This study reports the findings of two classes of corneal afferents excited by drying of the cornea (dry responses) in isoflurane-anesthetized rats; cold-sensitive (CS; 87%) and cold-insensitive (CI; 13%) neurons. Compared to CI neurons, CS afferents showed significantly higher firing rates over warmer corneal temperatures (~31-15°C), greater responses to menthol, drying and wetting of the cornea but lower responses when hyperosmolar solutions are applied to the ocular surface. We proposed that the dry responses of these corneal afferents derive from cooling and an increased osmolarity of the ocular surface, leading to the production of basal tears. An ocular application of the TRPM8 antagonist, BCTC (20µM), decreased the dry responses by ~45-80% but failed to completely block it, while TRPA1 antagonist, HC030031, did not influence the responses to drying of the cornea or hyperosmolar tears. Furthermore, the responses produced by cold stimulation of the cornea accounted for only 28% of the dry responses. These results support the view that the stimulus for basal tearing (corneal dryness) derives partly from cooling of the cornea that activates TRPM8 channels, but that non-TRPM8 channels also contribute significantly to the dry responses and to basal tearing. Finally, we hypothesize that activation of TRPM8 by cooling in CS corneal afferents not only gives rise to the sensation of ocular coolness but also to the “wetness” perception (Thunberg’s illusion) while a precise role of the CI neurons in basal tearing and other ocular dryness-related functions such as eye blink and the “dryness” sensation remain to be elucidated.
Keywords: Dry Eye, Basal Tearing, Corneal Afferents, Transient Receptor Potentials Ion Channels, Temperature Sensitivity

INTRODUCTION

Millions of Americans suffer from dry eye disease (DED), especially women and aged population (Dogru and Tsubota 2011). DED is a chronic ocular disorder characterized by dryness, discomfort and a burning sensation in the eye. DED is thought to result from a disturbance of the lacrimal functional units (LFUs), which comprise sensory afferents and secretory efferents as well as their satellite tissues such as the cornea, conjunctiva, lacrimal and meibomian glands (Anonymous 2007). Normally together LFUs maintain a healthy tear film to protect the ocular surface and provide optical acuity. A dysfunction of any one component of these LFUs (such as the corneal nerves) is presumed to result in DED. We recently reported (Hirata and Meng 2009; Hirata and Meng 2010) that a special type of corneal afferent is strongly excited by the physiological stimuli considered to be important in basal tear production (e.g., drying and gentle cooling of the cornea, as well as evaporation and hyperosmolar tears covering the anterior eye). The data therefore suggest that these primary afferent neurons are involved in basal tear production and constitute the afferent limb of the lacrimation reflex (a component of the LFU). Also because these neurons are such an exquisite sensor of cooling and menthol applied to the cornea, we hypothesize that a member of transient receptor potential (TRP) channels, TRPM8, must reside in these nerve endings to detect ocular cooling.

Para et al. (Parra et al. 2010) recently reported that nerve terminal impulses recorded from the cold-sensitive neurons in the mouse-isolated cornea showed cooling and menthol sensitivities similar to the afferents we identified as the afferent limb of the lacrimation reflex (Hirata and Meng 2010). This study also reported that the responses to these ocular stimuli were largely absent in TRPM8-knockout (KO) mice and could be reduced by TRPM8 blocker, BCTC, in normal animals. Furthermore, the basal tearing measured at the normal corneal temperature (30.6°C) was significantly diminished in TRPM8-KO mice compared with wild type (WT) mice. These data support the hypothesis that TRPM8 mediates the detection of small temperature fluctuations at the corneal surface and its activation by temperature changes provides the necessary stimulus for basal tearing. However, it is not known if the responses to other physiological ocular stimuli (such as drying and hyperosmolar stimuli that occur during the evaporation of tears and presumably trigger basal tearing) are
also mediated by TRPM8. We previously proposed (Hirata and Meng 2010) that the responses to drying of the cornea result from cooling and hyperosmolar stimulations of the ocular surface since the process of evaporation (i.e., drying of the cornea) should result in gentle cooling and hyperosmolar stimulations at the corneal surface. Thus, one goal of the present study was to assess the contribution of TRPM8 to the dry responses by attempting to block them with an ocular application of the TRPM8 antagonist, BCTC. Also because it was reported recently that human embryonic kidney cells expressing TRPA1 were activated by hypertonic saline (Zhang et al. 2008), we sought to determine if the responses to drying of the cornea and hyperosmolar tears are also mediated by TRPA1. Our initial goals to pharmacologically characterize the cold-sensitive (CS) corneal afferents have led serendipitously to one of our novel findings of the cold-insensitive neurons in the present study.

