This study reveals that the cold-sensitive (CS) + dry-sensitive (DS) corneal afferents reported in a previous study consist of two types: 1) low threshold (LT)-CS + DS neurons with less than 1°C cooling sensitivity, and 2) high threshold (HT)-CS + DS neurons with a wide range of cooling sensitivities (~1-10°C cooling). We also found DS neurons with no cooling sensitivity down to 19°C [cold-insensitive (CI) + DS neurons]. LT-CS + DS neurons showed highly irregular discharge patterns during the dry cornea characterized by numerous spiking bursts, reflecting small temperature changes in the cornea. Their receptive fields (RFs) were mainly located in the cornea’s center, the first place for tears to ebb from the surface and be susceptible to external temperature fluctuations. HT-CS and CI + DS neurons showed a gradual rise in firing rate to a stable level over ~60s after the dry stimulus onset. Their RFs were located mostly in the cornea’s periphery, the last place for tears to evaporate. The exquisite sensitivity to cooling in LT-CS + DS neurons was highly correlated with heat sensitivity (~45°C). There was a perfect correlation between noxious heat sensitivity and capsaicin responsiveness in each neuron type. The high sensitivity to noxious osmotic stress was a defining property of the HT-CS and CI + DS neurons, while high sensitivity to menthol was a major characteristic of the LT-CS + DS neurons. These observations suggest that three types of DS neurons serve different innocuous and nociceptive functions related to corneal dryness.
INTRODUCTION

Homeostasis is a ubiquitous biological process that manifests itself in a variety of often tissue-specific cellular and molecular identifiers. The lacrimal functional unit (LFU), a homeostatic mechanism of the anterior eye, is an aggregate of ocular tissues that maintains a healthy tear film and is an important mechanism for the integrity of the entire eye because the anterior eye is the first defense against external threats (Stern et al. 1998; Stern et al. 2004). Dry eye disease (DED) is a clear example of the breakdown of this protective mechanism. Although the most recent international workshop on DED (DEWS 2007) acknowledged lack of tear production as an initiating and/or perpetuating factor for DED, little was known about the specific cellular and molecular mechanisms that regulate tearing until recently (Hirata and Meng 2010; Parra et al. 2010).

Since the original discovery of the innocuous cold thermoreceptors in the cornea (Lele and Weddell 1959), a theoretical debate about their functional significance has persisted (Belmonte and Gallar 2011). Unlike the thermal sensory role for the thermoreceptors in the skin, there was no evidence that the ocular thermoreceptors serve any temperature detection of the atmosphere (Belmonte and Gallar 2011). Then almost simultaneously two reports have appeared to demonstrate their role in tearing. A special class of corneal afferents (dry-sensitive, DS, neurons) was found that are driven by the types of ocular stimuli thought to be important for basal tearing such as drying, cooling, evaporation, and hyperosmolar stimulation of the cornea (Hirata and Meng 2010). It was hypothesized that they serve as the “afferent limb” of the LFU, and their dysfunction may lead to the pathogenesis of DED. The second study reported more comprehensive evidence that innocuous cold thermoreceptors function as producers of basal tears via activation of TRPM8 ion channels (Parra et al. 2010), suggesting the innocuous nature of their tearing functions. However, more recently, we demonstrated (Hirata and Oshinsky 2012) that the DS corneal afferents comprise heterogeneous groups of neurons containing TRPM8 and non-TRPM8 receptors, and that some of these neurons are activated by noxious cold stimuli, indicating that they may play a nociceptive role in tearing and/or ocular sensations. Although our previous study (Hirata and Oshinsky 2012) found two classes of DS corneal afferents defined by their sensitivities to ocular-dryness and cold stimuli, their responses to innocuous and noxious levels of the...
temperature and chemical stimuli were not systematically examined and quantified. Also, other response properties such as pattern of discharges and receptive field (RF) location on the cornea critical in defining their functional significance were not explored. Thus, it became necessary to thoroughly characterize the different types of DS neurons to innocuous and noxious ocular stimuli. In the first step toward understanding their functional roles and in the hope of illuminating the mechanisms, the present study examined quantitatively the responses of these DS corneal neurons to various ocular stimuli and found unique sets of homeostatic identifiers that suggest different sensory and reflex roles.

MATERIALS AND METHODS

Surgery and Recordings

Under 2.5% isoflurane (in 100% oxygen) anesthesia, the femoral vein and artery of male Sprague-Dawley rats (380-600g) 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 animal was artificially respired (Harvard Rodent Ventilator Model 55-3438, MA) and the end tidal CO$_2$ was monitored with a CO$_2$ 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 electrophysiological 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). A neuron displaying on-going discharge when the cornea was dry and suppressed when the cornea was wet was 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 (100 mg/kg, i.p.). The experimental room
was air conditioned with HVAC system at ~21°C and maintained at ~40% humidity throughout the experiments. All procedures were approved by the Thomas Jefferson University IACUC.

