Ambient Air Currents Activate Corneal Nerves During Ocular Desiccation in Rats: Simultaneous Recordings of Neural Activity and Corneal Temperature

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Submitted: April 19, 2018
Accepted: July 5, 2018

Citation: Hirata H, Dallacasagrande V, Mizerska K, Ivakhnitskaia E, Rosenblatt MI. Ambient air currents activate corneal nerves during ocular desiccation in rats: simultaneous recordings of neural activity and corneal temperature. *Invest Ophthalmol Vis Sci*. 2018;59:4031–4043. https://doi.org/10.1167/iovs.18-24604

Purpose. Previously we found two types of corneal neurons that we hypothesized to play an important role in tearing. One type is called low threshold–cold sensitive plus dry sensitive (LT-CS + DS), and the other is termed high threshold–cold sensitive plus dry sensitive (HT-CS + DS). The present study examined critical stimuli influencing the activity of these neurons to elucidate environmental factors that may trigger this ocular reflex.

Methods. Single corneal neurons were extracellularly recorded from the trigeminal ganglia in response to ocular stimuli that mimic environmental conditions one encounters in daily life. They included an ocular desiccation and slight air currents and were presented while simultaneously monitoring the ocular surface temperatures (OST) in rats.

Results. The results showed that the changes in steady state (SS) activity of the neurons closely followed the changes in SS OST: during the sustained ocular desiccation, neural firing displayed numerous small sudden increases in activities ("spiking"); these "spiking" activities of LT-CS + DS neurons were replicated by a minute air current that induced slight ocular surface cooling of approximately 0.2–0.1°C; and the responses of HT-CS + DS neurons showed an inconsistent relationship to the changes in SS OST or exhibited little evidence for "spiking" activities.

Conclusions. These results suggest that LT-CS + DS neurons play a role in the afferent trigger of tearing as we face the environment, exposing the cornea to prevailing air currents that produce a slight cooling of the ocular surface. By contrast, HT-CS + DS neurons may serve to protect the eyes from extreme dryness by eliciting nociception-evoked tearing when the OST or osmolarity of tears becomes injurious.

Keywords: dry sensitive corneal afferents, electrophysiology, ocular surface temperature

Although cold-sensitive fibers comprise only a small proportion of corneal afferents, their functions in temperature and osmolarity detection implicate these fibers as one of the major determinants of basal tearing and ocular protective reflexes like blinking.[1–5] We have previously shown that cold-sensitive corneal nerves have exquisite sensitivity to cooling of the ocular surface and are also excited by drying of the cornea.[6,7] During drying of the cornea (i.e., tear evaporation), at least two types of ocular stimulation are expected to occur. One is the minor cooling of the ocular surface and the other is an increase in osmolarity of the tear film as the water evaporates, raising the salt concentration. We have demonstrated previously[8] that one type of corneal neurons, high threshold–cold sensitive plus dry sensitive neurons (HT-CS + DS), is best suited to monitoring the tear osmolarities and that another type, low threshold–cold sensitive plus dry sensitive neurons (LT-CS + DS) is an exquisite cooling sensor. Although different sensory roles have been proposed for these neurons, such as basal tearing and eye blinking, and some of their pharmacological properties have been characterized,[8–11] other response properties that might elucidate their additional sensory functions remain uncertain. One of these uncertainties was the attributes of the environmental stimuli that drive these neurons. Thus, one of the purposes for this study was to better understand the nature of the physiological stimuli that evoke the responses of these corneal neurons to ocular surface conditions that could give rise to tearing or eye blinking. We accomplished this by simultaneously recording ocular surface temperatures (OSTs) and the neural responses evoked during drying of the cornea. In addition, although the response profiles of cold thermoreceptors in the cutaneous tissues and cornea have been thoroughly studied,[1,12–16] one unique activity profile only seen in LT-CS + DS corneal neurons during drying of the cornea has not been fully explained. In our previous studies,[6,7] we observed distinct neuronal spike patterns ("spiking") during drying of the cornea for LT-CS + DS neurons compared with HT-CS + DS neurons. However, the sources of these "spiking" activities during corneal dryness are not known. Thus, our other purpose for this study was to uncover the potential environmental stimuli that gave rise to these "spiking" patterns of the corneal neurons.

Materials and Methods

Surgery and In Vivo Electrophysiology

Under deep isoflurane anesthesia (3%–4% in 100% O_2), the male Sprague-Dawley rats (2–4 months old) were fitted with femoral venous and arterial catheters for fluid injections and blood pressure monitoring, respectively. After tracheotomy, the animals were then placed in a stereotaxic instrument and their heads were firmly fixed in place with mouth and ear bars, and finally a partial craniotomy of the parietal bone was performed to expose the brain surface. Tungsten microelectrodes (5 Mohms; FHC, Inc., Bowdoin, ME, USA) were then lowered through the opening in the skull into the left trigeminal ganglion for the purpose of extracellularly recording from the single neurons innervating the cornea. Single neurons were identified, captured, and analyzed using a commercial hardware and software program (CED Micro1401, Spike2 v. 8; CED, Cambridge, England). Before electrophysiological 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 small animal respirator (model 693; Harvard Apparatus, Holliston, MA, USA, or model SAR-830; CWE, Ardmore, PA, USA). For the entire duration of the recording session, all physiological parameters, such as mean arterial pressure, rectal temperature, and end tidal CO_2, were monitored and maintained within a normal physiological range. The experimental protocol was approved by the Weill 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." These criteria have also been used in our previous studies.[6,7] The LT-CS + DS corneal neurons respond to slight cooling (<2°C). The small temperature fluctuations of the corneal surface occurring during drying of the cornea activate these neurons and presumably stimulate the lacrimal gland to produce "basal tears." The basal tearing driven by corneal nerve activation may therefore be due to the small degree of cooling that occurs during air flow experienced during the normal activities of daily living.

In contrast, HT-CS + DS corneal neurons do not respond to the small cooling fluctuations, but respond better to hyperosmolar stimuli applied to the ocular surface; thus, they are primarily "pure" osmotic-pressure sensors.[6,8] 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 these two types of neurons react very differently to corneal dryness: LT-CS + DS neurons are excited by slight cooling (<2°C),[17] whereas HT-CS + DS neurons respond to changes in tear osmolarity.

Ocular Stimuli

A wet environment was applied to the ocular surface, respectively, by placing an eye cup (approximately 8 mm diameter plastic tube) over the eye that would be filled with artificial tears (ATs) (approximately 200 μL), and dry environments created by removing the eye cup and ATs.[7] The wet stimulus (ATs) covered the entire surface of the anterior eye, including the cornea and conjunctiva, for 5 minutes. After detaching an eye cup from the eye, the dry stimulus was applied by placing the filter papers around the eye edges to completely draw out and, thereby, remove the ATs for 2 minutes (dry stimulus). Our "dry stimulus" was meant to serve as a process of drying of the cornea and may not represent the complete loss of water from the corneal surface, as we did not measure the quantity of the fluids remaining on the surface. An eye cup containing ATs was placed again onto the eye to present the controlled temperature stimuli.[6,7,9,10]

Controlled Temperature Stimulation. For the determination of the neuronal classes based on their cooling threshold[6] and also to provide steady state temperature stimuli in the current study, a more controlled type of cooling stimulus was applied to the ocular surface via fluids that flowed into the eye cup (bath). The fluids were drawn out from a reservoir (50-mL beaker) via polyethylene tubing by the use of a peristaltic pump at a rate of 1.3 mL/min and passed through a thermoelectric, Peltier-based device, and pumped into the eye cup (bath). The temperature of the fluids in contact with the ocular surface was regulated by the Peltier-based device (Temperature Controller; Warner Instruments, Hamden, CT, USA), which was placed approximately 1 cm proximal to the eye cup. The cooling stimulus was a 12°C drop from a 31°C adapting temperature of the bath down to 19°C and back to 31°C that took approximately 51 seconds. The rate of cooling was, on average, 0.20°C/s (range, 0.17–0.24) for a 12°C change. The ATs in mM were composed o: NaCl 106.5, $NaHCO_3$ 26.1, KCl 18.7, $MgCl_2$ 1.0, NaH_2PO_4 0.5, $CaCl_2$ 1.1, HEPES 10, pH 7.45.[18] The osmolarities of the solutions were measured with an osmometer (μ OSMETT; Precision System Inc., Natick, MA, USA).