**MATERIALS AND METHODS**

*Surgery and Recordings*

Under 2.5% isoflurane (in 100% oxygen) anesthesia, the femoral vein and artery of male Sprague-Dawley rats (350-570g) were catheterized, respectively, for fluid injections and mean arterial pressure (MAP) recordings. The animal was placed in a stereotaxic instrument that held its head firmly with mouth and ear bars, and the tracheal tube was connected to a ventilator after tracheostomy. A partial craniotomy was performed to expose the brain overlying the left trigeminal ganglion (TG). The animals were artificially respired (Harvard Rodent Ventilator Model 55-3438, MA) and the end tidal CO₂ was monitored with a CO₂ analyzer (4-5%; CWE, PA). The core temperature was maintained at 37-38°C with a feedback-controlled regulator (FHC Inc, ME). Just before the recordings, the isoflurane concentration was decreased to and maintained at 1.5% throughout the experiment. After checking for noxious stimulation-evoked withdrawal reflexes, pancuronium bromide (0.6 mg/kg/hr) was infused continuously during electrophysiological recordings. A tungsten microelectrode (5-9 Mohms; FHC) was lowered into the left TG to search for a spontaneously active neuron. After an amplification and discrimination with template matching software (CED, Cambridge, England), the neural spike outputs, MAP and the temperatures during the corneal thermal stimulation were acquired and analyzed by CED Power 1401 and Spike2, v5.21 (Cambridge, England). Neurons that responded to room temperature (RT) saline solution applied to the eye (cooling stimulus; 21-23°C) with a brief (<4s duration) burst
of activity were isolated. Receptive fields (RFs) were identified on the cornea with an ice-cooled dental metal probe (tip diameter ~1 mm). At the end of the experiment, each animal was euthanized with sodium Euthasol (200 mg/kg, i.p.). The experimental protocol was approved by the Thomas Jefferson University IACUC.

Dry and Wet Corneal Stimulation

After locating the RF, the discharge rate of each unit was recorded during two conditions of corneal fluid status. The wet cornea condition (5 min) occurred when the cornea was moistened with 100-200 µl of rat artificial tears (ATs) via a sterile pipette dropped into a plastic well that enclosed an entire anterior eye. The dry cornea condition (1-2 min) occurred after the well was detached from the eye and the excess ATs removed with a piece of filter paper. There were two types of stimulation protocols: short (5 min) drug application and prolonged (1 hr) drug application. In the short experiments (Figures 5 and 7a), each stimulus pair (wet and dry stimulus) was presented three times before the antagonists or their vehicles were applied. Antagonists were left on the cornea for 5 min and then removed with a filter paper to provide the dry stimulus for 1-2 min. Then after washing with ATs (~5 ml), the same stimulus pairs were again applied 3 times to determine the recovery from the drug effects. In the prolonged experiments (Figures 6a and 7c), each stimulus pair was applied three times before the drug, and then the ocular surface was moistened with the drug for 5 min. The drug was then removed to provide the dry stimulus for 1-2 min, which was followed by re-application of the drug (instead of washing the drug) for 15 min (i.e., total duration of 20 min of drug application). Subsequently, the drug was reapplied two more times each lasting 20 min interspersed with 1-2 min of dry stimuli. The experimental room was air conditioned with HVAC system at ~21°C and maintained at ~40% humidity throughout the experiment.

Chemical Stimulation

Chemical stimuli (50µM menthol, 585mOsm mannitol) were applied to the ocular surface in 100-200 µl solutions dropped into the plastic well with a sterile pipette. These were left on the cornea for 30-40sec. The chemical solutions were then washed by flushing the eye with copious amounts of ATs (~5 ml). Ten min later the ATs were removed with a filter paper to be replaced with the antagonist to bathe the anterior eye for 5 min. Then the antagonist solutions were removed and replaced with the cocktail solutions of antagonist and chemical stimuli for 30-40 sec. Fifteen min after washing the cocktail solution, the chemical stimuli without antagonists were again applied into the well to assess the recovery from the antagonist (Figure 8a). The concentrations of chemical stimuli were chosen because they represented the middle range of dose-response
functions previously observed (Hirata and Meng 2010). Menthol (Sigma-Aldridge) was dissolved in 40% ethanol to make a 10 mM stock solution, which was then diluted to the required concentrations with ATs on the day of the experiment. The solutions of mannitol (Sigma-Aldridge) were prepared by dissolving them in ATs and their osmolarities were measured with an osmometer (µ OSMETT, Precision System Inc., Natick, MA). The composition of ATs in mM was: NaCl 106.5; NaHCO₃ 26.1; KCl 18.7; MgCl₂ 1.0; NaH₂PO₄ 0.5; CaCl₂ 1.1; HEPES 10; pH 7.45 (Kessler et al. 1995).

**Thermal Stimulation**

Temperature stimuli were applied to the ocular surface via fluids that bathed the anterior eye. The temperatures of the fluids were regulated by a Peltier-based device (the Temperature Controller; Warner Instruments, Hamden, CT) placed between the reservoir and the plastic well that enclosed the anterior eye. The fluids descended from the reservoir (60 ml syringe) by gravity via P.E. tubing through the temperature controller down into the plastic well, and escaped from the well into the collecting flask situated underneath the animal. Because the flow rates of the fluids depended on gravity and the temperature changes depended on the flow rates, the fluids filled the reservoir at the same height each time the temperature changes were initiated to ensure the same rates of flow. In the steady state (SS) temperature series, cooling stimuli were presented to the cornea beginning with a 31°C adapting temperature followed by consecutive 2°C incremental decreases in temperature to 10-11°C followed in some experiments by a final temperature of 6-7°C, with each temperature lasting for 60s (Figure 1). The rates of temperature change were, on the average, 0.15°C/sec (range = 0.13-0.18°C/sec).

