral reticular formation of the brainstem.

In a subset of animals, we also removed the trigeminal ganglia, placed them in 4% paraformaldehyde for 30 minutes at room temperature, rinsed in 0.1 M PB, and then placed them in 30% sucrose in 0.1 M PB at 4°C until cryosectioning. Each trigeminal ganglion was covered in Tissue-Tek® optimum cutting temperature (O.C.T.) compound and frozen. Trigeminal ganglia were sectioned (20 μ m) on a Leica CM1950 cryostat (Leica Microsystems, Inc., Buffalo Grove, IL) and mounted directly onto room temperature SuperFrost® Plus microscope slides (Fisher Scientific, Pittsburgh, PA) slides. Sections were allowed to dry and stored at -20°C.

2.4.2. Immunocytochemistry—Brainstem sections were processed as free-floating as previously described with modifications (see sections 2.4.3 and 2.4.4) (Aicher et al., 2003; Bailey et al., 2006; Hegarty et al., 2010; Winkler et al., 2006). Trigeminal ganglia sections were processed on slides (see section 2.4.5). Prior to immunocytochemical processing in all studies, sections were incubated in a 1% sodium borohydride solution for 30 minutes. The primary antibodies, their immunogens, catalog numbers, sources, the experimental dilutions and the secondary antibody with which each primary antibody was paired are listed in Table 1. For clarity, we will use the current TRPV1 nomenclature when referring to the primary antibodies that were raised against the “VR1” protein or the “capsaicin receptor” (See Table 1).

2.4.3. Single-label peroxidase immunocytochemistry—Tissue sections were incubated with goat anti-TRPV1 primary antibody (Neuromics, Edina, MN; Table 1) in 0.1% bovine serum albumin (BSA, Sigma-Aldrich) and 0.25% Triton X-100 (TX, Sigma-Aldrich) in 0.1 M Tris-buffered saline (TS) for two nights at 4°C. Bound primary antibody was visualized by incubating tissue sections in biotinylated horse anti-goat secondary antibody (1:400; Vector Laboratories, Inc., Burlingame, CA), followed by incubation in avidin-biotin (Elite Vectastain ABC kit, Vector) and then in diaminobenzidine-hydrogen peroxide solution. Tissue sections were mounted on gelatin-coated slides, dehydrated in ethanol and then xylenes, and coverslipped with DPX mounting medium (Sigma-Aldrich).

2.4.4. Immunofluorescent immunocytochemistry—Tissue sections were incubated for 2 nights at 4°C in a cocktail of primary antibodies in 0.25% TX and 0.1% BSA in 0.1 M TS. Tissue sections from capsaicin-treated, vehicle-treated and anesthesia control animals were incubated in goat anti-CTb, rabbit anti-c-Fos and guinea pig anti-TRPV1 (Table 1). Brainstem tissue sections from rats designated for CTb and TRP channel co-localization analysis were incubated in one of the following primary antibody cocktails: 1) goat anti-CTb and guinea pig anti-TRPV1; 2) rabbit anti-CTb and goat anti-TRPV1 (Santa Cruz); 3) goat anti-CTb and rabbit anti-TRPM8; or 4) goat anti-CTb and rabbit anti-TRPA1 (Table 1). After primary antibody incubation, tissue sections were rinsed and incubated in a cocktail of fluorescent secondary antibodies for 2 hours, light-protected at room temperature (Table 1).

In a subset of animals used for TRPV1 channel co-localization analysis ($n = 3$), we performed immunocytochemistry using sequential incubation in primary and secondary antibodies, instead of incubation in a cocktail of primary antibodies followed by a cocktail of secondary antibodies.

Tissue sections from capsaicin-treated, vehicle-treated and anesthesia control animals were rinsed and then incubated in NeuroTrace™ (NT) 435/455 blue fluorescent Nissl stain (Invitrogen/Life Technologies, Grand Island, NY), light-protected, for 20 minutes at room temperature. In all studies, sections were rinsed and then mounted on gelatin-coated slides, coverslipped with Prolong Gold™ Antifade reagent (Life Technologies) and stored at –20°C to preserve labeling.

2.4.5. Immunofluorescent immunocytochemistry on slides—Immunofluorescent studies on slides containing sections of trigeminal ganglia from a subset of animals ($n = 3$)

were performed using sequential incubation in primary and secondary antibodies. Slides containing cryosectioned trigeminal ganglia sections were thawed at room temperature and then sections were circumscribed with a hydrophobic barrier (ImmEdge pen, Vector). Sections were subjected to the same sequential immunocytochemical protocol as described above except that reagents were applied to the sections on each slide within the hydrophobic barrier. Trigeminal ganglia tissue sections were incubated in NT 530/615 fluorescent Nissl stain in 0.1 M PB (Invitrogen/Life Technologies), light-protected, for 20 minutes at room temperature. Sections were rinsed, air-dried, coverslipped with Prolong Gold™ Antifade reagent (Life Technologies) and stored at -20°C to preserve labeling.