Air Current. To recapitulate the air flow experienced by the cornea during daily activities, we devised laboratory procedures that could create a cooling stimulus of the order seen in these commonly experienced conditions. "Gentle" air flow was generated by moving the cupped hands in nasal to temporal direction, whereas more "brisk" air flow was applied by a moderately forced exhalation of approximately 1 second at approximately 15 cm in front of the left eye. The temperature changes associated with each stimulus were carefully determined as described below. However, the forces of each air flow stimulus were not measured, as it has been done previously by others via the use of Belmonte's esthesiometer,[19] given the purpose of this study was to monitor the magnitudes and the rate of temperature changes, and to correlate these parameters with the activity of the corneal neurons.

Ocular Thermography

The OSTs were recorded using an infrared camera (model T420; FLIR, Wilsonville, OR, USA) during the neural recording session. FLIR camera had an accuracy of ± 2% at 25°C (nominal). Thermographic video filming began approximately 30 seconds before the ocular dryness stimulus was applied and ended approximately 30 seconds after the dryness stimulus was removed (i.e., when the wet stimulus commenced). The temperature signals from the video camera were converted digitally (30 frames per second [fps]), copied to an Excel spread sheet, and displayed as an Excel chart (either 30 fps or 3 fps). When calculating the OST with T420, the emissivity of the tear film (mainly water) (0.95) was considered to reflect that of the corneal surface[20,21] when calculating the OST with the T420. The thermographic images were taken simultaneously with the electrophysiological recordings but were captured as two different inputs (i.e., the inputs were not led into the electrophysiological capture hardware). However, a synchronization of two inputs was achieved by inserting the notes on

the Spike2 that indicate when the thermography was turned on or off and when other procedures were performed (e.g., dry, wet).

Data Analysis

Neural discharges from corneal neurons were analyzed as a mean frequency with the Spike2 software program. The peak activities to stimuli (e.g., ocular dryness) were continuously displayed as frequency per sec (Hz) and the averages during the last 30 seconds of dry and temperature stimuli were also analyzed using this software. The ocular surface cooling produced by gentle or strong winds over the eye surface was generated, monitored, and recorded by the thermographic camera. We read the rates and the magnitudes of cooling so produced from the Excel Spread Sheets (30 frames [points] per second) and calculated manually from these numbers. The corresponding peak spike activities were then compared with the magnitudes or rates of cooling for each measurement for all units. We performed statistical analyses for the effects of the cooling or dry stimuli on neural discharges with ANOVA (GraphPad Prism5; GraphPad, La Jolla, CA, USA) with or without repeated measures. We used Bonferroni multiple comparison tests for post hoc analyses of individual comparisons. Also, *t*-tests were used to evaluate the differences between the two sample populations. All average graphs were displayed with the standard errors of the mean (SEM).

Results

A total of 31 dry-sensitive (DS) neurons (21 LT-CS + DS and 10 HT-CS + DS) were examined in the present study via simultaneous recordings of neural responses and OSTs. The average cooling thresholds for LT-CS + DS and HT-CS + DS neurons, respectively, were −0.55 ± 0.1°C and −4.64 ± 1.11°C from a starting temperature of 31°C. None of the LT-CS + DS neurons had a threshold colder than −2°C from a 31°C adapting temperature, and none of the HT-CS + DS neurons showed thresholds warmer than 2°C, fulfilling the defining criteria for these neuronal classes.[6]

LT-CS + DS Neurons But Not HT-CS + DS Neurons Display Steady State (SS) Activity Levels That Reflect the OSTs

Figure 1A shows a typical simultaneous recording of the neural response and the OST for an LT-CS + DS neuron during wet and dry cornea conditions. When the ocular surface was wet with ATs, the relatively SS temperature of the surface was maintained at an average of approximately 25.7°C. But when the ATs were removed to begin the ocular dryness stimulation, the temperature suddenly elevated to approximately 27.4°C, whereupon the temperature slowly declined over the next 15 to 20 seconds (thick blue horizontal line). This temperature change was associated with increasing SS neural firing frequency. During this changing OST, there were also small but sudden increases (*) and decreases (thin blue arrow) that were associated with the decrease (*) and increase in neural activity. Then, the OST increased (thick magenta horizontal line), accompanied by decreasing neural activity until the temperature appeared to stabilize at approximately 26.9°C for the remainder of the ocular dryness (green bracket). The profiles of the initial temperature changes (approximately first 30 seconds) at the start of the dryness state before the stabilized period were different for different animals: in some animals, after an initial elevation of OST on beginning the dryness, there was a simple slow continuous increase, whereas in others there was a continuous decrease until a stable temperature was reached. The reason for this difference is not likely to be due to the experimental room temperature and humidity, as they displayed only a very small daily variation (24 ± 0.5°C), or due to the core body temperature of the animals, which was maintained almost precisely at 38°C (see Materials and Methods). Regardless of this difference, however, there was a close inverse relationship between the changing of the SS OSTs and the SS neural activities within an individual (i.e., the decreasing temperature correlating with the increasing activity level of the LT-CS + DS neurons, and vice versa, Fig. 1A). Figure 1A shows, also, that sudden (dynamic) changes in OST do not appear to be perfectly correlated with the neural activation. This is presumably due to the slow time scale used to generate the OST records (i.e., normal 30 fps thermographic records were averaged and converted to 3 fps), which made the sudden changes difficult to detect. The relationship between dynamic OST changes and neural activity levels are described in more detail later.

In comparison, for an HT-CS + DS neuron, Figure 1B shows that although initial slow cooling (thick blue line) after the start of the desiccation produced the expected slow increase in SS neural activity, the warming (thick magenta line) that followed was not associated with a decrease in SS activity, unlike LT-CS + DS neurons. Regardless of the temperature changes, the neural firing frequency appeared to have simply kept increasing until the SS activity level was achieved. This lack of correlation between SS temperature changes and SS neural activities was also observed in all other HT-CS + DS neurons examined in this study.

In contrast to the varying initial temperature changes that were recorded for the different animals described above, the stabilized OSTs observed during sustained desiccation were generally present in all the animals. Figures 1A and 1B show that this stabilized temperature was associated with generally stable neural activity (green horizontal line; 13.89 Hz at 26.9°C for an LT-CS + DS neuron and 15.14 Hz at 28.4°C for an HT-CS + DS neuron) except for the "spiking activities" in the LT-CS + DS neuron, which is described below. The average OST during this stabilized period across all neurons was 27.5 ± 0.4°C ($n = 20$: range = 24.9–31.4°C) and 28.6 ± 0.46°C ($n = 10$: range = 26.1–30.3°C), respectively, for LT-CS + DS and HT-CS + DS neurons, which was not significantly different ($P = 0.0914$). The respective neural frequencies were 10.99 ± 1.14 Hz (range = 3.4–21.38 Hz) and 7.56 ± 1.06 Hz (range = 4.44–14.00 Hz). The difference in activity levels between these two neuron populations was marginally significant ($P = 0.0485$). The average activity could not be measured in 1 LT-CS + DS neuron due to unstable OSTs.

