Hyperosmolar Tears Enhance Cooling Sensitivity of the Corneal Nerves in Rats: Possible Neural Basis for Cold-Induced Dry Eye Pain

Harumitsu Hirata^{1,2} and Mark I. Rosenblatt²

¹Department of Neurology, Thomas Jefferson University, Philadelphia, Pennsylvania, United States

²Department of Ophthalmology, Weill Cornell Medical College, New York, New York, United States

Correspondence: Harumitsu Hirata, Department of Ophthalmology, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, USA; hah2016@med.cornell.edu.

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PURPOSE. Tear hyperosmolarity is a ubiquitous feature of dry-eye disease. Although dry-eye patients' sensitivity to cooling is well known, the effects of tear hyperosmolarity on a small amount of cooling in the corneal nerves have not been quantitatively examined. Recently reported corneal afferents, high-threshold cold sensitive plus dry-sensitive (HT-CS + DS) neurons, in rats is normally excited by strong ($>4^{\circ}\text{C}$) cooling of the cornea, which, when applied to healthy humans, evokes the sensation of discomfort. However, corneal cooling measured between blinks does not exceed 2°C normally. Thus, we sought to determine if these nociceptors could be sensitized by hyperosmolar tears such that they are now activated by small cooling of the ocular surface.

METHODS. Trigeminal ganglion neurons innervating the cornea were extracellularly recorded in isoflurane-anesthetized rats. The responses of single corneal neurons to cooling stimuli presented in the presence of hyperosmolar (350–800 mOsm NaCl) tears were examined.

RESULTS. The HT-CS + DS neurons with thresholds averaging 4°C cooling responded to cooling stimuli presented after 15 minutes of hyperosmolar tears with thresholds of less than 1°C . The response magnitudes also were enhanced so that the responses to small (2°C) cooling emerged, where none was observed before.

CONCLUSIONS. These results demonstrate that after exposure to hyperosmolar tears, these nociceptive corneal neurons now begin to respond to the slight cooling normally encountered between blinks, enabling the painful information to be carried to the brain, which could explain the cooling-evoked discomfort in dry eye patients.

Keywords: dry eye disease, corneal nerves, dry eye discomfort, rats, trigeminal ganglion

Dry-eye disease (DED) is a chronic anterior eye disorder that afflicts approximately 20% of the population worldwide, depending on age and sex.^{1–3} Dry-eye disease, which is characterized by symptoms of ocular discomfort and pain, is the most common complaint from patients visiting eye care clinics. One prominent feature of DED is the ubiquitous presence of hyperosmolar tears in dry-eye (DE) patients.⁴ Although long-term deleterious effects of hyperosmolar solutions on the corneal epithelial cells^{5–7} and the effects of chronic dry eye, which are presumed to be the results of chronic tear hyperosmolarity, on corneal nerves studied by in vivo confocal microscopy^{8,9} have been known for some time, the effects of tear hyperosmolarity on specific functions of the corneal nerve are entirely unknown. Recently a special class of corneal nerves that could be excited robustly by drying of the cornea ("dry-sensitive" corneal afferents) has been strongly implicated in the production of tears and a sensation of ocular discomfort.^{10–12} One class of these dry-sensitive corneal afferents, low-threshold cold-sensitive plus dry-sensitive (LT-CS + DS) neurons, is exquisitely sensitive to a small cooling ($<1^{\circ}\text{C}$) of the ocular surface and is responsible for TRPM8-mediated basal tear formation.¹³ The second type of dry-sensitive corneal neurons, high-threshold cold-sensitive

plus dry-sensitive (HT-CS + DS), is excited exclusively by noxious levels of cooling of the cornea ($\sim 4^{\circ}\text{C}$ on the average).^{10,12} Human psychophysical experiments demonstrated that greater than 4 to 5°C cooling is required to evoke ocular sensations of stinging and irritation.^{14,15} Thus, it has been hypothesized that some cold-sensitive neurons, the so-called high-threshold cold fibers, may provide the unpleasant and painful cold sensations, different from the burning sensation presumably evoked by the activation of polymodal fibers.¹⁶ However, normally the corneal surface temperatures do not fall below approximately 2°C from the preblink level between eye blinks.^{17–19} We therefore sought an explanation as to how such a small temperature change (cooling of the ocular surface) could provoke discomfort and pain in DE patients. We hypothesize that in DE patients the thresholds for activation by cooling are significantly lowered due to the presence of hyperosmolar tears. Thus, the goal of this study was to determine if the dry-sensitive corneal afferents that normally require strong cold stimuli (average of $>4^{\circ}\text{C}$ cooling) for activation could be sensitized by a short-term (15-minute) application of a hyperosmolar solution to the eye so that milder cooling stimuli ($<2^{\circ}\text{C}$) now activate these neurons and/or produce a greater response magnitude.

