Dry eye modifies the thermal and menthol responses in rat corneal primary afferent cool cells

Masayuki Kurose\(^1\) and Ian D. Meng\(^2\)

\(^1\) Division of Oral Physiology, Department of Oral Biological Sciences, Niigata University, Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Niigata 951-8514, Japan

\(^2\) Department of Biomedical Sciences, College of Osteopathic Medicine, University of New England, 11 Hills Beach Rd, Biddeford, ME 04005 USA

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Corresponding author:
Ian D. Meng, PhD
Department of Biomedical Sciences
University of New England
11 Hills Beach Rd.
Biddeford, ME 04005
Phone: (207) 602-2195
Fax: (207) 294-5931
E-mail: imeng@une.edu
Dry eye syndrome is a painful condition caused by inadequate or altered tear film on the ocular surface. Primary afferent cool cells innervating the cornea regulate the ocular fluid status by increasing reflex tearing in response to evaporative cooling and hyperosmoticity. It has been proposed that activation of corneal cool cells via a TRPM8 channel agonist may represent a potential therapeutic intervention to treat dry eye. This study examined the effect of dry eye on the response properties of corneal cool cells, and the ability of the TRPM8 agonist menthol to modify these properties. A unilateral dry eye condition was created in rats by removing the left lacrimal gland. Lacrimal gland removal reduced tears in the dry eye to 35% when compared to the contralateral eye, and increased the number of spontaneous blinks in the dry eye by over 300%. Extracellular single-unit recordings were performed 8-10 weeks following surgery in the trigeminal ganglion of dry eye animals and age-matched controls. Responses of corneal cool cells to cooling were examined after the application of menthol (10µM-1.0 mM) to the ocular surface. The peak frequency of discharge to cooling was higher and the cooling threshold was warmer in dry eye animals when compared to controls. The dry condition also altered the neuronal sensitivity to menthol, causing desensitization to cool-evoked responses at concentrations that produced facilitation in control animals. The menthol-induced desensitization of corneal cool cells would likely result in reduced tearing, a deleterious effect in individuals with dry eye.
Introduction

Tears provide nourishment to the anterior eye and are necessary for its protection from potentially damaging stimuli (Dartt 2009). Corneal primary afferent neurons involved in noxious stimulus-evoked tearing include both polymodal and mechanoreceptive neurons (Acosta et al. 2001a; Acosta et al. 2004). Increased tearing caused by activation of these afferents is accompanied by irritation or pain (Acosta et al. 2001a; Acosta et al. 2001b; Chen et al. 1995). In addition to polymodal and mechanoreceptive afferents, the cornea is innervated by neurons that are sensitive to innocuous cooling (Belmonte and Giraldez 1981; Brock et al. 2001; Hirata and Meng 2010). These corneal cool cells are also activated by menthol, an agonist to the transient receptor potential melastatin 8 (TRPM8) channel, and by hyperosmotic stimuli (Acosta et al. 2001a; Gallar et al. 1993; Hirata and Meng 2010; Madrid et al. 2006). Corneal cool cells are involved in a reflex that promotes tear production in response to drying of the ocular surface (Parra et al. 2010; Robbins et al. 2012). Activation of this tearing reflex does not appear to be accompanied by irritation or pain, which has led to the suggestion that TRPM8 agonists may be useful for the treatment of dry eye (Hirata and Meng 2010; Parra et al. 2010; Robbins et al. 2012).

Dry eye syndrome, a painful condition caused by an inadequate or altered tear film, may be the result of an inability of the lacrimal glands to produce an adequate quantity of tears with the proper composition (Abelson et al. 2009; Barabino and Dana 2007). Alternatively, dry eyes may result from an inability of sensory afferent neurons to monitor the corneal surface, resulting in insufficient neuronal drive to produce a sufficient quantity of tears (Dartt 2004; 2009; Mathers 2000; van Bijsterveld et al. 2003).
Furthermore, even if the initial cause of dry eye is dysfunction of the lacrimal gland, it has been suggested that the dry eye condition itself may affect corneal afferents involved in tear regulation, initiating a vicious cycle that may lead to a further deterioration in lacrimal gland function and a worsening of the condition (Mathers 2000).