Figures 1A and 1B also show that the temperatures during the wet period (25.6°C and 26.9°C) produced neural activities (7.18 Hz and 0 Hz) that were much lower than those evoked during the stabilized dry period, suggesting the temperature-dependent characteristics of the SS neural activity. It has been well known that the cold thermoreceptors in the skin exhibit stimulus (temperature)-response (neural activity) functions that depended on the surface temperatures.[22,23] Thus, to more clearly analyze the relationship between the wide range of static temperatures that may be encountered in daily life and the corresponding neural activity level, we recorded from 10 LT-CS + DS neurons and 3 HT-CS + DS neurons while subjecting them to controlled temperature stimulation using flowing fluid stimulation with the Peltier device (Materials and Methods section) and compared them with the response profiles seen during drying of the cornea. Examples from these experiments from two LT-CS + DS neurons are shown in Figure 2.

The left sides of Figures 2A and 2B depict the neural activities and the OSTs during ocular desiccation, showing that

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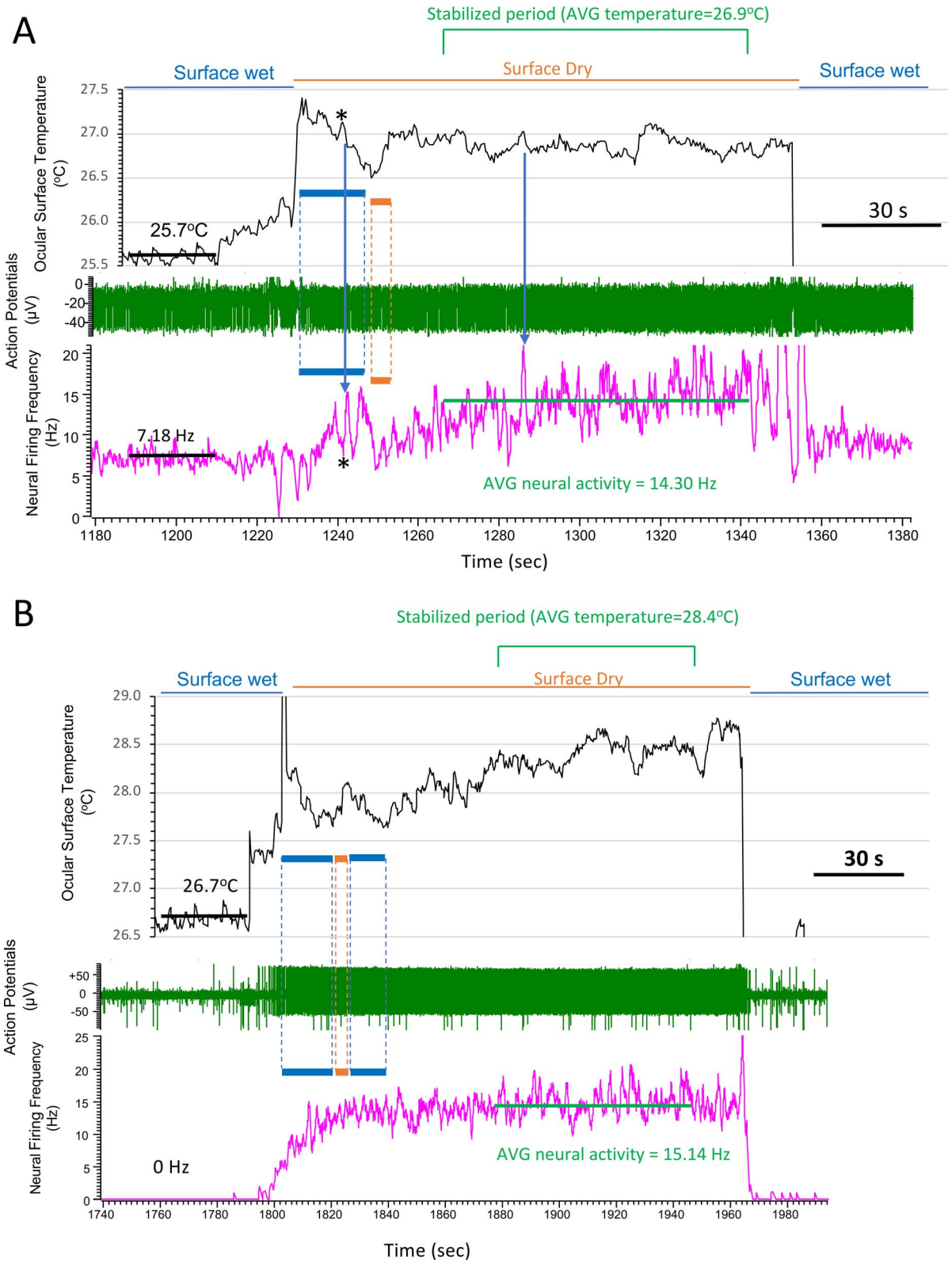

Figure 1. Simultaneous recording of the neural activity and OST before and after the cornea was dried. (**A**) The recording was made from an LT-CS + DS neuron with a threshold to cooling of 0.4°C from a 31°C adapting temperature (i.e., an absolute temperature of 30.6°C). The temperature and activity values indicate the averages taken during the periods represented by *horizontal bars* shown below the values. (**B**) Similar recording from an HT-CS + DS neuron with a threshold to cooling of 3.2°C from an adapting temperature of 31°C (absolute temperature of 26.8°C).

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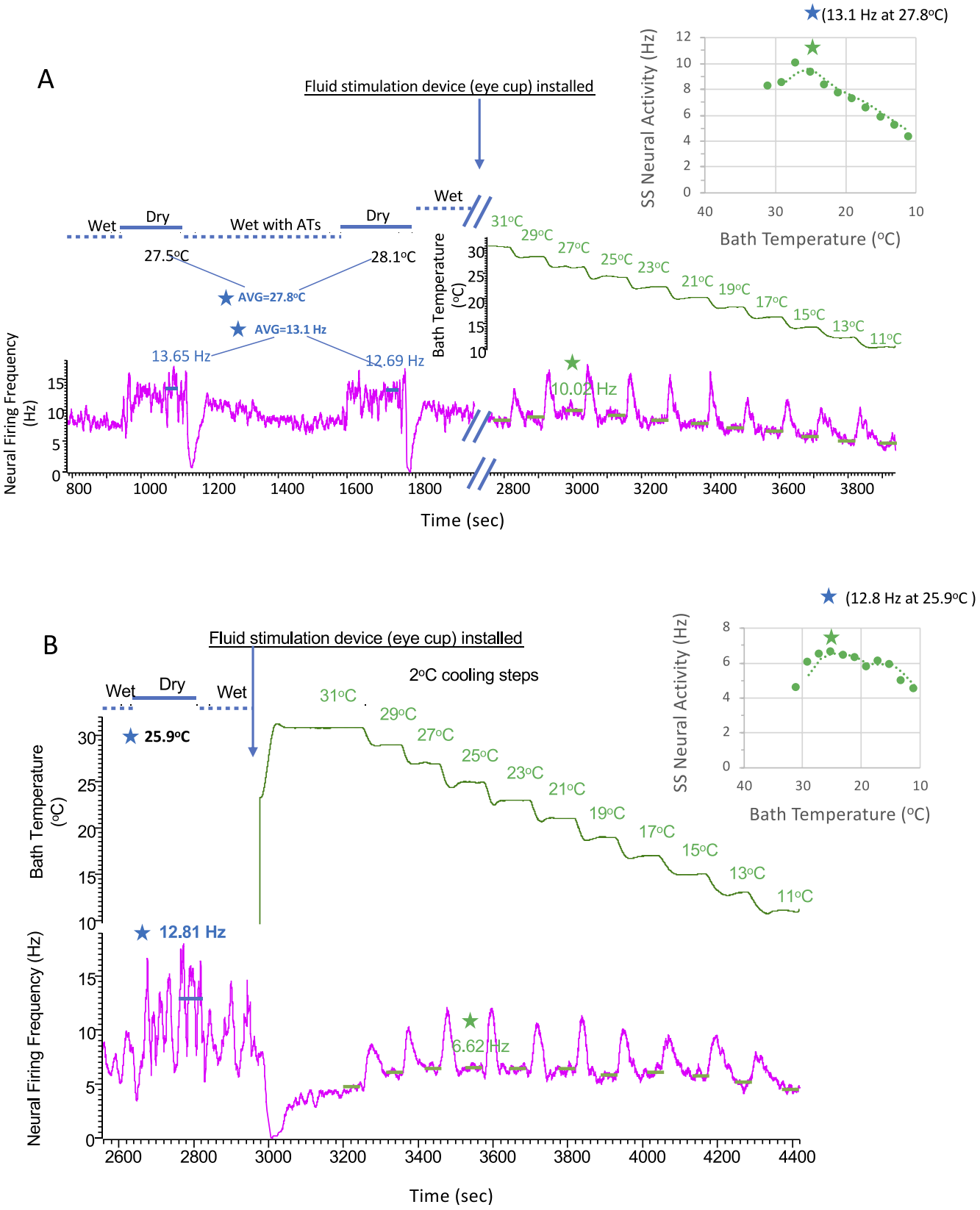