METHODS

The detailed methods for surgery, in vivo electrophysiology, and corneal stimulation have been previously described^{10–12} and are therefore briefly provided here. Under 3.0% isoflurane (in 100% oxygen), male Sprague-Dawley rats purchased from Charles River Laboratories (Taconic Farms, NY, USA and Raleigh, NC, USA; 430–600 g) were surgically fitted with venous and arterial catheters and tracheal tubes. The animals then were placed in a stereotaxic instrument and a partial craniotomy was performed for easy penetration of the recording electrodes into the left trigeminal ganglion (TG). Just before the recordings, the isoflurane concentration was decreased to and maintained at 1.5% to 2.0% throughout the experiment. After checking for pinch-evoked withdrawal reflexes to ensure an adequate plane of anesthesia, pancuronium bromide (0.6 mg/kg/h) was infused continuously and animals were artificially respired. All vital signs remained within physiological range (arterial pressure > 100 mm Hg; end-tidal CO₂ 4%–5%; body temperature 37–38°C) throughout the recording sessions. A tungsten microelectrode (5–9 Mohms; FHC, Brunswick, ME, USA) was used to record extracellularly from a single corneal neuron in the left TG, exhibiting spontaneous activity during the dry cornea stage (see below). After amplification and discrimination with template matching software (CED, Cambridge, England), the neural spike outputs and the temperatures during the corneal thermal stimulation were acquired and analyzed by CED 1401 hardware and Spike2 software (CED). Receptive fields (RFs) of neurons were identified on the cornea with an ice-cooled dental metal probe (tip diameter ~1 mm) or with a von Frey filament (2 g) when the mechanical sensitivity was present. At the end of the experiment, each animal was euthanized with sodium Euthasol (pentobarbital sodium 100 mg/kg, intraperitoneally). The experimental protocol was approved by the Thomas Jefferson University and Weill Cornell Medical College Institutional Animal Care and Use Committees and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

General Experimental Protocol

After locating the RF to identify the neuron as dry sensitive, the discharge rate of each unit was recorded during two conditions of corneal fluid status. The wet cornea condition (wet stimulus; 5 minutes) occurred when the cornea was moistened with 200 μ L of rat artificial tears (ATs) dropped into a plastic well that enclosed an entire anterior eye. Next, the dry cornea condition (dry stimulus; 2 minutes) occurred after the well was detached from the eye and the excess ATs removed with a piece of filter paper. Each stimulus pair (wet and dry stimuli) was presented a total of three times to obtain averaged response magnitudes (wet and dry responses). The dry-sensitive corneal afferents were defined as those units that were excited by drying of the cornea and quieted by wetting; rates during dry stimulus were generally more than 20% greater than those during wet stimulus.¹¹ Then, a series of cooling stimuli was presented before, and 3 and 15 minutes after the application of the hyperosmolar solutions. The cooling stimulus was applied to the ocular surface via fluids that bathed the plastic well placed gently over the anterior eye. The temperature of the fluids was regulated by a Peltier-based device (Temperature Controller; Warner Instruments, Hamden, CT, USA), which was placed between the reservoir and the plastic well. The fluids were drawn from the reservoir by a peristaltic pump at a rate of approximately 1 mL per minute via polyethylene tubing through the temperature controller down into the plastic well. The thermistor was placed in the plastic

well approximately 1 mm above the ocular surface to monitor the temperatures of the chamber. The tip of the outlet from the Peltier device was approximately 1 mm above the thermistor. The cooling stimulus was 12°C changes from a 31°C adapting temperature to 19°C and back to 31°C, which took approximately 71 seconds. The rates of temperature change were, on the average, 0.15°C per second (range, 0.13–0.18°C per second) for 12°C change and 0.20°C per second (range, 0.21–0.19°C per second) for 16°C change. The hyperosmolar solutions used were 350, 450, 600, and 800 mOsm and were prepared by adding mannitol or NaCl (Sigma-Aldrich, St. Louis, MO, USA) to the ATs (305 mOsm). Their osmolarities were measured with an osmometer (μ OSMETT; Precision System, Inc., Natick, MA, USA). 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 7.45.²⁰