** Drugs**

The drugs were the TRPM8 antagonist, N-(4-tert-butyphenyl)-4-(3-chloropyridin-2-yl)piperazine-1-carboxamide (BCTC; Biomol International, Plymouth Meeting, PA) and TRPA1 antagonists, HC-030031 (Tocris Bioscience). BCTC and HC 030031 were dissolved in 100% dimethyl sulfoxide (DMSO) to make 10 mM stock solutions, aliquoted and kept in a -20°C freezer until the day of the experiment. A 10 mM solution was diluted to the desired concentration with ATs on the day of the experiment. Normally one antagonist was applied to the ocular surface per neuron and only one neuron was studied per animal, unless 2 simultaneously recorded neurons were encountered in an animal (Figures 1a and 1b). When two antagonists or two concentrations of an antagonist were used per unit, at least one hour elapsed between drug applications, since the drug effects
faded away within one hour (see for example Figures 5). Capsaicin (CAP; Sigma) was dissolved in ethanol and Tween-80 to prepare a 10 mM solution and diluted to the desired concentration with ATs.

Data Analysis

Neural discharges were analyzed based on 1 sec bin acquired with Spike2 software. The discharge rates (# of spikes/sec) during dry corneal conditions (dry responses) were based on the averages of the last 30s of a 1-2 min dry cornea period. This period was chosen since our previous studies (Hirata and Meng 2010) showed that the dry responses after the ATs were removed (start of the dry period) gradually increased over ~30sec to a more stable level thereafter. The discharge rates during wet cornea conditions (wet responses) were based on the averages of 30s immediately preceding the beginning of the dry periods. The chemical stimuli (menthol, mannitol) were kept at RT ([21-23°C], which was colder than the average corneal temperature of the rats [31-33°C]). Therefore, when applied to the ocular surface, these stimuli evoked responses attributable to both “cold” and chemical stimulation of the ocular surface. Thus, the evoked responses to chemical stimuli in this study were defined as the total number of spikes during the stimulus minus the activity produced by the vehicle (ATs or antagonist solutions). The activity during the steady state (SS) cooling series (Figure 1) was calculated based on the last 30s of each of the 1 min temperature stimuli. This period was chosen because they represented the SS discharge rates after the dynamic responses to temperature changes subside. Statistical analyses for the effects of the drugs (antagonists) on neural discharges were performed with ANOVA (GraphPad Prism5) with or without repeated measures. Post-hoc analyses were done with a Bonferroni multiple comparison tests for individual comparisons. T-tests were also used to evaluate two sample populations.

RESULTS

General Properties

Cold-sensitive (CS) vs cold-insensitive (CI) corneal afferents: We found a total of 103 corneal afferents from 91 animals that were excited by drying of the cornea. Of these, 90 neurons (87%) responded to ocular application of the room temperature (RT) saline solutions (21-23°C) and their receptive fields (RFs) could be easily identified as restricted to the cornea with an ice-cold metal probe. They were designated as cold-sensitive (CS) neurons. In addition, the present study found 13 neurons (13%) that were unresponsive to the RT saline eye
drops used for the initial attempt to identify a unit (see Methods). Also their RFs were difficult to identify due to
their diminished or missing sensitivities to the ice-cold metal probe used to locate them on the cornea: they
were classified as “cold-insensitive” (CI) neurons. However, in 3 of these neurons their RFs could be located
within the corneal-limbus border with an ice-cold probe while ten could not be driven by this stimulus. The CI
neurons were found because of their high discharge rates during the dry corneal conditions and near silence
during the wetting of the cornea.

To assess possible functional significance of CS and CI afferents, we recorded their responses to
different ocular stimuli shown in Figures 1-3. Figure 1 illustrates the sharp contrasting response profiles to
temperature stimuli, and dry and wet stimuli applied to the ocular surface. Figures 1a and 1b show the peri-
stimulus time histograms (PSTHs) in responses to 2°C step cooling in one CS and one CI afferent. This CS
neuron displayed dynamic and static responses observed in a typical innocuous cold thermoreceptor (Hensel
1974) while CI afferents show very little dynamic or static discharges throughout the temperature ranges (31-
10°C). Figures 1c and 1d illustrate the differences in their responses to drying and wetting of the cornea.
During the wet cornea condition, there was considerable discharge in this CS neuron but only a trickling of the
firing was seen in CI neuron. The average and individual temperature responses for 8 CS and 7 CI neurons (3
of these responded to the ice-cold probe) are shown in Figure 1e-h. Unlike the CS units, none of the CI
neurons displayed a noticeable activity (~1 spikes/sec) until the temperatures reached extreme cold (<10-
13°C). These temperatures were well below that of the RT saline solutions (21-23°C) and therefore could not
excite CI afferents as described above.

Figure 2 shows further the comparisons between CI and CS neurons in their responses to four types of
ocular stimuli. The average graphs shown in Figure 2h demonstrate that the responses to menthol were
considerably weaker for the CI neurons than the CS afferents (p=0.0003, t-test, two tailed): 31.75 ± 21.27
spikes/stimulus for CI (N=6) vs. 169.71 ± 17.41 spikes/ stimulus for CS neurons (N=25). By contrast, the
responses to mannitol (the hyperosmolar stimulus) were marginally greater for CI units than the CS units
(p=0.0480, t-test, two tailed): 227.67 ± 28.59 spikes/stimulus for CI (N=6) vs. 124.5 ± 17.24 spikes/stimulus for
CS neurons (N=15). Furthermore, the dry response was slightly larger for CS units than CI units (p=0.0461, t-
test, two tailed): 12.97 ± 0.60 spikes/sec for CS neurons (N=60) vs. 9.83 ± 1.13 spikes/sec for CI (N=10),
whereas the response to the wet stimulus was much greater for the CS neurons than CI neurons ($p=0.0064$, t-test, two tailed): $2.54 \pm 0.35$ spikes/sec for CS neurons ($N=60$) vs. $0.14 \pm 0.06$ spikes/sec for CI ($N=10$).

