Cold-Sensitive Corneal Afferents Respond to a Variety of Ocular Stimuli Central to Tear Production: Implications for Dry Eye Disease

Harumitsu Hirata^{1,2} and Ian D. Meng¹

PURPOSE. To investigate the response characteristics of the corneal afferents that detect ocular conditions critical to the activation of the “afferent limb” of the lacrimation reflex.

METHODS. In isoflurane-anesthetized male rats, trigeminal ganglia were explored extracellularly in vivo to identify the corneal neurons that can be activated by ocular stimuli important to lacrimation. After verifying their receptive field loci to be restricted to the cornea, neural response properties were characterized with a variety of stimuli, such as drying and wetting of the cornea, by applying and removing artificial tears, temperature changes (35°C–15°C and 39°C–51°C), menthol (10–100 μ M), and hyperosmolar solutions (NaCl, sucrose; 297–3014 mOsm), applied to the ocular surface.

RESULTS. A specific type of corneal afferent was identified that responded to drying of the ocular surface. These neurons were classified as innocuous “cold” thermoreceptors by their responses to steady state and dynamic temperature changes applied to the cornea. In addition to drying and slight cooling (<1°C) of the corneal surface, these neurons were excited by evaporation of tears from the ocular surface and hyperosmolar tears. Moreover, these neurons were activated by noxious thermal stimulation and menthol applied to the corneal surface.

CONCLUSIONS. These results demonstrate that innocuous “cold” cornea thermoreceptors are activated by drying of the ocular surface and hyperosmotic solutions, conditions that are consistent with a role in tear production. The authors hypothesize that the dysfunction of these corneal afferents and the lacrimation reflex pathway they activate lead to some forms of dry eye disease. (*Invest Ophthalmol Vis Sci.* 2010;51:3969–3976) DOI: 10.1167/jovs.09-4744

Dry eye disease (DED) is a chronic disorder that results in part from inadequate or altered tear film on the anterior ocular surface. Earlier studies have shown that sensory stimulation of the cornea and conjunctiva is essential in initiating the lacrimation reflex in humans.^{1,2} Several types of corneal and conjunctival afferents have been identified.^{3–6} Activation of two classes of corneal primary afferents, polymodal and mechanoreceptive neurons, elicits reflex tearing, blinking, and pain. The neural regula-

tory mechanisms responsible for maintaining normal tear film, through basic (basal) tear production, are still unknown. Understanding these regulatory mechanisms is especially important because dysfunction of the corneal sensory nerves^{7–10} and of the neurons in the central nervous system¹¹ involved in basic tearing has been hypothesized to contribute to the pathogenesis of DED. Because the “cold” sensitive ocular thermoreceptors are the only sensory afferents that exhibit spontaneous activity, it was speculated that the discharge of these afferents is necessary for the production of basic tears.¹² However, the detailed response properties of these neurons to ocular stimuli important to lacrimation have not been examined. Furthermore, a previous study¹³ reported the existence of neurons in the rostral spinal trigeminal nucleus caudalis that monitor the fluid status of the ocular surface and modulate tear production in rats. Because the evoked tears are produced and blocked, respectively, by glutamate and muscimol injections into the vicinity of these neurons, which previously were shown to project to superior salivatory and facial nuclei,¹⁴ it was hypothesized that these neurons form a central component of the lacrimation reflex circuit. However, it has not been determined what specific cornea afferents activated these central neurons.

The present study identifies and characterizes the primary afferent neurons innervating the cornea that respond to the types of ocular stimuli that will produce tears and, therefore, may serve as the “afferent limb” of the lacrimation reflex. We report that these corneal afferents can be classified as cold neurons, and we discuss the potential contribution to and significance of these afferents to DED.

METHODS

Surgery and Recordings

Under 2.0% to 2.5% isoflurane anesthesia, the femoral vein and artery of male Sprague-Dawley rats were catheterized, respectively, for fluid injections and mean arterial pressure (MAP) recordings. Each 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). Animals were artificially respired (Rodent Ventilator Model 683; Harvard Apparatus, Boston, MA), and the end tidal CO₂ was monitored with a CO₂ analyzer (4%–5%; CWE, Ardmore, PA). Core temperature was maintained at 37° to 38°C with a feedback-controlled regulator. Just before the recordings, the isoflurane concentration was decreased to and maintained at 1.25% to 1.5% throughout the experiment. After checking for noxious stimulation-evoked withdrawal reflexes, pancuronium bromide (0.6 mg/kg/h) was infused continuously before and during electrophysiological recordings. Any significant elevation in MAP resulted in an increased concentration of isoflurane. A tungsten microelectrode (5–9 M Ω ; FHC, Bowdoin, ME) was lowered into the left TG to search for a spontaneously active neuron after amplification and discrimination of the neural signals (BAK Electronic, Inc., Mt. Airy,

From the ¹Department of Physiology, College of Osteopathic Medicine, University of New England, Biddeford, Maine; and the ²Department of Neurology, Thomas Jefferson University, Philadelphia, Pennsylvania.

