Cooling and menthol-mediated inhibition of itch
ABSTRACT
Popular treatments for itch include innocuous cooling and creams containing the active anti-itch ingredient, menthol. While these anti-itch treatments have been used for decades, the molecular mechanisms for cooling and menthol-mediated itch inhibition remains to be understood. The discovery of the transient receptor melastatin 8 ion channel, TRPM8, as the molecular sensor for cold temperatures and menthol motivated our investigation of the role of TRPM8 in cooling and menthol-mediated itch inhibition. Furthermore, we hypothesized the activation of TRPM8 may provide an effective treatment to alleviate itch. Additionally, the effects of cooling and menthol on itch behavior have yet to be tested and confirmed in a rodent experimental model. Our investigation demonstrated that cooling and menthol significantly decreases the amount of itch behaviors in response to histamine and chloroquine-evoked itch observed in mice. This suggests that cooling and menthol inhibit itch in mice. Furthermore, this demonstration of cooling and menthol-mediated itch inhibition in a rodent experimental model, allowed us to conduct preliminary experiments to investigate the role of TRPM8 in cooling and menthol-mediated itch inhibition. Our preliminary results suggest that cooling and menthol-mediated itch inhibition occurs through a TRPM8-mediated pathway.
INTRODUCTION
The somatosensory system is responsible for our sensation of touch (Basbaum et al., 2009). Our skin is innervated by many different populations of sensory neurons that transduce specific sensations in response to temperature, pressure and chemical stimuli (Basbaum et al., 2009). Afferent sensory neurons carry this information to the dorsal horn of the spinal cord where they synapse with neurons that relay this information to higher brain centers to evoke a physical response (Ross, 2011).

Expression of certain molecular sensors on distinct populations of sensory neurons enables specific responses to environmental stimuli (McKemy et al., 2002). A distinct family of molecular sensors, transient receptor potential (TRP) channels is the primary detectors of thermal and painful stimuli (McKemy et al., 2002). Different populations of TRP channels are activated at different temperatures and therefore transduce different thermosensations, ranging from noxious cold to noxious hot. The McKemy laboratory focuses research on the transient receptor potential melastatin 8 ion channel, TRPM8. TRPM8-expressing neurons allow a depolarizing current to enter the cell, beginning at 26˚C, when they are stimulated with cold ramps (McKemy et al., 2002). Similarly, exposure to the cold-mimetic compound, menthol, provokes a depolarizing effect in TRPM8-expressing neurons (McKemy et al., 2002). Furthermore, studies have shown that TRPM8 has an essential role in transducing thermosensations, ranging from the pleasant cooling sensation activated by menthol to the noxious cold sensation activated at extremely low temperatures (McKemy et al., 2002).

Recent research has suggested the essential role of TRP channels in mediating the sensation of itch (Ross, 2011). Histamine, a well-known pruritogen, activates the transient receptor potential cation channel subfamily V member 1 (TRPV1), via activation of a G-coupled protein receptor H1R (Kim et al., 2004; Ross, 2011). Alternatively, chloroquine, another well-known pruritogen, activates the transient receptor potential cation channel member A1 (TRPA1), via activation of a G-coupled protein receptor MrgrpC11 (Ross, 2011). These findings propose the potential of TRPV1 and TRPA1 antagonists as effective anti-itch treatments (Kim et al., 2004). However, TRPV1 and TRPA1 have an essential role in transduction of heat and pain sensations in response to high temperatures and a variety of molecular agonists (Ross, 2011). Therefore, a potential
drawback of developing pharmaceutical antagonists of TRPV1 and TRPA1 is the inhibition of these essential sensations of heat and pain.

Antihistamines are another popular treatment for itch. Antihistamines present researchers with the potential of improving itch management by targeting the H1 receptor of histamine (Twycross et al., 2003). However, with the recent discovery of numerous molecular pathways mediating the sensation of itch (Ross, 2011), the potency and effectiveness of antihistamines as anti-itch treatment has become disputable. In addition, many individuals experience adverse side effects and allergic reactions to antihistamines (Twycross et al., 2003). Thus, itch management is an important aspect of healthcare that lacks much research.