The effect of dry eye on the corneal epithelium has been examined using several different animal models, including lacrimal gland removal (Barabino and Dana 2004; Fabiani et al. 2009; Kaminer et al. 2011). While lacrimal gland removal has been used to assess the effectiveness of tear replacement therapies in treating the corneal barrier disruption, little is known regarding the effect of the dry eye condition on the properties of neurons innervating the cornea (Fujihara et al. 2001; Higuchi et al. 2012; Higuchi et al. 2010). Understanding the effect of dry eye on corneal cool cells is particularly important, since activation of these neurons has been suggested as a potential treatment for dry eye syndrome. In this study, we examined the effect of lacrimal gland removal-induced dry eye on the response properties of corneal cool cells and determined the ability of the TRPM8 agonist menthol to modify these properties.

**Materials and Methods**

**Animals.** Male Sprague-Dawley rats (Charles River), weighed 200-250 g at the time of surgery and 350-450 g at time of recording. Animals were housed in an environment with a controlled 12hr light/dark cycle and allowed free access to food and water. Animals were treated according to the policies and recommendations of the National Institutes of Health guidelines for the handling and use of laboratory animals. All procedures were approved by the Committee on Animal Research at the University of New England.
Surgery. Under isoflurane anesthesia, a unilateral dry eye was created by removing the left exorbital and infraorbital lacrimal glands (Venable and Grafflin 1940). Carprofen (5 mg/kg, s.c.) was administered once per day for 3 days to provide postsurgical analgesia.

Tear measurements. In dry eye animals, tears were measured 4 and 8 weeks post-surgery. Using fine forceps, cotton phenol red threads (Zone-Quick, MA) were placed in the lateral canthus of the eye for 30 s in unanesthetized animals. One measurement was taken from each eye. After removal, the length of color change on the phenol red threads was measured under a microscope to the nearest 0.1 mm.

Blink measurements. Blinks were monitored every other week beginning the second week post-surgery in dry eye animals. Rats were placed in a 24x45x20 cm (LxWxH) chamber. After a 15 min acclimation period, spontaneous blinks in each eye were counted for 5 min.

Fluorescein staining. Corneal fluorescein staining was performed in order to assess the degree of corneal damage. A 1% fluorescein solution (10µl) was applied to the cornea in isoflurane anesthetized dry eye animals. After 3 min, the eye was flushed with artificial tears to remove excess fluorescein and examined using cobalt blue light from a slitlamp (PocketScope, Welch Allyn, NY). The degree of staining was scored based on the 0-4 grading system (Lemp 1995; Nakamura et al. 2005; Suwan-apichon et al. 2006). A score of 0 indicated an absence of punctate fluorescein staining, a score of 1 indicated one eighth or less of the corneal surface showed staining, a score of 2 indicated one eighth to one fourth of the corneal surface showed staining, a score of 3 indicated one quarter to one half of the corneal surface showed staining, and a score of 4 was given
when greater than one half of the corneal surface was stained. Corneas were examined every other week beginning the first week after surgery.

Electrophysiological recordings. Corneal responsive neurons were recorded from the trigeminal ganglion 8-12 weeks after removal of the lacrimal gland and in age-matched controls. Under isoflurane anesthesia, the femoral artery and vein were catheterized to monitor blood pressure and deliver drugs, and a tracheotomy was performed for ventilation. Urethane/chloralose anesthetics (500 mg/kg urethane and 50 mg/kg chloralose, i.v.) were delivered to replace the isoflurane prior to recording. This was done to provide consistency with recordings performed in an earlier experiment (Kurose and Meng 2013). End-tidal CO₂ was continuously monitored and maintained between 3.5 and 4.5%. Rats were placed in a stereotaxic apparatus and a partial craniotomy was performed to allow for electrode penetration of the trigeminal ganglion (approximately 1.8-2.2 posterior and 1.6 lateral to Bregma). Extracellular single unit recordings were carried out using platinum-coated tungsten microelectrodes 500 KΩ; FHC, Inc, Bowdoinham, ME). Corneal cool cells were identified by responses that could be evoked by the placement of a cold metal probe (tip diameter ~1 mm) placed near the receptive field. While checking the cool-responsive receptive fields of corneal cool cells, the tip of the metal probe was kept 1-3 mm from the corneal surface.