Supported by National Institutes of Health Grants K02DA018408 and R01DA021193 (IDM) and supported in part by National Institutes of Health Grant NINDS-R01NS061571.

Submitted for publication October 7, 2009; revised January 6 and February 8, 2010; accepted March 7, 2010.

Disclosure: **H. Hirata**, None; **I.D. Meng**, None

Corresponding author: Harumitsu Hirata, Department of Neurology, Thomas Jefferson University, JAH 398, 1020 Locust Street, Philadelphia, PA 19107; harumitsu.hirata@jefferson.edu.

MD). Discriminated spike outputs, MAP, and temperatures during the corneal thermal stimulation were acquired and analyzed (Micro 1401 mkII and Spike2, v5.19; Cambridge Electric Design Ltd., Cambridge, UK). Neurons that responded to room temperature saline solution applied to the eye with a brief (<4-s 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). A bipolar silver electrode was placed on the RF to activate the nerve terminal of the neuron (A360 and Pulsemaster A300; WPI Inc., Sarasota, FL) to measure afferent conduction velocity (CV). The CV was calculated by dividing the conduction distance by the latency of the action potential to electrical stimulation of the RF. The average conduction distance from the center of the cornea to the center of TG was 17.5 mm ($n = 3$). Based on the calculated CV, neurons were categorized as either A δ (1.5–15.0 m/s) or C-fibers (<1.5 m/s).¹⁵ At the end of the experiment, each animal was euthanized with sodium pentobarbital (200 mg/kg, IP). The experimental protocol was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the IACUC at the University of New England.

Dry and Wet Corneal Stimulation

After determining the afferent fiber type, the discharge rate of each unit was recorded during two conditions of corneal fluid status. The wet cornea condition (5 minutes) occurred when the cornea was moistened with 20 μ L rat artificial tears (ATs) with a sterile pipette.¹⁶ This volume was sufficient to cover the entire anterior ocular surface. The dry cornea condition (1 minute) occurred after the ATs were removed with a piece of filter paper.

Chemical Stimulation

Chemical stimuli (i.e., menthol, NaCl, sucrose) were applied to the ocular surface in 20- μ L drops immediately after removal of ATs with filter paper and were left on the cornea for approximately 1 minute. Chemical solutions were then washed off by flushing the eye with copious amounts of ATs (~4 mL).

Thermal Stimulation

Thermal stimuli were applied to the corneal surface with a Peltier-based thermal stimulator (TSII; Medoc Ltd., Ramat Yishai, Israel), which was placed using an operating microscope (Carl Zeiss, Oberkochen, Germany) next to, but not directly in contact with, the surface of the cornea. This method allowed the ATs (~50 μ L) to remain by capillary action between the ocular surface and the thermode surface, keeping it moist and bathing the nerve terminals. In addition, this method kept the position of the thermal probe constant relative to the cornea, thus providing consistent temperatures throughout a number of stimulus applications. Two temperature stimulation protocols were used: a steady state temperature series and a dynamic temperature series. In the steady state temperature series, cooling stimuli were presented to the cornea beginning with a 35°C adapting temperature followed by consecutive 2°C incremental decreases in temperature to a final temperature of 15°C, with each temperature lasting for 60 seconds (see Fig. 3). After the cooling stimulus sequence, a warming series back to 35°C was presented, also in 2°C steps. In the dynamic temperature stimulus series, 4°C step ramp cooling or heating stimuli were applied to the cornea from the adapting temperature of 35°C to 15°C or to 51°C, respectively (see Fig. 4). Each stimulus ramp lasted 20 seconds, with an inter-stimulus period of 20s, except for the noxious heat stimuli of 47°C and 51°C, which had an interval of 100 seconds, to avoid heat-induced sensitization. The rates of temperature changes depended on the magnitudes of the temperature changes and ranged from 1°C/s to 2.7°C/s for 4° step cooling and to 2°C/s to 3°C/s for 4° step heating.

Drugs

Menthol (Sigma-Aldrich, St. Louis, MO) was dissolved in 40% ethanol to make a 10-mM stock solution. The stock solution was then diluted to the required concentrations with ATs on the day of the experiment.

The solutions of NaCl and sucrose (Sigma-Aldrich) were prepared by dissolving in 0.9% saline (vehicle), and their osmolarities were measured with an osmometer (Osmomat 030; Gonotec GmbH, Berlin, Germany). The control solution for menthol was 0.4% ethanol, which was the equivalent concentration contained in the highest menthol dose (100 μ M) and was made by diluting 40% of each solution with ATs. The composition of ATs was 106.5 mM NaCl, 26.1 mM NaHCO₃, 18.7 mM KCl, 1.0 mM MgCl₂, 0.5 mM NaH₂PO₄, 1.1 mM CaCl₂, 10 mM HEPES, pH 7.45.