To improve itch management, our laboratory focused on investigating the underlying molecular mechanism of itch inhibition. Innocuous cooling and topical application of creams containing the active anti-itch ingredient, menthol are popular treatments for itch (Than et al., 2013; Bromma et al., 1995). Although numerous studies have supported the effectiveness of these treatments for itch management, previous studies have suggested these treatments act through a numbing effect to inhibit the sensation of itch (Cliff & Green, 1994). These findings propose both menthol and cooling inhibit itch through a general, overall desensitization of sensory neurons (Cliff & Green, 1994). However, our laboratory proposes that itch inhibition occurs through a more specific, potent pathway. Identification of TRPM8 as the molecular detector of cold temperatures and menthol, motivated our investigation of the plausible role of this channel in mediating itch inhibition. We hypothesize the activation of TRPM8 will provide an effective treatment to alleviate itch.

In addition, although the effects of cooling and menthol have been demonstrated on human models (Bromma et al., 1995), the effects of these anti-itch treatments in rodent experimental models have yet to be confirmed. In the present study, we tested the hypothesis that cooling and menthol inhibit itch in mice. To determine the effect of cooling on itch, we conducted a cold-plate assay that compared itch behavioral responses in wild-type mice injected with a solution of histamine (100 μg) or chloroquine (200 μg) in their hind paw across a range of temperatures. To determine the effect of menthol on itch, we conducted a cheek assay that compared itch
behavioral responses in wild-type mice injected with histamine to those injected with histamine and menthol in their cheek. We also assessed whether cooling exerted its inhibitory effects on itch in an application-dependent manner. After establishing this necessary and significant foundation of a reliable experimental rodent model, we conducted preliminary experiments investigating the role of TRPM8 in itch inhibition through trpm8 +/- studies.

RESULTS

Temperatures below 20°C suppress histamine and chloroquine-evoked itch in wild-type mice

To investigate if cooling inhibited itch in mice, a cold-plate assay was performed on wild-type mice. To provoke itch, the pruritogens, chloroquine (200 μg) or histamine (100 μg), were injected subcutaneously into one hind paw of the mice. To test the effect of cooling on pruritogen-provoked itch, the mice were placed on cold plates of temperature 30°C (histamine, N = 6; chloroquine, N = 6) 24°C (histamine, N = 3; chloroquine, N = 8), 20°C (chloroquine, N = 5, histamine, N=6), 17°C (histamine, N = 8; chloroquine, N = 7) or 10°C (histamine, N = 8; chloroquine, N = 4). The effect of cooling on itch was measured as the duration of itch behaviors observed over 30-minutes post-injection. In both the histamine (Figure 1A) and chloroquine (Figure 1B) evoked itch cold plate assays, itch durations were significantly reduced in mice placed on plates below temperatures of 20°C. The significant decrease in duration of itch behavior observed below temperatures of 20°C suggests cold temperatures significantly suppress the sensation of itch in mice.
Cooling is an application-dependent temporary inhibitor of itch

Previous research suggests that cooling must be directly applied to the area of itch to produce its inhibitory effect. Cold plate assays were implemented to investigate this application-dependent component that we hypothesized to be essential to the cooling-mediated itch inhibition.

Chloroquine injections were administered to the hind paw of mice to ensure contact with the cold-plate and therefore, demonstrate application dependency. To determine whether cooling was an application-dependent inhibitor of itch in mice, we conducted a transition cold plate assays that observed itch behavioral responses in wild-type mice. The mice were placed on a plate that increased in temperature from 20°C to 24°C (N = 9). The plate increased temperature after 15 minutes, and itch behavioral responses were observed for a total 30-minute duration. To provoke itch, wild-type mice were injected with chloroquine (200 μg) subcutaneously in their hind paw. The effect of direct application of cooling was measured as a comparison between the duration itch behavioral responses observed in the first 15 minutes (0-15 minutes) versus the last 15 minutes (15-30 minutes). We observed a significant increase in the duration of itch behavioral responses after 15 minutes, when the temperature of the plate increased to 24°C (Figure 3A). Thus, upon removal of the cold stimulus with the increase in temperature from 20°C to 24°C, a significant increase in itch behavior was observed. This suggested that cooling
acts in an application-dependent manner to inhibit itch. Therefore, cooling-mediated itch inhibition is dependent on direct contact of the origin of the itch with a cooling surface.