2.4.6. Antibodies—The specificity of the primary and secondary antibodies has been previously validated in our laboratory and others. We used two different antibodies raised against CTb, made in different species (Hegarty et al., 2010) to allow for combination with other antibodies of interest. For both CTb antibodies, the discrete pattern of CTb immunoreactivity in the ventrolateral Vc ipsilateral to the cornea that received CTb tracer matched our previous study (Hegarty et al., 2010) and another study using horseradish peroxidase (HRP) to label corneal afferents (Marfurt and Del Toro, 1987). PreadSORption of the goat anti-CTb antibody with its immunogen abolished all immunolabeling (Llewellyn-Smith et al., 1995). Preabsorption with antigenic peptides was also used by others to validate the rabbit anti-c-Fos antibody (Howorth et al., 2009), the guinea pig anti-TRPV1 antibody (Amadesi et al., 2009) and both goat anti-TRPV1 antibodies (Czaja et al., 2008; Yeo et al., 2010). Immunoreactivity for the goat anti-TRPV1 antibody and the rabbit anti-TRPA1 antibody was absent in TRPV1 and TRPA1 null mutant mice, respectively (Brierley et al., 2009; Yeo et al., 2010). The specificity of the secondary antibodies was confirmed by omitting the primary antibodies. There was no immunolabeling in any of the omission experiments.

2.5. Microscopy and analysis

2.5.1. Light microscopy and analysis—Brainstem sections labeled with goat anti-TRPV1 (Neuromics) for peroxidase and sections throughout the trigeminal ganglia were imaged on an Olympus BX51 microscope equipped with a DP71 camera (Olympus America, Center Valley, PA). The trigeminal ganglia were examined under epifluorescence and the ganglion section with the highest concentration of CTb-labeled neurons was chosen for TRPV1 expression analysis. Low magnification images were taken throughout the length of one ganglion section from each animal ($n = 3$) using B (CTb), G (NT) and IY (TRPV1) excitation. Analysis was performed using Adobe Illustrator (Adobe Systems, San Jose, CA). NT-stained neurons that contained CTb immunoreactivity were circled and numbered first. In order for a neuron to be included in this analysis, the nucleus had to be visible in the NT channel. After the CTb-labeled neurons were marked and numbered, the numbers were transferred to the TRPV1 image, and each neuron was evaluated for TRPV1 expression. Light images used for publication were adjusted for optimal brightness and contrast in Adobe Photoshop (Adobe Systems).

2.5.2. Confocal imaging—Triple-labeled immunofluorescent tissue sections with NT staining from the capsaicin experiments were imaged with either a Zeiss LSM 710 (Carl

Zeiss MicroImaging, Thornwood, NY) or a Leica AOB5 (Leica Microsystems, Inc., Buffalo Grove, IL) confocal microscope. Similar results were produced from both confocal microscope systems. Dual-labeled immunofluorescent tissue sections were imaged with a Zeiss LSM 510 META confocal microscope.

In all cases, Z-stacks of 0.4 μ m optical sections were bounded by the vertical extent of immunoreactivity for the antibodies used and images were captured using the single pass, multi-tracking format. Two 40 μ m sections were chosen for analysis based on darkfield landmarks and peak CTb-ir corneal afferent input in the ventrolateral region of Vc at the caudal and rostral transition zones (Aicher et al., 2013; Aicher et al., 2014; Hegarty et al., 2010). Confocal micrographs used for publication are projections of several 0.4 μ m thick optical sections that were adjusted for optimal brightness and contrast using Zeiss ZEN software.

2.5.3. Confocal analyses—Images from animals included in the capsaicin-induced c-Fos immunocytochemical experiments were first analyzed for the number of c-Fos-ir neuronal soma in the ventrolateral caudal and rostral transition zones of Vc. C-Fos-ir neuronal somata were identified by the presence of c-Fos immunoreactivity within the boundaries of fluorescent NT Nissl-stained somata in at least two consecutive optical sections. We then examined both c-Fos-ir somata and somata that did not contain c-Fos immunoreactivity that received an apposition from CTb-ir corneal varicosities. Varicosities were operationally defined as approximately circular punctate enlargements that were present in at least two consecutive optical sections (Aicher et al., 2013; Bailey et al., 2006; Hegarty et al., 2010). An apposition was identified by the presence of a CTb-ir varicosity directly adjacent to a NT Nissl-stained soma in two consecutive optical sections (Aicher et al., 2013). All animals included in these analyses met the minimum number of CTb-ir varicosities (25 caudal, 50 rostral) that we set in previous studies (Aicher et al., 2013; Hegarty et al., 2010). Of those CTb-ir varicosities that made an apposition with a soma, we then examined whether that varicosity also contained TRPV1. A CTb-ir varicosity had to contain TRPV1 immunoreactivity in two consecutive optical sections in order to be considered immunoreactive for both markers.

In order to examine TRPV1 (n = 7), TRPM8 (n = 5) and TRPA1 (n = 7) localization in CTb-ir corneal afferents, CTb-ir varicosities were identified and numbered on optical sections of ventrolateral caudal and rostral Vc known to contain both CTb and TRP immunoreactivity, but were selected with the TRP signal turned off. All animals included in the co-localization study also met the minimum threshold for CTb varicosity labeling at each level (25 caudal, 50 rostral). Each varicosity was then examined for co-localization of CTb and the TRP channel. TRP immunoreactivity had to be contained within the CTb-ir varicosity in at least two consecutive optical sections to be considered co-localized. All appositional and co-localization analyses were verified by two independent observers to ensure objectivity.