Statistical Analysis

Because the chemical stimuli (menthol, sucrose, NaCl) were kept at room temperature (21°C–24°C), which was colder than the average corneal temperature of the rats (31°C–33°C), responses during the first 4 seconds after the stimulus application were considered to be due to cold stimulation of the ocular surface. See Figure 5a for a depiction of these initial bursts of activities (arrow) that were constant in magnitude and duration across all doses of menthol and vehicle (0.4% ETOH). In contrast, the chemical stimulus-induced responses were slower to develop, sustained for at least 30 seconds, and dose dependent. Thus, the evoked responses to chemical stimuli in this study (response magnitude [Rmag]) were defined as the total number of spikes for 5 to 35 seconds after the stimulus application after subtracting the background activity (mean $\pm$ 2 SD based on the 30-second period preceding the stimulus). Steady state activity to 2°C cooling or warming (see Fig. 3c) was calculated based on the last 30 seconds of each of the 1-minute temperature stimuli. The dynamic responses to 4°C step cooling and noxious heating of the cornea (see Fig. 4c) were defined as the total discharges that exceeded the background activity during the 20-second stimulus periods (i.e., Rmags based on the 20-second period before stimulus). Discharge rates during dry and wet corneal conditions were, respectively, based on the averages of the last 30 seconds of a 1-minute dry cornea period and the 30 seconds immediately preceding the dry period. Statistical analyses for the effects of ocular stimulation on neural discharges were performed with ANOVA (PPC 6.5.1 [GB-STAT, Silver Spring, MD]; 12.0 [SPSS, Chicago, IL]) with or without repeated measures. Post hoc analyses were conducted with Newman-Keuls tests for individual comparisons.¹⁷

RESULTS

Receptive Fields of Corneal Afferents

The cold-sensitive RFs of the TG neurons on the cornea were identified and found to be restricted to the cornea (Fig. 1). Although the locations of the RFs were widely distributed over the corneal surface, clusters of RFs were also observed at several loci (striped oval). Excluding the units on the equatorial line ($n = 9$), twice as many cold-sensitive neurons were found on the inferior cornea ($n = 26$) as on the superior cornea ($n = 13$). However, RFs on the temporal side of the cornea ($n = 20$) were approximately equal in number to those on the nasal side ($n = 21$).

Response to Drying and Wetting of the Cornea

Discharges of all 48 cold-sensitive TG neurons were increased by drying of the cornea and depressed by wetting of the cornea with AT. Figures 2a and 2b illustrate the activity profiles of two units during dry and wet corneal surface conditions. After the removal of the ATs (upward arrows), the discharge rates gradually increased over the next 20 to 25 seconds to more stable levels. Overall, the difference between the average discharge rates during the dry (15.8 ± 0.9 spikes/s) and the wet (6.0 ± 0.7 spikes/s) conditions for all neurons was highly significant (Fig. 2c; $P < 0.01$). When, based on CV, units were grouped into A δ and C fibers, the rates of activity for the A δ fibers during the dry cornea conditions were greater than those for the C fiber afferents (17.7 ± 1.4 spikes/s vs. 13.8 ± 1.1 spikes/s; $P <$

FIGURE 1. RF locations for cold-sensitive TG neurons on the left cornea. RFs were defined by responses to a cold metal probe because all neurons were unresponsive to mechanical stimulation. The *striped lines* divide the cornea into four quadrants. Symbol sizes do not represent the extent of nerve terminals.

0.05); however, activity during the wet cornea condition for these two types of afferents was not significantly different (5.7 ± 1.1 spikes/s and 6.4 ± 0.7 spikes/s for A δ and C fibers,

respectively; $P = 0.65$). Additionally, we examined two mechanically sensitive but cold-insensitive units on the cornea and three cold-sensitive neurons with RFs on the conjunctiva for responses to drying. None of these five units exhibited differential discharge rates to dry and wet ocular conditions (data not shown).

Response to Thermal Stimulation

Forty-two cold-sensitive TG neurons were tested with quantitative thermal stimulation of the corneal surface. Figure 3a shows the typical responses to temperature changes, which were seen in 37 of the 42 units. These units displayed dynamic increases in activity in response to cooling, followed by stable rates of activity that depended on the steady state (SS) temperature (dynamic + static units). All units that exhibited dynamic increases in discharge to cooling rapidly adapted during the SS temperature within 10 seconds. In three units, one of which is shown in Figure 3b, SS temperatures were not encoded by changes in neuronal activity, though their dynamic responses to temperature changes were still present (dynamic-only units). In another two units, the dynamic components were missing, but SS activities were present (static-only units; not shown). The average SS responses to consecutive 2°C step temperatures between 35°C and 15°C were bell-shaped curves with maximum discharge occurring between 25°C and 27°C (Fig. 3c). The overall SS discharge rates were significantly higher in the cool-