**Experimental protocol**

After locating the RF, the discharge rates of each unit were recorded during two conditions of corneal fluid status. The wet stimulus (5 min) occurred when the cornea was moistened with 200 µl of rat artificial tears (ATs) via a pipette dropped into a plastic well that enclosed an anterior eye. The dry stimulus (2 min) occurred after the well was detached from the eye and the excess ATs removed with a piece of filter paper from the ocular surface. The cooling and heating stimuli were applied next to the ocular surface via fluids that bathed the anterior eye in a plastic well. 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. The fluids were drawn from the reservoir by a peristaltic pump at a rate of ~1 ml/min, and via polyethylene tubing flowed through the temperature controller down into the plastic well. The cooling stimuli were 12°C changes from a 31°C adapting temperature to 19°C followed by warming back to the 31°C adapting temperature. Approximately 1 min later heating stimuli from 31°C to 44-45°C (~50s) were applied followed by cooling back to the 31°C adapting temperature. Then the adapting temperature was raised to 35°C, which was maintained for at least 1 min, and the cooling stimuli began from this temperature down to 19°C. The rate of temperature change was, on the average, 0.15°C/sec (range = 0.13-0.18°C/sec).

In our previous paper (Hirata and Oshinsky 2012), the chemical stimuli, 585 mOsm mannitol (hyperosmolar solution), 50µM menthol (cooling stimulus), and 10µM capsaicin (CAP) were applied to a neuron at room temperature (~21-23°C), which produced both chemical and temperature stimulation to the cornea. Therefore, to eliminate the confounding effect of temperature stimulation induced by the “cold” chemicals, in the present study the chemical stimuli were applied at a bath temperature of 31°C using the corneal perfusion system described above. The stimulus was 2 min in duration, and the inter-stimulus interval (ISI) was at least 5 min. The mannitol and menthol stimuli were presented to a neuron in a random order and CAP was always used at the end of the experiment only once.

**Chemicals**
L-Menthol (Sigma-Aldrige) 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 D-Mannitol (Sigma-Aldridge) were prepared by dissolving it in ATs and the osmolarities were measured with an osmometer (µ OSMETT, Precision System Inc., Natick, MA). CAP (Sigma) was dissolved in ethanol and Tween-80 to prepare a 10 mM solution and diluted to the desired concentration with ATs. 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). The osmolarity of the ATs was near iso-osmolar (305 mOsm).

**Data Analysis**

Neural discharges were analyzed based on 1 sec bin. The discharge rates during dry (dry responses) and wet corneal conditions (wet responses) were based on the averages of the last 30s of 2-min dry stimulus periods and the averages of 30s immediately preceding the beginning of the dry periods, respectively. Our data indicated that the responses to chemical stimuli were not sustained for the duration of the stimulus in most afferents: they adapted to smaller response magnitudes at various times after 30 sec (Figure 5B-C, and 7D, F). Therefore, the evoked responses to menthol, mannitol, and capsaicin were defined as the total number of spikes in the first 30 sec following the stimulus onset that exceeded the mean + 2 SD (background activity), which were based on the activity over the 30s preceding the stimulus. In some cases (Figure 7A-D), these total counts were transformed into spikes/sec by dividing them by the period used for counting (30s). The evoked responses to cooling and heat were similarly defined: however, here the total activities during the entire stimulus periods were measured. The total activities were chosen because the responses to temperature ramp stimuli always ceased at the end of the stimuli (e.g., warming following 12°C cooling stimuli or cooling following the heat; see Figure 2A-D). In order for the evoked response to be counted as an onset of the response (threshold), at least 3 consecutive bins after the stimulus had to exceed the background. Statistical analyses for the effects of the stimuli on neural discharges were performed with ANOVA (GraphPad Prism5) with or without repeated measures. Post-hoc analyses were done with a Bonferroni multiple tests for individual comparisons. T-tests and Pearson correlations were used to evaluate two sample populations, and chi square analyses were also performed for non-parametric statistical comparisons.

**RESULTS**
**Discharge patterns and the RF locations:** A total of 54 TG neurons excited by drying of the cornea (DS neurons) were studied in the present report. All but one were mechanically insensitive to scratch or indentation of the corneal surface with a calibrated von Frey monofilament (2-4 g). For 47 of the 54 neurons, we could classify them into three types based on their discharge patterns during dry and wet cornea, and their temperature sensitivities, described later. Figures 1A-C show the patterns of discharges observed in three types of DS neurons, and Figure 1D illustrates their averages. The first type, the low threshold cold-sensitive (LT-CS) + DS neurons (18/47; 38.3%; Figure 1A), showed a highly irregular pattern of discharge during dry cornea, characterized by numerous sudden increases in activity (asterisks). The increase in the discharge rate began almost immediately after the removal of ATs (the dry stimulus onset) seen more clearly in averaged graph in Figure 1D (black). In stark contrast, the wet responses were extremely regular (typically 1-2 spike variations between bins), and significantly smaller in magnitudes than the dry response (respectively, 7.91 ± 0.95 and 14.49 ± 1.21 spikes/sec; p<0.0001). Figure 1E shows that their RFs (solid circles) were mostly located in the central part of the cornea (11/18 neurons, 61.1%).