Mice placed on a plate of temperature 24°C served as our control for this experiment (N = 8). If we observed a similar increase in duration of behavioral responses across a 30-minute period in mice placed on a plate of a constant temperature of 24°C, then we would not be able to attribute the increase in temperature as the apparent cause for the increase in itch behaviors. Furthermore, we would not be able to assess whether the inhibitory effects of cooling were application-dependent. However, we observed a decrease in the duration itch behaviors between the first 15 minutes compared to last 15 minutes (Figure 3B). This observation of a decrease, rather than an increase in the duration of itch behavior in the control group confirms the findings of our experimental group.

Our observation of an decrease in the duration of itch behaviors in the control group compared to an increase in itch behaviors in the experimental group led us to propose that cooling acted as a temporary suppressor of itch. To investigate this hypothesis, we compared the total duration of itch behaviors observed in the two sets of mice. We did not observe a significant difference in the total duration of itch behaviors between the two groups (Figure 3C). If there was a significant difference in the totally duration of itch behaviors, we would consider cooling a permanent disruptor of itch. However, the lack of difference in total duration of itch behaviors suggests that cooling acts to temporarily inhibit the sensation of itch.
Preliminary results suggest cooling-mediated itch inhibition is TRPM8-dependent

After establishment of a reliable rodent model that confirmed the inhibitory effects of cooling on itch, we began investigating the role of TRPM8 in cooling-mediated itch inhibition. To provoke itch, histamine (100 μg) was subcutaneously injected into the hind paw of wild-type and trpm8 -/- mice. Trpm8 -/- mice have been genetically depleted of any functional TRPM8 channels. To investigate the effects of cooling on itch, mice were placed on a plate set to either a temperature of 24°C or 20°C (Figure 4). To investigate the role of TRPM8 in cooling-mediated itch inhibition, itch behavioral responses of trpm8 -/- mice were compared to itch behavioral responses of wild-type mice. We observed a significant increase in itch behavioral responses in trmp8 -/- mice compared to wild-type mice. The significant increase in itch behavioral responses of trpm8 -/- mice suggest the inability of these mice to suppress itch. Thus, these findings suggest cooling-mediated itch inhibition is dependent on TRPM8 expression. Completion of experiments on trpm8 -/- mice injected with histamine is necessary to compare itch behavioral responses in trpm8 -/- mice to wild-type mice across the various plate temperatures (30°C, 24°C, 20°C, 17°C or 10°C). Upon completion of these comparisons, we will conclude whether cooling-mediated itch inhibition is TRPM8-dependent.
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8 μg of menthol inhibits low concentrations of histamine-evoked itch

To investigate whether menthol inhibited itch in mice, a cheek assay was performed on wild-type mice. To provoke itch, histamine (100 μg) was subcutaneously injected into the cheeks of wild-type mice (N = 5). To determine the effect of menthol on itch, a solution of histamine (100 μg) and menthol (8 μg) was subcutaneously injected into the cheeks of a second group of wild-type mice (N = 5). Itch inhibition was measured as the number of scratch bouts that was observed over the duration of 40-minutes post-injection. To determine the effects of menthol on histamine-evoked itch, we compared the number of scratch bouts in the group of wild-type mice injected with histamine to the group of wild-type mice injected with both histamine and menthol. Wild-type mice in the two groups did not show a significant difference in the number of scratch bouts in response to an injection of histamine compared to an injection of histamine and menthol.

We hypothesized that this lack of inhibitory effect on itch could be influenced by the relatively high concentration of histamine. This high concentration could have provoked an itch response that exceeded the inhibitory effects of menthol. To test this hypothesis, we lowered the concentration of histamine to 20 μg. We observed a significant decrease in the number of scratch bouts in the group of wild-type mice injected with 20 μg-histamine with 8-μg menthol compared to the group of wild-type mice injected with 20 μg-histamine alone. These results
suggest that menthol only inhibits low concentrations of histamine-evoked itch. While confirming our initial hypothesis that menthol inhibits itch, these results suggest that menthol does not exert the necessary potency to demonstrate the inhibitory effects that were shown in the cold plate assay for 100 μg of histamine. These results, therefore, advocate the investigation for more potent and effective anti-itch molecular agonists that perhaps exert this inhibition of itch through activation of TRPM8.

To ensure the inhibitory effect of menthol was significant across various quantification measurements, we analyzed the duration of itch behaviors. We observed a significant decrease in the duration of itch behaviors in mice injected with a solution of histamine (20 μg) and menthol (8 μg) compared to mice injected with histamine (20 μg) alone (Figure 5B). These results agreed with our previous quantifications of itch behaviors measured by the number of scratch bouts. The agreement of both quantification measurements serves to confirm the trend of menthol inhibition of itch at low concentrations of histamine.