FIGURE 2. Neuronal activity evoked by drying the ocular surface. (a, b) Peristimulus time histograms (PSTHs) of two cold-sensitive TG neurons during the period when the cornea was wet with ATs (wet) and dried by removal of the ATs with filter paper (dry). Brief spike increases (*upward* and *downward* arrows) are artifacts caused by the removal and application of ATs. *Insets*: ongoing action potentials of these neurons during wet and dry corneal conditions and were taken during the periods indicated between the two diagonal lines. (a) It took several seconds for the unit to reach a stable level of activity after the removal of the ATs (i.e., dry eye condition). In other units, this period was much shorter (e.g., 2–3 seconds; see b). (b) The unit demonstrated periodic bursts of activity during the dry condition (*asterisks*). In all figures, bin width for PSTHs is 1 second. (c) Average discharge rates of cold-sensitive neurons during wet and dry corneal conditions for A δ - ($n = 25$) and C afferent ($n = 23$) fibers. * $P < 0.05$, ** $P < 0.01$ (two-way ANOVA). Graph displays mean $\pm$ SEM in all figures.

FIGURE 3. Dynamic and static discharges during 2°C step temperature sequences. (a) Activity of a cold-sensitive neuron that responds during both the dynamic and the static stages of 2°C step cooling and warming stimuli. (b) Activity of another cold-sensitive neuron that responds only during the dynamic phase of 2°C step cooling stimuli. (c) Average steady state activity evoked by 2°C step cooling and warming stimuli. $n = 10$. * $P < 0.05$, ** $P < 0.01$ vs. 35°C; two-way ANOVA with repeated measures.

ing direction than in the warming direction (two-way ANOVA; $P < 0.001$). This fact, which has been termed hysteresis, has been observed previously for innocuous cold trigeminal afferents in monkeys.¹⁸ Furthermore, the SS discharge rates at 33°C to 15°C during the cooling series and at 17°C to 29°C during the warming series were significantly greater than activity at the adapting temperature of 35°C (one-way ANOVA; $P < 0.05$ and $P < 0.01$).

During the dynamic 4°C step temperature stimulus series (Fig. 4a), dynamic activity and SS neuronal activity were similar to those observed during 2°C cooling temperatures (Fig. 3a). In addition, increases in evoked activity were observed at 47°C and 51°C (Fig. 4a at arrows; so-called paradoxical discharges to noxious heat in innocuous cold neurons¹⁹). These paradoxical discharges were observed in 36 of 40 cold-sensitive units tested with 51°C. Average responses to 4°C step ramp cooling and heating stimuli for 30 cold-sensitive units are shown in Figure 4b. The responses to 27°C or colder temperatures were significantly higher than activity at the adapting temperature (35°C),

whereas only the evoked responses to 51°C were significantly different compared with activity during the adapting temperature. The R_{mag} to the 51°C stimulus was approximately four times greater than for the 47°C stimulus.

Threshold temperature for dynamic activation of cold-sensitive neurons tested with a 4°C cooling stimulus from the 35°C adapting temperature was $<1^\circ\text{C}$ (e.g., Fig. 4c). The overall average threshold among the 26 units tested for dynamic responses to 4°C cooling was $34.3^\circ\text{C} \pm 0.2^\circ\text{C}$.

Response to Menthol

Of the 36 cold-sensitive TG neurons tested with application of menthol to the cornea, 32 showed significant increases in activity above background (total $R_{mag} > 10$ spikes) by either the 50- or 100- μM concentration (Fig. 5). Four units were not excited by the highest concentration of menthol used in this study (100 μM) and were considered menthol insensitive. Average R_{mags} to increasing concentrations of menthol were dose dependent, and higher concentrations of menthol produced progressively shorter laten-

FIGURE 4. Responses to 4°C step cooling and heating sequences. (a) Example of a representative neuron demonstrating responses to both during 4°C step ramp cooling and heating stimuli. All dynamic increases in activities were in response to cooling stimuli (*asterisks*), but at the *arrows* the increases were in response to heat. (b) Average evoked responses (Rmag/stim) to 20-second 4°C step ramp cooling and heating stimuli. $n = 25$. ** $P < 0.01$ vs. 35°C; one-way ANOVA. (c) Temperature responses of a cold-sensitive afferent to 4°C step ramp cooling stimulus applied to the ocular surface. *Upper trace*: temperature change. *Lower trace*: neural activity on the same time scale. *Arrowheads*: the threshold temperature to cooling stimulus for this unit was 34.7°C. Notice that the temperature change from 31°C back to adapting temperature of 35°C (warming) produced complete cessation of activity.

cies to fire (Figs. 5a, 5b). Only 1 of 17 units tested (Fig. 5b, small cell) produced any noticeable activity to 0.4% ETOH (vehicle solution) above the pre-drug level, whereas 16 units responded to a 10- μ M or greater concentration of menthol with a total Rmag of greater than 10 spikes/stimulus.