Figure 2. (**A**, **B**) Comparisons of firing frequencies and ocular temperature in two LT-CS + DS neurons during ocular dryness (*left portion* of each figure) and controlled-step thermal stimulation (*right portion* of each figure), which began when the eye cup was placed on the eye and the fluid stimulus device was installed (bath temperature). The inset shows the graphic function plotting SS neural firing frequencies against SS temperatures. The *dotted line* on the function is the moving average across *green dots* (data series). The *blue and green stars* indicate, respectively, the average neural firing frequency obtained during ocular dryness (with associated average OST measured thermographically) and the maximum SS frequency obtained during the optimal SS temperature. Two *oblique lines* in (**A**) signify the break in the recordings: the length of the break can be estimated from the *y*-axis (seconds).

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the blue lines across neural firing frequencies indicate the last 30 seconds of ocular dryness periods where the average dry responses were calculated. The values for neural frequencies and the temperatures were derived from these periods. In contrast, the right sides of Figures 2A and 2B show the relationship between SS temperatures of the ocular surface (bath temperatures) and the neural activities attained at each temperature level. When the temperature was suddenly decreased by 2°C, the dynamic changes in activity (dynamic responses) were elicited each time. Thereafter the level of the activity became stable (SS activity, green horizontal lines across neural activity records), which was dependent on the SS temperatures. The stimulus-response functions shown in the insets of Figure 2 demonstrate that the relationship is nonlinear but bell-shaped (inverted U-shaped). The maximum SS neural activities across all LT-CS + DS neurons were attained at different temperatures for different neurons, which ranged 15–29°C with activity levels ranging from 6.39 to 10.29 Hz. However, as shown in Figures 2A and 2B, comparison of the average neural activity levels (SS response) during the ocular dryness stimulation (blue horizontal lines across neural activity records in the left sides of the figures) and those during SS temperatures (with a controlled stimulation in the right sides of the figures) revealed significant mismatches between these two values. The ocular dryness activity (next to the blue star, 13.1 Hz in Fig. 2A) was significantly greater than the maximum activity levels attained by the optimum SS temperature (next to the green stars, 10.02 Hz in Fig. 2A).

Figure 3 shows the averaged stimulus (SS bath temperatures) − responses (neural activities) functions for LT-CS + DS and HT-CS + DS neuron populations. The average neural activity levels at the stabilized OST during the ocular dryness period (blue stars) in both classes of neurons were significantly higher than those predicted from the optimum SS temperature obtained via the controlled (flowing fluid) stimulation (green stars), suggesting that factors other than the SS temperatures were inducing the average neural activity during the ocular dryness. The differences, however, were statistically significant only for the LT-CS + DS neurons. Nonetheless, it is noteworthy in this regard that there was a marked difference in the spike patterns observed during the ocular dryness and SS temperature stimulations. The response profiles achieved during the former stimulation tended to be more "spiky" (or bursting) than those observed during the SS temperature stimulation. This was especially prominent in the LT-CS + DS neurons, which was reported previously by us.[6] The spiking appeared to render average activity level higher during the ocular dryness period, compared with activity levels recorded during the SS temperature stimulation. We hypothesized that the mechanisms underlying these spiking patterns may be related to a minuscule movement of the air occurring sporadically on the ocular surface, producing a slight but rapid cooling, hence inducing the spiking of the neuronal activities. Thus, if we diminish this air movement by covering the left frontal face with an aluminum foil barrier (with enough space between the foil and the eye to allow the ocular dryness to proceed), we speculated that we should be able to reduce both the spiking activity and the elevation of the average firing. This is indeed what we observed, as shown in Figure 4.

Figures 4A and 4B show, respectively, neural recordings from an LT-CS + DS neuron and an HT-CS + DS neuron during ocular dryness when the frontal face was screened with an aluminum foil barrier (eye covered) to prevent air turbulence from striking the ocular surface (as opposed to unscreened, or eye exposed). The numerous large "spiking" activities are seen only during the exposed eye period, and only for the LT-CS + DS neuron. Notice a marked difference in the average firing frequencies between these two ocular conditions: Compare the values above the horizontal lines crossing the neural activity records. The upper panels in Figures 4A and 4B show the "line" representations of the discriminated signals, which are derived from the activity of a single neuron (i.e., each vertical line represents one action potential). Note that there are many more clusters (bursts) of activity (black blocks) during the eye-exposed periods in this line figure only from LT-CS + DS neuron. The average (± SEMs) graphs depicted in Figure 4C show that statistically significant differences in the coefficient of variation (CV) and the average responses between these two atmospheric conditions were obtained only for the LT-CS + DS neurons.

The LT-CS + DS Neurons Display Numerous Sudden Shifts (Spiking) in Firing Frequencies (Dynamic Firing Rates) During a Stabilized Period of Ocular Dryness That Show a Positive Linear Correlation With the Rates and the Magnitudes of Cooling

As seen in Figure 1A for LT-CS + DS neurons (but not in Fig. 1B for HT-CS + DS neurons), during the apparently stabilized ocular temperature, frequent small and sudden (dynamic) decreases and increases of temperature along with the "spiking" neural activities were observed. Figure 1 also shows that although the temporal correspondence was not perfect (with some delay of 0.5–1.5 seconds), there appears to be a very close relationship between the magnitudes of the dynamic (sudden) cooling or warming monitored at the ocular surface and the activity increases or decreases (long arrows intersecting temperature and neuronal activity traces). This suggests that a sudden slight temperature drop (<0.2°C) is an adequate stimulus for the activation of LT-CS + DS neurons. We had been noticing for quite some time during the course of previous experiments that these sudden shifts in OST often coincided with episodes when someone walked into the room or in front of the recording table, or when the room air conditioner turned on, presumably causing tiny changes in the room air circulation. To examine this possibility that a slight degree of air turbulence produces sudden but very small temperature changes on the ocular surface, we generated a light breeze by waving hands or exhaling near the ocular surface approximately 15 cm in front of the left eye (to produce smaller or greater cooling on the area, respectively), while simultaneously recording the neuronal activity. The magnitudes and the rates of cooling were measured with infrared thermography. The exemplary results from these experiments are shown in Figure 5.