The second type of DS neurons, high threshold cold-sensitive (HT-CS) + DS neurons (24/47, 51.1%), had markedly different dryness-induced patterns as shown in Figure 1B for the individual and Figure 1D for the averaged data (light gray). Upon removing the ATs from the ocular surface (dry stimulus onset), the activity gradually increased over 30-60 sec until a stable activity level was achieved. One-way ANOVA across all neurons was used to predict when there were no differences in the spike counts between bins during the dry stimulus period: the numbers of spikes per bins were not statistically different from 62-120 sec (i.e., the activity became stable, p>0.05). The average wet response for HT-CS + DS neurons, 0.72 ± 0.22 spikes/sec (light gray in Figure 1D), was significantly smaller than that for LT-CS + DS neurons (t-test, two tailed; p<0.0001). However, the average dry responses for HT-CS + DS neurons (12.41 ± 1.15 spikes/sec) were not statistically different from LT-CS + DS neurons (t-test, two tailed; p=0.2274). Figure 1E shows that nearly four times as many RFs of these neurons (squares) were found in the periphery than in the center (19 vs. 5). The chi-square analysis demonstrated that LT-CS + DS neurons had significantly more RFs in the center of the cornea than HT-CS + DS neurons (p=0.0016, with Fisher’s exact test). Conversely, significantly more RFs of HT-CS + DS neurons were found in the periphery than the center of the cornea compared with those of LT-CS + DS neurons.
The third type of DS neurons, cold-insensitive (CI) + DS neurons (5/47; 10.6%), showed little or no wet response followed by either slow or rapid increases in activity until it reached a stable dry response (Figure 1C). The pattern of dryness-induced discharge was indistinguishable from HT-CS + DS neurons, but we separated these two classes based on entirely different temperature sensitivities, as described below. The average dry responses of the CI + DS neurons (10.18 ± 3.08 spikes/sec; Figure 1D) were not statistically different from LT-CS or HT-CS + DS neurons (p=0.2550, One-way ANOVA with Bonferroni post-hoc tests), while the wet responses were significantly smaller than LT-CS + DS neurons (p<0.0001, One-way ANOVA with Bonferroni post-hoc tests) but not from HT-CS + DS neurons (p>0.05). The RF of only one CI + DS neuron (solid triangle), which was located in the cornea’s periphery, could be identified using an ice-cold metal probe (Figure 1E), confirming their insensitivity to the cold stimulus.

In addition, as described in the beginning of the results, seven neurons could not be classified as any of the three types described above. These neurons showed dryness-induced patterns very similar to HT-CS or CI + DS neurons while their responses to cooling and heating stimuli were very much like those of LT-CS + DS neurons. One of the unclassified neurons showed mechanical sensitivity to scratching of the cornea with 0.16 g von Frey filament. Their RF locations were all in the periphery of the cornea (Figure 1E).

Responses to thermal stimuli: Three types of DS neurons displayed distinctly different sensitivities to cooling and heating stimuli applied to the cornea. These differences are shown in Figures 2A-F (individuals) and in Figure 2G (average). LT-CS + DS neurons showed the lowest thresholds (-0.19 ± 0.04°C) and the largest magnitudes of evoked response (724.18 ± 74.87 spikes/stimulus) to the cooling stimulus (Figure 2B), while CI + DS neurons had no thresholds (>−12°C change) and no evoked responses to cooling (Figure 2F). The values for the HT-CS + DS neurons were between those of LT-CS + DS and CI + DS neurons (−3.59 ± 0.59°C and 188.17 ± 34.75 spikes/stimulus, respectively). Figure 2G shows that the neuronal types greatly impacted the magnitudes of cold-evoked responses (p<0.0001, one-way ANOVA) with post-hoc analyses indicating the significant differences between LT-CS and HT-CS or CI + DS neurons but not between HT-CS + DS and CI + DS neurons. For the threshold, the differences between LT-CS and HT-CS + DS neurons were highly significant (p<0.0001, t-test, two-tailed): the statistics were not performed for CI + DS neurons since they did not show any response to cooling (no threshold; indicated by “?” in Figure 2G).

There were also differences in the heat-evoked responses. The evoked responses to heat were
observed in all 14 LT-CS + DS neurons tested with 44-45°C (Figure 3A), whereas only 6 of 20 HT-CS + DS
(Figure 3B) and 1 of 5 CI + DS neurons (Figure 3C) showed responses to heat. Figure 3D demonstrates that
the neuronal types were a significant factor in determining the response magnitudes to heat: greater response
in LT-CS + DS than HT-CS + DS or CI + DS neurons (respectively, 491.43 ± 92.99, 35.25 ± 28.47, 31.80±
35.55 spikes/stimulus) (p<0.0001; one-way ANOVA). The post-hoc analysis indicated that the differences in
heat-evoked response magnitudes were significant between LT-CS + DS and HT-CS + DS (p<0.0001) or CI +
DS neurons (p<0.001) but not between HT-CS + DS and CI + DS neurons. The thresholds for heat-evoked
responses were lower for the LT-CS + DS neurons (42.58 ± 0.57°C) than for the HT-CS + DS (43.89 ± 0.79°C)
and CI + DS neurons (44.34°C) but the difference between LT-CS + DS and HT-CS + DS neurons was not
statistically significant (p=0.1927, t-test, two-tailed). Because all LT-CS + DS and only a small number of HT-
CS + DS and CI + DS neurons showed the heat-evoked responses, we sought to determine if the sensitivities
to cooling and heating were related. The Pearson correlation coefficient (Figure 3E) revealed that indeed the
greater cool-evoked responses tended to produce greater heat-evoked responses for LT-CS + DS neurons
(p=0.006, n=14) or LT-CS + DS and HT-CS + DS neurons combined (p<0.0001; n=20), suggesting a common
mechanism underlying cold- and heat-evoked responses. However, the correlation for HT-CS + DS neurons
alone was not significant (p=0.1426; N=6) and was not performed for one CI + DS neuron.

In addition, LT-CS + DS neurons occasionally exhibited bursting discharges during cooling and heating
stimuli as reported for the CS corneal afferents in other studies (Carr et al. 2003; Parra et al. 2010). These
response characteristics were not observed in HT-CS + DS neurons. Also in LT-CS + DS neurons when the
cooling pulse ended and the warming pulse began, there was a near cessation of the activity followed by a
gradual return to pre-stimulus level of activity during the steady state 31°C adapting temperature (Figure 2A
and 3A).