2.5.4. Statistical analyses—All statistics were performed using SigmaPlot 12.0 software (Systat Software, Inc., San Jose, CA). A t-test was performed for each behavioral assessment to compare the mean number of eye blinks, eye wipes or bouts of spontaneous grooming between the groups of unanesthetized animals that received ocular application of

0.1% capsaicin and vehicle. A one-way ANOVA with a Holm-Sidak post hoc test was used to compare mean numbers of c-Fos-ir cells in each group (anesthesia control, vehicle control and capsaicin-treated) in both the caudal and rostral transition regions. The proportions of CTb and TRP channel dual-labeled varicosities to total varicosities in the caudal Vc were compared to the proportions in the rostral Vc for each TRP channel by using the z-test, in which a high z value indicates that the proportions being compared are different and in which a P value less than 0.05 was considered significant (Hegarty et al., 2010).

3. RESULTS

3.1. Ocular capsaicin application increases acute nociceptive behaviors and suppresses spontaneous behaviors

In order to demonstrate that acute nociceptive behaviors are evoked by noxious corneal stimulation, we counted eye blinks and eye wipes in unrestrained and unanesthetized rats after ocular application of 0.1% capsaicin or vehicle. A single application of 0.1% capsaicin evoked an increase in ipsilateral eye blink responses (Fig. 1, left graph, black bar) in the first 15 minutes after application as compared to application of vehicle (Fig. 1, left graph, gray bar). Ocular application of capsaicin also evoked ipsilateral forelimb eye wipes in rats (Fig. 1, middle graph, black bar), while application of vehicle solution did not evoke eye wipes (Fig. 1, middle graph).

During the same 15 minutes following capsaicin or vehicle application, spontaneous grooming behaviors were also assessed. Capsaicin suppressed grooming behaviors in treated animals (Fig. 1, right graph, black bar) as compared to control animals that received vehicle (Fig. 1, right graph, gray bar). These results confirm that the dose of capsaicin utilized in these studies was sufficiently noxious to evoke acute nociceptive behaviors and suppress spontaneous behaviors in unanesthetized rats.

3.2. Neurons in trigeminal nucleus caudalis are activated by ocular application of capsaicin

We used c-Fos immunoreactivity to assess activated neurons in trigeminal dorsal horn after ocular application of capsaicin (Fig. 2 and 3). We examined two regions of ventrolateral trigeminal nucleus caudalis (Vc) that receive corneal afferents: caudal (Fig. 2A, white box, 2B) and rostral (Fig. 2C, white box, 2D) (Aicher et al., 2013; Aicher et al., 2014; Hegarty et al., 2010; Marfurt and Del Toro, 1987). A one minute ocular application of capsaicin induced significant c-Fos activation of neurons in the rostral trigeminal dorsal horn (Fig. 2D) and this was greater than the c-Fos induction seen in caudal trigeminal dorsal horn in the same animals (Fig. 2B). Group data are shown in Figure 3. Capsaicin (black bars) evoked a significant increase in c-Fos-ir neurons in rostral Vc compared to caudal Vc (Fig. 3). There were no differences in total number of c-Fos neurons in trigeminal dorsal horn between anesthesia controls and animals that received vehicle applied to the ocular surface (Fig. 3, Anesthesia control, white bars; Vehicle control, gray bars). These findings show that ocular application of capsaicin specifically and preferentially activated neurons in the rostral pole of Vc.

3.3. Capsaicin-activated neurons in trigeminal nucleus caudalis receive direct input from corneal afferents

In order to determine if the c-Fos-ir neurons seen after capsaicin stimulation in trigeminal dorsal horn in rostral Vc received direct afferent input from corneal afferents, we counted the number of NeuroTrace (NT)-stained neurons that received direct somatic appositions from CTb-ir corneal afferent varicosities and then assessed whether these neurons contained c-Fos immunoreactivity (Fig. 4). For comparative purposes we conducted similar analyses in both control groups. In animals that only received anesthesia, $9.2 \pm 5.3\%$ ($n = 3$ rats) of rostral Vc neurons receiving appositions from CTb-ir corneal afferents were c-Fos-ir. In cases where the rats were under anesthesia and received ocular application of vehicle, the percentage of neurons that received CTb-ir corneal afferents and were also c-Fos-ir increased to $24 \pm 6.6\%$ ($n = 3$ rats). Finally, when anesthetized animals were stimulated with ocular capsaicin, we found that half of the neurons receiving appositions from CTb-ir corneal afferents (Fig. 4, arrowheads) in rostral Vc expressed c-Fos immunolabeling ($53 \pm 0.1\%$; $n = 3$ rats; Fig. 4, blue arrows). These data demonstrate that some neurons in rostral Vc respond to anesthesia or non-noxious corneal stimulation, but the effect of capsaicin is greater than these other components of the stimulation paradigm.