Figure 5A demonstrates that the slight cooling during ocular dryness had significant effects on the neural activation. Even before the air currents were generated (red arrowheads), the long blue arrows show a near perfect temporal correspondence between a small temperature decrease (approximately 0.1–0.2°C) and the jumps in neural activation ("spiking"). When the air currents were produced, the magnitudes of neural activation were larger than those in response to the smaller cooling before the breezes were generated, suggesting a linear relationship between temperature decreases and neural activity. More remarkably, the spiking activities were seen to follow faithfully the degrees of cooling generated after each breeze stimulus (red arrowhead). The expanded view of Figure 5B (derived from the blue box shown in Fig. 5A) revealed a more detailed relationship between the ocular temperature changes and the neuronal responses. The exquisite sensitivity to dynamic changes in OST (approximately 0.1–0.4°C in thermographic images of I–IV) is evident in the almost instantaneous increases in neural activity after slight cooling (blue arrows) as well as in the "humps" in activity

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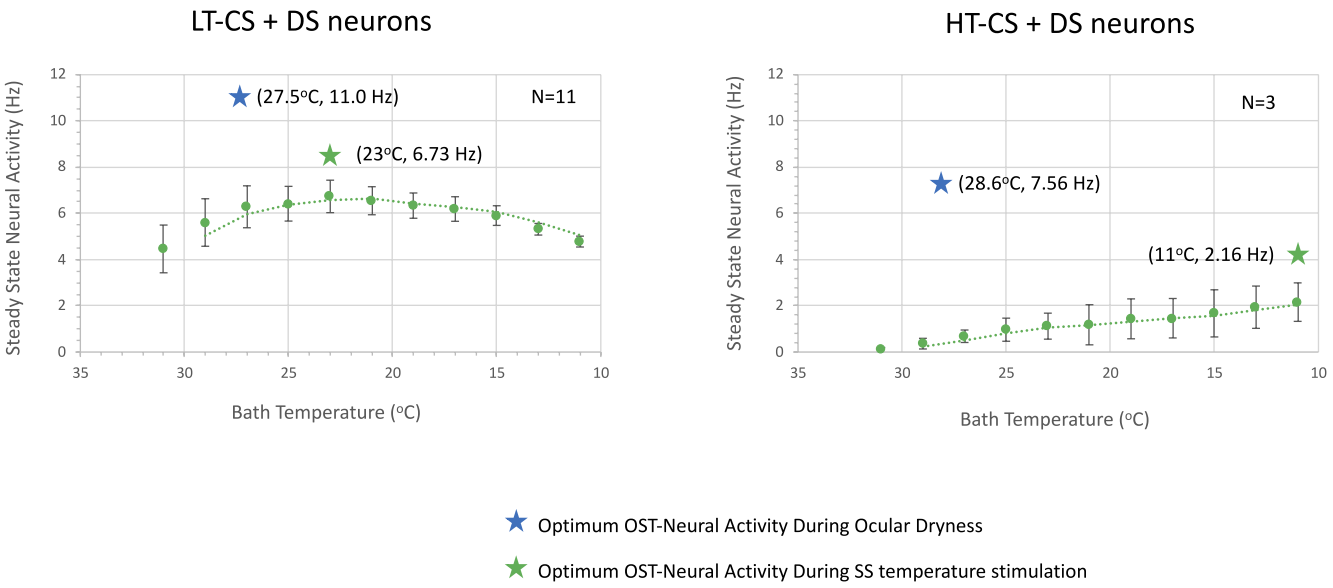

Figure 3. The stimulus (bath temperature)-response (neural activity) functions from all neurons studied. The *green dots* represent average neural firing frequencies (± SEM) obtained at each temperature based on all LT-CS + DS and HT-CS + DS neurons analyzed in this study. The *blue and green stars* denote the same parameters as in Figure 2.

(green arrows) seen when the cooling is interrupted by a minimal warming, which was then followed almost instantaneously by much cooler temperatures due to initiation of air flow that produced further neuronal activity increases. A degree of warming that produced humps was too brief and too small to have led to visible decreases in activities, such as those seen next to the asterisks. It should be noted that a warming of the ocular surface associated with the decreases in neuronal activity (asterisks in Fig. 5B) is one of the response characteristics of all cold thermoreceptors thus far examined.[22,24] As described above, Figure 5 shows that there appears to be a cooling intensity–dependent activation of the neuronal spikes. To quantify the relationship between the magnitudes or the rates of cooling achieved during the air current production (waving hand or exhaling breath) and the magnitudes of the neural activations recorded, we analyzed these parameters using the methods shown in Figure 6.

Figure 6A depicts the simultaneous recording of the neural firing frequencies and the associated temperature changes produced by air currents from an LT-CS + DS neuron. The more detailed views shown in Figures 6B and 6C revealed relationships between the cooling magnitudes (vertical side of the hypotenuse) or rates (slope of the hypotenuse) and the neural activity levels in expanded scales.

Figure 7 shows the results when the neural activity levels were plotted as a function of the cooling magnitudes (Figs. 7A, 7C) or cooling rates (Figs. 7B, 7D): the resulting regression lines were highly significant only for the LT-CS + DS neuron population. Each dot (incident, e.g., at the blue arrow) represents a datum from the time when the air current was produced (with associated cooling) and the neural activity was evoked. Of 4 LT-CS + DS neurons tested, significant correlations between cooling magnitude and firing frequency were obtained in all four neurons, whereas the correlation between the cooling rates and the firing frequency was significant in three of the four neurons. For four HT-CS + DS neurons tested, the correlations between the magnitudes of cooling and the neural activity levels were not statistically significant. Detailed analyses of HT-CS + DS neurons revealed that if the cooling intensity was low (i.e., produced by waving hands), the activity to those stimuli was not above the background level (data not shown). However, occasionally in some neurons, if the cooling stimulus was strong enough (i.e., blowing air by exhaling), the responses of the neurons were elicited, suggesting that for HT-CS + DS neurons to be activated requires strong cooling (or sudden increases in tear osmolarity due to evaporation), such as generated by blowing breath over the eyes. This is consistent with the classification criteria of HT-CS + DS neurons (Materials and Methods).

Discussion

General Summary

The present study demonstrated that one of the critical stimuli influencing the activity of LT-CS + DS neurons is the very slight change in temperature that occurs at the ocular surface when the cornea begins to dry after fluids (tears) start to evaporate. One common environmental stimulus associated with ocular surface cooling is air flow over the eye when individuals move around their environment. Even the very slight turbulence in the air flow produced by simply walking could generate such a stimulus. The LT-CS + DS corneal nerves merely require a sudden decrease of approximately 0.1–0.2°C in the OST to increase their firing activity above baseline (Fig. 1). The numerous "spiking" activities observed during the corneal dry state in the present study were found to be the results of these small shifts in OST (Fig. 2). Also, when these spiking events are initiated, the average neural activities were much higher than would be predicted to occur during comparable SS temperatures (Fig. 3). The neuronal signals so produced are then likely to be transmitted as an afferent signal of the lacrimation reflex (or eye-blink reflex) to trigger basal tearing and/or eye blinking. The spiking (bursting) nature of primary afferent neurons, such as those observed in the present study during ocular desiccation, has been known to contribute to a greater postsynaptic activation of the central neurons[25] and is likely to deliver stronger signals to the neurons responsible for the tearing or eye-blink reflexes compared with signals associated with more regular patterns of spikes. When local air turbulence during ocular desiccation conditions was prevented (Fig. 4), the neurons largely failed to produce "spiking,"

