Estimating the Osmolarities of Tears During Evaporation Through the "Eyes" of the Corneal Nerves

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Purpose. A population of corneal neurons in rats preferentially sense and monitor the hyperosmolar conditions of tears when the tears begin to evaporate during corneal dryness. The present study exploited this ability in an effort to estimate tear osmolarities by comparing the responses to corneal dryness to their responses to hyperosmolar stimuli.

Methods. Extracellular recordings were performed from single neurons in the trigeminal ganglia innervating the corneas of rats. To determine the extent to which the corneal neurons' responses to drying of the cornea were induced via the activation by hyperosmolar stimuli, we assessed the responses to ocular instillation of 500 and 600 mOsm/L, and a graded series of hyperosmolar stimuli ranging from 350 to 1000 mOsm/L.

Results. The magnitudes of the responses to drying of the cornea were matched almost exactly to those induced by the ocular instillation of the 600 mOsm/L stimuli but not the 500 mOsm/L solutions. The response magnitudes to a graded series of hyperosmolar solutions were nearly linear from the 350 to the 600 mOsm/L stimuli, but reached a plateau or declined slightly thereafter.

Conclusions. Our results demonstrate that the tear osmolarity in rats could reach 600 to 1000 mOsm/L during ocular dryness. Furthermore, a spontaneous eye blink could be generated at a tear osmolarity of approximately 400 mOsm/L if the blink is solely determined by hyperosmolar tears, but ocular surface cooling also can become a major factor if hyperosmolar tears occurring during ocular dryness lower the threshold of activation of the neurons.

Keywords: corneal nerve, dry eye disease, tear hyperosmolarity, rat, eye blink

Tear hyperosmolarity is thought to play a critical role in the initiation and/or perpetuation of dry eye disease (DED) as the core mechanism that could have consequences ranging from inflammation to corneal nerve abnormality.[1] It also is regarded as a "gold standard" among diagnostic tools for DED.[1–5] Because of these implications in the pathogenesis of DED, there has been a multitude of studies estimating the values of tear osmolarities in normal and dry eye (DE) patients with a variety of techniques. For example, earlier methods using freezing point depression[6–8] and vapor pressure techniques[9] yielded generally a narrow distribution ranging from approximately 284 to 316 mOsm/L in normal individuals compared to a much wider distribution curve ranging from approximately 286 to 364 mOsm/L in DE patients.[10] More recent studies by means of user-friendly, low volume sampling methods (TearLab Corporation, San Diego, CA, USA) also have demonstrated similar ranges of values that have been claimed to show a high correlation with DED severity.[11] However, the results using the latter method have been a source of controversy for producing great variability among the DE patient population.[12,13] More importantly, because the samples are collected from tears in the lower meniscus in all these studies, the measurements do not fully reflect the values of tear osmolarities present on large parts of the corneal surface, where dynamic and more significant effects are presumed to be taking place. The changes in tear film instability directly over the ocular surface are thought to have a significant role in inducing the major dry eye symptoms, like discomfort and pain.

In contrast, entirely different approaches to attempt to measure tear osmolarity on the cornea have been applied in studies[14–18] that took advantage of the ability in human tears to break up and/or thin as they evaporate from the corneal surface and of the mathematical models derived from these measurements. The results from these studies of normal individuals have provided much higher values of tear osmolarities, ranging from approximately 600 to 900 mOsm/L, than those using the other methods described above. The present study was an attempt to contribute to these discussions from an entirely different perspective that has not been explored previously to our knowledge. We asked if the activity of the corneal nerves can inform us of the osmolarity of the tears that bathe the corneal nerve endings in the epithelial cell layers of rats. It is particularly pertinent to show the osmolarity of the intraepithelial environment, since our recent findings indicated that 600 to 1000 mOsm/L hyperosmolar solutions (HOS) treatment of the eyes induces severe functional and anatomic changes in corneal nerves,[19] resembling the symptoms of DED in humans.[20–22]

Our attempt to estimate tear hyperosmolarity in our rodent model was first conceived when we recently found[23–25] that one type of corneal neurons was extremely adept at detecting corneal dryness and osmotic pressure in the cornea, and serve

primarily as faithful sensors of the osmolarity of their extracellular environments. Thus, these neurons could be used to measure the osmolarity of the tears that immerse the nerve endings. Also, some of the ideas that led to the present study derived from the report by Liu et al.,[26] which demonstrated that the pain sensations evoked by the ocular instillation of HOS and the sensations evoked during prolonged eye opening in humans could be used to estimate tear osmolarities. Thus, instead of using the sensation as a common signal to determine tear hyperosmolarity, in the present study we have used the corneal nerve activity as a similarly mutual signal to estimate osmolarities during the evaporation of the tear fluid from the ocular surface.

Methods

Under deep isoflurane anesthesia (3%–4% in 100% O_2), Sprague-Dawley rats were fitted with venous and arterial catheters (femoral vein and femoral arteries, respectively) for fluid injections and blood pressure monitoring. After tracheostomy, the tracheal tube was connected to the ventilator (Model 683; Harvard Apparatus, Holliston, MA, USA) and artificially ventilated at 1.5 to 2.5 cc/stroke and approximately 70 strokes/minutes. The animals then were placed in a stereotaxic instrument and their heads were firmly fixed in place with mouth and ear bars, and finally a partial craniotomy was performed to expose the brain surface. Tungsten microelectrodes (5–9 Mohms; FHC, Inc., Bowdoin, ME, USA) then were lowered through the opening in the skull into the left trigeminal ganglion for the purpose of recording from single neurons innervating the cornea under isoflurane anesthesia (1.5% to 2.0% in 100% O_2). Single neurons were identified, captured, and analyzed using a commercial hardware and a software program (CED Micro1401; Spike2 v. 8; CED, Cambridge, England). Before electrophysiologic recordings began, the animals were paralyzed with a continuous intravenous infusion of a neuromuscular blocker, pancuronium bromide (0.6 mg/kg/h), and artificially ventilated with a Harvard Apparatus small animal respirator. For the entire duration of a recording session, all physiologic parameters, such as mean arterial pressure, rectal temperature, and end tidal CO_2, were monitored and maintained within a normal physiologic range. The experimental protocol was approved by the Cornell Medical College Institutional Animal Care and Use Committee and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Once proper isolation of a single neuron was achieved, each neuron was identified first as "dry-sensitive" when the activity of the neurons was increased during drying of the cornea and suppressed during wetting of the cornea. Then, each neuron was further classified as a low threshold-cold sensitive plus dry sensitive (LT-CS + DS) corneal afferent or "high-threshold cold-sensitive plus dry-sensitive (HT-CS + DS) corneal afferent." The LT-CS + DS corneal afferents respond to a cooling stimulus applied to the corneal surface of less than 2°C from an adapting temperature of 31°C. In contrast, when the neurons required greater than a 2°C cooling for their activation, they were classified as "HT-CS + DS corneal afferents." These criteria also have been used in our previous studies.[23,24]

Rationales for Testing HT-CS + DS Neurons and Not LT-CS + DS Neurons

We previously found[23] that the trigeminal ganglia (TG) of rodents contained the two types of neurons innervating the cornea whose activity was increased during drying of the cornea and decreased during wetting of the cornea, the so-called dry-sensitive neurons. In addition to their "dryness" sensitivity, they also responded to ocular surface cooling to different degrees. One type, termed LT-CS + DS corneal neurons, responds to slight cooling (<2°C). The small temperature fluctuations of the corneal surface occurring during drying of the cornea activate these neurons and stimulate the lacrimal gland presumably to produce "basal tears." This is why, we hypothesized, that as we walk around the environment and a gentle wind cools the ocular surface slightly during "drying of the cornea," basal tears are produced constantly during our waking hours. Figure 1 shows the ocular surface temperatures monitored during drying of the cornea, demonstrating only a minor cooling that would excite only LT-CS + DS corneal neurons.