A custom-made chamber was placed around the eye with inflow and outflow portals to allow for the maintenance of a constant environment of artificial tears on the ocular surface. Controlled thermal stimulation with a 5 mm² contact thermode was used to examine responses to cooling stimuli (TSAII, Medoc Ltd., Ramat Yishai, Israel). The thermode was positioned directly on the surface of the chamber, approximately 0.4 mm.
from the surface of the cornea. From a holding temperature of 35 °C, the cooling stimulus consisted of a ramp down to 20 °C at a rate of 1.5 °C/s. The total duration of the cooling stimulus was 150 s. From a holding temperature of 35 °C, the heating stimulus consisted of a ramp up to 52 °C at a rate of 3.4 °C/s. The total duration of the heating stimulus was 20 s. Data were acquired by a CED Micro 1401 and isolated neurons were analyzed with Spike2 (Cambridge, England).

*Menthol application.* After control thermal stimulation of the cornea, the fluid in the chamber was removed and replaced with either vehicle or menthol (0.01- 1.0 mM). A maximal concentration of 0.5 mM menthol was applied in dry eye animals, since preliminary experiments showed that 1 mM menthol in dry eye animals produced complete and long lasting (> 20 min) inhibition of activity. Cooling was applied 5 min after menthol application. Following the cooling stimulus, the chamber was drained and flushed several times with artificial tears. A minimum of 30 min was allowed between successive applications of menthol in order to allow for cold-evoked activity to return to baseline levels. Menthol (Sigma-Aldrich, St. Louis, MO) was made from a 10 mM stock solution in 40% ethanol and then diluted with artificial tears to the desired concentration. Vehicle consisted of 0.4% ethanol in artificial tears (in mM: NaCl 106.5; NaHCO₃ 26.1; KCl 18.7; MgCl₂ 1.0; NaH₂PO₄ 0.5; CaCl₂ 1.1; HEPES 10; pH 7.45). In preliminary pilot studies, we found that high concentrations of ethanol (>10%) often suppressed cool cell activity; however, lower concentrations (4% ethanol) had no effect.

*Statistical analysis.* Baseline activity was defined as the ongoing unit activity at the 35 °C holding temperature for 30 s prior to the onset of each stimulus. To assess the responses evoked by cooling, the average frequency of cold-evoked activity during the
beginning (0-30s), middle (60-90s) and end of the thermal stimulus (120-150s) was calculated after subtracting the baseline activity. To determine the effect of menthol on the average cold-evoked discharge during these three time periods, the frequency of activity during these time points in a pre-drug artificial tear control trial was subtracted from the frequency of activity evoked post-vehicle or menthol application. The peak frequency was recorded as the highest frequency (spikes/s) value recorded during the cooling ramp. The cooling threshold was defined as the temperature in degrees Celsius at which the average number of spikes/s increased to a value greater than the mean frequency of the ongoing baseline activity plus 3 times its standard deviation (Parra et al. 2010). Heat-evoked discharge was calculated by subtracting the average baseline activity from the average activity evoked during the stimulus. Menthol evoked discharge was determined using the response magnitude (Rmag), which was calculated by subtracting the mean baseline activity + 2SD from the activity evoked 0-30 and 30-120s after drug application (Robbins et al. 2012).

Comparisons between multiple groups were made using either a one-way or two-way ANOVA with repeated measures after it was determined that the dataset conformed to a normal distribution with equal variances followed by Tukey’s multiple comparison post-hoc tests. In cases where the normality or equal variance tests failed, two groups were compared using the Mann-Whitney Rank Sum test, and multiple group comparisons were made using Kruskal-Wallis one-way analysis of variance on ranks test followed by Dunn’s multiple comparison post-hoc tests for non-parametric data. The association between the treatment group and the type of heat-response (facilitation versus inhibition) was evaluated by Pearson's Chi-square test. Facilitation was defined as an Rmag greater
than zero during the heat stimulus. Analysis was performed using SigmaStat version 3.5 (Systat Software, Chicago IL). In all cases, data are represented as the treatment group mean ± SEM and p < 0.05 was considered to be statistically significant.

Results

Dry eye condition. After lacrimal gland removal, spontaneous blinking, aqueous tear production, and fluorescein staining of the cornea was examined in order to assess the dry eye condition. Spontaneous blinking was monitored in alternate weeks beginning the second week after surgery and continuing over the course of 8 weeks. A significant difference in the total number of blinks recorded during the 5 min observation period was found between the left and right eyes (Fig. 1A, p < 0.001, 2-way ANOVA with repeated measures). At the two-week time point, the number of blinks on the dry eye side was over four times higher than blinks recorded from the contralateral eye, an increase that persisted over the 8-week observation period.