Responses to Hyperosmolar Solutions

In one cold-sensitive afferent, we observed that the high ambient temperature and low humidity in the experimental room produced the discharge profiles shown in Figure 6. Low spontaneous activity, seen immediately after the ATs were dropped on the cornea (arrow), gradually increased over a 5- to 15-minute period until the cornea was again wet with ATs (star). The dramatic increase in discharge over this period appears to be the result of evaporation of the ATs from the ocular surface, which would have increased the osmolarity of the tears. If this were the case, the cold-sensitive afferents would also be excited by hyperosmolar solutions applied to the ocular surface. Figure 7 demonstrates that hyperosmolar solutions of both NaCl and sucrose caused significant increases in neuronal activity in a concentration-dependent manner (one-way ANOVA; $P < 0.01$). The average stimulus-response (S-R) relationship of Figure 7b could be best fit by a logarithmic function, regardless of whether the two highest osmolarity values were included [dotted curve, $y = 151 \ln x - 832$; $R^2 = 0.957$]. The S-R function for osmolarities up to 611 mOsm may be also described by a linear function (not shown; $y = 0.45 \times -117$; $R^2 = 0.9692$). Furthermore, two additional neurons were tested with osmotic solutions using a nonpermeant substance, mannitol; again, the increases in osmolarity were associated with increases in afferent activity.

DISCUSSION

This is the first study to comprehensively characterize the response properties of the specific type of corneal afferents to ocular stimuli that regulate tearing. Two principal types of tears have generally been recognized: basic tears and reflex tears. Basic tears are considered to arise from subtle stimulation of the anterior eye such as cooling and drying caused by evaporation.²⁰ Reflex tearing, on the other hand, is thought to be produced by stronger, noxious stimulation of the ocular¹³ and extraocular tissues such as the nasal mucosa.²¹ Strong cooling stimuli (-4.5°C), but not subtle cooling stimuli (-1°C), applied to the cornea significantly increased reflex tear secretion above and beyond basic tears,¹² indicating that high-intensity temperature stimulation of the ocular surface produces reflex tears and, conversely, that low-intensity stimuli are important to basic tears. Furthermore, instillation of hyperosmolar solutions to the eyes (~ 450 mOsm and higher) was reported to evoke ocular discomfort and pain in humans,²² which should provide a strong and necessary stimulus for tear production. These observations are consistent with the conclusion that the corneal afferents reported here play a key role in activating the afferent limb of the lacrimation reflex important in basic tear production. In addition, the negative feedback model of a nervous system reflex predicts that the opposite stimulations (i.e., warming and wetting of the cornea and the isosmolar tears) should lessen or even silence the activation of these neurons. Our results are consistent with this prediction. However, the possibility that they may also play a role in the eye blink reflex cannot be excluded because some stimuli that activate these afferents, such as a decrease in ocular temperature, also evoke blinking.^{23,24} Furthermore, the involvement of other ocular afferents innervating the cornea and conjunctiva, such as polymodal nociceptors^{6,25} in the tearing reflex, remains to be elucidated.

The corneal afferents in the present study were identified as innocuous cold thermoreceptors because they exhibited temperature response characteristics similar to those observed for previously described innocuous cold thermoreceptors on the skin^{26,27} and thermosensitive units innervating the orofacial tissues.^{3,28-30} Consistent with the properties of cold corneal

FIGURE 5. Menthol-evoked activity in cold-sensitive corneal afferents. (a) Action potential discharges from two units (*large action potential; #small action potential) in response to vehicle (ethanol) and menthol application to cornea. A drop of solution (cold stimulation) on the cornea evoked bursts of activity (arrow) that were not related to the menthol doses. In contrast, the menthol-related discharges were delayed, clearly separable in time, and dose dependent. (b) PSTHs from two discriminated units shown in (a). Horizontal striped lines: approximate response durations. Sizable spikes at the beginning of the stimuli are due to cold stimulation. (c) Average dose-response function for cold-sensitive corneal afferents ($n = 16$) in response to vehicle and menthol applications to the cornea. $n = 16$; ** $P < 0.01$ vs. ETOH (one-way ANOVA with repeated measures).

afferent reported by Gallar et al.,³ the cold afferents recorded in the present study displayed spontaneous activity, were exquisitely sensitive to dynamic changes in temperature, and responded to hyperosmotic stimuli. In contrast, however, our afferents in rats were able to encode the corneal SS temperatures (Fig. 3c) though cold afferents in the cat failed to do so.³ Our corneal afferents appeared to resemble the corneal cold sensitive fibers that were reported to detect cooling of -1°C and to respond to 200 μM menthol.³¹ However, the detailed responses of these units to thermal stimuli or corneal drying

were not described.³¹ Finally, innocuous cold thermoreceptors reported in the present study were clearly different from the polymodal nociceptors because our afferents responded to small temperature decreases ($<1^{\circ}\text{C}$) and showed substantial spontaneous activity yet failed to respond to CO_2 applied to the cornea ($n = 5$; data not shown).