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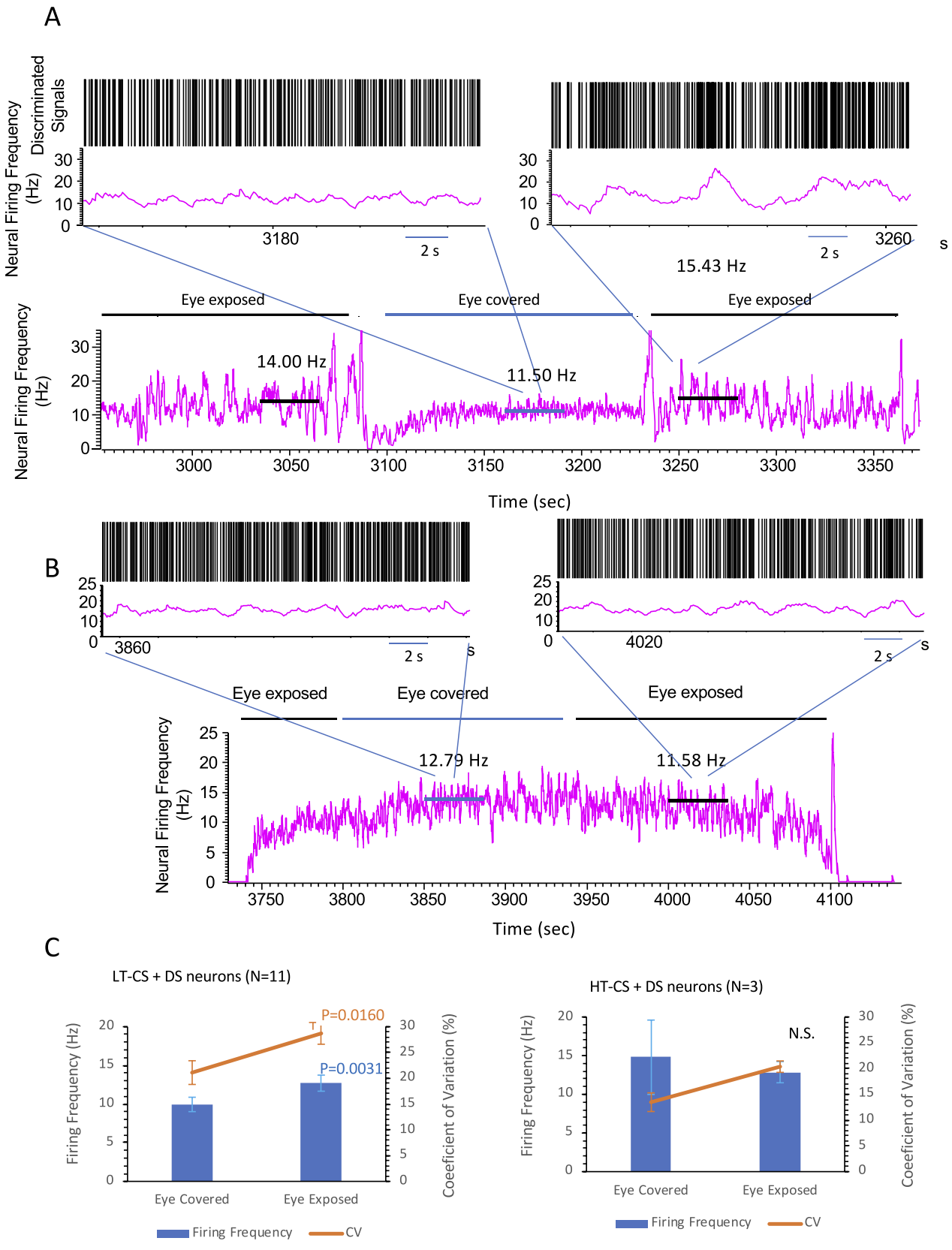

Figure 4. (**A**, **B**) Neural recordings from a LT-CS + DS neuron (**A**) and a HT-CS + DS neuron (**B**) during ocular dryness when the frontal face was screened with an aluminum foil barrier (eye covered) to prevent air turbulence from striking the ocular surface (as opposed to unscreened, or eye exposed). (**C**) Average (± SEMs) graphs of neural firing frequencies and CVs when we exposed the eyes and when we protected the eyes with an aluminum foil cover. The statistical results (probability, *P*) were based on *t*-tests between the two ocular conditions.

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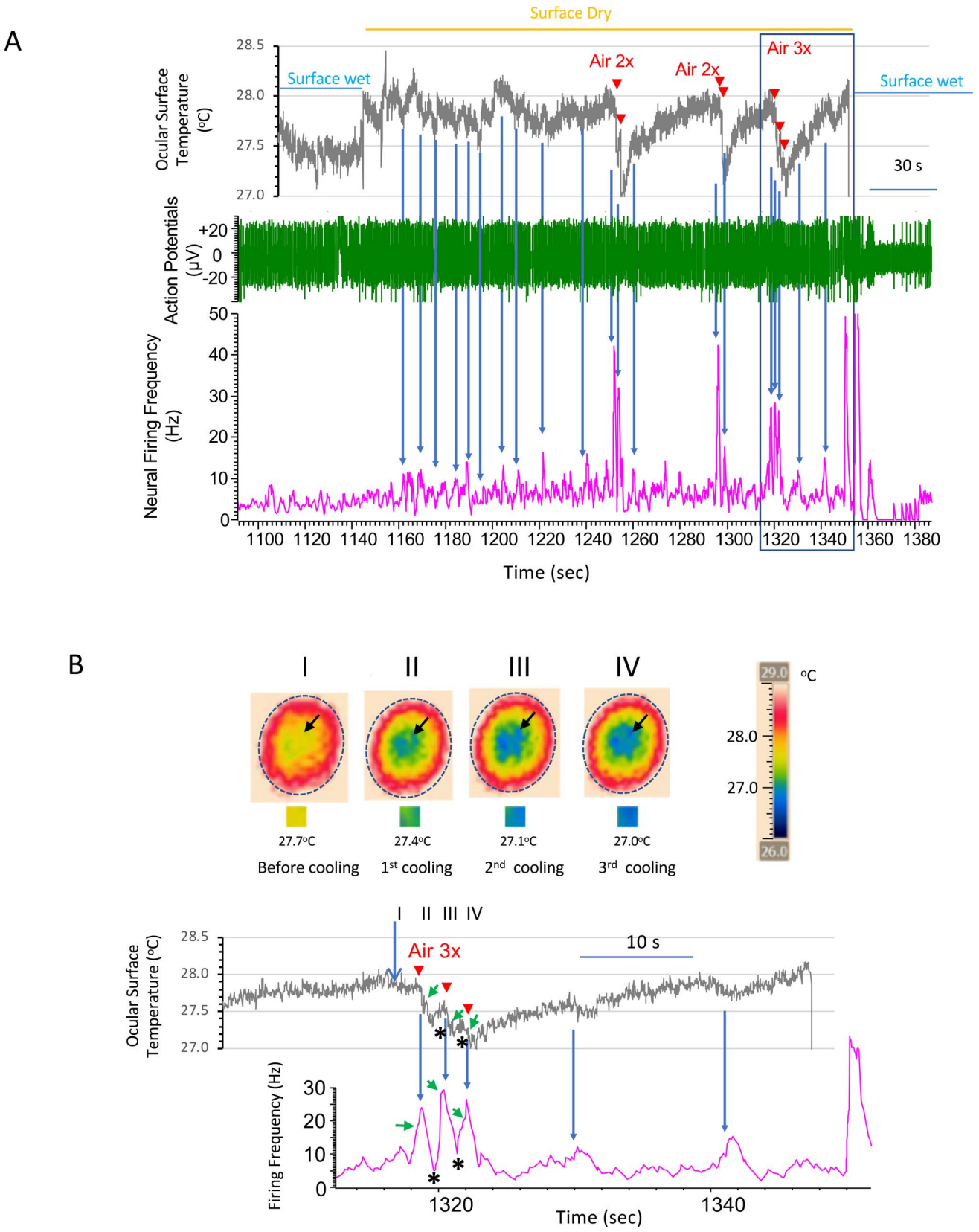

Figure 5. (**A**) Simultaneous recording of the OST and neuronal responses of another LT-CS + DS neuron during the spontaneous drying of the cornea and stimulation by air currents generated by fanning hands in front of the animal's eye. The instances when the hands were moved and breezes generated are indicated by inverted *red triangles* above the OST monitors (Air). (**B**) Close-up view of the OST and neuronal responses from the record, shown inside the *blue box* in (**A**). The thermographic color photos at the times indicated by Roman numerals (four insets, I–IV) demonstrate the degrees of OST. The *small black arrows* on the photos point to the location of the neuronal receptive field (nerve terminals in the cornea), from which the thermal recordings were made. The *dotted ovals* in I to IV indicate the limbal/conjunctival borders.