3.4. Capsaicin-transducing corneal afferents do not contain TRPV1 centrally

Since we found a high degree of c-Fos activation in corneal-receptive second-order neurons in trigeminal dorsal horn after corneal capsaicin stimulation, we determined if CTb-ir corneal afferent varicosities that contacted these neurons contained the TRPV1 channel that is thought to transduce capsaicin stimulation at the periphery. TRPV1 immunoreactivity is present in varicose fibers (Fig. 5, white arrows and black arrowheads) within the outer laminae of both caudal (Fig. 5A, 5C) and rostral (Fig. 5B, 5D) ventrolateral Vc. However, we were unable to detect TRPV1 in any of the CTb-ir varicosities that apposed rostral capsaicin-activated c-Fos-ir neurons (0/31 CTb-labeled varicosities apposing c-Fos-ir neurons from 3 rats).

3.5. Corneal afferents demonstrate differential TRPV1 expression in their cell bodies versus their central terminals

One explanation for the low incidence of co-localization of TRPV1 immunoreactivity in CTb-ir corneal afferents that contacted c-Fos-ir neurons was that there might be a capsaicin-induced change in the central expression of the TRPV1 channel. Using a similar approach to our previous study (Hegarty et al., 2010) we used CTb as an anterograde tracer to look at TRPV1 co-localization in the central terminals of corneal afferents in a population of animals that did not receive corneal stimulation. We found that while TRPV1 was abundantly expressed in varicosities and fibers in caudal and rostral Vc (Fig. 5 and 6A - 6C), it was rarely found in corneal afferents of unstimulated animals (2%, 7/300 varicosities, $n = 4$ rats; Fig. 7, Total, black bar). An immunocytochemical study using sequential application of the primary and secondary antibodies was consistent with our previous study, finding no CTb-ir varicosities that contained TRPV1 (0/225 total varicosities from 3 rats).

Another possibility for the low incidence of co-localization is that corneal afferent terminals that are labeled by the CTb tracer may not express TRPV1. We tested this hypothesis by

performing immunocytochemistry on the trigeminal ganglia of the unstimulated animals. We found that $30 \pm 4.3\%$ of CTb-ir corneal neurons ($n = 3$ rats) in the trigeminal ganglion contained TRPV1 immunoreactivity (Fig. 8, yellow arrows) although only 2% of the CTb-labeled central terminals from unstimulated animals contained TRPV1 (see above). These data suggest differential peripheral and central trafficking of TRPV1 channels in corneal afferents.

3.6. Small number of corneal afferents contain other transducer channels at their central terminals

We wanted to determine whether the low incidence of TRPV1 expression in CTb-ir corneal afferent terminals was exclusive to TRPV1 or if it was also the case for other TRP channels, such as TRP melastatin 8 (TRPM8) and TRP ankyrin 1 (TRPA1) that transduce physiologically relevant stimuli from the cornea to the brain. In animals that received CTb, but did not receive corneal stimulation, TRPM8 and TRPA1 were also assessed in caudal and rostral Vc (TRPM8: Fig. 6D - 6F; TRPA1: Fig. 6G - 6I). The proportion of CTb-ir varicosities that contained each TRP channel in Vc (caudal plus rostral) was calculated separately for each animal and then averaged (Fig. 7, Total). We found TRPA1 to be the most abundant TRP channel in corneal afferents at both caudal and rostral levels of Vc, with an overall prevalence in 10% of corneal afferents (52/525 varicosities, $n = 7$ rats; Fig. 7, Total, white bar). TRPM8 was found in 2% of corneal afferents (TRPM8: 8/375 varicosities, $n = 5$ rats; Fig. 7, Total, gray bar). There were no statistically significant differences in the proportion of TRP localization in caudal versus rostral Vc corneal afferent varicosities for any TRP channel examined (Figure 7), thus the data from rostral and caudal Vc were pooled for Total.

4. DISCUSSION

The major findings of the current study are: 1) ocular application of capsaicin results in changes in acute and spontaneous behaviors in rats and activates neurons in rostral trigeminal nucleus caudalis (Vc); 2) about half of the neurons in rostral Vc that receive direct input from labeled corneal afferent fibers are activated by ocular capsaicin; but 3) very few of the corneal afferents that contact capsaicin-activated neurons contain TRPV1. Our findings suggest that the transmission of capsaicin-induced corneal nociception involves a pathway from the cornea to second order neurons in rostral Vc and that the central aspect of this pathway does not appear to utilize TRPV1.

In a comparative analysis, only a small to moderate number of corneal afferent terminals in Vc contain the transduction molecules TRPV1, TRPM8 or TRPA1 with TRPA1 being most abundant. These results are in contrast to our previous studies showing that CGRP and vesicular glutamate transporters 1 and 2 are found in a substantial proportion of corneal afferent central terminals (Hegarty et al., 2010). These findings support the notion that TRP channels are involved in peripheral transduction, but not central transmission of nociception.