Using the cotton thread test, quantification of tears after lacrimal gland removal revealed a significant difference between the two eyes. Tears in the left eye were almost three times lower than those measured from the right eye at four weeks post-surgery and remained suppressed at the 8-week time point (Fig. 1B, p <0.001, 2-way ANOVA with repeated measures). Fluorescein staining was examined prior to recording to determine the degree of corneal damage. All animals had greater than one eighth of the cornea stained positively with fluorescein, with corresponding scores between 1 and 4 on the rating scale (Fig. 1C, D).
Thermal responses in corneal cool cells. A total of 22 neurons in 22 dry eye animals and 27 neurons in 25 lacrimal gland intact control animals were recorded from the trigeminal ganglion with corneal receptive fields. Receptive fields consisted of a small region of the cornea (<0.5 mm²), and did not cross over to the conjunctiva. As is typical of cool cells, neurons had ongoing activity at room temperature and at the chamber holding temperature of 35 °C with a peltier thermode. At this holding temperature, neurons recorded in dry eye animals did not display any difference in ongoing activity when compared to controls (4.2 +/- 1.0 spikes/s versus 5.3 +/- 0.7 spikes/s for dry eye and control animals, respectively; p>0.05, Mann-Whitney rank sum test).

All neurons responded to cooling of the receptive field with an increase in activity. Cold-evoked activity consisted of a robust dynamic phase during the change in temperature (phasic period) followed by a relatively lower degree of activity during the temperature plateau (static period). Neurons recorded in both control and dry eye animals displayed a similar temporal pattern of activity to the cooling stimulus (Fig. 2A, B). The dynamic and static phases were examined by analyzing activity at three different time periods from the start of the cooling stimulus: 0-30 s, 60-90 s, and 120-150 s. A comparison of activity in dry eye and control animals indicated a difference only during the first epoch (Fig. 2C). The average number of spikes during the dynamic phase of cooling was significantly greater in dry eye animals when compared to controls (17.7 ±1.1 spikes/s versus 13.6 ± 1.1 spikes/s, respectively, p<0.05, 2-way ANOVA with repeated measures). No difference in evoked activity during the static phase of cooling was found (Fig. 2C).
Additional analysis was consistent in demonstrating an increased sensitivity to the cooling stimulus in dry eye animals. The peak frequency of activity, which occurred during the dynamic phase of cooling, was 1.4 times greater in dry eye animals when compared to controls (Fig. 2D, p < 0.05, Mann-Whitney rank sum test). Furthermore, the cooling threshold in dry eye animals of 31.5 ± 0.5 °C was significantly higher than the threshold of 29.9 ± 0.5 °C recorded in controls (Fig. 2E, p < 0.05, Mann-Whitney rank sum test).

The response of cool cells to noxious heat was compared in dry eye and control animals. Although noxious heat often inhibits cool cells (Fig. 3A, top panel), they are sometimes activated by noxious heat after a brief period of inhibition, often referred to as the ‘paradoxical’ heat response (Long 1973; 1977). Neurons recorded in dry eye animals were significantly more likely to be activated by noxious heat, which was defined as a positive Rmag value (p < 0.01, Chi-square analysis). In control animals, 18.5% (5/27) of the neurons were activated by noxious heat, whereas 54.5% (12/22) of the neurons recorded in dry eye animals were activated by the same stimulus. Neurons recorded in dry eye animals, however, still displayed a brief period of inhibition prior to their activation (Fig. 3A, lower panel). Overall, the mean frequency of the heat response in dry eye animals was significantly greater than the heat response in control animals (11.9 ± 3.9 spikes/s versus 2.5 ± 1.5 spikes/s, respectively, p < 0.05, Mann-Whitney rank sum test, Fig. 3B). The cooling temperature thresholds did not differ between heat-sensitive and heat-insensitive neurons. Cooling temperature thresholds in heat insensitive and heat sensitive neurons recording from control animals were 29.9 ± 0.6 °C and 30.0 ± 0.8 °C,
respectively, while these thresholds in heat insensitive and heat sensitive neurons in dry eye animals were 31.9 ± 0.6 °C and 31.1 ± 0.8 °C, respectively.