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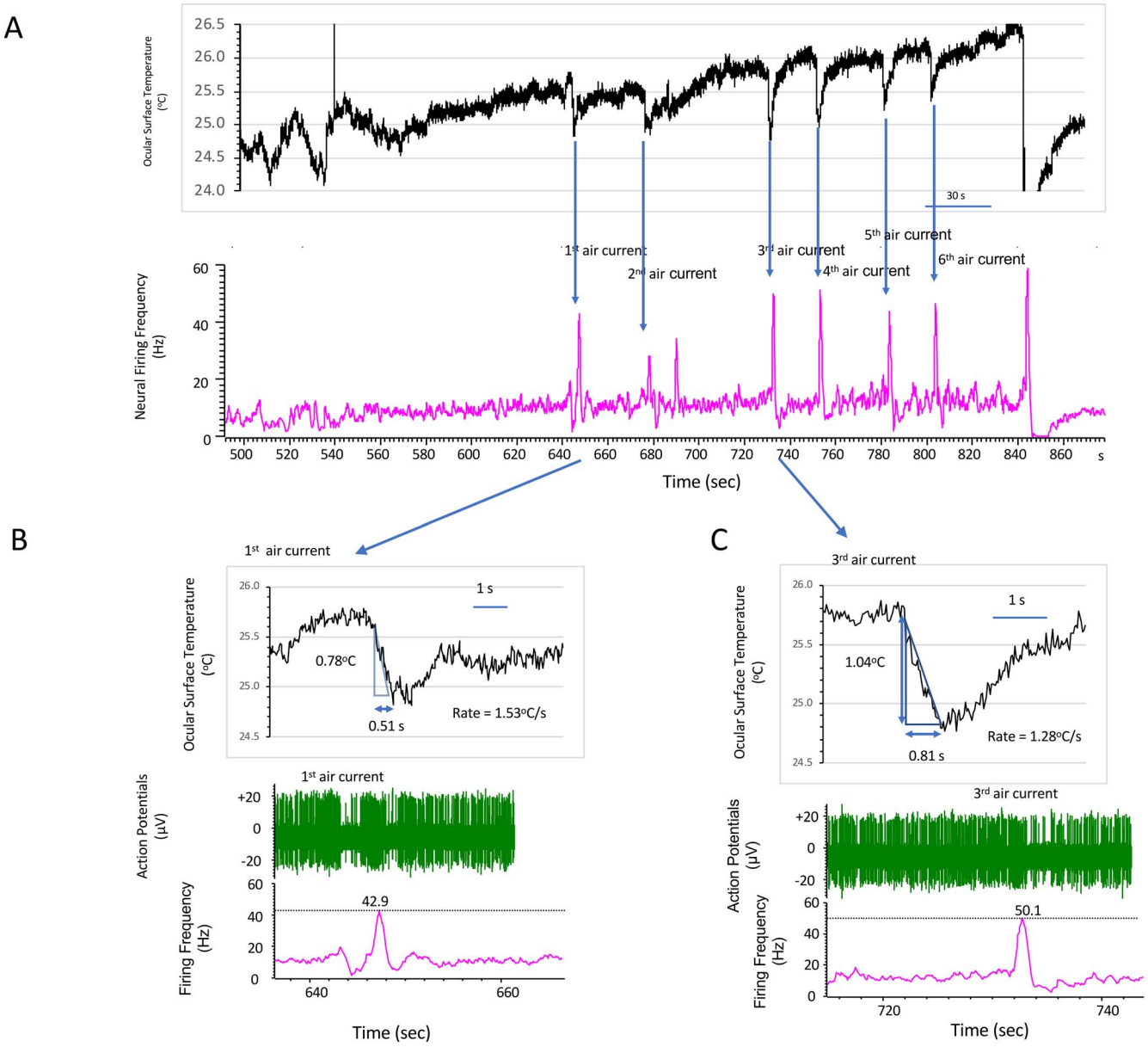

Figure 6. (**A**) Simultaneous recording of the neural firing frequencies and the associated temperature changes from an LT-CS + DS neuron. The first and second air currents were generated by moving hands briskly, and the third and fourth airs by exhaling the breath over the eyes. (**B**, **C**) Detailed views extracted from the recording areas shown with *blue arrows* for the first air current (**B**) and third air current (**C**) are also depicted. The triangles display the methods used to calculate cooling magnitudes, the time (horizontal scales) it took to reach the magnitudes (vertical scales) and rates (slopes) that derived from these two values. The numbers above the black horizontal lines in the neuronal records (*lower panels* in **B** and **C**) indicate the peak firing frequencies associated with the cooling generated by air currents. The results of these calculations for all neurons are shown in Figure 7.

resulting in a significantly lower average activation. This finding indicates that a sporadic large volume of tearing may be superimposed on a less voluminous continuous tear flow, which is presumably produced by the SS neural activity after the fluids (tears) begin to evaporate. SS temperatures rarely exist in nature and are unlikely to be the factor determining neural activity except in cases in which the cornea is wet with copious tears, as reflected in the extremely stable wet response of LT-CS + DS neurons.[6]

In contrast to LT-CS + DS neurons, the activity of the HT-CS + DS neurons exhibited inconsistent or weak relationship to the changing OST (Figs. 1, 7). We previously hypothesized that the changes in their activities are associated more with the local increases in the osmolarities of the extracellular milieu than the cooling of the ocular surface.[8] This is consistent with the present observation that, unlike LT-CS + DS neurons, when the ocular dryness began, initial changes in surface temperature (first approximately 60 seconds) after the surface fluids were removed were not consistently related to the neural activity levels (Fig. 1B). However, during this initial stage, the tear osmolarities are expected to slowly increase, which was directly reflected in the slow activity increase observed in most HT-CS + DS neurons in our previous[6] and current studies. One function speculated for HT-CS + DS neurons is production of desiccation-induced ocular pain in response to increases in tear osmolarities. In the present experiment, the activity of these neurons was seen to gradually increase over 30 to 60 seconds of continued ocular desiccation (Fig. 1B). A recent report corroborates this