In contrast, the other type, called "HT-CS + DS corneal neurons," does not respond to these small cooling fluctuations, but responds better to hyperosmolar stimuli applied to the ocular surface; thus, they are primarily "pure" osmotic pressure sensors. Figure 2 shows graphically the basis for our rationale for using HT-CS + DS neurons in estimating tear osmolarity during corneal dryness. The HT-CS + DS neurons express a strong response to an evaporation-induced increase in the osmolarities of the tears, suggesting their crucial role as osmo-sensors. We proposed, therefore, that during corneal dryness, which produces the effect of a slight cooling (<2°C),[27,28] as well as an evaporation-induced increase in tear osmolarity, these two types of neurons react very differently to corneal dryness: LT-CS + DS neurons are excited by slight cooling and HT-CS + DS neurons are responding to changes in tear osmolarity. Thus, to estimate the osmolarity of the tears present in the vicinity of the nerve terminals (the intraepithelial cell layers), we reasoned that using HT-CS + DS neurons would be a more appropriate system for the purpose of the present study by not adding the confounding effect of cooling, which would have happened if we had analyzed LT-CS + DS neurons instead. Therefore, we compared the magnitude of the neural response to corneal dryness with the magnitude of the same neuron to the hyperosmolar stimulus that causes the activation of the corneal neurons.

Ocular Stimuli

Wet and dry stimuli were applied to the ocular surface, respectively, by placing an eye cup (approximately 8 mm diameter plastic tube) over the eye, which would be filled with artificial tears (ATs; approximately 200 μl), and then removing the eye cup and ATs.[24] The wet stimulus (ATs) covered an entire surface of the anterior eye including cornea and conjunctiva, for 5 minutes. After detaching an eye cup from the eye, dry stimulus was applied by placing the filter papers around the edges of the palpebral aperture to completely draw out and thereby remove the ATs for 2 minutes. Our "dry stimulus" was meant to serve as a process of drying of the cornea and not the complete loss of water from the corneal surface as we did not measure the quantity of the fluids remaining on the surface. Thus, "dry," "drying," and "evaporation" were interchangeably used throughout this study. Then, an eye cup was placed again onto the eye to present cooling stimuli.[19,23–25] The cooling stimulus was applied to the ocular surface via fluids that flowed into an eye cup (bath). The fluids were drawn out from the reservoir (50 ml beaker) via polyethylene (PE) tubing by the use of a peristaltic pump at a rate of 1.3 ml/minute and passed through a thermoelectric (Peltier-based) device, and pushed into the eye cup. 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 eye cup. The cooling stimulus was a 12°C drop from a 31°C adapting temperature down to 19°C and back to 31°C, which

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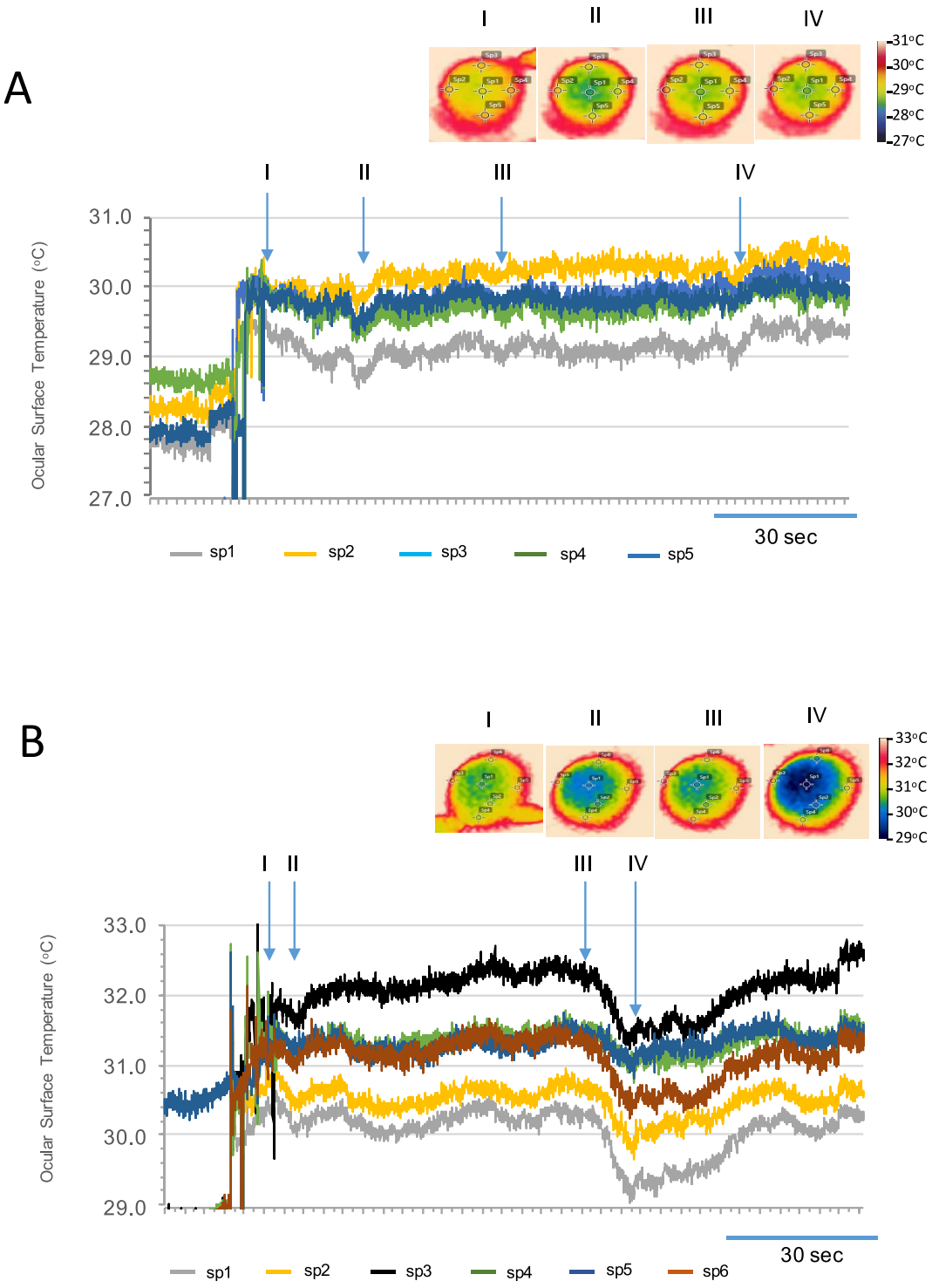