![Figure 5A](image1.png) **Figure 5A.** 8 μg of menthol inhibits histaminergic itch in wild-type mice at low concentrations of histamine (20μg). Itch inhibition was measured as the number of scratch bouts across a 40-minute duration.

![Figure 5B](image2.png) **Figure 5B.** 8 μg of menthol inhibits histaminergic itch in wild-type mice at low concentrations of histamine (20μg). Itch inhibition was measured as the duration of itch behavior across a 40-minute duration. Across two different quantification measurements (number of scratch bouts versus duration of itch behaviors), both results demonstrate a significant decrease in itch response in the group of mice injected with 20 μg histamine with menthol.
DISCUSSION

*Menthol and cooling significantly suppress itch in wild-type mice*

Our results suggest that cooling and menthol significantly reduce itch behavioral responses in wild-type mice. These findings confirm our initial hypothesis that cooling and menthol inhibit itch in mice. Previous research has shown that histamine and chloroquine provoke itch through two independent distinct pathways (Ross, 2011). Therefore, since cooling significantly reduces itch behavior in both histamine and chloroquine-evoked itch, our results suggest that cooling may be amongst the most effective, currently available itch medications. Furthermore, our results demonstrated cooling acts in an application-dependent manner to inhibit itch. Alternatively, the limited ability of menthol to inhibit itch in mice only at low concentrations of histamine-evoked itch suggest the significance of identifying or developing compounds that may exert a more potent itch inhibition.

*The temporary inhibitory effects of cooling to suppress itch and the mechanism of cross-stimulation suggest inhibition may occur through an inhibitory interneuron-mediated pathway*

The results of the cold-plate assay demonstrated cooling acts in an application-dependent manner to inhibit itch (Figure 3A). Upon removal of the cold stimulus (the change of plate temperature from 20°C to 24°C), itch behaviors significantly increased in wild-type mice injected with chloroquine (Figure 3A). This temporary, application-dependent suppression of itch by cooling provides insight to a plausible mechanism in which cooling suppresses itch.

Recent studies have revealed the general circuit for the transduction of itch (Ross, 2011). The sensation of itch occurs through the release of the excitatory neurotransmitter, glutamate, from a pruritoceptor, a sensory neuron activated by itch-causing stimuli (Ross, 2011). Stimulated by glutamate, this itch sensation is relayed to our brain (Ross, 2011). Therefore, the existence of glutamate in the synapse can be attributed to the duration of itch responses.

While past research revealed itch inhibition is multi-faceted, there are multiple explanations for specific itch-inhibition pathways. The mechanism of cross-stimulation provides one plausible explanation for itch inhibition. Many inhibitory and excitatory interneurons exist in the dorsal horn that facilitates and modifies the transduction of sensory information (Ross, 2011). While
specific populations of afferent fibers respond to and relay specific sensory information, this sensory information does not remain segregated once the afferent fibers enter the dorsal horn (Ross, 2011). This arrangement allows for the cross-talk, or interaction and convergence, of multiple sensory pathways. This interaction among multiple populations of sensory neurons allows for counter-stimulation to occur, or the ability of one stimulus to counter, or suppress another. Considering this concept of cross-stimulation, we propose cooling may act through such a mechanism to inhibit itch.

The lack of significant difference in total duration of itch responses between control and experimental groups in the transition cold-plate assay proposes that cooling-mediated inhibition does not remove the excitatory stimulus of glutamate from the synapse. If glutamate was removed from this synapse, we would expect to observe a significant decrease in total duration of itch behaviors in the experimental group because of the initial suppression of itch at 20˚C. Instead, itch sensation may be temporarily suppressed by an inhibitory interneuron that suppresses the transduction of the sensation of itch to the central nervous system. This proposed mechanism of itch inhibition via an inhibitory interneuron would have no effect on depleting glutamate in the synapse nor disrupting the transduction of itch at the level of the pruriceptor. Instead, this proposed mechanism suggests that an inhibitory interneuron mediates interneuronal transmission at the dorsal horn. This proposed pathway is supported by our findings that itch response returns upon the extinguishment of the cooling contact.

We propose that cooling stimulates a cross-stimulation pathway to inhibit itch. Our proposition suggests TRPM8’s inhibitory role via activation of an interneuron that acts to counter and suppress the sensation of itch.