Data Analysis

Neural discharges were analyzed based on 1-second bin acquired with Spike2 software. The spontaneous (ongoing) discharge rates were based on 30-second periods (number of spikes per second). The evoked responses to cooling stimuli were defined as the total number of spikes following the stimulus onset that exceeded the mean + 2 SD; mean and SD were based on the activity over the 30 seconds preceding the stimulus. The end of the evoked response was defined as the beginning of the period in which the activity level fell consistently below the prestimulus or predrug rates, when the cooling ramp was followed by warming back to the adapting temperature (Fig. 1). Furthermore, during the 12°C cooling pulse, the response to the initial 2°C cooling (i.e., from 31°C to 29°C) was analyzed because hyperosmolar stimulus-induced changes in the cooling response to the small changes were of particular interest in this study (Fig. 2). To quantify the evoked responses to 2°C cooling, the total numbers of spikes during the first 2°C drop (from 31 to 19°C of the fluids bathing the cornea) were counted (see the numbers in parentheses in Fig. 5). Statistical analyses for the effects of hyperosmolar solutions on the neural discharges were performed with ANOVA (GraphPad Prism5, GraphPad Software, Inc., La Jolla, CA, USA) with or without repeated measures. Post hoc analyses were done with Bonferroni multiple comparison tests for individual comparisons; *t*-tests also were used to evaluate the differences between two sample populations. Because the primary goal of this study was to assess the effects of hyperosmolar stimulus on nociceptive neurons, the HT-CS + DS afferents¹⁰ (thresholds > 2°C cooling with average cooling thresholds of approximately 4°C from a 31°C adapting temperature) were searched and analyzed in this study. Thus, the LT-CS + DS afferents¹⁰ that had thresholds less than 1°C (typically approximately 0.1°C), when encountered during a unit search, were not studied further.

RESULTS

Time-Dependent Effects of Hyperosmolar Stimuli on Ongoing Discharges

Application of the hyperosmolar NaCl solutions to the cornea caused an immediate increase in activity in all neurons (Fig. 1). The magnitude of this dynamic response (peak response in Fig. 1E) depended on the osmolarities of the solutions. The latency of the peak response in seconds was 11.83 ± 0.96 for 800 mOsm ($n = 6$), 12.13 ± 2.00 for 600 mOsm ($n = 6$), 24.30 ± 3.16 for 450 mOsm ($n = 9$), and 60.25 ± 6.90 for 350 mOsm ($n = 5$); the latency to peak after 350 mOsm NaCl

FIGURE 1. (A–D) Peri-stimulus time histograms (PSTHs) in response to ATs and hyperosmolar stimuli in four HTCS+DS neurons. Neural discharge increased immediately after the application of hyperosmolar stimuli and then adapted over several minutes. *Top*, temperature changes; *middle*, action potentials (raw data); and *bottom*, PSTHs (1-second bins). The cooling stimuli were applied 3 and 15 minutes after the hyperosmolar solutions. The values of the calibration bars are shown in (D) and apply to other graphs here as well as other figures that follow. (E) Average graph depicting changes in ongoing activities before, and 3, 10, and 15 minutes after hyperosmolar stimuli. The averages were based on 30 seconds before the times indicated on the *x*-axis. Peak responses occurred at variable latencies after application of the hyperosmolar stimuli that depended on osmolarities (see text). Statistical symbols at each time point indicate the significant levels, from *top* to *bottom*, for 800 ($n=6$), 600 ($n=6$), 450 ($n=9$), and 350 mOsm NaCl ($n=5$). **** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$; NS, not significant versus before.

solutions in one neuron could not be determined because activity levels after hyperosmolar stimulus did not exceed one spike per second. Figure 1 also shows that after the peak activity, the discharge level began to decrease (adaptation; defined as at least 20% reduction from the peak activity within 3 minutes after the hyperosmolar stimulus) and continued to decrease until it became relatively stable by 10 to 15 minutes.

This adaptation of the discharge under continued hyperosmolar stimulation was observed in all units tested. Two-way ANOVA was performed to evaluate the overall effects of *hyperosmolarity* and *time after* the hyperosmolar stimuli on neural activities; both factors had significant influences on neural activities ($P < 0.05$ and <0.0001 , respectively). In addition, one-way ANOVA performed separately for each