Figure 1. Thermographic records of continuous changes in the ocular surface temperatures across several spots (sp) on the corneas during 2-minute corneal dryness in isoflurane-anesthetized, paralyzed rats. (**A**–**B**) Ocular surface temperatures from 2 animals recorded from sp1 to sp5 (**A**) or sp1-sp6 (**B**) of the cornea illustrated in the *insets* above the line records. The record in (**A**) is typical of most of the animals showing numerous episodes of mild cooling (II-IV in [**A**], 0.1°–0.3°C) seen throughout the ocular dryness. Slow but sizable cooling was seen during the first approximately 30 seconds after the eye cup was removed and drying of the cornea began (**I**) on the central cornea (sp1). In rare occasions, a large drop in surface temperature such as IV in (**B**) (0.7°–1.4°C) was observed. The *insets* on the right show the calibration color palettes corresponding to the temperatures. Ocular thermographic photos in (*I*) to ([*V*], *insets*) were taken at the times indicated with *arrows* on the *line graphs*. The border between the *red* and *yellow* zones in *insets* (**I**–**IV**) delineates the approximate limbal region.

took approximately 51 seconds. The rate of cooling was, on the average, 0.20°C/s (range = 0.17–0.24) for a 12°C change. Approximately 5 minutes later, a 600 mOsm/L HOS stimulus was applied to the anterior eye using the flowing fluid system described above. Artificial tears were first applied for at least 5 minutes and then the fluids were switched to a HOS stimulus by placing the PE tubing from ATs to 600 or 500 mOsm/L solutions. To avoid mixing of the two solutions, air (approx-

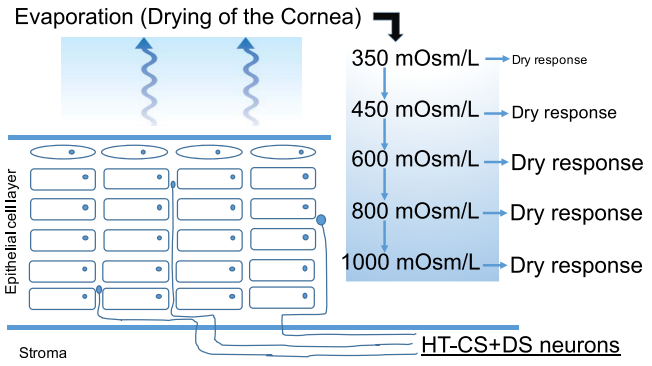

Figure 2. A diagram illustrating the proposed principle behind the calculation of the osmolarity of the extracellular fluids surrounding the corneal nerve terminals of the HT-CS + DS neurons (*lines* tipped with *bulbous endings*), where transductions of dry and HOS stimuli into the action potentials (responses) are presumed to take place. In this study, the dry stimulus is expected to result in an evaporation of the precorneal tear film, which in turn leads to progressive increases in the osmolarity of the fluids surrounding the nerve terminals, producing a gradual increase in the dry response (indicated by the size of the letters) until it achieves stable activity (see also Fig. 7).

imately 100 µl) was interjected when the tubing was switched. The temperature of the bath solutions was kept constant at approximately 31°C. In another series of experiments, the cornea was stimulated with an ascending order of HOS, 350, 450, 600, 800, and 1000 mOsm/L solutions with an interstimulus interval of at least 5 minutes (see Fig. 5). The hyperosmolar solutions were prepared by adding NaCl (Sigma-Aldridge Corp., St. Louis, MO, USA) to the ATs (approximately 305 mOsm/L). Their osmolarities were measured with an osmometer (µ OSMETT; Precision System, Inc., Natick, MA, USA). The ATs in mM were composed of: NaCl 106.5, $NaHCO_3$ 26.1, KCl 18.7, $MgCl_2$ 1.0, NaH_2PO_4 0.5, $CaCl_2$ 1.1, and HEPES 10; pH 7.45.[29]

Thermography

Ocular surface temperatures were recorded in 5 animals using an infrared camera (model T420; FLIR, Wilsonville, OR, USA) under the same experimental conditions used in the electrophysiology recording (i.e., animals anesthetized with isoflurane and paralyzed during a 2-minute corneal dryness period).

Data Analysis

Neural discharges were analyzed based on 1-second bin acquired with Spike2 software from HT-CS + DS corneal neurons. The evoked responses to cooling stimuli were defined as the total number of spikes following the stimulus onset that exceeded the mean + 2 SD, which were based on the activity over the 30 seconds preceding the stimulus. The end of the evoked response was defined as the consistent level of activity at or below the level observed before the stimulus, when the cooling ramp was followed by warming back to the adapting temperature. The responses to drying of the cornea (dry response) and HOS stimuli were defined as number (#) of spikes/s averaged over the 30-second period preceding the end of the dry or HOS stimulus (approximately 1.5–2 minutes after the onset of stimulus; Figs. 3–5). Statistical analyses for the effects of HOS or dry stimulus on neural discharges were performed with ANOVA (GraphPad Prism5; GraphPad Software, Inc., San Diego, CA, USA) with or without repeated measures. Post hoc analyses were done with Bonferroni multiple comparison tests for individual comparisons, and *t*-tests also were used to evaluate the differences between the two sample populations (Figs. 3E, 4D).

Results

A total of 34 HT-CS + DS neurons, 20 from the new data and 14 from our previously obtained data (Fig. 4), was used for analyses in the present study. The cooling thresholds of our sample were all greater than 2°C cooling, satisfying the definition of HT-CS + DS neurons.[23]

Comparison Between the Responses to Dry and Single-Dose HOS Stimuli

In our other ongoing studies investigating the effects of debridement on the responses of the corneal neurons to a variety of ocular stimuli, including HOS (600 mOsm/L), we observed a striking parallel in the magnitudes (steady state activity) of the dry and HOS responses in many of the HT-CS + DS units tested. These two responses were markedly different from the minor responses to cooling stimuli in the same neurons as shown in Figures 3A to 3C. Therefore, we examined 10 neurons that we collected from these studies, and compared the magnitudes of the responses to dry and HOS (600 mOsm/L) stimuli. Figures 3A to 3C showed that, following the applications of dry and HOS stimuli, there was an increase in the activity level (slowly increasing for the dry response and rapidly increasing in the HOS response) that appears to stabilize after approximately 30 to 50 seconds of continued stimulation. Thus, we analyzed these steady state activities to both of these stimuli. The individual curves shown in Figure 3D revealed that of the 10 neurons examined, the magnitudes of the dry responses were greater than those of the HOS responses in 6 neurons but smaller in 4 neurons. However, the average dry and HOS responses, shown in Figure 3B, were similar (respectively, 6.68 ± 0.44 and 7.35 ± 0.86 spikes/s), and were not significantly different from each other (P = 0.4375, paired *t*-test, 2-tailed), suggesting that the dry responses might be produced by an extracellular HOS stimulus whose intensity is close to 600 mOsm/L.