We acknowledge the technical limitations in our studies. The ocular stimuli used to activate these corneal thermoreceptors are not mutually exclusive: drying of the cornea leads to cooling of the ocular surface and increases the osmolarity of the tear film. We propose that the dry response in innocuous cold thermoreceptors on the cornea is based on two distinct mechanisms: temperature and osmotic changes. To determine which stimulus is more relevant in producing the dry response would require a direct measurement of the changes in temperature and osmolarity that occur during corneal drying. However, we were unable to perform such measurements. Nonetheless, it is interesting to note that the maximum level of discharge attained by the optimum cornea temperature (25°C – 27°C ; Fig. 3) is only part of the activity achieved by drying of the cornea (Fig. 2), indicating that the excitation of these afferents in response to drying of the cornea involves more than simply the change in temperature. Our preliminary data showed that the dry response of the cornea afferents was partially blocked by the antagonist of the membrane receptor, TRPM8 [N-(4-*t*-Butylphenyl)-4-(3-Chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide], suggesting that these membrane channels are important in detecting the dryness of the cornea and in activating the lacrimation reflex. The existence of TRPM8 on the nerve terminals of the cold thermoreceptors on the cornea is consistent with our present findings that these afferents responded to stimulation by menthol and cool temperatures.^{32–36} Other TRP channels, such as TRPV1,^{37,38} may also be involved in sensing osmotic stress during cornea drying.

FIGURE 6. PSTH of a cold-sensitive neuron showing changes in spontaneous activity presumably caused by the evaporation of ATs. After the second wetting of the cornea (arrow), the ATs were not replenished until fresh ATs were reapplied (star) approximately 18 minutes later, at which time activity returned to its lower level.

FIGURE 7. Sensitivity of cold-sensitive afferents to the hyperosmolar solutions. (a) Action potential discharges from a single neuron in response to vehicle and two different hyperosmolar solutions applied to the cornea (arrows above action potential traces). Numbers in parentheses are osmolarities of the solutions used to activate the neuron. (b) Average concentration-response functions of neurons to ocular application of NaCl and sucrose solutions. Normal saline (0.9%) was a vehicle solution for all concentrations of both NaCl and sucrose. NaCl ($n = 16$); sucrose ($n = 9$). Rmags to all hyperosmolar solutions were significantly different compared with vehicle ($P < 0.01$; one-way ANOVA).

The importance of the corneal afferents in contributing to the vicious circle that perpetuates DED has been known for some time^{39,40} but has recently been rediscovered.^{7,9,10} Denervation of the sensory afferents (destruction of the ophthalmic division of the TG) produces severe consequences on the ocular surface system: it alters protein synthesis in^{41,42} and protein secretion from⁴³ the lacrimal gland and leads to changes in the corneal epithelial function.⁴⁴ However, lack of information about the specific population of corneal afferents that stimulates exocrine function has slowed progress in understanding the cellular and molecular mechanisms underlying the lacrimation reflex and its importance in DED processes.

Our present studies are the first step in elucidating the pathways for tear production and providing important insights into the role that the lacrimation reflex plays in the perpetuation of DED. If dysfunction of the lacrimation reflex contributes to DED, certain abnormalities are expected in dry eye (DE) patients. First, a molecular underpinning for this reflex is expected to be impaired. Although morphologic alterations of the corneal nerves are frequently observed in DE patients,^{7,45–49} it is not known what specific neural mechanisms are impaired. Our preliminary results suggest that TRPM8 membrane channels are likely involved in this process. Second,

ocular stimuli that normally produce tears, such as drying, cooling the cornea, and hyperosmolar tears, would be expected to evoke abnormal sensations and to produce diminished tears. Significantly impaired sensation to subtle cooling of the cornea was reported in DE patients.⁵⁰ Furthermore, a patient with mild DE reported a rapid and more intense discomfort sensation induced by corneal instillation of hyperosmolar solutions.²² Patients with SS display an inability to make reflex tears, consistent with autoimmune loss in lacrimal gland function, whereas patients with non-SS type DE are characterized by an ability to still produce reflex tears with a concurrent reduction in basic tears.²⁰ In addition, abnormal activity of corneal sensory afferents seen after photorefractive keratectomy in animals⁵¹ has been suggested as evidence for the origin of dry eye sensations often reported after this surgery.⁵² Finally, the central nervous system integration of external and internal inputs important to tear production may provide clues to the pathogenic mechanisms underlying DED. Hirata et al.¹³ found complex interplays of inputs from the periorbital, nasal, and ocular tissues impinging on the central neurons involved in modulation of the lacrimation reflex. From this, we hypothesize that dysfunction of central nervous system integration plays a critical role in an alteration of the lacrimation reflex and ultimately may contribute to some forms of DED.