FIGURE 2. Peri-stimulus time histograms showing the changes in thresholds and magnitudes of responses to 12°C and 2°C cooling of the cornea in an HT-CS + DS corneal afferent after 350 mOsm hyperosmolar stimulus. (A) Continuous records (temperature, action potentials, and PSTH from *top*, *middle*, and *bottom*) before and after application of hyperosmolar solution. (B, C) Enlarged view of the response to cooling before (B) and after (C) hyperosmolar stimulus. The numbers within cooling pulses correspond to the cooling threshold and apply to Figures 3 through 5. Notice that the responses to both 12°C (*numbers without parentheses*) and 2°C cooling (*numbers in parentheses*) showed enhanced sensitivity; that is, lowered threshold (*arrows with temperatures*) and increased response magnitudes. The *black boxes* on temperature traces indicate the 2°C cooling shift. The *vertical dotted lines* point to the areas on PSTH (*brackets*) that show the evoked responses to 2°C cooling (*number of spikes in parentheses*). Notice that there was no response to 2°C cooling before but the response emerged (seven spikes) after the hyperosmolar stimulus. The designations here apply to all figures that follow.

osmolarity group revealed that the peak response (1.67 ± 0.37 , 10.44 ± 1.52 , 13.83 ± 2.90 , 19.67 ± 3.93 spikes per second for 350, 450, 600, and 800 mOsm groups, respectively) was significantly higher than the ongoing discharge rates before the application of the hyperosmolar solution (0.04 ± 0.05 , 0.19 ± 0.1 , 0.03 ± 0.02 , and 0.05 ± 0.06 spikes per second for 350, 450, 600, and 800 mOsm groups, respective-

ly) regardless of the osmolarity ($P < 0.0001$). The activities produced by 450 mOsm at all time points (3, 10, and 15 minutes) were significantly different from that before the application, whereas those induced by 600 and 800 mOsm stimuli showed significant departures only at 3 minutes after. This is due presumably to variability in activity levels by these stimuli. On the other hand, the comparisons of the discharge

FIGURE 3. Peri-stimulus time histograms showing the changes in thresholds and magnitudes of responses to 12°C and 2°C cooling of the cornea in another HT-CS + DS corneal afferent after 450 mOsm hyperosmolar stimulus. **(A)** Continuous records before and after application of hyperosmolar solution. **(B, C)** Enlarged view of the response to cooling before **(B)** and after **(C)** hyperosmolar stimulus. The sensitization of the cooling-evoked responses similar to that shown in Figure 3 is also evident here.

rates at 3, 10, and 15 minutes after the applications revealed that they were not significantly different from each other in all groups.

Acute Application of Hyperosmolar NaCl Solutions Lowers the Thresholds and Enhances the Magnitudes of the Cooling-Evoked Responses in HT-CS + DS Corneal Afferents

Figures 1A through 1D also demonstrate that the responses to 12°C cooling stimuli (top traces) applied 3 minutes after the hyperosmolar stimulus occurred during the dynamic decrease in discharges, which rendered the calculation of the cooling-evoked response unreliable because of the inconsistent mean

and SD from which the cooling-evoked responses were derived (see definition in Methods). On the other hand, the discharge rate adapted to a relatively stable level by 15 minutes after the hyperosmolar stimuli. Therefore, we computed the responses to the cooling stimuli presented at 15 minutes after the hyperosmolar stimulus onset so that the evoked-response could be reliably measured. Figures 2 through 5 show that the low (350 mOsm) to high (800 mOsm) NaCl solutions provoked considerable changes in responses to 12°C and 2°C corneal cooling in four separate HT-CS + DS neurons, a phenomenon observed in all other units as well. Three significant results could be noted. First, the thresholds for activation by corneal cooling decreased significantly 15 minutes later in the presence of the hyperosmolar stimuli (i.e., compare graphs B and C in each

FIGURE 4. Peri-stimulus time histograms showing the changes in thresholds and magnitudes of responses to 12°C and 2°C cooling of the cornea in an HT-CS + DS corneal afferent under 600 mOsm NaCl solutions. Notice that unlike after the 350 and 450 mOsm hyperosmolar stimuli, the response magnitude to 12°C cooling was reduced to under 600 mOsm, whereas the response to 2°C cooling still showed enhanced sensitivity (lowered threshold in activation temperature from 27.9°C to 30.6°C and heightened magnitude from 0 to 20 spikes). Notice also that the response to 12°C cooling reached peak much earlier (shorter latency to peak) under hyperosmolar solutions (numbers in brackets), which could partially explain the reduced total evoked spikes from 200 to 60 spikes for 12°C cooling-evoked response.

figure). This is more clearly shown in the averaged graph of Figure 6A. Two-way ANOVA indicated the highly significant differences between before and after hyperosmolar stimuli ($P < 0.0001$). Average thresholds for all osmolarities combined were 26.9°C before and 30.4°C after the hyperosmolar stimuli. Regardless of the osmolarity group, hyperosmolar stimuli produced decreases in thresholds from approximately