The adapting temperatures and the neuronal classification.

Because it has been known that the cooling sensitivity of innocuous cold thermoreceptors depends on the
adapting temperature from which the cooling stimulus is presented (Kenshalo and Duclaux 1977), we applied
the cooling stimuli from two different adapting temperatures in the select numbers of DS neurons. Figure 4A-B
show that regardless of the adapting temperature, the ranges of thresholds to cooling that defined three types
of neurons were mostly non-overlapping with 4 exceptions (arrows). Figure 4A shows that when using the
31°C adapting temperature the ranges of cooling threshold were 30.6-30.9°C (0.01-0.25°C changes) for LT-CS + DS neurons (units 1-13), 20.5-30.0°C (1.0-10.5°C changes) for HT-CS (units 14-24), and <19°C (greater than 12°C changes) for CI + DS neurons (units 25-28). The same neurons then were tested with cooling from the 35°C adapting temperature and their thresholds are shown in Figure 4B: the range of thresholds was 33.5-34.9°C (0.1-1.5°C changes) for LT-CS + DS neurons, 23.8-34.1°C (0.9-11.2°C changes) for HT-CS + DS, and <19°C (greater than 12°C changes) for 2 CI + DS neurons, but for the other 2 CI + DS neurons we could observe the thresholds at 23.5 and 27.7°C (rightmost panel in Figure 4B). One CI + DS neuron was not tested with 35°C adapting temperature. Two HT-CS + DS neurons, as classified by cooling from the 31°C adapting temperature (arrows in Figure 4B), may be considered LT-CS + DS neurons if the 35°C adapting temperature were used. Similarly, two CI + DS neurons (solid squares) may become HT-CS + DS neurons. Furthermore, regardless of the adapting temperatures, the neuron type was a significant factor in determining the cooling thresholds (p=0.0004, two-way ANOVA with repeated measure), justifying the present classification scheme.

Also Figure 4C illustrates that the differences in thresholds to cooling from two adapting temperatures were not significant for LT-CS + DS neurons (p=0.078, paired t-test, two tailed) but were marginally significant for HT-CS + DS neurons (p=0.041, paired t-test, two tailed), requiring stronger cooling from a 35°C adapting temperature.

Responses to osmotic (mannitol) and menthol stimuli

Our previous study (Hirata and Oshinsky 2012) indicated that CS + DS neurons showed much greater responses to menthol than mannitol, compared to CI corneal afferents. However, in that study not every neuron received the applications of both chemicals and moreover the temperatures of the stimulus was not maintained constant in a previous study. Also, we now find that the previously identified CS + DS neurons consist of two types. Therefore, to evaluate the differences in chemical sensitivities of all three types of DS neurons more clearly, the same doses of menthol (50 µM) and mannitol (585 mOsm) were applied to all neurons at a 31°C bath temperature. Figure 5A-D shows that in all three types of neurons, there was a reciprocal relationship between menthol- and mannitol-evoked responses (i.e., the neurons with the greater menthol response displayed the smaller mannitol response and vice versa) with a minor exception (1/12 LT-CS + DS neurons, 2/19 HT-CS + DS and 0/5 CI + DS neurons; see dotted lines in Figure 5D). Moreover, this relationship depended highly on the neuronal types as demonstrated in Figure 5D and 5E (p=0.0390; one-way
ANOVA). The average mannitol responses were significantly smaller than the menthol responses for LT-CS + DS neurons (77.08 ± 15.92 and 410.33 ± 61.17 spikes/stimulus, respectively; p<0.0003, t-tests, two-tailed), whereas the respective values for HT-CS and CI + DS neurons yielded the opposite relationship [259.43 ± 31.25 and 86.00 ± 34.10 spikes/stimulus for HT-CS + DS neurons (p=0.0004, t-tests, two-tailed), and 262.40 ± 70.03 and 11.20 ± 12.52 spikes/stimulus for CI + DS neurons (p=0.0176, t-tests, two-tailed)]. However, two-way ANOVA (neuron types x chemicals) showed that the neuronal types, although close, were not a significant factor in determining the overall chemical responses (p=0.0771) presumably due to the similarity between HT-CS and CI + DS groups (Figure 5D and 5E).

The relationship between dryness-induced vs. mannitol- or menthol-evoked response

To infer the mechanisms underlying the dry response in three types of DS neurons, we performed multiple correlations between the dry response and the mannitol-, cooling- or menthol-evoked response to determine which stimuli contribute more significantly to the dry response. Figure 6 shows that only one statistically significant correlation among these variables was observed: the correlation was highly significant between the dry and mannitol-evoked responses when the HT-CS + DS population alone (p=0.0023, Pearson correlation; Figure 6B), or the HT-CS and CI + DS neurons combined were analyzed (p=0.0003, Pearson correlation; graph not shown). The following comparisons were not statistically significant: between the dry and mannitol-evoked responses in LT-CS + DS population (Figure 6A), or between the dry and menthol- or cold-evoked responses in any of the three populations (Figure 7C-F; data for CI + DS neurons not shown).