Because we found no difference between the responses to dry and 600 mOsm/L HOS stimuli, we speculated that if we tested lower osmolar stimuli, we might obtain a significantly different response magnitude from that to the dry response. Consequently, we performed a retrospective analysis using our previous data from the studies in which we collected the responses to dry and 500 mOsm/L HOS stimuli.[23] Individual examples shown in Figures 4A and 4B revealed that, in contrast to the 600 mOsm/L HOS, we found that the dry response was much stronger than the responses to the 500 mOsm/L NaCl HOS solutions. Again, the responses to cooling were negligible in these neurons. Figure 4C demonstrated that, of 14 neurons examined, in all but one neuron the dry responses were much stronger than the responses to the 500 mOsm/L stimuli with highly significant average differences (Fig. 4D) between the dry and the HOS responses (respectively, 8.19 ± 0.31 vs. 6.09 ± 0.30 spikes/s; P = 0.0020, paired *t*-test, 2-tailed), suggesting that the dry responses were produced by the extracellular HOS stimuli whose intensities are greater than 500 mOsm/L (e.g., 600 mOsm/L), as suggested by Figure 3.

Comparison Between the Responses to Dry and Multiple Graded HOS Stimuli

The results from a single dose HOS described above implied that the responses to ocular dryness at stable levels (definition

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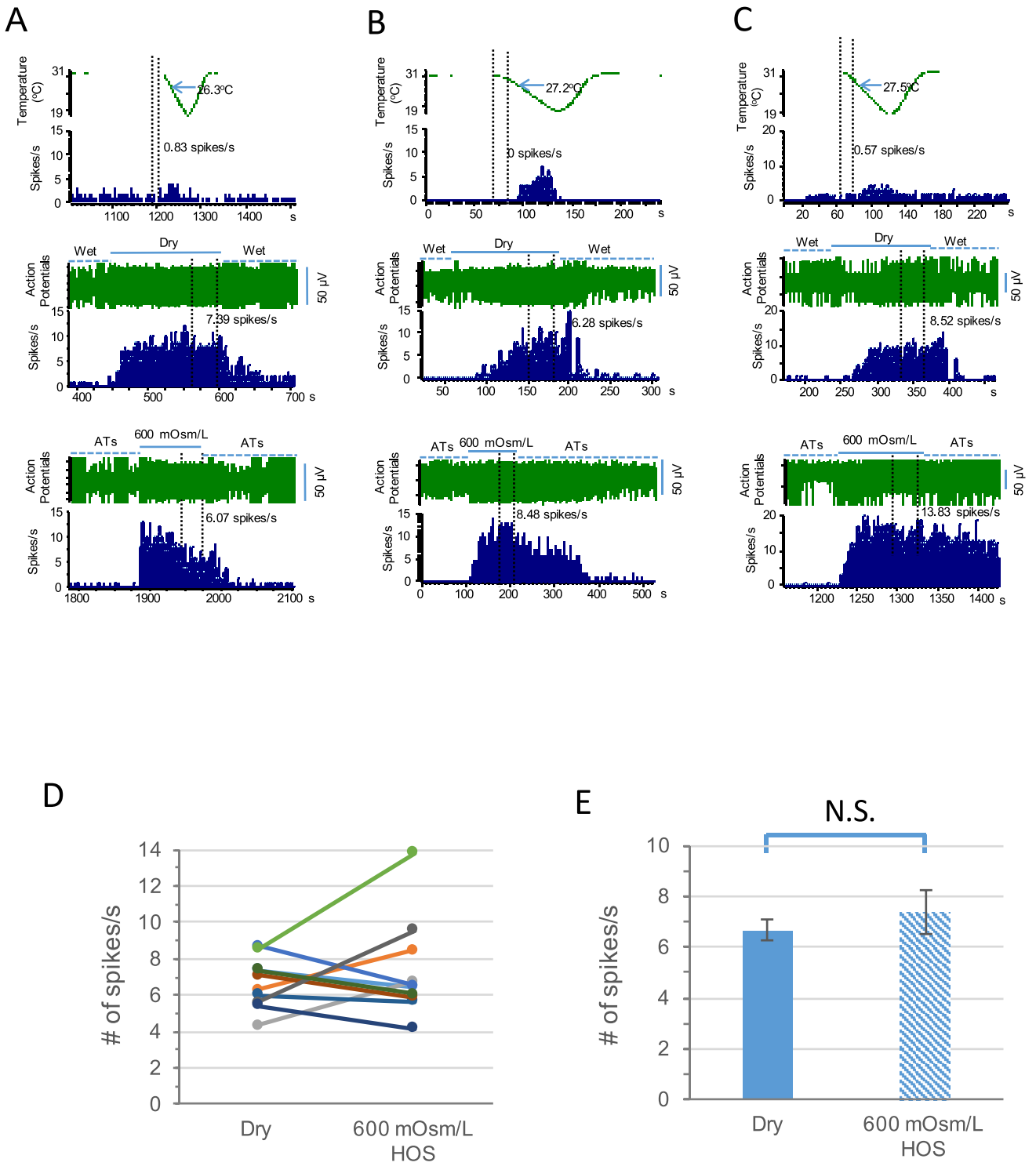

Figure 3. (**A–C**) Peristimulus time histograms (PSTHs) from 3 HT-CS + DS corneal neurons in response to the cooling, dry, and hyperosmolar stimuli (600 mOsm/L). *Upper*: In all 3 neurons, the responses to 2°C cooling (indicated by *vertical lines*) were minor compared to the responses to the dry and HOS stimuli. The responses to the 2°C cooling stimuli were analyzed because during the 2-minute ocular dryness, the temperature changes did not exceed 2°C (Fig. 1). *Arrows* with temperature values indicate the thresholds to the cooling stimuli for the neurons. *Middle* and *bottom panels*: The magnitudes of responses to the dry and HOS stimuli shown next to two *vertical lines* represent the steady state activity level during the last 30 seconds of the approximately 2-minute stimuli. (**D**) A comparison of the response magnitudes between the dry and HOS responses among individual HT-CS + DS neurons. (**E**) Average magnitudes of the responses (± SEM) to dry and HOS stimuli. Wet, ATs; N.S., non significance ($P > 0.05$, paired *t*-tests; $n = 10$).