Ocular pain is mediated through the ophthalmic branch of the trigeminal nerve and requires transduction of stimuli by nociceptive fibers followed by activation of central pain pathways (Belmonte et al., 2004; Marfurt and Del Toro, 1987). Centrally, activation of second-order

neurons in the trigeminal dorsal horn is necessary, but not sufficient to evoke pain behaviors. The nociceptive signal must reach supraspinal centers to evoke pain behaviors. In the present study we first sought to confirm that the capsaicin stimulus we applied to the ocular surface would evoke appropriate nociceptive behaviors. Previous studies from other laboratories have demonstrated that rodents exhibit different acute behaviors in response to distinct classes of stimuli when applied to facial skin (Klein et al., 2011; Shimada and LaMotte, 2008). For example, following the application of stimuli that evoke itch in humans to trigeminal receptive fields on the face, rodents will scratch the injected area with their hind limb (Klein et al., 2011; Shimada and LaMotte, 2008). In contrast, stimuli that evoke a sensation of pain in humans evoke wiping behavior instead of scratching. Specifically, noxious stimuli applied to trigeminal receptive fields of rodents evoke face and eye wiping behaviors that are performed with the forelimb (Klein et al., 2011; Neubert et al., 2008; Price et al., 2004; Shimada and LaMotte, 2008). Our studies show that our selected dose of capsaicin applied directly to the ocular surface evoked the stereotypic nociceptive eye wiping behavior, but did not evoke scratching responses. Thus, we saw only nocifensive behaviors after corneal capsaicin stimulation. Corneal application of capsaicin also evoked ipsilateral eye blinks, but surprisingly this response was of only modest frequency. Eye blinks were also occasionally evoked by application of vehicle while eye wipes were not.

In addition to evoked behavioral responses, painful stimuli can also cause suppression of normal exploratory and grooming activity. In fact, recent studies suggest that the suppression of normal spontaneous behaviors may represent a superior index of a pain state compared to acute behavioral responses that are often brief and elicited (Negus et al., 2006). In the present study we found that capsaicin applied to the ocular surface caused suppression of normal grooming behavior. This measure is complimentary to the measurements of acute nociceptive responses, supporting the notion that these behaviors accurately reflect a nociceptive state that would be considered painful in humans.

Having established a behaviorally relevant dose of capsaicin for our studies, we next used c-Fos activation to analyze the pattern of neurons activated by ocular capsaicin stimulation. We observed selective activation of neurons in the ventrolateral region of rostral Vc that preferentially receives input from corneal afferents (Aicher et al., 2013; Hegarty et al., 2010). In some cases, we observed a small number of c-Fos-ir neurons located in other areas of Vc, but we confined our analyses to the ventrolateral areas of Vc in which we have previously found peak levels of CTb-ir corneal afferent input (Aicher et al., 2013; Hegarty et al., 2010). The number of capsaicin-induced c-Fos activated neurons observed in rostral Vc are consistent with a study from another laboratory that also used ocular capsaicin to activate neurons in Vc (Chang et al., 2010). However, when we looked in caudal Vc, we were surprised that the number of c-Fos activated neurons in the capsaicin-treated animals was not significantly different from that of the two control groups in the current study. Our results do not support the notion that caudal Vc is responsible for the sensory-discriminative aspects of corneal nociception while rostral Vc is involved in encoding intensity-independent noxious and innocuous sensory information (Chang et al., 2010; Hirata et al., 2004; Meng et al., 1997). If this hypothesis were true, our dose of capsaicin should have evoked higher numbers of c-Fos activated neurons in ventrolateral caudal Vc than in the

control groups. In contrast, our results point to a more prominent role for rostral Vc in capsaicin-induced corneal nociception than caudal Vc.

We used anesthetized animals to isolate c-Fos activation driven purely from capsaicin stimulation of the ocular surface from neuronal activation induced by the acute nociceptive behaviors evoked by capsaicin. We maintained our animals at an anesthetic depth sufficient to prevent acute nociceptive behaviors after capsaicin application; therefore, it is possible that c-Fos activation of neurons in Vc was reduced by anesthesia. Isoflurane has been shown to decrease c-Fos activation in lumbar spinal cord after noxious electrical (Sommers et al., 2008) and mechanical (Jinks et al., 2002) stimulation to the hind paw. However, we were still able to evoke strong c-Fos activation by capsaicin in ventrolateral rostral Vc, a region that we have shown preferentially receives corneal afferent fibers (Aicher et al., 2013; Hegarty et al., 2010).

In animals that received application of the vehicle control solution, we saw corneal-receptive neurons that were also c-Fos-ir in rostral Vc. This suggests that rostral Vc neurons may also respond to innocuous stimuli, such as the physical application of a liquid to the cornea. It also supports the notion that some rostral Vc neurons are involved in initiating eye blink responses to corneal stimulation (Henriquez and Evinger, 2007). A clear incremental effect of anesthesia, vehicle, and capsaicin stimulation was observed in rostral Vc neurons that receive direct corneal input. Capsaicin clearly activated an additional subset of cells that responded to the noxious stimulus and not just to anesthesia or the innocuous application of liquid to the cornea. This is not consistent with previous studies that suggested that rostral Vc neurons encoded corneal stimuli independent of intensity (Chang et al., 2010; Hirata et al., 2004; Meng et al., 1997). The graded response by rostral Vc neurons in the current study supports a more sensory-discriminatory role for rostral Vc neurons in corneal nociception than previously thought.