Response to capsaicin

Because we observed a robust heat-evoked response in all LT-CS + DS but only a few in HT-CS + DS and CI + DS neurons, we investigated to see if the heat response in our DS neuronal population is due to TRPV1 activation. Fourteen DS neurons were subjected to an ocular application of 10µM capsaicin (CAP) at a 31°C bath temperature. Figure 7A-F shows the responses to cooling and heating along with their CAP sensitivities in three types of DS neurons. Of the six LT-CS + DS neurons that showed vigorous responses to heat (thresholds of 39.0-44.9°C), 10µM CAP excited all neurons (Figure 7A-B). Two HT-CS + DS neurons that showed heat-evoked responses were also activated by CAP (Figure 7C-D) and the other three HT-CS + DS neurons that did not show heat responses did not produce CAP-evoked responses. One CI + DS neuron that showed a small heat response was also activated by CAP (Figure 7E-F) while two CI + DS neurons that did
not produce heat responses were not excited by CAP either (not shown). The differences in proportions of
the CAP responders vs. non-responders for three types of neurons were statistically significant (p=0.0195, Chi-
square, Fisher’s exact test). Despite the observations that the CAP-evoked responses were only present in
neurons with the heat-evoked responses, the magnitudes of responses to heat among the responders (N=9)
were not correlated with magnitudes of responses to CAP when all types were analyzed (Figure 7G;
p=0.6532).

DISCUSSION

The main finding of the present study is that the corneal afferent neurons excited by drying of the cornea (DS
neurons) are divided into three types based on the combined criteria of their patterns of discharge during the
dry and wet cornea states, and their temperature sensitivities. Our previous study (Hirata and Oshinsky 2012)
presumably placed LT-CS + DS and HT-CS + DS neurons classified in the present study into one big category,
cold-sensitive (CS) neurons. However, the use of a high-resolution thermal stimulator in the present study
made it evident that LT-CS + DS and HT-CS + DS neurons displayed sufficiently dissimilar sensitivities to
cooling and therefore deserved different categories. Also the patterns of discharge and their responsiveness to
chemical stimuli clearly separated LT-CS + DS neurons from HT-CS + DS and CI + DS neurons. LT-CS + DS
neurons showed greater sensitivity to heat than the HT-CS + DS and CI + DS (Figure 3D). The distinction
between HT-CS + DS and CI + DS neurons, however, was subtle because they shared similar patterns of
discharge, RF locations, and chemical sensitivities. However, the complete lack of responses in CI + DS
neurons to 19°C or even to 6°C in some cases (Figure 2E) and to menthol were so different from LT-CS + DS
and HT-CS + DS neurons that they warranted the separate categorization. We acknowledge that classifying
the neurons into cold-insensitive purely on the basis of temperature sensitivity is not a trivial endeavor because
the definition relies entirely on the ability of the thermal stimulator to achieve desired temperatures. Our
temperature controller had a limit of 45°C at the higher end and ~6°C for the coldest temperature. Adding to
this complexity is the findings of the cutaneous nociceptors responding to sub-zero temperatures of the skin,
e.g., -18°C (Simone and Kajander 1997), suggesting some of our CI neurons may become CS, had we used
sub-zero temperatures. Moreover, another unresolved issue is that we found seven unclassified DS neurons
that showed the combination of features that defined the three types discussed above. These neurons
demonstrated the cooling sensitivity like LT-CS + DS neurons (less than 1°C change) while their discharge
patterns and RFs loci were similar to HT-CS + DS or CI + DS neurons. It is possible, therefore, that neurons with mixed characteristics such as these could be found in future studies. It is important to note, however, that none of the neurons with the discharge pattern of LT-CS + DS neurons had the cooling thresholds larger than 1°C changes, indicating the tight relationship between discharge pattern and cooling threshold in this class of DS neurons.

The detailed electrophysiological characterization of LT-CS (primarily TRPM8-mediated) and HT-CS (primarily potassium channel-mediated) neurons in the cultured TG neurons in vitro has appeared previously (Madrid et al. 2009). However, the differences between this and the present study should be noted. First, Madrid et al. used a warmer adapting temperature (35°C) from which the cooling stimulus was presented to determine their thresholds to define neuronal classes, while 31°C was the adapting temperature of the present study. This could have influenced the cooling thresholds and therefore the classifications. The results, however, showed that the differences in adapting temperatures did not fundamentally alter the classification scheme we employed (Figure 4A-B). Furthermore, the range of cooling threshold for LT-CS neurons in the Madrid et al. study was much broader than ours: 34-25°C (i.e., 1-10°C cooling from a 35°C adapting temperature), compared with 34-33°C (i.e., less than 2°C cooling from 35°C adapting temperature). Thus, only a small subset of the LT-CS neurons (a population with the lowest thresholds to cooling) would have been the LT-CS + DS neurons and that a much larger population of the LT-CS neurons would be considered HT-CS + DS corneal afferents. Also the sample in Madrid et al. study came from the TG neurons in culture, which likely included non-corneal population. These observations suggest that DS neurons in the present study are probably the subset of the CS neurons reported by Madrid et al. and that HT-CS + DS neurons may be further subdivided into types that can be differentiated by varying expression levels of the molecular sensors such as TRPM8 and K⁺ channels (Madrid et al. 2009).