**Menthol responses in corneal cool cells.** The effect of topical menthol application on the ongoing activity was compared in dry eye and control animals. In control animals, menthol concentrations at or above 50 µM evoked sustained discharge for several minutes (Fig. 4A). Menthol produced a similar effect in dry eye animals at lower concentrations (Fig. 4B); however, at the highest concentration (500 µM), menthol increased discharge for a brief period of time, followed by a longer period of inhibition (Fig. 4B, bottom panel). This difference between control and dry eye animals in their response to menthol was examined by analyzing the response magnitude from 0-30 s and 30-120 s after menthol application. In control animals, menthol produced a concentration-dependent increase in activity during the initial 30 s period when compared to vehicle (Fig. 4C, p<0.05, Kruskal-Wallis). The initial response to menthol in dry eye animals also consisted of increased discharge beginning at the 50 µM concentration, although the responses to higher concentrations were blunted (Fig. 4D, p<0.05, Kruskal-Wallis). The increase in response magnitude was sustained in control animals, even at the highest menthol concentrations tested, as determined by analysis of the 30-120 s time period after menthol application (Fig. 4E, p<0.05, Kruskal-Wallis). In contrast, discharge in dry eye animals was maintained only for the lower concentrations of menthol (50 and 100 µM, p<0.05, Kruskal-Wallis), whereas discharge after the highest concentration of menthol was not greater than vehicle during the 30-120 s time period (Fig. 4F, p>0.05, Kruskal-Wallis).
Cold-evoked discharge after menthol. The response to corneal cooling was examined 5 min after menthol application in dry eye and control animals (Fig. 5). Breaking down the cooling response pattern into the initial phasic (0-30 s) and static (120-150 s) periods revealed significant differences between control and dry eye animals after menthol application. During the phasic period of cooling, 100 µM menthol produced a significant increase in activity in control animals (Fig. 6A, p < 0.05, Kruskal-Wallis, see Fig. 5A for example). In contrast, this same concentration of menthol in dry eye animals produced a decrease in discharge (Fig. 6B, p < 0.05, Kruskal-Wallis, see Fig. 5B for example). Decreased discharge was also the result after application of the highest concentration of menthol in both control and dry eye animals (Fig. 5A & B, p < 0.05, Kruskal-Wallis). Menthol-induced changes in peak frequency discharges were generally consistent with those changes observed during the phasic period of cooling (Fig. 6C & D). During the static period, menthol did not significantly affect cold-evoked activity in control animals (Table 1, p > 0.05, Kruskal-Wallis). However, in dry eye animals the highest concentration of menthol decreased cold-evoked activity (Table 1, p < 0.05, Kruskal-Wallis).

Menthol also produced concentration-dependent changes in temperature thresholds. In control animals, both 50 µM and 100 µM menthol produced a decrease in the cooling threshold when compared to vehicle, as neurons responded to the cooling stimulus at warmer temperatures (Fig. 6E, p < 0.05, Kruskal-Wallis). However, no change in threshold occurred after the application of either the lowest (10 µM) or highest (1.0 mM) menthol concentrations. In dry eye animals, menthol failed to produce significant changes in cooling thresholds, although a trend towards an increase in
threshold after 10 µM menthol and a decrease in threshold after 100 and 500 µM menthol was observed (Fig. 6F, p>0.05, Kruskal-Wallis).

**Discussion**

In this study, we examined the effect of a dry eye condition on the response properties of corneal cool cells, a set of afferent neurons that mediate a tearing reflex in response to evaporation of the tear film. As expected, and consistent with previous reports (Fujihara et al. 2001; Higuchi et al. 2012; Higuchi et al. 2010; Kaminer et al. 2011), removal of the lacrimal glands reduced tear production, increased blinking, and caused an increase in epithelial cell fluorescein staining. The chronic dry eye condition also sensitized corneal cool cells, evidenced by an increase in cool-evoked responses, and an increase in the temperature activation threshold. The response to menthol was also altered in dry eye animals in a manner consistent with an increase in sensitivity to TRPM8 activation. In control animals, menthol caused a dose-dependent change in cool-evoked responses, increasing activity at lower concentrations but decreasing activity at the highest concentrations. A similar trend was observed in dry eye animals; however, under the dry eye condition menthol decreased cool-evoked responses at concentrations 10-fold lower than those that decreased responses in control animals.