The TRPV1, TRPM8 and TRPA1 channels are expressed peripherally in the cornea, in the trigeminal ganglion neurons and centrally in Vc (Bae et al., 2004; Guo et al., 1999; Hiura and Nakagawa, 2012; Kim et al., 2010; Ma, 2002; Murata and Masuko, 2006; Nagata et al., 2005; Nakamura et al., 2007; Parra et al., 2010; Yamamoto et al., 2009; Yeo et al., 2010). TRPV1 is the transducer of capsaicin stimulation to the periphery (Caterina et al., 1997; Caterina et al., 2000); therefore, we expected that c-Fos neurons activated by ocular capsaicin stimulation would receive direct contacts from corneal afferents that expressed TRPV1 immunoreactivity. We limited our analysis to direct somatic contacts since c-Fos activation is confined to the neuronal cell body and does not extend into the dendritic arbor. We found that co-localization of TRPV1 and CTb immunoreactivity very rarely occurred in either capsaicin-treated animals or naïve animals. We tested whether our standard method of simultaneous antibody incubation may have lead to competition between the CTb and TRPV1 antibodies, resulting in artificially low numbers of varicosities that expressed both markers. We found that immunocytochemical studies using sequential application of the primary antibodies were consistent with studies using simultaneous antibody application. These data suggest that the low incidence of co-localization was not due to technical aspects of this study.

Another possibility is that TRPV1 channels are not expressed in corneal afferents that transport the tracer CTb. In the present study, as well as our previous studies, we chose CTb as the tract tracer based on its compatibility with the heptanol epithelial abrasion method and its ability to transport transganglionically and effectively label the central projections of corneal neurons (Aicher et al., 2013; Aicher et al., 2014; Hegarty et al., 2010). We have found that other tract tracers, including FluoroGold and wheat germ agglutinin-horseradish peroxidase (WGA-HRP) do not fit all of these criteria. It is thought that CTb selectively traces myelinated primary afferents (Todd et al., 2003) and that TRPV1 immunoreactivity is primarily contained in unmyelinated axons in the sensory root proximal to the trigeminal ganglion (Yeo et al., 2010). These studies would suggest that TRPV1 and CTb would be expected to be found in separate populations of afferents. However two lines of evidence from this laboratory do not support this notion. First, our recent ultrastructural study of vagal afferents projecting to the nucleus tractus solitarii (NTS) demonstrates that CTb is present in both myelinated and unmyelinated vagal axons and terminals in NTS (Hermes et al., 2014). Furthermore, in our present study, we found a substantial percentage of CTb-labeled trigeminal ganglion neurons (30%) that also contained TRPV1 immunoreactivity. This is similar to a previous study looking at TRPV1 expression in trigeminal ganglion neurons labeled from the cornea with CTb (Murata and Masuko, 2006). TRPV1 expression has also been detected in dorsal root ganglion (DRG) neurons labeled with CTb from the sciatic nerve (Ma, 2002). These data suggest that CTb is not exclusively labeling myelinated axons and that neurons that are labeled by CTb are capable of expressing TRPV1 channels.

Previous studies have concluded that co-localization of markers in ganglion neurons does not necessarily mean that the peripheral and central ends of the afferent neurons will express these same markers at the same frequency. For example, Guo and others found that despite moderate to high levels of co-localization of TRPV1 with the peptides calcitonin gene-related peptide (CGRP) and substance P (SP) in DRG neurons, these markers did not co-localize with the same frequency in central lumbar spinal cord nerve terminals or peripheral corneal fibers (Guo et al., 1999). Another study demonstrated that even under inflammatory conditions, TRPV1 protein expression in central terminals does not increase as it does in spinal DRG neurons and in peripheral nerve terminals innervating the hind paw (Ji et al., 2002). This idea of differential central and peripheral TRPV1 trafficking may be one reason why the co-localization of TRPV1 and CTb in corneal trigeminal ganglion neurons (Murata and Masuko, 2006) is not reflected in their central afferent fibers in the current study, even after corneal capsaicin application.

The present study supports the notion that while TRPV1 channels are present in CTb-labeled corneal neuron cell bodies, and play a prominent role in the peripheral transduction of noxious heat and capsaicin stimulation (Caterina et al., 1997; Caterina et al., 2000; Patapoutian et al., 2009), they may be playing a different role centrally (Kim et al., 2014; Largent-Milnes et al., 2014; Patapoutian et al., 2009; Patwardhan et al., 2009). Unlike our previous study of vesicular glutamate transporters and neuropeptides in corneal afferents (Hegarty et al., 2010), we did not find substantial expression of TRP channels in CTb-ir corneal afferents, nor any topographical differences between caudal and rostral Vc for any of the TRP channels in the present study. The central expression of TRP channels in the area of corneal afferent input may be due to TRP expression by other non-corneal trigeminal

afferents or by postsynaptic neurons in the trigeminal dorsal horn as seen in spinal cord dorsal horn (Bae et al., 2004; Valtschanoff et al., 2001; Zhou et al., 2009). Recent studies have suggested that central TRPV1 channels play a modulatory role in pain transmission, perhaps contributing to central sensitization during chronic neuropathic (Kim et al., 2014) and inflammatory (Patwardhan et al., 2009) pain states.