4°C to less than 1°C cooling, as shown in Figure 6A. Second, this threshold decrease resulted in marked changes in the magnitudes of the 2°C cooling-evoked responses after the hyperosmolar stimuli. For units in Figures 2 through 5, as in all other units, the hyperosmolar stimulus simply induced the *emergence* of the response to this small cooling (numbers in parentheses). The average graph of Figure 6B shows this

FIGURE 5. Peri-stimulus time histograms showing the changes in thresholds and magnitudes of responses to 12°C and 2°C cooling of the cornea in another HT-CS + DS corneal afferent under 800 mOsm NaCl solutions. The same sensitivity changes described in Figure 4, including the shortened latency to peak, are also evident here.

notable effect observed with all hyperosmolar stimuli (two-way ANOVA between before and after hyperosmolar stimuli: $P < 0.0001$). Figure 6B also demonstrates that the maximum enhancements of the response magnitudes to 2°C cooling occurred at osmolarities between 450 and 800 mOsm NaCl solutions: the response to 2°C cooling with 800 mOsm was clearly much smaller than that with 600 mOsm.

Third, in contrast to the 2°C cooling-evoked response, Figures 4 and 5 show that the responses to 12°C cooling (numbers without parentheses in B and C) were not enhanced, but instead reduced after 600 and 800 mOsm hyperosmolar stimuli compared with those before, even though the

responses to 2°C cooling were still heightened under the same hyperosmolar stimuli (Fig. 6A). This effect with high osmolarities, shown in Figure 7A for average, is reflected in two other response parameters: (1) the latencies to peak response, which were much shorter than those observed before the hyperosmolar stimuli (see the numbers in brackets in Figures 4 to 5); and (2) the responses that followed the peaks were greatly diminished, accounting partly for the decreased total spike counts in response to 12°C cooling. However, the primary source for the reduction in total counts of the evoked responses after high hyperosmolar stimuli was the large increase in background activity that occurred after these high

FIGURE 6. Average changes in responses to cooling of the cornea. (A) Thresholds to cooling stimuli before and after hyperosmolar stimuli. (B) Response magnitudes to 2°C cooling applied to the cornea. Statistical symbols (*t*-tests): **P* < 0.05; ***P* < 0.01; NS, nonsignificant versus before. Statistical symbols in (A) are from top to bottom for 350, 450, 600, and 800 mOsm, respectively. Graph displays mean ± SE. *n* = 5, 6, 5, and 5, respectively, for 350, 450, 600, and 800 mOsm groups.

osmolar stimuli (Fig. 1), which was subtracted to compute the cooling-evoked responses (see Methods). Although Figures 4 and 5 show that the shortening of the peak latency appeared to have been observed only with 600 and 800 mOsm, Figure 7B shows that the average latencies for the peak responses to 12°C cooling were also shorter after 350 and 450 mOsm hyperosmolar stimuli as well. The sample sizes for Figure 7B were small in low osmolarity (350 and 450 mOsm) groups; therefore, the statistics were not performed.

The Hyperosmolar Mannitol Solutions Produce a Similar Sensitization of the Cooling-Evoked Response to That With Hyperosmolar NaCl Solutions

Because the change in extracellular NaCl ($[Na^+]_e$) is known to influence the driving force of the action potentials that might have produced superfluous effects in addition to those by pure osmotic pressure, we presented hyperosmolar mannitol (non-

FIGURE 7. Graph showing the dose-dependent changes in response magnitudes to 12°C cooling stimuli applied to the ocular surface (A) and the decrease in latency to peak responses (B) after hyperosmolar stimuli. Overall, hyperosmolarity failed to significantly influence the response magnitudes to 12°C cooling (2-way ANOVA, *P* > 0.05), and also individual comparisons (Bonferroni tests) did not reveal significant differences, either. NS, no significance versus before. (B) Dose-dependent shortening of the latency to peak under hyperosmolar stimuli, which were depicted in Figures 4 and 5. *n* = 3, 4, 5, and 2, respectively, for 350, 450, 600, and 800 mOsm NaCl solutions. The lines for the 600 and 800 mOsm groups were almost completely overlapped and therefore not easily recognizable. Statistics were not performed due to the small number of samples in some groups (see text).