There were also differences in their responses to heat (~43°C): all 6 CS units responded ("paradoxical" responses) (Long 1977; Parra et al. 2010) but none of 5 CI units had responses to this stimulus. The examples are shown in Figure 3. Interestingly, despite the relative insensitivity to cold stimuli among the CI neurons depicted in Figure 1, their responses to warming were similar to CS afferents: it inhibited the firing (Figure 3b). Also similar to the CS neurons reported previously (Hirata and Meng 2010), the CI units did not exhibit a mechanical sensitivity. Moreover, 6 CS and 1 CI neurons were tested with 10µM capsaicin (CAP): 5 CS and 1 CI neurons did not respond to CAP while one CS neuron showed responses to CAP (data not shown). In addition, as reported earlier (Hirata and Meng 2010) one CS unit showed a "dynamic response only" profile to the temperature stimuli with little or no SS discharges (Figure 3a). This unit showed disproportionately large responses to the 43°C heat stimulus compared with the cooling responses (Figure 3a) but CAP was not tested. Furthermore, the patterns of discharges were not affected by various stimuli or antagonists (Figure 2a-g) except for the bursting patterns occasionally observed during cold-stimulation in some CS neurons as reported earlier (Hirata and Meng 2010). These observations indicate that the CS neurons, whose response characteristics are consistent with those of classic innocuous "cold" thermoreceptors, are better suited for small temperature discrimination perhaps utilizing TRPM8 as a molecular sensor than CI neurons.

Predicting the corneal temperatures during wet and dry cornea conditions.

To determine if the corneal temperatures are the exclusive determinant of the dry and the wet responses in the CS and CI afferents, we predicted the corneal temperatures during wet or dry corneal conditions by comparing for each unit the average 30-sec discharge rates during the SS temperature stimuli to the wet or dry responses. If the dry or wet responses were driven by the temperature fluctuations of the corneal surface alone during this period, the discharge rates of the dry or wet responses should match those produced by temperature stimulation of the cornea. For example, the comparison of the discharge rates between Figure 1a and Figure 1c indicates that the discharge rate during the wet corneal condition (3.73 spikes/sec) was similar to the rate during 27°C SS temperature (2.94 spikes/sec) for this CS unit: the exact bath temperature at which the wet response (3.73 spikes/sec) would have been observed was extrapolated to be 26.3°C. In this manner, the corneal temperatures during the wet and dry corneal conditions could be predicted in 9 of 15 units.
in response to wet conditions (Figure 4a) and none of 15 afferents in response to dry conditions (Figure 4b).

Figure 4a shows that the predicted corneal temperatures during the wet cornea conditions appear to cluster around 18-21°C and 26-28°C. However, the exact corneal temperatures could not be determined for 2 CI units because the same discharge rates during the wet cornea (0 spikes/sec) were observed at temperatures between 31-21°C. Also the corneal temperatures could not be established in 4 CS afferents because their rates during the wet cornea states were much higher than those observed at any SS temperature tested. This was also the reason for all 15 units (8 CS and 7 CI neurons) whose corneal temperatures during the dry cornea conditions could not be predicted (Figure 4b): the dry responses of all 15 units were considerably higher than the discharge rates to SS temperature stimuli. Figure 4b also shows that the optimum temperatures that produced maximum discharges were below 15°C for all CI and above 15°C for all CS neurons, justifying the partition of these neurons into two classes. The average rates for the responses to temperature and dry stimuli are shown in Figure 4c (11.5 ± 1.17 spikes/sec for dry response and 4.62 ± 0.90 spikes/sec for SS temperature response). The SS temperature response accounted for only 28% of the dry response. Additionally, 1 CS unit, which displayed only the dynamic responses to temperature changes, had a substantial dry response (10.97 spikes/sec) but little or no SS discharge rate at any temperature (Figure 3a). These results indicate that while the wet responses may be accounted for by the corneal temperature fluctuations in the majority of the afferents, the dry responses could not have been produced solely by the temperature stimulation of the cornea. The additional mechanisms would have to be invoked to explain the totality of the dry responses.

Effects of BCTC on Dry and Wet Responses

To ascertain the molecular mechanisms underlying the dry and wet responses of CS and CI neurons, TRPM8 antagonist, BCTC, was applied to the ocular surface to attempt to block the responses to drying and wetting of the cornea. Figures 5a-c shows the examples from 3 CS corneal afferents in responses to a series of drying and wetting of the cornea before and after a short (5 min) application of BCTC and its vehicle DMSO. As shown here, 20µM but not 10 µM BCTC was able to decrease significantly the responses to both drying (dry response; p<0.0001, one way ANOVA) and wetting (wet response; p<0.0035, one way ANOVA) of the cornea. The average reductions by 20µM BCTC were 45% (Figure 5d) from the pre-drug control responses to the 3rd dry stimulus (range 14 – 104%) and 54% (Figure 5e) from the responses to the 3rd wet stimulus (range
The average activities recovered to 74% of the pre-drug level for dry response and 95% for wet responses within 15 min after the wash. While neither 10 µM BCTC nor 0.2% DMSO attenuated the dry responses significantly (p>0.05, one way ANOVA), the wet responses were decreased greatly by 10µM (p<0.0180, one way ANOVA), as shown in Figure 5e (compare the responses to wet stimulus 3 vs 4 in Figure 5b). When the wet responses were taken into account in calculating the evoked responses to drying of the cornea (i.e., subtracting the wet from the dry responses), the overall effects of BCTC were similar (Figure 5f).