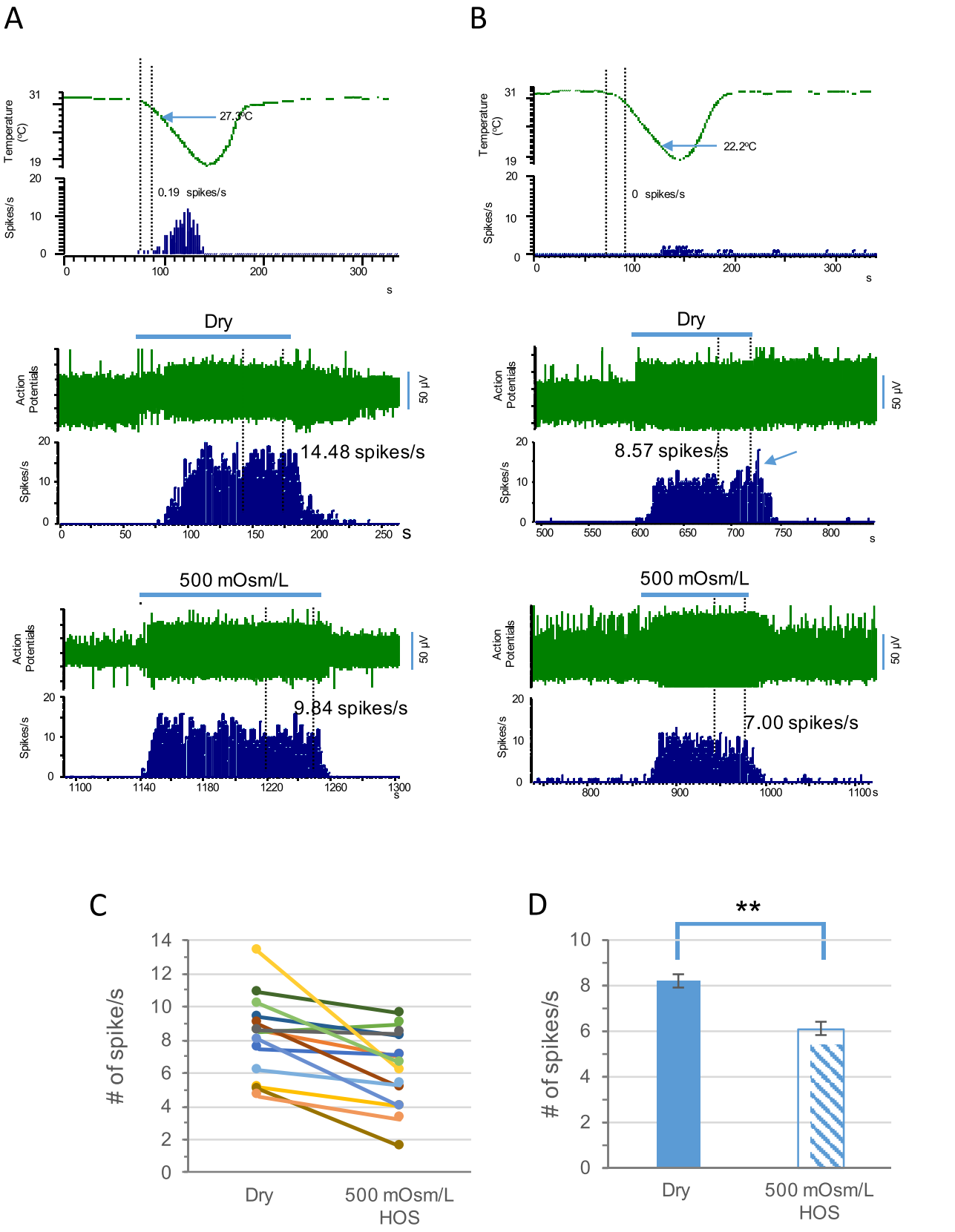

Figure 4. **(A–B)** Examples from 2 HT-CS + DS neurons in responses to the cooling, dry and hyperosmolar stimuli (500 mOsm/L NaCl). All designations in this figure were the same as in Figure 3. **(C)** A comparison of the response magnitudes between the dry and HOS responses among individual HT-CS + DS neurons. **(D)** Average graph showing the magnitudes of the responses to the dry and HOS stimuli with a highly significant difference between these responses. $^{**}P = 0.0020$, paired *t*-tests; $n = 14$.

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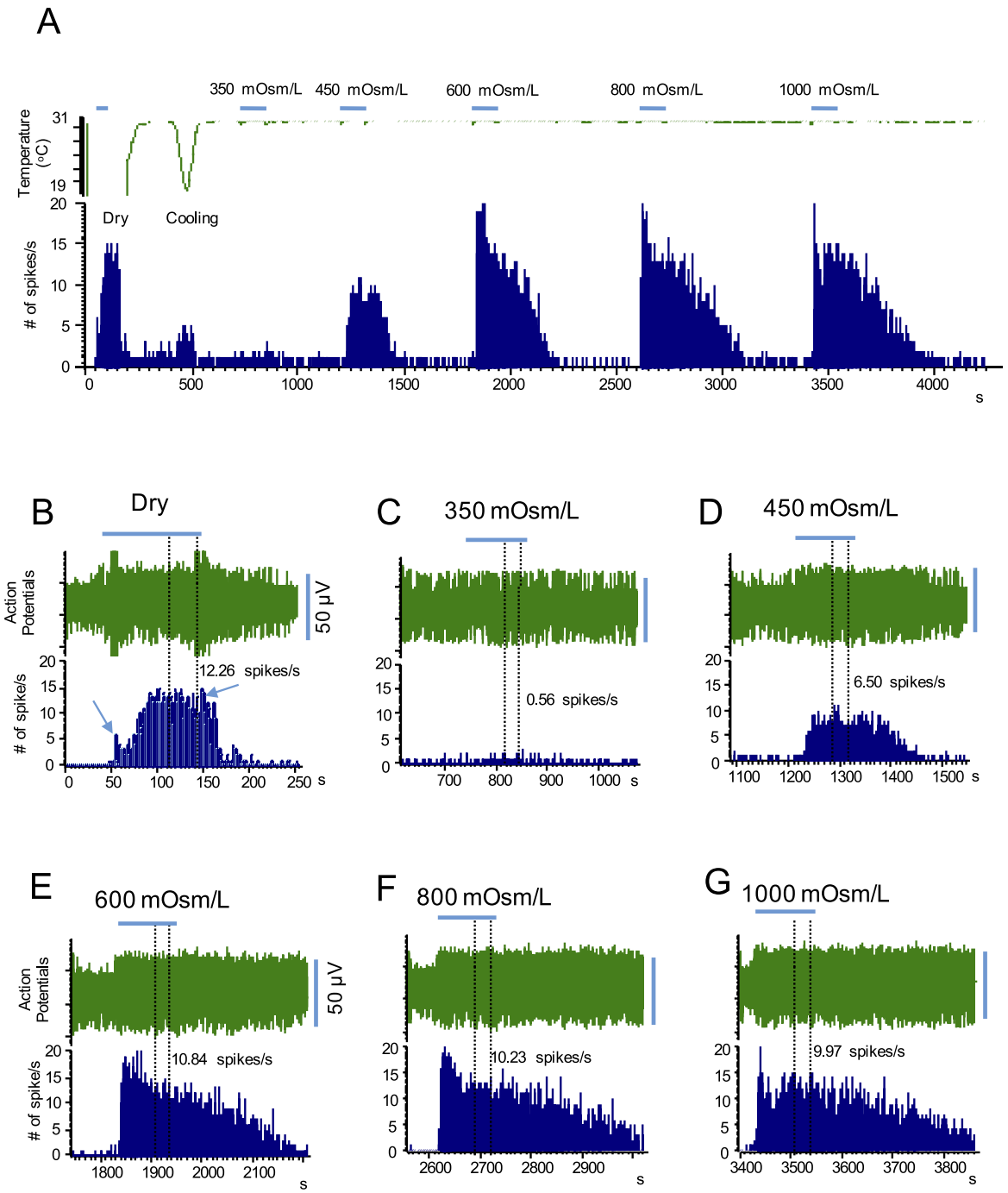