Previous studies have used lacrimal gland removal to produce a dry condition on the ocular surface (Fujihara et al. 2001; Higuchi et al. 2012; Higuchi et al. 2010; Kaminer et al. 2011). Using this dry eye model, investigators have examined the central circuitry underlying spontaneous blinking, which involves processing in the spinal trigeminal nucleus and input from the basal ganglia (Kaminer et al. 2011). This model has also been
used to assess the ability of eye drop treatments to reduce corneal fluorescein staining and other indicators of corneal epithelial barrier disruption (Fujihara et al. 2001; Higuchi et al. 2012; Higuchi et al. 2010). Alterations in corneal epithelial cells in dry eye, however, may not be indicative of changes in the properties of corneal primary afferent neurons. In the present study, characterization of corneal cool cells under these conditions has revealed significant alterations, including an increase in the sensitivity to cooling.

The mechanism of sensitization in corneal cool cells in dry eye animals remains unknown. Cold allodynia has been reported in nerve injury-induced chronic pain models, yet the sensitization of primary afferent cool cells under these conditions has not been described (Cha et al. 2012; Chichorro et al. 2006; Rossi et al. 2012). In one study that examined the properties of cultured trigeminal ganglion neurons after infraorbital nerve injury, both cold and menthol-evoked responses were diminished after nerve injury, which was in contrast to the increase in heat responses found under similar conditions (Schmid et al. 2011). Dry eye, however, may have more in common with a chronic inflammatory condition on the ocular surface than a nerve injury state.

Induction of dry eye in rodents, produced by either environmentally controlled arid conditions or the injection of choleratoxin B into the lacrimal gland, increased the corneal expression of inflammatory cytokines (Dursun et al. 2002; Lee et al. 2012; Okanobo et al. 2012; Shim et al. 2012; Stevenson et al. 2012; Zhu et al. 2009; Zhu et al. 2012). However, just as nerve injury reduced cold and menthol-evoked activity, inflammation also appears to inhibit TRPM8-mediated responses. In cultured dorsal root ganglion (DRG) neurons, the inflammatory mediators bradykinin and prostaglandin E2 initially increased calcium influx in a subset of cold and menthol sensitive neurons (Linte
et al. 2007). However, these neurons were subsequently less sensitive to cooling, demonstrating a reduction in the overall amplitude in response to cooling as well as lower cold temperature thresholds.

Inflammatory mediators have also been shown to produce inhibitory effects on cold responses recorded from corneal primary afferent terminals (Zhang et al. 2012). Furthermore, in this same study, carrageenan-induced inflammation of the tongue inhibited cold-evoked activity in lingual afferent nerve fibers. Based on the results from these studies, inflammatory mediators appear unlikely to account for the increase in cold-evoked responses recorded in dry eye animals. Instead, other mediators, such as neuronal growth factors, may be involved in sensitizing corneal cool cells in the dry eye condition.

Nerve growth factor (NGF) levels are increased in dry eye disease and NGF increased cold sensitivity and the percentage of menthol sensitive DRG neurons in culture (Babes et al. 2004; Lambiase et al. 2011).

Inflammatory mediators may play more of a role in increasing the heat-evoked responses observed in dry eye animals. Several studies have demonstrated inflammation-induced sensitization of heat-evoked activity in polymodal nociceptive neurons (LaMotte et al. 1992; Liang et al. 2001; Szolcsanyi 1987) through the phosphorylation of noxious heat sensitive TRPV1 channels (Mizumura et al. 2009; Moriyama et al. 2005; Sugiura et al. 2002). Similarly, an inflammatory soup applied to the cornea has been demonstrated to increase heat-evoked responses in cool afferent fibers even while inhibiting cold-evoked responses (Zhang et al. 2012). These apparently disparate results may be due to the fact that a number of signaling pathways that sensitize the heat-activated TRPV1 channels desensitize TRPM8 channels (reviewed by (Patapoutian et al. 2009)).
Alternatively, the increase in heat-evoked responses may be caused by changes in the properties of TRPV1 positive polymodal nociceptors that leave them responsive to cooling. Along these lines, an increase in TRPM8 expression and menthol sensitivity in capsaicin sensitive neurons has been reported in acutely dissociated DRG neurons following chronic constriction nerve injury (Xing et al. 2007).