permeant) solutions to HT-CS + DS afferents and compared their results with the same osmolarities obtained after NaCl solution. In the first set of experiments, we applied both mannitol and NaCl solutions (500 mOsm) to each of 14 neurons in a counterbalanced order. Application of both solutions increased ongoing discharges immediately to the peak, followed by an adaptation in much the same way described previously (Fig. 1). However, the response after mannitol application showed significantly longer latency to first spike (12.06 ± 1.91 vs. 8.33 ± 1.66 seconds), and smaller peak response (10.22 ± 1.40 vs. 12.78 ± 1.58 spikes per second) than NaCl (both at *P* < 0.001 *t*-tests); however, the latency to peak did not differ between two populations (18.72 ± 2.08 vs. 17.94 ± 2.81 seconds). In the second set of studies using separate groups of neurons, we applied 450 mOsm (*n* =

FIGURE 8. Peri-stimulus time histograms showing the changes in thresholds and magnitudes of responses to 12°C and 2°C cooling of the cornea in another HT-CS + DS corneal afferent after mannitol (600 mOsm) hyperosmolar stimulus. (A) Continuous records before and after application of hyperosmolar solution. (B, C) Enlarged view of the response to cooling before (B) and after (C) hyperosmolar stimulus. The sensitization of the cooling-evoked responses similar to that shown in Figure 3 for NaCl hyperosmolar stimulus is also evident here.

6) and 600 mOsm ($n = 3$) mannitol hyperosmolar solutions and assessed their ongoing discharges and the responses to cooling stimuli after the hyperosmolar mannitol solutions. Figure 8 shows that the changes in ongoing discharges, and thresholds and evoked responses to cooling after 600 mOsm mannitol solutions were identical to those described earlier for NaCl hyperosmolar solutions. Thus, the average graphs of Figure 9 demonstrate that the ongoing spontaneous discharge rates adapt following the increases to peak (Fig. 9A). The thresholds to cooling were also lowered (Fig. 9B) and the evoked response magnitudes to 12°C as well as 2°C cooling were enhanced (Figs. 9C, 9D) after 450 and 600 mOsm mannitol solutions. However, the detailed analyses indicate some differences: after 15 minutes of mannitol solutions, the

ongoing discharge rates were much lower (0.92 ± 0.59 spikes per second and 1.44 ± 0.41 spikes per second for 450 mOsm and 600 mOsm, respectively) than those after comparable NaCl (3.93 ± 1.77 and 4.07 ± 1.77 spikes per second, from Fig. 1), although statistical significances were not attained ($P > 0.05$ for both 450 and 600 mOsm; t -tests). The cooling-evoked responses to 12°C cooling were greater, albeit not significant, after mannitol (121.8 ± 32.4 and 130.0 ± 21.6 spikes per stimulus for 450 and 600 mOsm, respectively) than after NaCl (87.2 ± 37.8 and 63.2 ± 26.9 spikes per stimulus for 450 and 600 mOsm, respectively). Similarly, the respective values for the 2°C cooling-evoked responses were 13.5 ± 5.2 and 18.3 ± 7.0 spikes per stimulus for mannitol, and 11.8 ± 3.9 and 12.4 ± 5.9 spikes per stimulus for NaCl. This is presumably due to a

FIGURE 9. Sensitivity changes of HT-CS + DS corneal afferents following hyperosmolar mannitol solutions applied to the cornea. **(A)** Graph illustrating the increased (peak) activity followed by adaptation of the ongoing discharges produced by ocular applications of 450 and 600 mOsm mannitol. Each neuron was tested with both solutions. $n = 14$. **(B)** Graph showing the lowering of the thresholds to corneal cooling. **(C)** Graph showing the changes in 12°C cooling-evoked responses. Notice that the response after 600 mOsm mannitol was not reduced, unlike that after NaCl. **(D)** Graph showing the changes in 2°C cooling-evoked responses. Statistical symbols: **** $P < 0.0001$; *** $P < 0.001$; * $P < 0.05$; NS, not significant versus before (paired t -tests); a, $P < 0.05$; b, $P < 0.0001$ versus peak; all other comparisons were not significant ($P > 0.05$). $n = 6$ for 450 mOsm and $n = 3$ for 600 mOsm in **(B–D)**.

smaller background activity produced by 15 minutes of continuous mannitol than NaCl as described above, on which cooling-evoked responses were measured.