There was 1 CI unit in each of the 10µM and 20µM BCTC groups, while no CI unit was in the DMSO group. The effects of BCTC on dry responses in these CI units were similar to the CS afferents (-29% with 20 µM and -14% with 10 µM BCTC) and the statistical results were the same whether the CI neurons were included or not.

To assess the degree to which the poor drug access might have contributed to the failure of short (5 min) BCTC application to completely abolish the dry responses, we applied and reapplied 20µM BCTC for a total duration of 1 hr in an effort to block the dry responses in 12 separate afferents. We divided these neurons into two types depending on whether the dry responses were above (weakly BCTC-sensitive) or below (highly BCTC-sensitive) 20% of the pre-drug level after 20 min BCTC application. Figure 6 shows that the prolonged application of BCTC does produce progressive decreases in dry responses over 5-60 min in all neurons but a substantial portion (43% of the pre-drug level) still remained in half of the afferents tested (weakly BCTC-sensitive) after 1hr. Interestingly, of the 6 weakly BCTC-sensitive neurons, 3 were CI afferents. Figure 6b shows that the average dry responses after 5, 20, 40 and 60 min of BCTC applications were, respectively, 45%, 24%, 16% and 22% of the pre-drug level (p<0.0001 vs the pre-drug dry responses [3rd dry response in Figure 6a], one-way ANOVA with repeated measure). Figure 6b also demonstrates that the average dry responses after 20 and 60 min of BCTC application were not different from the dry responses after 5 min of BCTC application. By contrast, Figure 6c shows that the wet response was virtually silenced by 20 µM BCTC in all neurons even after 5 min of application and remained quiet over 20-60 min of BCTC presence. The average wet responses after 5, 20, 40 and 60 min of BCTC applications were, respectively, 6%, 3%, 6% and 2% of the pre-drug level (p<0.0001 vs the pre-drug dry responses, one-way ANOVA with repeated measure).

The results from short and long BCTC application demonstrate that TRPM8 channel activation may not explain all of the dry response magnitudes while the wet responses presumably are mediated entirely by the TRPM8.
Effects of HC030031 on Dry and Mannitol Responses

To determine if additional mechanisms beside TRPM8 might be responsible for the production of the dry and wet responses, we applied a specific TRPA1 antagonist, HC030031, to the ocular surface to attempt to block these responses. Figure 7 shows that both short (5 min, Figure 7a-b) and long (1hr, Figure 7c-d) application of HC030031 had no effect on the responses to drying or wetting of the cornea (p>0.05, One-way ANOVA with repeated measure). For the 5 min application, the average decreases in dry and wet responses (to 4th stimuli) by HC030031 were 13% and 11% from the control (to 3rd stimulus), respectively. For a long application, although there was a small progressive decline in the dry responses after a 20-60 min application (11-17% decreases) this was not statistically significant either from the pre-drug level (p>0.05, one-way ANOVA with repeated measure) or in comparison with the DMSO control group (p>0.05, two-way ANOVA with repeated measure). Similar comparisons for the wet responses showed that HC030031 was also without a significant effect (p>0.05, one-way or two-way ANOVA with repeated measure; data not shown).

In addition, because we hypothesized previously (Hirata and Meng 2010) that the dry responses might derive partly from the hyperosmolar stimulation of the cornea, we tested the responses to mannitol application before and after HC030031. Figure 8 demonstrates that HC030031 had no significant influence on the responses to hyperosmolar stimuli, 585 mOsm mannitol (p=0.3952, One-way ANOVA with repeated measure). The average response magnitudes to hyperosmolar stimuli before, during and after washing HC030031 were 111.7±33.3, 112±22.6 and 241.8±85.2 spikes/stimulus, respectively.

DISCUSSION

The present study found that the corneal afferents that detect dryness of the cornea could be divided into two types based on their temperature sensitivities: cold-sensitive (CS) and cold-insensitive (CI) neurons. Although the biophysical and electrophysiological properties of CS and CI had been described previously for the cultured trigeminal ganglion (TG) cell population (Viana, de la Pena, Belmonte 2002), to our knowledge the present study is the first to report the presence of CI neurons among the corneal afferents. These two classes of neurons in the present study were distinguished by their ability (CS) or inability (CI) to respond clearly to room temperature (RT; 21-23°C) saline drop and an ice-cold probe. The CI neurons in our study are very similar to the CI neurons defined by Viana et al (Viana, de la Pena, Belmonte 2002) for not responding to a
ramp cooling to ~19°C or to menthol, although their later study (Madrid et al. 2009) demonstrated that some of these CI neurons became CS when an extreme cooling stimulus down to ~8°C was employed.