In addition to altering thermal stimulation-evoked responses, the dry eye condition also shifted the concentration response to menthol. A biphasic response to menthol has previously been described (Klein et al. 2010; Klein et al. 2012). In these studies, low concentrations of menthol produced an increase in sensitivity to cold stimuli and enhanced cold-evoked neuronal discharge from spinal cord dorsal horn neurons, while high concentrations caused a decrease in cold sensitivity and inhibited cold-evoked discharge from dorsal horn neurons. In the dry eye animal, the desensitization following 500 µM menthol is similar to an effect previously described after 50 mM menthol in control animals (Robbins et al. 2012). This effect is also reminiscent of the capsaicin-induced desensitization via TRPV1 in polymodal nociceptors (Koplas et al. 1997; Mohapatra and Nau 2003). The shift in sensitivity to menthol concentration in the dry eye condition was also reflected by alterations in cold-evoked activity following menthol application. While lower concentrations of menthol increased cold-evoked activity and cooling thresholds in control animals, similar concentrations of menthol produced the opposite effect, causing a decrease in cold-evoked responses and thresholds.

The increased sensitivity of cool cells to the desensitizing effects of menthol may be the result of increased TRPM8 channel expression, which has been reported after nerve injury (Xing et al. 2007). These results may also reflect changes in the regulation
of TRPM8 channels in the dry eye condition. The regulation of TRPM8 channels, a
calcium dependent process, has been suggested to play a role in the rapid adaptation to
prolonged cooling stimulation and involves several factors (Abe et al. 2006; Daniels et al.
2009; Sarria and Gu 2010; Sarria et al. 2011; Thut et al. 2003). TRPM8 activity is
increased by the membrane phospholipid, PIP2, and reduced by phospholipase C-
mediated hydrolysis of PIP2 (Daniels et al. 2009; Rohacs et al. 2005; Yudin et al. 2011;
Yudin and Rohacs 2012). Of note, these studies have all been performed in vitro, often
in DRG cultured neurons, and the involvement of similar factors in TRPM8 regulation in
vivo still requires verification. Additionally, alterations in the properties of other cold
sensitive channels, including members of the KCNK channel family or TRPA1, may play
a role in the sensitization of cool cells in the dry eye condition (Thut et al. 2003).

Activation of TRPM8 channels has been proposed as a treatment for dry eye,
offering potential benefits over current treatments that involve the repeated application of
lubricating artificial tears (Hirata and Meng 2010; Parra et al. 2010; Robbins et al. 2012).
While the application of menthol under control conditions may increase tear secretions
without producing unwanted side effects, our results suggest that this may not be the case
in the dry eye condition. Instead, TRPM8 activation in dry eye could result in the
desensitization of neurons involved in the production of tears. Thus, menthol, contained
in some over-the-counter eye drops, may have detrimental effects in dry eye sufferers.

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Long RR. Cold fiber heat sensitivity: dependency of "paradoxical" discharge on body

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Figure Legends.

Figure 1. The effect of lacrimal gland removal on blinking, tearing, and corneal fluorescein staining. (A) Spontaneous blinking counted over a 5 min observation period was elevated in the eye ipsilateral to the side of gland removal (closed circles) compared to the contralateral eye (open circles) over an 8-week period. (B) Tear measurements taken with a cotton thread in unanesthetized animals (see Methods) were lower on the side of lacrimal gland removal (closed circles) compared to the contralateral side (open circles). (C) A photomicrograph taken from a control animal demonstrating the absence of corneal fluorescein staining. (D) Corneal fluorescein staining in an animal 8-weeks after lacrimal gland removal. In this case, the area of positive staining is greater than 50% of the corneal surface, representing a score of 4 on the fluorescein rating scale. ***p < 0.001 versus measurements taken from the contralateral side.

Figure 2. The effect of the dry eye condition on cold-evoked activity. Examples of cold evoked discharge in a (A) control, and (B) dry eye animal. Similar temporal patterns of activity were found in both groups of animals. Activity was greatest during the change in temperature (phasic period) and decreased to a stable level at the temperature plateau (static period). (C) Average discharge evoked during the cooling stimulus, divided into three epochs: 0-30 s, 60-90 s, and 120-150 s. Cold-evoked activity was increased in dry eye animals (closed circles) compared with controls (open circles) only during the initial, phasic period of cooling. (D) The group average peak frequency of activation, which occurred during the phasic period of the cooling stimulus, in control and dry eye animals. (E) The average temperature threshold for activating cool cells in control and dry eye
animals. N = 27 units in control animals and 22 units in dry eye animals. * p < 0.05, *** p < 0.001.