Figure 5. **(A)** Continuous PSTHs from one HT-CS + DS neuron showing the responses to all the stimuli used in computing the HOS dose response functions shown in Figure 6. The marked difference between the response magnitudes to the dry and cooling stimuli is evident, while the response magnitudes to the 600 mOsm/L stimulus are very similar to the dry responses. **(B–G)** The expanded views of the responses to the stimuli shown in **(A)**. The designations in all the Figures were the same as in Figures 3 and 4. The *arrows* in **(B)** indicate the examples of inadvertent inclusion of the artifacts due to removal of the eye cup.

of the dry response) were likely initiated by a hyperosmolar stimulus intensity greater than or equal to 600 mOsm/L, suggesting that during corneal dryness the osmolarity of the extracellular solutions surrounding the nerve terminal might equal or exceed 600 mOsm/L. This is because the average dry response magnitudes were significantly greater than the responses to the 500 mOsm/L HOS but not to the 600 mOsm/L HOS. So, to determine precisely where along the axis of the HOS intensity scale the magnitudes of the dry responses would equal the response magnitudes evoked by the HOS stimuli, we presented multiple HOS doses (350, 450, 600, 800, and 1000 mOsm/L in ascending order) in 10 additional neurons. An example from one HT-CS + DS neuron in Figure 5 illustrates the sequence of stimuli applied (dry, cooling, and an ascending series of graded HOS stimuli) and the responses to these stimuli. Two observations are immediately obvious. First, there was little or no response to the 350 mOsm/L stimulus, and second, there appeared to be no additional

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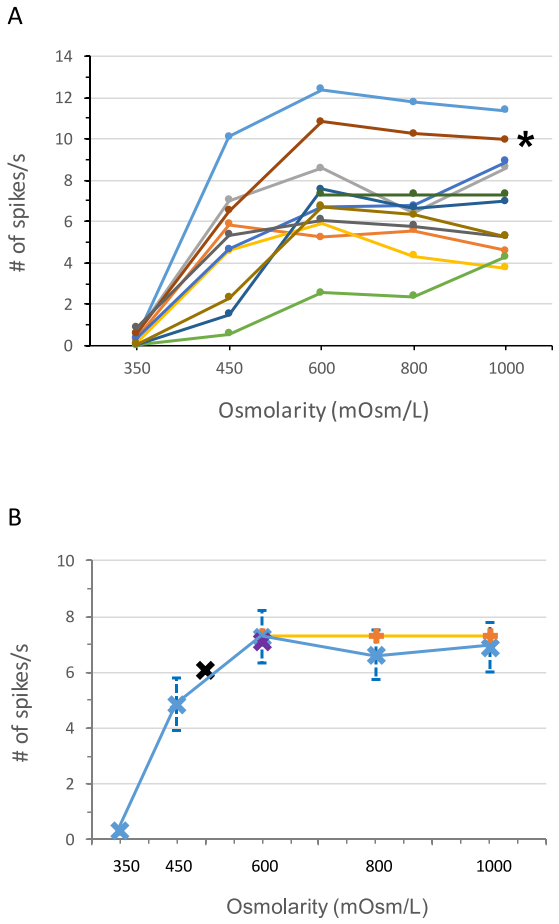

Figure 6. **(A)** Individual dose (HOS) response functions. A *curve* with an *asterisk* was from the neuron shown in Figure 5. **(B)** The average dose response function is shown in the *blue line*. Also represented for comparison are the average responses to the 500 mOsm/L (*black cross*) and 600 mOsm/L (*purple cross*) stimuli from Figures 3 and 4 (single dose experiments). The *yellow* line intersecting 600 to 1000 mOsm/L is the average dry response (7.36 spikes/s) drawn to demonstrate the closeness to the HOS responses (600–1000 mOsm/L).

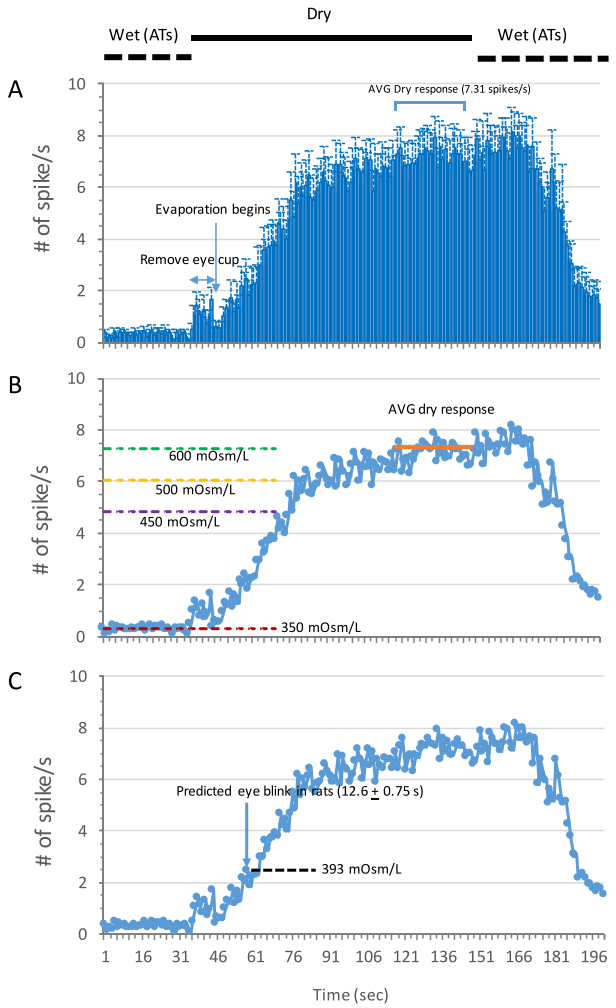

Figure 7. **(A)** Averaged PSTHs in response to the wet and dry stimuli (± SEM) based on 10 HT-CS + DS neurons in the graded HOS experiments. Some important events associated with these stimuli also are depicted: the eye cup removal in preparation for the dry stimulus presentation when the evaporation began. The time period where the dry responses were calculated (approximately last 30 seconds of the dry stimulus) also is shown. **(B)** Line graph representation of the same response profiles shown in **(A)** without SEM for clarity. The *dotted color lines* are the average responses to the HOS stimuli shown next to the *lines*. **(C)** The *line graph* as in **(B)** in association with the time and osmolarity when the rat eye blink is expected to occur (see text for explanation).