LT-CS + DS neurons had highly irregular discharge patterns during the dry cornea while displaying a very regular high rate of discharge during the wet cornea conditions. Their responsiveness to gentle cooling is the characteristic reminiscent of the classical "innocuous" cold thermoreceptors found in cutaneous regions of the body (Hensel 1974). Their role as a producer of basal tears has only recently been demonstrated (Parra et al. 2010). The present study further corroborates that conclusion. The discharge pattern of the LT-CS + DS neurons suggests that their activation is rather immediate upon and throughout corneal dryness, as seen more
clearly in the average data (Figure 1D). Numerous sudden increases in activity during dry corneal conditions may indicate that they are extremely sensitive to a rapid rate of minute cooling on the corneal surface as the dynamic responses of the “cold” thermoreceptors are largely determined by the rate of temperature change (Molinari and Kenshalo 1977). Thus, these spikes may well reflect the sudden change in local temperature that occurs when cool room air is blown over the RFs. Their extremely stable wet response (Figure 1A) means that a small deviation can be used as a significant signal (i.e., dry response) for eliciting an output of tears, a characteristic suited for basal tearing. The location of their RFs (the center of the cornea) is also indicative of their role as a basal tear producer since these areas are the first to be exposed to the air when dryness begins, as the meniscus will pull the tears toward more peripheral parts of the cornea (King-Smith et al. 2008). Our previous study (Hirata and Oshinsky 2012) showed that the units with lower thresholds to cooling (CS neurons) were more susceptible to the TRPM8 antagonist, BCTC, than Cl neurons, suggesting that TRPM8 activation is most likely the molecular counterpart underlying the dry response of the LT-CS + DS neurons (see Figure 6B in our previous study). TRPM8 as a molecular sensor of gentle cooling of the environment has been firmly established (Bautista et al. 2007; Colburn et al. 2007; Dhaka et al. 2007; McKemy et al. 2002). It was surprising, therefore, to discover that cooling or menthol response was not significantly correlated with the dryness-induced activity of the LT-CS + DS neurons (Figure 6C-F), suggesting that cooling and menthol stimulation play a minor role in the production of the dry response. It is possible that the intensity of cooling (12°C change) and concentration of menthol (50µM) used in this study might have stimulated additional receptors such as TRPA1 (Karashima et al. 2007) to mask the TRPM8 contribution. TRPA1 is unlikely a significant player, however, as our previous study demonstrated that the TRPA1 antagonist failed to block the dry response of the DS neurons (Hirata and Oshinsky 2012), pointing to other thermo-sensitive or menthol-sensitive channels as additional contributors of the dry response in LT-CS + DS neurons.

The present study found that 100% of the LT-CS + DS neurons as defined in this report responded to heat stimuli (44-45°C; paradoxical response). A proportion of cutaneous innocuous cold thermoreceptors that showed a “paradoxical” response to heat (45°C) in earlier studies varies from 20% (Long 1973) to 40% (Kenshalo and Duclaux 1977). Recent findings indicate that these values could be much higher (72%) with lower thresholds for activation (Parra et al. 2010). The differences in the proportions between Para et al. study and the current study suggest that LT-CS + DS neurons may represent a subset of the CS neurons reported in
Para et al. study (i.e., those displaying the lowest thresholds for cooling and heat stimuli). Furthermore, the present study found that 100% of LT-CS + DS neurons showing the paradoxical discharges to heat were also responsive to capsaicin, suggesting the existence of both TRPV1 and TRPM8 receptors on corneal nerve terminals. This conclusion is also supported by the present observation that the exquisite sensitivity of the LT-CS + DS neurons to cooling was highly correlated with the sensitivity to heat as well (Figure 3E). The evidence for co-localization of TRPV1 and TRPM8 in sensory ganglia (Abe et al. 2005; Babes et al. 2004; Dhaka et al. 2008; McKemy et al. 2002; Parra et al. 2010; Reid et al. 2002; Takashima et al. 2007; Viana et al. 2002; Xing et al. 2006) has been in existent for some time, although other studies showed the extent of the co-expression to be insignificant (Kobayashi et al. 2005).

The second type of DS neurons, HT-CS + DS neurons, with their slowly increasing activity to an eventual stable level of response during the dry cornea condition, implicates a different function. They are likely the instruments of a last-ditch effort by the anterior eye to protect itself. Such a mechanism should be strongly activated by the nociceptive stimuli: HT-CS + DS neurons show exceptional sensitivity to hyperosmolar tears. The osmolarity of the mannitol solutions used in our study was clearly in the noxious range (Liu et al. 2009). Their sensitivity to colder temperatures, compared to the LT-CS + DS neurons (Figure 2G), is also consistent with their nociceptive function. Many of these neurons are located in the periphery of the cornea, which is the last place on the cornea to be exposed to the air and is also expected to hold hyperosmolar tears. These observations suggest that they are suited to perform functions such as nociception-driven tearing to complement the first mechanism via LT-CS + DS neurons (basal tearing), to replenish the healthy tears and to dilute the unwanted (hyperosmolar) tears. Hypertonic saline leads to breakdown of the proteostasis (Burkewitz et al. 2011) and the formation of inflammatory mediators at cellular level (Luo et al. 2005).

The present study revealed that the only stimulus significantly contributing to production of the dry response was the mannitol stimulus in the HT-CS + DS neurons (Figure 6), indicating that the response to the hyperosmolar stimulus largely explains the dry response of HT-CS + DS neurons. It was also found that the dry response reaches the plateau after 30-60 sec (Figure 1B-D), suggesting that the osmolarity of the tears under a prolonged dry condition of the cornea does not continue to climb much beyond 500-600 mOsm. Our previous studies demonstrated, however, that DS neurons are capable of responding to osmolarities beyond 1000 mOsm (Hirata and Meng 2010). Furthermore, the activity plateau during the corneal dryness was seen
even when the tears were continually removed from the corneal surface with a filter paper in the same experimental conditions (unpublished observation). These results suggest that mechanisms other than tear production by the lacrimal glands are regulating the osmolarity of the extracellular milieu from becoming an injurious stimulus. Corneal epithelia and endothelia possess aquaporin (AQP) 5 and AQP 1 (Thiagarajah and Verkman 2002), respectively, to transport water from the epithelial and endothelial cell layers, respectively. This process can potentially maintain the osmolarity equilibrium in the immediate extracellular environment of the nerve terminals and thus the neural activity plateau seen in our preparations.