**Figure 3.** Responses to noxious heat in control and dry eye animals. (A) An example of an inhibitory response to noxious heat recorded in a control animal (top panel), and an excitatory response by the same stimulus in a dry eye animal (bottom panel). (B) Overall, mean discharge evoked during heating was greater in dry eye animals when compared to control animals. N = 27 units in control animals and 22 units in dry eye animals. * p < 0.05.

**Figure 4.** Menthol-evoked discharge in control and dry eye animals. (A) An example of neuronal activity following application of vehicle, 100 µM menthol, and 1.0 mM menthol in a control animal. (B) Neuronal activity in a corneal unit following vehicle, 50 µM menthol and 500 µM menthol in a dry eye animal. (C) The average response magnitude (Rmag) of units recorded in control animals calculated from 0-30 s after the application of menthol. (D) The average Rmag 0-30 s after menthol application in dry eye animals. (E) The average Rmag recorded 30-120 s following menthol application in control animals. (F) The average Rmag calculated 30-120 s following menthol application in dry eye animals. In control animals, n = 27, 8, 11, 19, and 6 for vehicle, 10 µM, 50 µM, 100 µM, and 1 mM menthol, respectively. In dry eye animals, n = 22, 8, 17, 20, and 9 for vehicle, 10 µM, 50 µM, 100 µM, and 500 µM menthol, respectively. * p < 0.05 versus vehicle. The number of neurons for each group is the same in Figure 6.
Figure 5. Examples of the response to cooling applied 5 min after the application of vehicle (top panels) and 100 µM menthol (bottom panels). (A) In a cool cell recorded from a control animal, menthol produced an increase in cool-evoked activity during the initial phasic period of cooling. (B) In the dry eye animal, the same concentration of menthol inhibited the response to cooling.

Figure 6. The effect of menthol on the response to cooling in control (left panels) and dry eye (right panels) animals. (A) Average discharge during the initial 30 s of cooling (phasic period) after vehicle and increasing concentrations of menthol in control animals. (B) Average discharge during the initial 30 s of cooling in dry eye animals. (C) Average peak frequency responses to cooling after vehicle and increasing concentrations of menthol in control animals. (D) Average peak frequency responses in dry eye animals. (E) Average cooling thresholds in control animals after vehicle or menthol application to the cornea. (F) Average cooling thresholds in dry eye animals after vehicle or menthol application. * p < 0.05 versus vehicle.
Figure 1

A) Number of blinks (5 min) vs. Time (weeks post-surgery)

B) Tearing (mm) vs. Time (weeks post-surgery)

C) and D) Images showing different conditions or procedures.
Figure 2
Figure 3

A) Activity (spikes/s) for control and dry eye conditions.

B) Average discharge (spikes/s) comparison between control and dry eye.

* indicates a statistically significant difference.
**Figure 4**

A) Control

B) Dry eye

C) Evoked response (Rmag 0-30 s)

D) Evoked response (Rmag 0-30 s)

E) Evoked response (Rmag 30-120 s)

F) Evoked response (Rmag 30-120 s)
Figure 5
Table 1. The effect of menthol on cold-evoked responses during the static period of cooling in control and dry eye animals.

<table>
<thead>
<tr>
<th>Menthol concentration</th>
<th>Vehicle</th>
<th>10 µM</th>
<th>50 µM</th>
<th>100 µM</th>
<th>500 µM</th>
<th>1.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.67 ± 0.53</td>
<td>0.39 ± 1.63</td>
<td>-0.22 ± 1.00</td>
<td>0.21 ± 0.88</td>
<td>-2.91 ± 2.23</td>
<td></td>
</tr>
<tr>
<td><strong>Dry eye</strong></td>
<td>0.55 ± 0.51</td>
<td>5.20 ± 2.35</td>
<td>0.57 ± 1.77</td>
<td>-2.66 ± 1.01</td>
<td>-5.09 ± 1.40*</td>
<td></td>
</tr>
</tbody>
</table>

Numbers represent the average discharge (spikes/s) evoked during the final 30 s of the cooling stimulus, calculated by taking the difference in cold-evoked discharge during a baseline stimulation trial from cold discharge evoked after either vehicle or menthol application (see methods for details). * p < 0.05 versus vehicle treatment, Kruskal-Wallis 1-way ANOVA on Ranks and Dunn’s methods.