Acknowledgments

The authors thank Suzuro Hitomi for technical help and Patricia Barry-Hirata and Michael Oshinsky for reading and editing the manuscript.

References

- Mutch JR. The lacrimation reflex. *Br J Ophthalmol.* 1944;28(7):317–336.
- Collins ET. The physiology of weeping. *Br J Ophthalmol.* 1932;16(1):1–20.
- Gallar J, Pozo MA, Tuckett RP, Belmonte C. Response of sensory units with unmyelinated fibres to mechanical, thermal and chemical stimulation of the cat's cornea. *J Physiol (Lond).* 1993;468:609–622.
- Acosta MC, Belmonte C, Gallar J. Sensory experiences in humans and single-unit activity in cats evoked by polymodal stimulation of the cornea. *J Physiol.* 2001;534(2):511–525.
- Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. *Exp Eye Res.* 2003;76(5):521–542.
- Belmonte C, Garcia-Hirschfeld J, Gallar J. Neurobiology of ocular pain. *Prog Retinal Eye Res.* 1997;16:117–156.
- Dastjerdi MH, Dana R. Corneal nerve alterations in dry eye-associated ocular surface disease. *Int Ophthalmol Clin.* 2009;49(1):11–20.
- Mathers WD. Why the eye becomes dry: a cornea and lacrimal gland feedback model. *CLAO J.* 2000;26(3):159–165.
- Dartt DA. Dysfunctional neural regulation of lacrimal gland secretion and its role in the pathogenesis of dry eye syndromes. *Ocul Surf.* 2004;2(2):76–91.
- Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res.* 2009;28(3):155–177.
- van Bijsterveld OP, Kruize AA, Bleyls RL. Central nervous system mechanisms in sjogren's syndrome. *Br J Ophthalmol.* 2003;87(2):128–130.
- Acosta MC, Peral A, Luna C, Pintor J, Belmonte C, Gallar J. Tear secretion induced by selective stimulation of corneal and conjunctival sensory nerve fibers. *Invest Ophthalmol Vis Sci.* 2004;45(7):2333–2336.
- Hirata H, Okamoto K, Tashiro A, Bereiter DA. A novel class of neurons at the trigeminal subnucleus interpolaris/caudalis transition region monitors ocular surface fluid status and modulates tear production. *J Neurosci.* 2004;24(17):4224–4232.
- Hirata H, Takeshita S, Hu JW, Bereiter DA. Cornea-responsive medullary dorsal horn neurons: modulation by local opioids and