DISCUSSION

The present study demonstrated that hyperosmolar tears within the ranges of osmolarity found in DE patients increase the sensitivity of the cold nociceptive (HT-CS + DS) neurons. And these nociceptors, which normally require more than 2°C cooling (average of 4–5°C cooling), are now activated by less than 1°C cooling of the corneal surface, thus providing the painful information to the brain and making it a possible neural

mechanism underlying the cooling-induced discomfort and pain reported by DE patients. In patients without dry eye, there is no hyperosmolar stress on the ocular surface; thus, small amounts of cooling elicited by evaporation or environmental conditions would not be expected to elicit a sensation of discomfort. However, in patients with DED, the constitutive and prolonged hyperosmolar environment alters the threshold for stimulation of the cold-sensing nociceptors such that even relatively small amounts of evaporation during the interblink interval or environmental cooling will lead to ocular discomfort. Clinically, this heightened sensitivity of DED patients to a cooling and drying environment has been noted. The Ocular Surface Disease Index, a validated clinical instrument for DED

assessment, has incorporated this finding by evaluating the discomfort patients experience in an air-conditioned room. The stimuli that exist in such an environmental condition are likely not simple; however, the potential sources include slight cooling of the ocular surface and a minor mechanical stimulus provided by blowing air over the eye. Whether or not this mechanical stimulus in other conditions, such as a windy day outdoors, is also a disturbing stimulus to the DE patients is not known. On the other hand, the ocular surface cooling in an open-air environment is a known source of discomfort for DE patients (copious tearing in a cold environment). Previous studies indicate the corneal sensitivity to be generally lowered (not enhanced) in DE patients, including the cooling sensation.²¹ There are, however, significant numbers of studies^{22–24} showing contrasting results (i.e., hyperesthesia of the cornea). The discrepancy has been attributed in part to the stages of DED.²⁵ It is also possible that for the slight cooling-induced pain to be experimentally measurable, tests must be administered at the time of their symptoms (i.e., when patients report the discomfort, or when presumably their tear hyperosmolarity is accentuated). It is important to note also that these studies used the sensory thresholds as a measure of sensitivity changes. It has been known for some time that the threshold changes are not a valid measure of *pain* sensitivity.^{26,27} The changes in cooling-induced or mechanically induced pain of the direct *scaling method*, which represents a valid measure of pain,²⁷ in these patients are not known from these studies. In our study, not only the threshold change in neural activation was evident after the hyperosmolar stimuli (Figs. 6A, 9B), but also the magnitudes of the response to slight cooling (2°C) were significantly altered (Figs. 6B, 9D). This “emergence of the response” to approximately 1°C cooling was dramatic even after the lowest osmolarity tested (350 mOsm), a likely level to be found in DE patients.²⁸ It could be argued, nonetheless, that such a small change in magnitudes of response may not reach the central nervous system for the perception of this stimulus. However, it must be noted that many (perhaps hundreds of) inputs from single corneal primary afferents are expected to converge to excite central neurons.²⁹ Our findings, therefore, clearly indicate that a slight ocular cooling encountered in everyday life, a normally nonpainful stimulus, should elicit the pain sensation.

Our observation that the neuronal discharge adapted to a low but steady-state level in 10 to 15 minutes of continuous application of the hyperosmolar stimulus (Fig. 1) suggests that this condition may represent the ocular conditions to which DE patients are perennially exposed, which also indicates the chronic state of sensitivity increases in these patients. In addition to cooling sensitivity changes, whether or not other sensory modalities, such as mechanical or heat senses are heightened, awaits further investigations. Although a burning sensation is a serious component of DED,⁴ in this study we did not explore the possibility of sensitization to heat (or warmth) after hyperosmolar stimuli because we detected that the use of heat (even 45°C) had unpredictable consequences on the neural responses to subsequent heat stimuli, making this method somewhat unreproducible across different treatments over short periods of time.

There has been an extensive discussion as to what the values of tear hyperosmolarity are in DE patients. Evaporation during ocular dryness under the conditions of aqueous tear deficiency is expected to lead to local spots of tear hyperosmolarity that can reach up to 900 mOsm,^{30,31} which can then become a source of ocular irritation and discomfort.³² It has been acknowledged that it is difficult to measure osmolarity by the current methods using a pipette or tissues to collect samples without disturbing the ocular surface, which inevitably initiates the tearing reflex, thus leading to values that

are somewhat underestimated. Theoretically, the values may reach as high as 900 mOsm^{31,33} or it has been estimated in the area of tear breakup to be as high as 560 mOsm.²⁸ Thus, our hyperosmolar values are well within the ranges expected in DE patients.