**Future experiments on trpm8 -/- mice and mice with ablated TRPM8 neurons will determine whether cooling and menthol-mediated itch inhibition is TRPM8-dependent**

To test our proposed TRPM8-mediated pathway of itch-inhibition, completion of future experiments on trpm8 -/- mice and mice with ablated TRPM8 neurons is necessary. If the TRPM8 channel is significant and essential for cooling or menthol-mediated inhibition of itch, a significant increase in itch behaviors is expected in the cold plate and histamine cheek assays,
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respectively, when compared to wild-type mice. Our laboratory has created a line of mice that upon injection with diphtheria toxin (DTx), are ablated of the population of TRPM8-expressing neurons. If these TRPM8 neurons are significant and essential for cooling or menthol-mediated inhibition of itch, a significant increase in itch behaviors is expected in the cold plate and histamine cheek assays, respectively, when compared to wild-type mice. Furthermore, comparison of these two populations of mice, trpm8 -/- and mice with ablated TRPM8 neurons, will generate insight into the molecular mechanism underlying the inhibition of itch.

Previous studies in our laboratory have shown differences in behavioral responses of mice ablated of TRPM8 neurons compared to trpm8 -/- mice. These findings suggest a mechanistic difference in the influence of the population of TRPM8 neurons versus the TRPM8 channel. Both mice ablated of TRPM8 neurons and trpm8 -/- mice display similar behavioral responses to evaporative cooling, cold-plate nocifensive behaviors and cold-induced analgesia (Knowlton et al., 2013). Mice ablated of TRMP8 neurons show little to no response to innocuous to noxious cold temperatures (Knowlton et al., 2013). Compared to trpm8 -/- mice, mice with ablated TRPM8 neurons have more profound deficits in cold behaviors (Knowlton et al., 2013). These behavioral differences suggest the plausible existence of TRPM8-independent transduction mechanisms in TRPM8 neurons, providing cold sensitivity in the absence of TRPM8 channels (Knowlton et al., 2013).

If mice with ablated of TRPM8 neurons show a significantly greater increase in itch behaviors in the cold plate or histamine cheek assays when compared to the trpm8 -/- mice and wild-type mice, these findings will suggest the significant role of TRPM8 neurons mediating the pathways for cooling and menthol itch inhibition. This finding would suggest the plausible existence of a TRPM8-indeendent pathway in TRPM8 neurons for the inhibition of cold. Alternatively, if trpm8 -/- mice show a significantly greater increase in itch behaviors in the cold plate or histamine cheek assays when compared to mice ablated of TRPM8 neurons and wild-type mice, this will suggest a TRPM8-dependent pathway for the inhibition of itch. Upon completion of these future experiments, we will identify the specific role of TRPM8 in itch inhibition and offer valuable insight to the molecular mechanism underlying the inhibition of itch.
More potent agonists of TRPM8 may improve clinical itch management

If we confirm our proposed TRPM8-dependent pathway of cooling and menthol inhibition of itch, then our findings can direct the development of more potent TRPM8-agonists for the treatment of itch. The results of the histamine-evoked cheek assay demonstrated the limitations of menthol to suppress itch at high concentrations of histamine. Compared to the inhibition demonstrated by cooling on histamine-evoked itch, these results advocate the investigation of alternative, more potent molecules to suppress itch. Other cold-mimetic compounds, such as icilin, activate TRPM8 and have been shown to produce a significantly higher intensity cold-sensation (Rossi et al., 2007). Furthermore, the development of more effective pharmaceuticals targeting TRPM8 may drastically improve itch management, while maintaining normal thermal and mechanical sensations in individuals.

METHODS

Wild type Trpm8DTR, trpm8 -/- and TRPM8-ablated mice

The Trpm8DTR line of transgenic mice was generated as outlined in the experiment of Takashima et al. (2007). TRPM8 neuron ablation was conducted as outlined in the experiment of Knowlton et al. (2013). A trpm8 bacterial artificial chromosome (BAC) clone was modified by homologous recombination, targeting the simian diphtheria toxin receptor (DTR) to sequences corresponding to the second exon in the trpm8 gene. These procedures ensured that only TRPM8-expressing neurons would express the DTR protein. Upon diphtheria toxin injection, only these TRPM8-expressing neurons were be selectively ablated.