Previously we reported that a special class of corneal afferents was excited by drying of the cornea as well as by other types of ocular stimuli thought to be essential for production of basal tears, such as gentle cooling of the cornea, evaporation, and hyperosmolarity of the tears (Hirata and Meng 2010). These response characteristics and their additional sensitivities to menthol are all consistent with those of classic innocuous “cold” thermoreceptors found in the oro-facial skin (Hensel 1974; Poulos and Lende 1970) and fall into the present classification of CS afferents. Initially we and others proposed (Hirata and Meng 2009; Hirata and Meng 2010; Parra et al. 2010) that activation of a mild temperature sensor by cooling in the CS neurons, particularly the TRPM8 channels, is crucial to the production of basal tears. However, the finding of CI afferents in the present study that were also robustly activated by drying of the cornea but displayed little or no sensitivity to normal ambient temperatures, indicates that a fluctuation of the corneal temperature may not be the sole stimulus for evoking basal tears. This conclusion is supported by our observation that a maximum firing of a neuron at its optimum SS corneal temperature fell far short of the discharge rates evoked by drying of the cornea (Figure 4). Had the corneal temperatures been the only determinant of the dry responses, the discharge rates produced by these two stimuli would have been identical. The SS temperature responses averaged over the 30 sec period were chosen to compare with the dry responses over the same period for each neuron partly because the amounts of basal tears are typically measured during 30 sec to 2 min of ocular surface exposures in animals (Barabino and Dana 2004).

Furthermore, our assertion that the cooling stimulus may only be a part of the stimulus that drives these innocuous “cold” thermoreceptors to drying of the cornea is also consistent with our observation that an application of the saturating concentration of BCTC (20 µM) failed to completely abolish the neural excitation produced by drying of the cornea (Figures 5 and 6). The blocking ability of BCTC on the cooling responses has not been consistent, however. Previous studies reported that a 10µM BCTC did not reduce the cold-evoked responses of the majority of the guinea pig corneal nerve endings, although it completely blocked the cold-evoked responses of TG neurons (Madrid et al. 2006). However, in ~one-third of the corneal nerve endings in this study, BCTC produced a small but statistically significant decrease in cold-evoked discharges. In our study a substantial portion (~40%) of the dry responses of some CS and of all CI neurons (weakly BCTC-sensitive
afferents) remained even after prolonged application of BCTC (Figure 6), while other neurons (highly BCTC-sensitive afferents) were affected greatly by a 1hr BCTC application. It has been argued that this variability might be the result of a poor permeability of the compound across the corneal epithelia (Parra et al. 2010), or variable depths of the receptors at the nerve endings (Parra et al. 2010). Thus, the possibility that an incomplete effect of BCTC on the dry response in our study may be due to a drug access problem cannot be excluded. However, it is important to note that in the present study the wet responses were nearly completely blocked by BCTC after a short application (Figure 6), suggesting that the drug might have effectively permeated to the receptor sites. Use of a more specific TRPM8 antagonist or TRPM8-deficient transgenic mice might resolve these issues in the future.

Previously we proposed (Hirata and Meng 2010) that drying of the cornea leads to gentle cooling and an ensuing increase in osmolarity of the tears as the tears evaporate from the corneal surface, and that the response to cooling of the cornea is mediated by TRPM8 channel activation and the response to hyperosmolar stimulus by TRPA1 channel activation. While our result of BCTC attenuating the dry response by ~45-80% is consistent with TRPM8-mediated processes, the effect of HC030031 on the dry response failed to support the latter hypothesis (Figure 8). Some evidence indicates that this hyperosmolar tear-induced activation of the corneal afferents may be based on the TRPV1 receptors and not on TRPA1. The arginine-vasopressin containing neurons in the hypothalamus are sensitive to an increase in osmolarity of extracellular fluid (330-360 mOsm), presumably to regulate body fluids in animals (Oliet and Bourque 1993; Oliet and Bourque 1996). Activation of these osmosensory neurons to osmotic stimuli was absent in TRPV1-knockout mice (Ciura and Bourque 2006; Sharif Naeini et al. 2006). Also, an increased afferent renal nerve activity produced by hypertonic saline (600 mOsm) was blocked by a TRPV1 antagonist, capsazepine, when perfused into the renal pelvis (Zhu, Xie, Wang 2007). However, BCTC is also an effective blocker of TRPV1 channels (Valenzano et al. 2003) and should have produced some effect on the dry response. Thus, the mechanisms underlying the responses to hyperosmolar stimulus are yet to be elucidated.

A revelation of the mechanisms underlying the dry response may come from the studies of CI afferents, which we were not able to investigate in any significant manner in the present study. For example, we found that the CI neurons were much more resistant to the TRPM8 antagonist (Figure 6b) than CS neurons, suggesting that the greater portion of the dry responses in the CI neurons may be mediated by non-TRPM8
(hyperosmolar stimulus-based) mechanisms than the low threshold CS afferents. This view is consistent with our present observation that CI neurons were more strongly activated by a hyperosmolar stimulus than CS neurons (Figure 2h). Evaporation during ocular dryness is expected to lead to local spots of hyperosmolarity (Kimball, King-Smith, Nichols 2010; King-Smith et al. 2008), which can then become a source of ocular irritation and discomfort (Wolkoff 2010). These non-TRPM8 mechanisms may play a significant role in the ocular sensations encountered in DE patients. It is entirely possible that CI (and not CS) neurons may become a major player in the processing of the dryness sensation under pathological conditions such as inflammation or in the aged population. It has been known for some time that mechanically insensitive cutaneous afferents become mechanically sensitive under inflammatory conditions (Andrew and Greenspan 1999; Davis, Meyer, Campbell 1993) or develop spontaneous activity in neuropathic states in humans (Orstavik et al. 2003).

Another important function for the CI neurons may be the mediation of tearing under extremely cold environment since these neurons do not become active until the ambient temperature reaches below ~10-13°C (Figure 3b).