The present study also found that both NaCl- and mannitol-based hyperosmolar stimuli were capable of sensitization of the corneal neurons to corneal cooling, indicating that the osmotic pressure on the nerves contributed to the changes in excitability of HT-CS + DS corneal afferents. Our rationale for primarily using an NaCl-based hyperosmolar solution was that NaCl, being the highest in concentration of the tears, would increase significantly more as evaporation proceeds and the tear films thin.²⁸ Thus, we hypothesized that hyperosmolar tear-induced sensitivity changes in excitability of the corneal neurons derives alterations from these electrolytes. In lacrimal gland disease, the increase in electrolytes is not uniform but sodium appears to increase unduly in the tear film as its secretion by the lacrimal gland increases.³⁴ Furthermore, Botelho and Martinez³⁵ found that at low flow rates of tear flows, Na⁺ concentration increased whereas K⁺ did not. This increase is a result of increased Na⁺ in lacrimal gland fluids. Recently, it has been reported that a major factor in determining the corneal epithelial cell viability was the changes in CaCl₂ of tears and not NaCl.³⁶ In our study, however, the concentration of Ca²⁺ was kept constant across all osmolarities tested; therefore, Ca²⁺ would not have contributed to the neural excitability changes observed.

The HT-CS + DS corneal afferents studied here appear to be very similar to the previously reported HT-CS-cultured TG neurons in rodents^{37,38} or the “cold nociceptors” of cats³⁹ in their sensitivity to strong cooling of the ocular surface. They may also be categorized as polymodal nociceptors like those found in the cat’s cornea based on their responsiveness to hypertonic saline.⁴⁰ However, some differences should be noted. Although the thresholds and the ranges of the temperatures that activated the HT-CS cultured neurons (27–18°C) and HT-CS + DS neurons in this study (29–19°C) are similar, it is not clear if the former group of corneal afferents responds to drying of the cornea and/or the hyperosmolar stimulus that so characteristically defined our HT-CS + DS neurons. The “cold nociceptors” in the cat’s cornea responded to an “air-jet” stimulus, which can be considered a “dryness” stimulus,³⁹ but they also displayed mechanical sensitivity, whereas our HT-CS + DS corneal afferents showed no mechanical response. Similarly, unlike polymodal nociceptors, the HT-CS + DS corneal neurons displayed no mechanical sensitivity (2 g) and only small numbers of neurons responded to 45°C heat (6 of 20 neurons).¹⁰ In addition, rather minor sensitivity to acids (10 mM acetic acids) or capsaicin has been found among populations of HT-CS + DS corneal afferents,¹⁰ whereas the defining characteristics of the classic polymodal nociceptors are their responsiveness to these two types of chemical stimuli. It is worth noting also that the temperature fluctuations during drying of the cornea (<1°C cooling; Hirata H, unpublished observation, 2013) does not activate HT-CS + DS afferents, as these neurons required more than 2°C of cooling of the ocular surface. This leads to the conclusion that the “physiological stimulus” for the HT-CS + DS afferents lies somewhere other than ocular cooling alone. We demonstrated previously that the most consistent stimulus that triggers HT-CS + DS corneal neurons is a hyperosmolar solution,¹⁰ and thus the increase in tear osmolarity that occurs during drying of the cornea (evaporation) is the likely candidate for this type of stimulus. It is also possible, however, that the combined activation by “subthreshold” (<1°C) cooling and a low-level hyperosmolarity (e.g., <350 mOsm NaCl solutions) together produces the “dry responses” in these HT-CS + DS neurons.

This peculiarity of the dry response (that requires both types of stimulations) can easily be tested experimentally in the future, and ultimately may determine the significance of these neurons both in terms of their functions and classifications. Whether our population of “dry-sensitive” neurons represents a unique set or class or is simply a variation of the putative cold-sensitive neurons remains to be seen. It is interesting, however, to speculate on the functional significance of these cold-sensitive plus dry-sensitive corneal afferents. It has been known for quite some time that the “cold thermoreceptors” in the upper airway have been suggested to function as a laryngeal air-“flow” detector⁴¹ and the corneal cold receptors as the basis for an unpleasant sensation of “dryness.”⁴²

The present study was not designed to reveal the precise cellular and molecular mechanisms underlying the tear osmolarity-induced sensitization reported here. However, the possible mechanisms include upregulation of TRPM8 channels⁴³ or control of the voltage-gated potassium channels (Kv1.1) reported earlier.³⁷ Both channels are well-established cooling sensors^{44,45} that can be regulated by a hyperosmolar stimulus.⁴⁶ Another interesting mechanism has been proposed previously.²⁸ This mechanism stipulates that the hyperosmolar tears present in dry-eye patients causes chronic dysfunction of the corneal epithelial cell barriers, leading to the exposure of the corneal nerve ending, which in turn produces hypersensitivity to a variety of ocular stimuli (hyperesthesia) in these patients. The answers to these questions may pave the way for a new therapy for the dry-eye-induced pain and discomfort that have been so prevalent and difficult to treat among the worldwide population.

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