We also found low levels of expression of the TRPM8 and TRPA1 channels in corneal afferents in naïve animals. Of the three TRP channels studied, we found more TRPA1-containing CTb-ir corneal afferents than TRPV1 or TRPM8. Previous studies have suggested that the TRPA1 channel is involved in the transduction and/or modulation of mechanical, cold, chemical and environmental irritant stimuli (Belmonte and Viana, 2008; Nagata et al., 2005; Patapoutian et al., 2009; Stucky et al., 2009; Tsunozaki and Bautista, 2009); these are all stimuli that are physiologically relevant to the cornea (Belmonte et al., 2004). This suggests that the TRPA1 channel may be playing a more prominent, perhaps modulatory, role in mediating signal transmission from corneal afferents to their central targets than TRPV1.

4.1. Conclusions

We have demonstrated that neurons in the ventrolateral region of rostral Vc are activated by ocular capsaicin application and that these neurons often receive direct axo-somatic contacts from corneal afferent fibers. Although the TRPV1 channel is the known transducer of peripheral capsaicin stimulation, we did not find TRPV1 expressed in the corneal afferents that contacted the capsaicin-activated Vc neurons. In fact, despite being co-localized with CTb in trigeminal ganglion neurons, TRPV1 was rarely found in the central CTb-ir corneal afferents in naïve animals. We also found small proportions of TRPM8 and TRPA1 in CTb-ir corneal afferents in ventrolateral Vc. Therefore central TRP channels may be playing a different role in the transmission of pain than their peripheral counterparts.

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ABBREVIATIONS

Vc	trigeminal nucleus caudalis
TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid 1
TRPM8	transient receptor potential melastatin 8
TRPA1	transient receptor potential ankyrin 1
CTb	cholera toxin subunit B
PB	phosphate buffer

BSA	bovine serum albumin
TS	Tris-buffered saline
TX	Triton X-100
NT	NeuroTrace
-ir	immunoreactive

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Highlights

The rostral Vc is a major substrate of capsaicin-induced corneal nociception.

Axo-somatic transmission of corneal capsaicin stimulation does not use TRPV1 in Vc.

Central TRP channels may play an alternative role in pain transmission.

Figure 1.

Ocular application of capsaicin increased acute nociceptive behaviors and decreased spontaneous grooming behavior in rats. Ipsilateral eye blinks (left side of graph) and eye wipes (middle) were counted for 15 minutes in rats that received either vehicle control solution (gray bars, $n = 3$) or 0.1% capsaicin (black bars, $n = 3$). The average number of grooming bouts performed with both forepaws were counted for 15 minutes after application of vehicle control solution (gray bars, $n = 3$) or 0.1% capsaicin (black bars, $n = 3$). Data are represented as mean number of behavioral responses (Blinks, Wipes) or grooming bouts (Grooming) $\pm$ standard error of the mean (SEM). * $P < 0.05$ as compared to vehicle.

Figure 2.

Corneal application of capsaicin induced the expression of c-Fos protein in neurons in ventrolateral caudal and rostral Vc. Panels A and C show low magnification darkfield images of coronal rat brainstem sections through caudal (A) and rostral (C) Vc. The white boxed areas in panels A and C approximate the regions shown in panels B and D, respectively. Panels B and D demonstrate c-Fos-activated neurons (yellow neurons) induced by corneal application of 0.1% capsaicin in both caudal (B) and rostral (D) ventrolateral Vc. A small number of c-Fos-ir neurons were activated in deeper laminae and more dorsally, but the current analyses were confined to the area that received peak levels of CTb-ir corneal afferents. The number of neurons activated by capsaicin in rostral Vc was greater than that in caudal Vc. Arrows in panel A indicate dorsal and medial directions for orientation on these coronal sections. Vc = trigeminal nucleus caudalis; Vi = trigeminal nucleus interpolaris; spV = spinal trigeminal tract. Scale bars: panels A, C = 1 mm; panels B, D = 200 μ m.

Figure 3.

Corneal application of capsaicin increased the number of neurons in ventrolateral rostral Vc that expressed c-Fos immunoreactivity. This graph shows the average number of c-Fos-immunoreactive (–ir) soma in the ventrolateral region of caudal and rostral Vc after corneal application of capsaicin (black bars) or vehicle (gray bars) or after anesthesia only (white bars). There were significantly more cells activated by capsaicin in ventrolateral rostral Vc (* $P < 0.05$, black bar, Rostral) as compared to the number of capsaicin-activated cells in caudal Vc (black bar, Caudal) and the numbers of c-Fos-ir cells induced by corneal application of vehicle (gray bars) and anesthesia alone (white bars) in both regions of Vc (Caudal, Rostral). Data are represented as mean numbers of c-Fos-ir soma $\pm$ SEM. $N = 3$ animals per treatment group; two 40 μ m sections (caudal and rostral Vc) per animal.

Figure 4.

Corneal afferents form appositions with somata of neurons activated by corneal application of capsaicin. Confocal micrographs demonstrate CTb labeling of corneal afferent varicosities (A, white) and NeuroTrace (NT) Nissl-stained neuronal somata (B, magenta), some of which are immunoreactive for c-Fos protein (B, blue arrows). Yellow arrowheads indicate CTb-ir varicosities apposed to c-Fos-ir neurons (A, C), while red arrowheads indicate CTb-ir varicosities apposed to non-c-Fos-ir neurons (A, C). Image is a Z projection

of 5 consecutive 0.4 μm thick optical sections for a total thickness of 2 μm . Scale bar = 20 μm .