While it is almost universally accepted that activity of the CS neurons, especially of those that had been classified as low threshold CS neurons (Madrid et al. 2009), leads to a sensation of gentle cooling via a TRPM8 channel activation (Bautista et al. 2007; Colburn et al. 2007; Dhaka et al. 2007; McKemy, Neuhausser, Julius 2002), there may be an additional intriguing possibility that the temperature sensation carried by CS neurons may in fact be interpreted also as an ocular “wetness”. Over a hundred years ago, Thunberg (Thunberg 1905) described a sensation of a “liquid” when the forehead is stimulated with a cold substance for an interval of 20 sec (Bershansky 1923; Carnahan, H., Dubrowski, A., and Grierson, LEM. 2010). Similarly, cold tears on the ocular surface caused by evaporation could feel “wet”. The ocular wetness sensation, however, is rarely reported in normal humans, perhaps because the sensation has adapted due to a continual presence of the basal tears. The converse (dryness), however, appears to be a very powerful sensation and is one of the major complaints among dry eye (DE) patients (Begley et al. 2003). It is possible that a lack of tears in DE patients and therefore a lack of the wetness sensation can be perceived as “dry”, and that an increased activity of the CS afferents may serve as a neural substrate for the “dryness” sensation (Belmonte and Gallar 2011).
In conclusion, the present study found two populations of corneal afferents that were strongly excited by drying of the cornea. The responses of these afferents to ocular dryness are likely mediated by both TRPM8 and non-TRPM8 mechanisms. Furthermore, the CI neurons were more strongly activated by hyperosmolar tears than the CS afferents, while the CS neurons were much more vigorously excited by cooling and menthol than CI units. Because these two stimuli (cooling and increased osmotic pressure) are intimately involved in the process of drying and both CI and CS neurons respond to drying of the cornea, we hypothesize that both afferents are germane to the ocular dryness-related functions such as tearing, dryness sensation and eye blink. Unraveling of the mechanisms underlying these functions is an important step toward understanding the cellular and molecular processes that culminate in the dry eye disease.

Acknowledgement: We thank Dr. Manuel Cavarrubias for reading the manuscript and Dr. Richard Horn for loaning us the temperature controller.

Grants: This work was supported by the Thomas Jefferson University Pilot Research Award and NIH Grant EY020667.

Disclosures: No conflicts of interest, financial or otherwise, are declared by the author(s).

Reference Lists


Figure Captions

**Figure 1.** a-d, Peri-stimulus time histograms (PSTHs) in response to steady state (SS) temperatures (a, b) and to dry and wet stimuli (c, d) applied to the cornea in cold-sensitive (CS) and cold-insensitive (CI) corneal afferents. CS and CI units were recorded simultaneously from the same microelectrode but were discriminated using software by the shape of the waveforms (shown above PSTHs, 10 superimposed spikes). The records in a and c were from one unit. Those in b and d were from another unit. The timescale in b applies also to a. e-h, Individual (e, g) and averaged (f, h) SS temperature response profiles of CS and CI corneal afferents. Average graphs in this and the following figures display AVG + SEM.

**Figure 2.** PSTHs of a CS (a-c) and a CI corneal afferent (d-g) in response to a variety of ocular stimuli. Notice that there was little or no response to menthol and temperatures while the response to dry and hyperosmolar stimuli were substantial. h, Average graphs illustrating the differences in response to four types of ocular stimuli in CS and CI corneal afferents. The sample sizes for CI and CS units, respectively, were 6 and 25 for menthol, 6 and 15 for mannitol, 10 and 60 for dry and for wet responses. *** p<0.001  ** p<0.01  * p<0.05

Mannitol (585 mOsm). Menthol (50 μM).

**Figure 3.** a, PSTH of a “dynamic response only” CS corneal afferent in response to 4°C step cooling of the cornea from 35°C to 15°C. Note the exaggerated response to heat. b, PSTH of a CI neuron in response to cooling and heating applied to the cornea. This corneal afferent was virtually unresponsive to cooling stimuli
until the temperatures were ~<13°C. Notice the inhibition of activity by warming from 7°C to 9°C (indicated by solid line).

**Figure 4.** a, Graph showing the relationship between the discharge rates during the wet cornea (wet responses) and the predicted corneal temperatures, which would have produced the wet responses. The corneal temperature during the wet condition was predicted for each neuron from the data shown in Figures 1e and 1g. b, Graph showing the relationship between maximum SS temperature responses and the dry responses for 7 CI and 8 CS corneal afferents. CI and CS units are represented by # 1-7, and 8-15 on the x-axis, respectively. The temperatures shown above solid circles indicate the temperatures at which the maximum discharge rates were observed. c, Average dry and SS temperature responses of 7 CI and 8 CS neurons taken from b. The vertical scale in b applies also to c.

**Figure 5.** a-c, PSTHs in response to series of dry and wet stimuli before and after short applications (5 min) of the BCTCs and the vehicle solutions from three CS corneal afferents. Note the decrease in wet response after 20 μM BCTC (* in b). The brief increases in spike activity at the beginning and end of dry stimuli due to artifacts and wetting of the cornea (cold stimulation, see Methods), which were present in original data, were removed for clarity in these PSTHs. d-f, Average dry, wet and “dry-wet” responses before and after BCTC and vehicle (DMSO). Numbers on x-axis (stimulus #) represent the repeated stimuli shown in a-c. * p<0.05; ** p<0.01 vs. stimulus 3.