increase in the responses beyond the 600 mOsm/L stimulus: the differences in responses from the 600 to 1000 mOsm/L stimuli are reflected not in the magnitudes of the steady state responses (the number of spikes/s between two vertical lines) but in the transient peak responses before the steady state levels were reached, as well as in the washout period following the stimuli (which was longer with the higher osmolar stimuli). The detailed comparisons shown in Figures 5B to 5G demonstrated that the magnitude of the dry response was clearly much greater than the responses to the 450 mOsm/L stimulus and more closely matched the responses to the 600 mOsm/L stimuli. Figure 6A shows the individual HOS responses plotted against the graded series of HOS stimuli and revealed that the functions were not linear but saturated beyond 600 mOsm/L. Of the 10 units tested, the maximum responses occurred at 600 mOsm/L in 7 neurons, at 1000 mOsm/L in 2 neurons, and at 450 mOsm/L in 1 neuron. The average function shown in Figure 6B revealed that with the 350 mOsm/L stimuli there was virtually no response above and beyond the response to the 300 mOsm/L (i.e., the wet response – response under ATs, 0.32 ± 0.02 spikes/s; see Fig. 7 also). Above 350 mOsm/L (0.32 ± 0.12 spikes/s) the HOS response increased precipitously to 7.26 ± 0.94 spikes/s at 600 mOsm/L through 450 mOsm/L (4.84 ± 0.95 spikes/s). Beyond 600 mOsm/L, however, the HOS response magnitudes appeared to either level off or slightly decrease at 800 to 1000 mOsm/L (6.62 ± 0.89 and 6.90 ± 0.89 spikes/s, respectively). One-way ANOVA confirmed that the osmolarity had highly significant effects on the responses produced by a HOS stimulus ($P < 0.0001$ with repeated measures on osmolarity) while post hoc Bonferroni multiple comparison analysis indicated that the response to 350 mOsm/L was significantly lower than the responses to all the other HOS stimuli, but other comparisons were not significant, except between 450 and 1000 mOsm/L ($P < 0.05$).

Time-Related Changes in the Dry Response: Relationship to Tear Osmolarity and Eye Blink

Figure 7A based on 10 neurons in the graded HOS experiments shows that during the 2-minute dry stimulus,

the response magnitudes (1s bins) were seen to slowly increase approximately over the first 30 to 50 seconds after the evaporation began, and then the responses appeared to stabilize for the remainder of the stimulus: slight elevations of the responses at the beginning and end of the dry stimulus were due to inadvertent inclusions of the artifacts generated, respectively, by removing and applying the eye cups in some neurons (see also the arrows in Fig. 4B, middle panel and Fig. 5B). This phenomenon (slow increase and eventual stable response) was first reported by us previously.[23] The dry response was defined as the responses during these stabilized periods over a 30-second epoch (bracket above the histogram in Fig. 7A; 73–102 seconds after the evaporation began), and was 7.31 ± 0.98 spikes/s on average based on the 10 units examined in Figure 6.

Figure 7B shows the relationship of the slow increase in responses during the initial application of the dry stimulus to the responses produced by the graded series of HOS stimuli for the 350, 450, and 600 mOsm/L stimuli. As seen here, the average response to the wet stimulus (presumably to the approximately 300 mOsm/L stimulus) matches perfectly the response magnitude produced by the application of 350 mOsm/L NaCl solutions (orange dotted line; 0.32 ± 0.12 spikes/s). The average HOS response to the 450 mOsm/L stimulus (purple dotted line; 4.84 ± 0.95 spikes/s) corresponded to the 4.86 spikes/s of the response to the dry stimulus which occurred 30s after the evaporation began (removal of the wet stimulus). With the 500 mOsm/L HOS stimulus obtained from single dose experiments in Figure 6 (yellow dotted line; 6.09 ± 0.30 spikes/s), this correspondence occurred at the dry response of 6.18 ± 0.91 spikes/s 34 s after the evaporation. The average HOS response to 600 mOsm/L (green dotted line; 7.26 ± 0.94 spikes/s) nearly matched the average dry response (orange solid line; 7.31 ± 0.98 spikes/s). The average response magnitude to ocular dryness closest to that of 600 mOsm/L was first reached 56 seconds after the evaporation (7.14 spikes/s). By contrast, the average responses to the 800 and 1000 mOsm/L stimuli (6.62 ± 0.89 and 6.90 ± 0.89 spikes/s, respectively) were slightly lower (although not statistically significant, Bonferroni post hoc comparison) than those in response to either the 600 mOsm/L or the dry stimulus, and were not drawn in this Figure for the purpose of clarity.

Figure 7C shows that during the slow rise in the magnitude of the responses to corneal dryness that presumably accompanied the slow increase in tear osmolarity, the average intereye blink interval in rats (12.6 ± 0.75 seconds)[30] would have occurred at the arrow that corresponded to approximately 393 mOsm/L, assuming a linear increase in osmolarity from 350 to 450 mOsm/L.

Discussion

The present study found that analyzing the corneal nerve activity could be used to estimate the osmolarity of the tears immediately above and within the epithelial cell layers in rats. Our results showed that the extracellular fluid osmolarity found during a period of 2-minute corneal dryness in normal rodents' eyes is likely greater than 450 mOsm/L because the average dose-response function (Fig. 6B) still was growing beyond 450 mOsm/L through 500 mOsm/L to 600 mOsm/L. Above 600 mOsm/L, however, the present study was not able to identify a specific osmolarity because the responses to 800 and 1000 mOsm/L were not significantly different from those to the 600 mOsm/L stimuli. However, the evidence (Fig. 6B) seemed to indicate that 1000 mOsm/L was probably higher than the true osmolarity values existing around the nerve terminals, since the function began to decline at the 800 to 1000 mOsm/L range. This conclusion was consistent with our recent observation that the ocular instillation of 1000 mOsm solutions caused deleterious effects on the corneal nerves and their functions.[19] The mechanism for this leveling of the sensory response may be related to the water transport from more posterior tissues to reduce these extreme osmolarities to protect the corneal environment.[23] Therefore, the likely maximum osmolarity that exists during tear film evaporation in our design is expected to be between 600 and 1000 mOsm/L. Another observation in support of this conclusion is that the average dry response (7.31 spikes/s) that likely reflects the neurons' responses to changing extracellular osmolarities almost precisely matched the average magnitude of response to the 600 mOsm/L HOS stimuli (7.26 spikes/s; Fig. 6B), suggesting that the likely osmolarity during the 2-minute corneal dryness would be close to 600 mOsm/L.