Figure 5.

Light micrographs demonstrate the distribution of peroxidase-labeled TRPV1 immunolabeling in caudal (A, C) and rostral (B, D) Vc. The black boxes in A and B delineate the ventrolateral regions of caudal (A) and rostral (B) Vc shown at higher magnification in C and D, respectively. TRPV1 immunoreactivity was seen in fibers (white arrows) and varicosities (black arrowheads). Scale bars (A, B) = 200 μm ; (C, D) = 100 μm .

Figure 6.

Confocal micrographs demonstrate expression of CTb-ir corneal afferents (white; A, C, D, F, G, I) and TRPV1-ir (red; B, C), TRPM8-ir (cyan; E, F) and TRPA1-ir (green; H, I) fibers in ventrolateral caudal Vc. Yellow arrows indicate CTb-ir corneal afferents that contain the TRPA1 channel. All images are Z projections of 12 consecutive 0.4 μm optical sections for a total thickness of 4.8 μm . spV = spinal trigeminal tract. Scale bar = 20 μm .

Figure 7.

The graph shows the mean percentages of CTb-ir corneal afferent varicosities that also contained TRPV1 (black bars), TRPM8 (gray bars) or TRPA1 (white bars) in ventrolateral caudal and rostral Vc. The percentages in the Total group were calculated from the sum of CTb-ir corneal afferents that contained a TRP channel in both caudal and rostral Vc. Data are represented as mean percentages $\pm$ SEM.

Figure 8.

A subset of CTb-labeled corneal neurons in the trigeminal ganglion express TRPV1. Epifluorescence micrographs demonstrate CTb-labeled corneal neurons (A, white), NeuroTrace Nissl-stained neuronal somata (B, magenta) and TRPV1-labeled neurons (C, red) in the trigeminal ganglion. Panel D is an overlay of panels A – C. Yellow arrows indicate a CTb-labeled corneal neuron that also expresses TRPV1, while white arrows indicate a CTb-labeled corneal neuron that does not express TRPV1. Red arrows point to trigeminal ganglion neurons that express TRPV1, but not CTb. Images were adjusted for optimal brightness and contrast in Adobe Photoshop. Scale bars = 100 μm.

Table 1

List of Primary and Secondary Antibodies Used for Immunocytochemistry

Primary Antibody	Primary Antibody Immunogen Dilution, Catalog No., Source	Secondary Antibody Dilution, Source
Goat anti-CTb	Purified cholera toxin B subunit 1:25000, Catalog No. 703, List Laboratories, Campbell, CA	Alexa Fluor® 488 Donkey anti-Goat 1:800, Invitrogen/Life Technologies, Grand Island, NY
Rabbit anti-CTb	Purified cholera toxin B subunit 1:10000, Catalog No. ab13612, Novus Biologicals, Littleton, CO	Alexa Fluor® 488/647 Donkey anti-Rabbit 1:800, Invitrogen/Life Technologies
Rabbit anti-c-Fos	N-terminus residues 3–16 of c-Fos of human origin (FSGFNADYEASSR) 1:3000, Catalog No. SC-52, Santa Cruz Biotechnology, Santa Cruz, CA	Alexa Fluor® 546 Donkey anti-Rabbit 1:800, Invitrogen/Life Technologies
Rabbit anti-TRPA1	Amino acid residues 1061-1075 of rat TRPA1 (EKQHELIKLIQKME) 1:5000, Catalog No. ab58844, Abcam, Cambridge, MA	Alexa Fluor® 647 Donkey anti-Rabbit 1:800, Invitrogen/Life Technologies
Rabbit anti-TRPM8	1 st cytoplasmic loop of rat TRPM8 conjugated to an immunogenic carrier protein; homologous with the corresponding sequence in mouse 1:2500, Catalog No. NB100-98879, Novus Biologicals	Alexa Fluor® 647 Donkey anti-Rabbit 1:800, Invitrogen/Life Technologies
Guinea pig anti-Capsaicin receptor (TRPV1)	C-terminus residues 817-838 of rat VR1 (YTGSLKPEDAEVFKDS MVPGEK) 1:6000, catalog No. AB5566, Millipore, Billerica, MA	Cy5 Donkey anti-Guinea pig 1:800, Jackson ImmunoResearch Laboratories, West Grove, PA
Goat anti-VR1 (P-19) (TRPV1)	N-terminus residues 38-57 of rat VR1 (PHIFTTRSRTRLFGKGDSEC) 1:500, Catalog No. SC-12498, Santa Cruz Biotechnology, Santa Cruz, CA	Alexa Fluor® 488 Donkey anti-Goat, 1:800, Invitrogen/Life Technologies
Goat anti-VR1 (TRPV1)	N-terminus residues 4-21 of VR1 (RASLDSEESPPQENSC) 0.75 µg/ml, Catalog No. GT15129, Neuromics, Edina, MN	Alexa Fluor® 647 Donkey anti-Goat, 1:800, Invitrogen/Life Technologies