Another possible function of HT-CS + DS neurons is their role in ocular pain induced by noxious level of cold stimulation of the cornea. These neurons, compared to LT-CS + DS neurons, require a stronger cooling, ~4°C on average, (Figure 2D, 2G) to be activated. The previous studies demonstrated that in normal humans a minimum of 4-5°C cooling on ocular surface was necessary for irritation and stinging sensations to arise (Acosta et al. 2001b; Acosta et al. 2001a). The same group of investigators also have found that this level of cooling was the minimum adequate stimulus for evoked tearing in humans above and beyond the basal tearing presumably elicited by the different mechanisms (Acosta et al. 2004).

CI + DS neurons showed dryness-induced activity very similar to HT-CS + DS neurons’ but the temperature sensitivity was quite unique. The inability of the CI + DS neurons to respond to the normal physiological range of environmental temperatures (~35-19°C), plus their intense sensitivity to hyperosmolar tears, will likely make them the “pure” osmosensors. Osmosensitivity in DS neurons may derive from mechanical responses since some osmo-sensitive neurons, particularly those responding to a relatively low hypertonicity (Zhang et al. 2008), are sensitive also to changes in cell volume (Bourque 2008). However, the HT-CS + DS and CI + DS neurons in the present study were unresponsive to mechanical stimulation, although in our most recent studies (unpublished observation) we found one DS neuron (unclassified) that showed a clear sensitivity to mechanical stimulation of the RF with a von Frey filament. This finding, however, should be interpreted against hundreds of DS corneal afferents examined thus far showing no mechanical sensitivity (Hirata and Meng 2010; Hirata and Oshinsky 2012). It is safe to conclude, therefore, that the mechanical response in these neurons is extremely rare. The functional significance of the osmosensitivity in CI + DS neurons cannot be known from this study but they may play a role in the tearing under extremely cold condition as speculated before (Hirata and Oshinsky 2012).
Finally, the present study recognizes tear hyperosmolarity as a critically important stimulus for the dry response in the DS corneal afferents (Figure 6) that likely contributes to tearing function in some significant way. It is ironic, therefore, that the dry eye (DE) patients known to possess hyperosmolar tears are not able to produce normal tears (DEWS 2007), adding to the already mounting evidence that perhaps the corneal nerves responsible for tearing such as DS neurons might be in a dysfunctional state in DE patients (Dastjerdi and Dana 2009; Erdelyi et al. 2007; Villani et al. 2007). In addition, although the corneal afferents have been extensively studied and characterized (Acosta et al. 2001a; Belmonte and Giraldez 1981; Brock et al. 2001; Gallar et al. 1993), the importance of the DS neurons to the ocular functions cannot be overstated enough. Ocular dryness is likely a significant stimulus for eliciting some forms of ocular pain in pathological conditions. Clinical evidence appears to indicate that preventing ocular dryness by wetting (Jacobs and Rosenthal 2007) or simply by closing the eyes (personal communication with Dr. Reza Dana of Harvard) often alleviates the pain of the DED, and conversely moving air over the ocular surface exacerbates the DE pain (Rosenthal et al. 2009).

In conclusion, our observations suggest the potential importance of these three classes of DS neurons in different sensory and reflex roles, possibly utilizing discrete sets of molecular sensors. The investigation into the molecular mechanisms underlying the dry responses in DS neurons may lead to unraveling the pathogenesis underlying various ocular disorders including DED.

**Acknowledgement:** We thank Ms. Marnie Cooper for technical help. This work was supported by the Thomas Jefferson University Pilot Research Award and NIH Grant EY020667. No conflicts of interest, financial or otherwise, are declared by the author(s).

**Reference Lists**


Figure Captions

Figure 1. A. Peri-stimulus time histogram (PSTH) of a LT-CS + DS neuron showing the irregular firing pattern during dry cornea, characterized by numerous sudden increases (*) in activity. Brief spiking at the beginning and end of the dry period in A and also in B were mostly the cold stimulation produced by removing and applying, respectively, the artificial tears to the anterior eye. They also included artifacts created by removing and placing the plastic fluid well. B. PSTH of a HT-CS + DS neuron showing a slow increase in firing over 30-60 sec to a stable level of activity after the dry stimulus onset. C. PSTH of a CI + DS neuron showing a similar pattern of activity to HT-CS + DS neuron in B during the dry cornea except that a stable discharge level was substantially lower. The neurons in A, B, C, here are the same neurons shown, respectively, in Figures 2A, C, E, and Figures 5A, B, C. D. The average PSTHs to dry and wet stimuli in LT-CS + DS (black), HT-CS + DS (light gray) and CI + DS neurons (dark gray). The error bars were omitted for clarity. E. Receptive field locations of three types of DS and unclassified neurons on the left cornea. The outermost circle represents the limbus-corneal border. The corneal surface is divided into peripheral and central zones (dotted circle), and superior, inferior, nasal, and temporal areas (interrupted lines).