Comparisons With Other Studies

Extensive clinical and basic research on tear osmolarity in humans found a wide range of osmolarities in normal (approximately 290–330 mOsm/L[12,13]) and DE patients (approximately 315–365 mOsm/L[31–34]), even 675 mOsm/L in one study.[35] Many factors, such as the measurement system used (freezing point to TearLab Corporation), repeated measurements,[13] and the time of day, and even changes within an individual, such as intereyes differences and environmental disparities,[36] are thought to contribute to these variabilities. It has been noted that these factors in clinical settings have not been standardized and will have to be addressed in the future.[36] Clinical and methodologic issues associated with the tear measurement systems have been raised recently by many investigators.[12,13,37–39] These do not, however, invalidate the importance of tear hyperosmolarity to the pathophysiology of DED.[38]

Studies using entirely different approaches have yielded much higher osmolarity values. By comparing the sensations evoked by the known HOS stimuli to the sensations obtained during an extended eye-opening in humans (approximately 10–90 seconds), Liu et al.[26] estimated the osmolarities to potentially reach between 600 and 800 mOsm/L. Also by a mathematical model based on tear film thinning rates (due to evaporation), Kimball et al.[40] predicted the osmolarity during tear film breakup to be 800 to 900 mOsm/L, or the even higher value of 1830 mOsm/L, assuming the higher rate of thinning in localized spots.[16,18] These studies were based on the very sizeable numbers of estimates from mathematical models and localized high rates of evaporation in those models, indicating that different methods produce high degrees of variability in osmolarity values between studies.

Our studies demonstrated that the osmolarity could reach 600 to 1000 mOsm/L during tear evaporation in rats (Figs. 6, 7), consistent with the approximations of higher osmolarities discussed above. However, these osmolarities may not be reached in normal circumstances, since the 55 seconds needed to generate these osmolarities seem much longer than the normal eye blink intervals in rats, which occur on average 12.6 seconds after the ocular dryness begins.[30] However, a 55-second duration used in the present study and the high osmolarities (600–1000 mOsm/L) that were derived from these calculations could potentially exist in rats' eyes. First, in rats, much longer intervals of approximately 500 seconds during which few or no blink occurrences were interspersed with many blinks, the so-called blink periodicity, have been reported.[30] In the same study, a close examination of the figure revealed that blink rates of 1 to 2 blinks per

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minute (i.e., 30–60-second interblink intervals) could be obtained (Fig. 8 in their study). Furthermore, in our study it is important to point out that the estimated osmolarity of the tears' fluids was derived from HT-CS + DS neurons, which were found mostly in the peripheral or limbal cornea (88% of the total sample).[23] Therefore, it is possible that the osmolarity of the tears in the central cornea could reach 600 mOsm/L in a much shorter time than the 55 seconds used in the present study. Moreover, an environment that favors low humidity and a high temperature could further accelerate the evaporation rate and result in a heightened tear osmolarity in a shorter time than that found in our experiments. Thus, tear osmolarity rising to 600 mOsm/L between blinks in rats is highly likely.

Signifying the Dry Response: The Relationship to the Spontaneous Eye Blink

Our primary goal for this study was to determine the maximum osmolarity of the extracellular tear fluids found during drying of the cornea by assessing the steady state responses of a population of corneal neurons selectively responsive to a hyperosmolar stimulus. As a corollary to this goal, however, we also discovered that we could estimate the osmolarities of the ocular surface fluids that give rise to the spontaneous eye blink (Fig. 7C). This was because before the steady state is reached (Fig. 7), the response to ocular dryness initially undergoes a slow rising phase, presumably indicating their responses to the slowly rising osmolarities surrounding the nerve terminals. Thus, we were able to estimate the osmolarity of the tears (approximately 390 mOsm/L; Fig. 7C) that might have evoked the spontaneous eye blink in rats (12.6 seconds).[30] This assumed that the spontaneous eye blink was solely caused by the hyperosmolar stimulus acting on the nerve terminals. This argument, however, may be too simplistic as it has been known for some time that ocular surface cooling and eye blinking are strongly associated.[41]

The recent evidence indicates that the mechanisms underlying the spontaneous blinks may be based on activation of the TRPM8-expressing corneal nerves and that the combined effects of cooling and hyperosmolar solutions on the ocular surface may be more relevant stimuli for this reflex.[42] These investigators found not only that TRPM8-expressing neurons were excited by a small increase in osmolarity but also that they are activated by cooling stimuli of a more physiological level (i.e., approximately 31°C on the skin) under the hyperosmolar conditions. We have shown previously[25] that HT-CS + DS corneal afferents with an average cooling threshold of 4°C or more under normal tears (approximately 300 mOsm/L) were activated by less than 2°C cooling under hyperosmolar tear conditions (450 and even 350 mOsm/L). Activation of HT-CS + DS corneal afferents, thus, could produce a blink reflex by these combined stimulations of the corneal surface. It is possible, furthermore, that both systems (TRPM8 and HT-CS + DS corneal afferents) may contribute to increased sensitivity to cooling of the ocular surface, thus leading to eye blinking.

Analytical Consideration

In our present studies, we have found that a small percentage of the responses attributable to HOS stimuli remained after epithelial debridement, indicating that the true responses to HOS stimuli might actually be slightly smaller than the responses reported for HT-CS + DS neurons (i.e., overestimating the responses to HOS stimuli). In the present study, 2 neurons out of 8 neurons tested in single-dose (600 mOsm/L) experiments showed few spikes even after the debridement. In one neuron 25% and in another neuron 33% of the responses to HOS stimuli persisted after debridement. Thus, the error due to these anomalous responses after the debridement, on the average, is 7% in total population, which would decrease the magnitude of the average HOS response from 7.35 to 6.81 spikes/s in Figure 3B. In effect, this corrected value approached even closer to the value of the average dry response (6.68 spikes/s; Fig. 3E), vindicating our hypothesis that the HOS response largely underlies the dry response in HT-CS + DS neurons. In contrast to the HOS response, the dry response always disappeared after debridement in all neurons, indicating that the origin of this response resides entirely in the epithelial cell layers.

We came to the present conclusion that the osmolarities of the extracellular tear environment reach 600 to 1000 mOsm/L by assuming that the dry response that slowly rose to a steady state activity level to a large extent underlies the neurons' responses to the slowly changing osmolarities of the tears. It is possible, of course, that the fundamental mechanisms underlying the responses to corneal dryness and to HOS stimuli in HT-CS + DS neurons are different. This possibility, however, is unlikely for the following reasons. First, the only known physical energies (stimuli) that drive the corneal neurons are mechanical, thermal, and chemical.[43,44] We excluded the first two as the temperature changes that occur during ocular dryness (<2°C cooling) do not activate our samples of neurons (HT-CS + DS neurons) and no mechanical sensitivity was found in any of our previous and present HT-CS + DS neurons. None of the neurons included in the present analysis were excited by a temperature change of less than 2°C cooling (Figs. 3, 4). Even with 12°C cooling, the responses were meager at best compared to the responses to ocular dryness or to hyperosmolar stimuli (see also Fig. 1 of our prior report[19]). The maximum cooling during drying of the cornea monitored by an infrared camera was approximately 1.4°C, but in most animals was less than 0.5°C (Fig. 1A). Thus, the variations in neural responses to temperature changes in this study would have contributed very little to the dry response. Second, all the neurons that responded to dryness also responded to HOS stimuli in a similar manner (i.e., the stable level of activity was reached after approximately 1 minute in both responses). And finally, in our previous studies[23] there was a compellingly strong correlation between the dry response and the HOS-evoked responses for the HT-CS + DS neurons, but not for the LT-CS + DS neurons, underpinning a common mechanism for HOS and dry responses in HT-CS + DS corneal neurons.

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