- projections to thalamus and brain stem. *J Neurophysiol.* 2000;84:1050-1061.
15. Strassman AM, Raymond SA, Burstein R. Sensitization of meningeal sensory neurons and the origin of headaches. *Nature.* 1996;384(6609):560-564.
  16. Kessler TL, Mercer HJ, Zieske JD, McCarthy DM, Dartt DA. Stimulation of goblet cell mucous secretion by activation of nerves in rat conjunctiva. *Curr Eye Res.* 1995;14(11):985-992.
  17. Winer BJ. *Statistical Principles in Experimental Design.* New York: McGraw-Hill; 1971.
  18. Poulos DA, Lende RA. Response of trigeminal ganglion neurons to thermal stimulation of oral-facial regions, I: steady-state response. *J Neurophysiol.* 1970;33(4):508-517.
  19. Long RR. Cold fiber heat sensitivity: dependency of "paradoxical" discharge on body temperature. *Brain Res.* 1973;7(63):389-392.
  20. Tsubota K. Tear dynamics and dry eye. *Prog Retin Eye Res.* 1998;17(4):565-596.
  21. Gupta A, Heigle T, Pflugfelder SC. Nasolacrimal stimulation of aqueous tear production. *Cornea.* 1997;16(6):645-648.
  22. Liu H, Begley C, Chen M, et al. A link between tear instability and hyperosmolarity in dry eye. *Invest Ophthalmol Vis Sci.* 2009;50(8):3671-3679.
  23. Nakamori K, Odawara M, Nakajima T, Mizutani T, Tsubota K. Blinking is controlled primarily by ocular surface conditions. *Am J Ophthalmol.* 1997;124:24-30.
  24. Tsubota K, Hata S, Mori A, Nakamori K, Fujishima H. Decreased blinking in dry saunas. *Cornea.* 1997;16(2):242-243.
  25. Belmonte C, Gallar J. Corneal nociceptors. In: Belmonte C, Cervero F, eds. *Neurobiology of Nociceptors.* New York: Oxford Press; 1996.
  26. Hensel H. Thermoreceptors. *Annu Rev Physiol.* 1974;36:233-249.
  27. Kenshalo DR. Comparison of thermal sensitivity of the forehead, lip, conjunctiva and cornea. *J Appl Physiol.* 1960;15:987-991.
  28. Hutchison WD, Tsoukatos J, Dostrovsky JO. Quantitative analysis of orofacial thermoreceptive neurons in the superficial medullary dorsal horn of the rat. *J Neurophysiol.* 1997;77(6):3252-3266.
  29. Hirata H, Poulos DA, Molt JT. Differences in thermal responses of cat trigeminal ganglion 'cold' receptors under urethane and pentobarbital anesthetization. *Brain Res.* 1984;292(2):387-389.
  30. Brock JA, Pianova S, Belmonte C. Differences between nerve terminal impulses of polymodal nociceptors and cold sensory receptors of the guinea-pig cornea. *J Physiol.* 2001;533(pt 2):493-501.
  31. Acosta MC, Tan ME, Belmonte C, Gallar J. Sensations evoked by selective mechanical, chemical, and thermal stimulation of the conjunctiva and cornea. *Invest Ophthalmol Vis Sci.* 2001;42(9):2063-2067.
  32. Voets T, Nilius B. TRPs make sense. *J Membr Biol.* 2003;192(1):1-8.
  33. Nilius B, Voets T. Neurophysiology: channelling cold reception. *Nature.* 2007;448(7150):147-148.
  34. Nilius B. TRP channels in disease. *Biochim Biophys Acta.* 2007;1772(8):805-812.
  35. Julius D, Basbaum AI. Molecular mechanisms of nociception. *Nature.* 2001;413(6852):203-210.
  36. Dhaka A, Viswanath V, Patapoutian A. Trp ion channels and temperature sensation. *Annu Rev Neurosci.* 2006;29:135-161.
  37. Sharif Nacini R, Witty MF, Seguela P, Bourque CW. An N-terminal variant of Trpv1 channel is required for osmosensory transduction. *Nat Neurosci.* 2006;9(1):93-98.
  38. Ciura S, Bourque CW. Transient receptor potential vanilloid 1 is required for intrinsic osmoreception in organum vasculosum lamina terminalis neurons and for normal thirst responses to systemic hyperosmolality. *J Neurosci.* 2006;26(35):9069-9075.
  39. Stern ME, Beuerman RW, Fox RI, Mircheff AK, Pflugfelder SC. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. *Cornea.* 1998;17(6):584-589.
  40. Stern ME, Beuerman RW, Fox RI, Gao J, Mircheff AK, Pflugfelder SC. A unified theory of the role of the ocular surface in dry eye. *Adv Exp Med Biol.* 1998;438:643-651.
  41. Nguyen DH, Beuerman RW, Meneray M, Toshida H. Sensory denervation modulates eIF-2 alpha kinase expression in the rabbit lacrimal gland. *Curr Eye Res.* 2006;31(4):287-295.
  42. Nguyen DH, Beuerman RW, Meneray MA, Maichouk D. Sensory denervation leads to deregulated protein synthesis in the lacrimal gland. *Adv Exp Med Biol.* 1998;438:55-62.
  43. Meneray MA, Bennett DJ, Nguyen DH, Beuerman RW. Effect of sensory denervation on the structure and physiologic responsiveness of rabbit lacrimal gland. *Cornea.* 1998;17(1):99-107.
  44. Beuerman RW, Schimmelpfennig B. Sensory denervation of the rabbit cornea affects epithelial properties. *Exp Neurol.* 1980;69(1):196-201.
  45. Tuominen IS, Kontinen YT, Vesaluoma MH, Moilanen JA, Helinto M, Tervo TM. Corneal innervation and morphology in primary Sjogren's syndrome. *Invest Ophthalmol Vis Sci.* 2003;44(6):2545-2549.
  46. De Paiva CS, Pflugfelder SC. Corneal epitheliopathy of dry eye induces hyperesthesia to mechanical air jet stimulation. *Am J Ophthalmol.* 2004;137(1):109-115.
  47. Tuisku IS, Kontinen YT, Kontinen LM, Tervo TM. Alterations in corneal sensitivity and nerve morphology in patients with primary Sjogren's syndrome. *Exp Eye Res.* 2008;86(6):879-885.
  48. Cousen P, Cackett P, Bennett H, Swa K, Dhillon B. Tear production and corneal sensitivity in diabetes. *J Diabetes Complications.* 2007;21(6):371-373.
  49. Xu KP, Yagi Y, Tsubota K. Decrease in corneal sensitivity and change in tear function in dry eye. *Cornea.* 1996;15(3):235-239.
  50. Bourcier T, Acosta MC, Borderie V, et al. Decreased corneal sensitivity in patients with dry eye. *Invest Ophthalmol Vis Sci.* 2005;46(7):2341-2345.
  51. Gallar J, Acosta MC, Gutierrez AR, Belmonte C. Impulse activity in corneal sensory nerve fibers after photorefractive keratectomy. *Invest Ophthalmol Vis Sci.* 2007;48(9):4033-4037.
  52. Belmonte C. Eye dryness sensations after refractive surgery: impaired tear secretion or "phantom" cornea? *J Refract Surg.* 2007;23(6):598-602.