**Figure 6.** a, PSTH of a CI afferent in response to dry and wet stimuli during the repeated, prolonged BCTC applications. After 3 pre-BCTC control stimuli, BCTC (thick line) was presented for a total duration of 1hr except when the dry stimuli (dotted lines) were applied. There was no wash out throughout the test series until after the last dry stimulus (4). 1, 2, 3, and 4 above the dotted lines are, respectively, the dry stimuli presented after 5, 20, 40 and 60 min of BCTC. b and c, Percent changes in dry (b) and wet (c) responses after prolonged BCTC applications. * p<0.0001 vs pre-drug control response (to 3rd dry or wet stimuli); a = p<0.01, b = p<0.05 vs dry response (1) after 5-min of BCTC.

**Figure 7.** a, PSTHs of a CS neuron in response to series of wet and dry stimuli before and after short ocular application of the TRPA1 antagonist, 20μM HC030031. b, Average dry (solid squares) and wet responses
(solid circles) before and after HC030031 (6 CS and 1 CI neurons). c, PSTHs of a CS neuron in response to series of wet and dry stimuli during the repeated, prolonged ocular application of the 20µM HC030031. 1, 2, 3, and 4 above the dotted lines are, respectively, the dry stimuli presented after 5, 20, 40 and 60 min of HC030031. d, Average dry responses to prolonged HC030031 (4 CS and 1 CI neurons) and vehicle (DMSO; 3 CS and 1 CI neurons) applications.

Figure 8. a, PSTHs from another CS neuron in responses to hyperosmolar stimuli (585 mOsm mannitol) before, during and after washing HC030031. Artifacts and the responses to cooling when mannitol was applied and washed (see Methods) were removed for clarity. b, Average graphs for the mannitol responses before during and after washing the HC030031 (6 CS neurons).
**Figure 1**

Steady State Temperatures (°C)

- CS (N=8)

- CI (Average)

**Graphs:**

- (a) CS unit
- (b) CI unit
- (c) CS unit
- (d) CI unit
- (e) CS (N=8)
- (f) CS (Average)
- (g) CI (N=7)
- (h) CI (Average)

Temperature:

- 31°C
- 29°C
- 27°C
- 25°C
- 23°C
- 21°C
- 19°C
- 10°C

# of spikes/sec

- 0
- 3
- 6
- 9
- 12
- 15

Time (sec)

- 0
- 100
- 200
- 300
- 400
- 500
- 600
- 700
- 800
- 900
- 1000
- 1100
- 1200
- 1300

CI unit

- 50 µV
- 1 ms

- 100 µV
- 1 ms
Figure 2

(a) and (b) show the number of spikes/sec for CS cells with and without menthol and mannitol treatments. (b) compares the same treatments on CI cells.

g) SS temperatures range from 6°C to 43°C.

h) Bar graph showing the total number of spikes/stim (left) and the number of spikes/sec (right) for CS and CI cells with treatments.

Figure 2
Figure 3

(a) CS unit

(b) Cl unit

Temperature vs. time and spikes/sec.
Figure 4

Discharge Rates During Wet Cornea

(\text{# of spikes/sec})

Predicted Corneal Temperature (°C)

- CS cells (N=4)
- CI cells (N=5)

Figure 4
Figure 5

- **a**
  - Wet Stimulus
  - Drugs: 10 µM BCTC
  - Wet Response
  - # of spikes/sec

- **b**
  - Wet Stimulus
  - Drugs: 20 µM BCTC
  - Wet Response
  - # of spikes/sec

- **c**
  - Wet Stimulus
  - Drugs: 0.2% DMSO
  - Wet Response
  - # of spikes/sec

- **d**
  - Dry Stimulus
  - Dry Response:
  - Drugs: 10 µM BCTC (N=10)
  - 20 µM BCTC (N=12)
  - 0.2% DMSO (N=12)

- **e**
  - Wet Stimulus
  - Wet Response:
  - Drugs: 10 µM BCTC (N=10)
  - 20 µM BCTC (N=12)
  - 0.2% DMSO (N=12)

- **f**
  - Dry – Wet Response
  - Drugs: 10 µM BCTC (N=10)
  - 20 µM BCTC (N=12)
  - 0.2% DMSO (N=12)
Figure 6

a) Graph showing the number of spikes per second for a CI neuron with a wet stimulus (ATs) and a dry stimulus. The graph compares the effects of 20µM BCTC on the wet and dry responses.

b) Bar graph comparing the dry response percentage of Pre-drug for weakly BCTC-sensitive (N=6), highly BCTC-sensitive (N=6), and total (N=12) neurons. The graph shows significant differences indicated by asterisks.

c) Bar graph comparing the wet response percentage of Pre-drug for weakly BCTC-sensitive (N=6), highly BCTC-sensitive (N=6), and total (N=12) neurons. The graph shows significant differences indicated by asterisks.

Figure 6
Short application of HC030031 on dry and wet response

![Graph a](image1)

Long application of HC030031 on dry and wet response

![Graph b](image2)

![Graph c](image3)

![Graph d](image4)

Figure 7
HC030031 on mannitol

**Figure 8**

(a) Graph showing the number of spikes per second over time for Pre-HC030031, During-HC030031, and Post Wash conditions with mannitol.

(b) Bar chart showing the mannitol response with N=6 for Pre, HC030031, and Wash conditions.