Genotyping via Polymerase Chain Reaction (PCR)

DNA preparation

Tissue from the tail of mice was placed in 400 μL of tail lysis buffer and incubated at 55°C overnight. Samples were centrifuged for 15 minutes at 14000 rpm to retrieve the supernatant. 400 μL isopropanol was added to the supernatant and centrifuged at 14000 rpm. After discarding the supernatant, 200 μL of 70% ethanol was added to wash the pellet. Samples were then centrifuged for 5 minutes. After allowing the pellet to significantly dry, 400 μL of TE buffer was added and samples were incubated at 4°C overnight.
**Polymerase chain reaction and gel electrophoresis**
Samples were prepared for PCR by creating the following mixture per 1 μL of DNA sample: 14.2 μL deionized H₂O, 2 μL of buffer, 0.8 μL of deoxynucleotide triphosphates, 0.8 μL of 9456 forward DTR primer, 0.8 μL 10908 reverse DTR primer and 0.4 μL Taq Advantage 2 Polymerase. Samples were placed in the PCR apparatus for 3 hours. The PCR products were run on a 2% agarose gel at 120 volts for 40 minutes. The bands were visualized using Labworks software.

**Histamine and chloroquine cold plate assays**
Mice were habituated to a temperature controlled chamber set to 24˚C for 15 minutes prior experimentation. Under mild isoflurane anesthesia, 20 uL of histamine or was subcutaneously injected into one hind paw of the mice. Mice were placed on a plate set to 30˚C, 24˚C, 20˚C, 17˚C or 10˚C. In the series of experiments investigating whether cooling was application-dependent, mice were placed on a plate that was initially 24˚C and lowered to 20˚C after 15 minutes. Pruriceptive responses were observed and quantified over a duration of 30 minutes post-injection.

**Histamine and histamine + menthol-injected cheek assays**
Mice were habituated in the observation chamber for 2-3 days. 10 μL of 100 μg histamine or 10 μL of 100 μg histamine plus 8 μg menthol were injected subcutaneously into the cheeks of wild-type mice. In the subsequent series of experiments, 10 μL of 20 μg histamine or 10 μL of 20 μg histamine plus 8 μg menthol were injected into the cheeks of wild-type mice. Pruriceptive responses were quantified according to the number and duration of scratch bouts over the duration of 40 minutes post-injection.

**Quantification software and classification of behavioral responses**
Behavioral assays were video recorded for quantification. Quantification software enabled the classification and number of behavioral responses, while tracking the current time elapsed in the video recordings of the behavioral assays. In the histamine-evoked itch cheek assay, the effect of menthol on itch was initially reported as the number of observed scratch bouts. We later measured the duration of itch behaviors and observed the same trend in itch behavior responses.
We decided the duration of itch behaviors, compared to the number of individual itch behaviors (i.e. the number of scratch bouts) would better characterize itch behavior (i.e. correlating stimuli evoking short, quick scratches versus stimuli evoking longer scratches) and potentially identify a phenotype for different itch stimulants. Measuring the effects of itch inhibition by analyzing the duration of behavioral responses seemed to generate a greater precision amongst the results generated from multiple researchers in our laboratory and avoided the potential pitfalls of having to distinguish distinct, separate behaviors (i.e. exact number of bites versus the duration of the entire bite).

In the cheek injection assay, we discriminated itch behavior as outlined by Shimada & Lamotte (2008). The motion of “hindlimb scratching,” where the mouse scratched its injected cheek with its ipsilateral hind paw was considered itch-specific behavior (Shimada & Lamotte, 2008). This itch-specific behavior was discriminated from pain-specific behavior, described as the motion of “forelimb wiping,” where the mouse wiped its injected cheek with the ipsilateral forearm (Shimada & Lamotte, 2008).

In the cold plate assay, itch-specific behavior was quantified as the mouse biting the injected hind paw. Pain-specific behavior was discriminated as the mouse licking the injected hind paw. In the cold plate assay, discrimination of itch and pain specific behavior was more ambiguous than the cheek assay. However, since the inhibitory effects of cooling are application-dependent, we were unable to continually administer a cooling compress to the cheek of mice without obstructing the surface to which we would observe the itch behavioral response (i.e. scratching of its cheek) and therefore, decided not to alternate to the experimental design of the cheek assay. We continued to implement the cheek assay for the investigation of menthol-mediated inhibition of itch because of the consistency with previous studies and the accuracy generated from easily discriminated itch behaviors.
REFERENCES