Figure 2. Temperature responses of a LT-CS + DS (A), a HT-CS + DS (C), and a CI + DS neuron (E) to cooling of the cornea to 19°C and heating to 44-45°C from 31°C (first two cooling pulses) and 35°C adapting temperatures (last cooling pulse). The upper panels in each graph are temperature traces. The thresholds for activation (vertical dotted lines) by cooling down to 19°C in the same neurons are shown in B, D and are
indicated by the temperatures beside the arrows. The temperature response of a CI + DS neuron in E was nil to even extreme cold stimuli (~6°C; the last cooling pulse). Notice the lowest temperature on the y-axis at 0°C in E compared to 10°C in A and C. G. The average evoked responses and the thresholds to cooling in three types of DS neurons. A question mark for the threshold of CI + DS neurons indicates that these neurons did not produce a response to the coldest temperature used (19°C). *** p<0.001; **** p<0.0001 vs. LT-CS neurons.

Figure 3. Temperature responses of a LT-CS + DS neuron (A), a HT-CS + DS neuron (B), and a CI + DS neuron (C) to cooling from 31°C to 19°C followed by heating from 31°C to 44-45°C. The arrows point to the heat-evoked responses. D. The average evoked responses and the threshold temperatures to heating in three types of neurons. The sample sizes are shown in parentheses. A lack of response was counted as zero to obtain the averages in HT-CS and CI + DS neuron groups. *** p<0.001; **** p<0.0001 vs. LT-CS neurons. E. A scatter graph illustrating the strong association between cool- and heat-evoked responses in LT-CS + DS and HT-CS + DS neurons. Arrows indicate the data derived from the neurons depicted in Figures A and B. The CI + DS neuron shown in C is not included in this scatter graph because, although it produced a heat-evoked response, the cool-evoked activity was not observed.

Figure 4. A. Threshold temperatures at which cool-evoked responses were observed from 31°C adapting temperature in thirteen LT-CS + DS, eleven HT-CS + DS, and four CI + DS neurons. Notice the non-overlapping distributions of thresholds for three populations. The data points for CI + DS neurons are not shown because all 4 neurons failed to produce the responses to cooling to 19°C (<19°C in the right most column). One CI-CS + DS neuron was not tested at the 35°C adapting temperature and therefore not shown in this graph. B. Thresholds for the same neurons shown in A to cooling from the 35°C adapting temperature. Notice except for 4 DS neurons (arrows), three populations defined in A did not overlap. C. The average thresholds (°C changes) to cooling from two adapting temperatures. N=13 for LT-CS, and N=11 for HT-CS neurons. NS p> 0.05; * p< 0.05
Figure 5. PSTHs of a LT-CS + DS (A), a HT-CS + DS (B), and a CI + DS neuron (C) in responses to hyperosmolar (585 mOsm mannitol) and cooling stimuli (50μM menthol). Notice that two stimuli were presented in different order. D. Graphs demonstrating the reciprocal relationships between mannitol- and menthol-evoked responses for individual LT-CS + DS vs. HT-CS + DS and CI + DS neurons with small exceptions (dotted lines). E. The average mannitol- and menthol-evoked responses for LT-CS+DS (N=12) vs. HT-CS + DS (N=14) and CI + DS neurons (N=5). * p< 0.05, ** p< 0.01, *** p< 0.001

Figure 6. Scatter graphs showing the relationships between dry responses and mannitol-, menthol- or cool-evoked responses in LT-CS + DS (A, C, E; N=12) and HT-CS + DS neurons (B, D, F; N=14). X-axis values in A-D were derived by dividing the total spikes/stimulus by 30 sec to yield spikes/sec. Dry responses in these graphs were the averages of the responses to three dry stimuli (See Methods). The graphs for the CI + DS neurons are not shown because of a small sample size (N=5). R² and P (probability) from Pearson correlation are also shown in each graph.

Figure 7. PSTHs illustrating the relationship between responses to heat stimuli (A, C, E), and to ocular application of capsaicin (B, D, F) in a LT-CS (A, B), a HT-CS (C, D) and a CI + DS neuron (E, F). Y-axis scales in C-D are different from the other figures to make the cold- and heat-evoked responses easier to view. G. A scatter graph showing the lack of relationship between heat- and CAP-evoked responses in all three types of DS neurons that responded to CAP.
Figure 1
Figure 2

Temperature (°C)

SPikes/sec

Time

Spikes/sec

Time

Evoked Response

Threshold

Figure 2

Cool-Evoked Response

DS Neuron Type

G

Evoked Response

Threshold

Cool-Evoked Response

DS Neuron Type

LT-CS + DS

HT-CS + DS

CI + DS

Cool Threshold (°C)

Evoked Response

Threshold

Cool-Evoked Response

DS Neuron Type

LT-CS

HT-CS

CI

(17)

(24)

(5)

(19)
Figure 3

A. LT-CS + DS

B. HT-CS + DS

C. CI + DS

D. Heat-Evoked Response

E. LT-CS + DS (n=14) and HT-CS + DS (n=6)

\[ R^2 = 0.61107 \]
Figure 5

A  LT-CS + DS

B  HT-CS + DS

C  CI + DS

D  HT-CS + DS

E  Evoked Response (Total Spikes/ Stimulus)

N=12

N=19

Mannitol

Menthol

***

***

*

Evoked Response

Menthol

Mannitol

LT-CS  HT-CS  CI

DS Neurons
Figure 6

A. LT-CS + DS

B. HT-CS + DS

C.

D.

E.

F.