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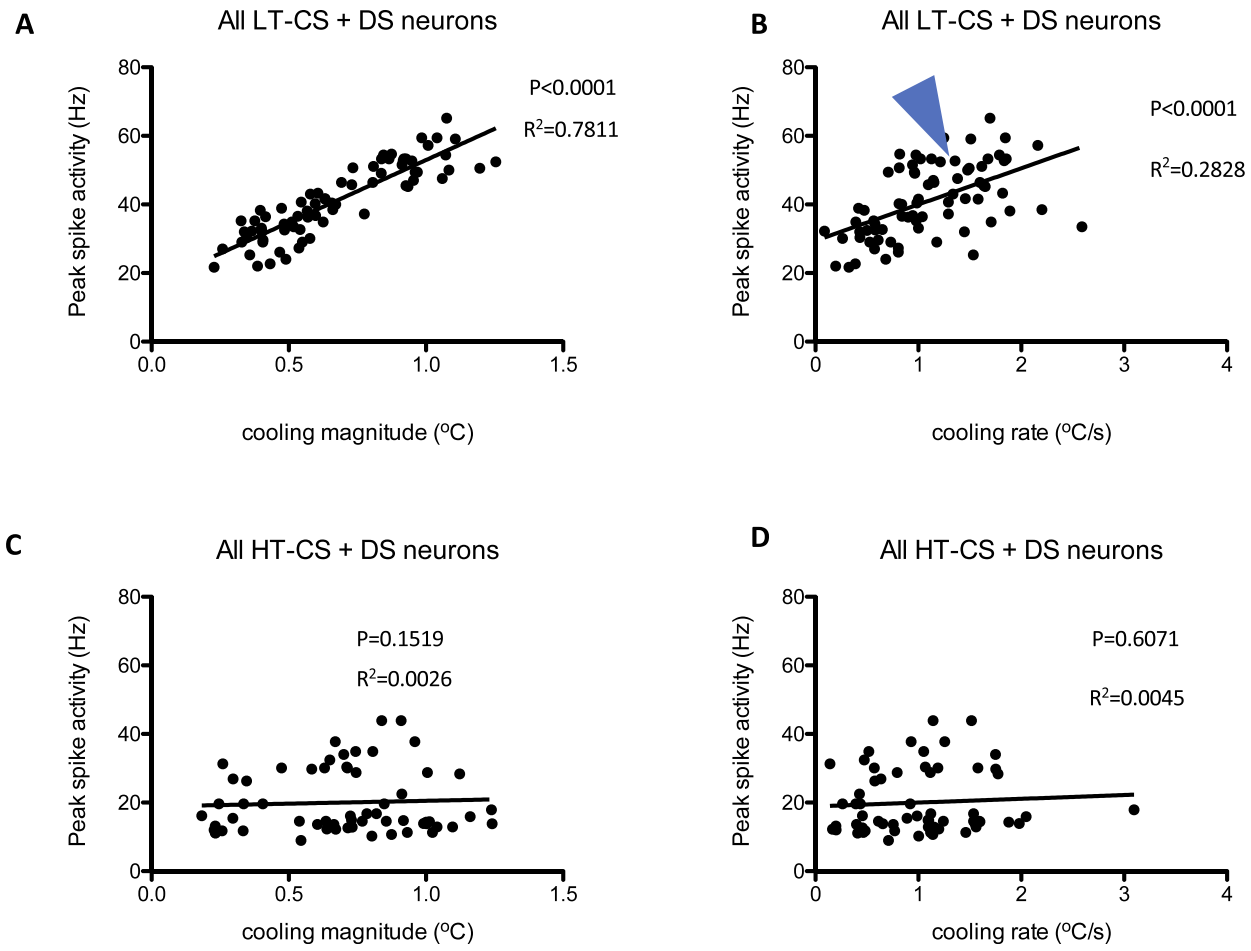

Figure 7. Graphs showing the correlations between cooling magnitudes (**A**, **C**) or cooling rates (**B**, **D**) and peak neural firing frequencies of LT-CS + DS neurons (**A**, **B**) and HT-CS + DS neurons (**C**, **D**). Probabilities for statistical significance (linear regressions) are also shown in each graph. The graphs are based on 73 pairs of *x*- and *y*-variables from four animals for LT-CS + DS neuron group and 61 pairs from four animals for HT-CS + DS neuron group. Each data point in these figures was derived from the calculation shown in Figure 6 (four data points were drawn from Figs. 6B and 6C). For example, the data point at a *blue arrow* was derived from the rate of cooling (1.33°C/s) and the peak activity (51.5 Hz) shown Figure 6C (fourth air current).

same finding in human subjects who went through forced-eye opening: the subjects' pain gradually increased over 10 to 30 seconds until it became intolerable and blinking began.[26] Additionally, there is evidence of a biphasic response to the ocular pain sensation during dry conditions, the second phase beginning 15 to 50 seconds after eye opening.[27] The spiking activity was observed in some HT-CS + DS neurons (data not shown), especially when the much stronger cooling was generated by strong wind. We expect that these spiking activities derived from the sudden increases in tear osmolarities with these stimuli. Thus, it is possible that these spiking activities of some HT-CS + DS neurons probably contribute to the strong reflexive responses (tearing or eye blinking), preventing any severe damage that could occur during continued ocular desiccation. It has been reported that a type of corneal nerves that express Transient Receptor Potential cation channel subfamily M member 8 (TRPM8) detects changes in tear osmolarities and could produce eye blinking in mice.[4] The specific molecular underpinning of the HT-CS + DS neurons, however, is currently unknown.

Comparison With Studies of Human Subjects

Under relaxed conditions, humans blink at an average of approximately every 4 seconds after opening their eyes.[28] This interval could increase to approximately every 15 to 30 seconds under experimental conditions, such as fixating on a computer display,[17,28–31] suggesting that the 2 minutes of ocular desiccation used in the current study might not arise in everyday life. This might make our study somewhat an artificial situation. However, the initial temperature changes we observed during the first 30 seconds in the current study (Fig. 1) have demonstrated that SS changes in OST between eye blinks in humans described above are essentially similar to our current results. The previous experiments showed an immediate increase in OST after the eyes opened, followed by a slow decrease.[29] In our study, a similar pattern was observed when the ocular desiccation began (Fig. 1). These results demonstrate that the relationship between the SS OST and the SS neural activity changes during this early period in our rodent model is nonetheless relevant to humans. Moreover, during these initial OST changes, one can still observe small but sudden increases or decreases in neural activity that were associated with, respectively, dynamic cooling or warming (Fig. 1A), suggesting that the mechanisms we proposed to underlie the "spiking" activity of the corneal neurons and their significance (i.e., cooling-evoked neural activation) should still apply during this early period of recording.

The individual differences in the SS temperature changes in the first approximately 30 seconds of ocular desiccation

observed in the present study (some simply increasing and others involving a more complex mixture of increasing and decreasing OST [Fig. 1]) could be due to several factors. Diurnal variation in body temperature has been known to influence differences in OST.[29] Room temperature is similarly correlated with OST, showing a typical increase of 0.15 to 0.2°C in OST per degree centigrade increase in room temperature.[29] These factors presumably contributed to the variability in the corneal surface temperatures reported in previous human studies.[30,32–34] However, in the present study, two of these factors (i.e., room temperature and body temperature) were well controlled, and hence unlikely to have contributed to the differences in OST and therefore the neural activity. One other factor that might have contributed to the individual differences in SS temperatures during the stabilized periods is the propagation of temperatures from the vascular limbal region. Although the body core temperature was maintained in a relatively constant status by the feedback-controlled device (Materials and Methods section), the local circulation probably varied considerably among individual animals[35,36] and also from the influence of internal conditions, such as inflammation. Dry eye patients experiencing symptomatic ocular inflammation have been found to exhibit a higher OST,[21,37] perhaps reflecting increased conjunctival hyperemia.

Functional Significance

It is possible that the SS activity in LT-CS + DS neurons underlies the trigger for basal tearing and that the spiking activities during the stable ocular temperature conditions may account for blinking, although these two reflexes (i.e., tearing and blinking) are very difficult to dissociate mechanistically. Previous studies demonstrated that similar factors, such as wind and exposed corneal surface areas, influenced both blink rates[30] and tear dynamics,[3,33,38] suggesting that these factors are in fact interdependent on each other, one influencing the other. For example, the blink rates themselves are important factors affecting tear dynamics.[33] These results suggest that the central neural mechanisms in the pons may receive convergent inputs using similar trigemino-facial (-lacrimal) connections in other areas of the brain,[39,40] which in turn provide the divergent outputs to the lacrimal glands and orbicularis muscles. From the point of view of triggering sources that share the same stimuli (cooling), this is a plausible explanation.

Are There Spontaneous (Basal) Tears?

Clinical investigations generally separate basal tears from reflex tears. However, our studies suggest that all tearing, including the so-called basal tearing described in clinical studies, is evoked by external stimuli acting on the ocular surface, whether these stimuli are light breezes or slight temperature changes due to air turbulence or temperature propagation from the limbal circulation.

Conclusions

To keep the eye protected from external insults, the ocular surface is normally covered with a thin film of tears. When, however, the tears begin to evaporate under arid conditions, nervous system feedback will react to produce more tears. Our current study demonstrated that one of the factors that triggers tearing is a slight cooling generated by atmospheric air turbulence that in turn differentially influences two types of dry sensitive corneal neurons. In particular, the sensitivity of the LT-CS + DS neurons to cooling is exquisite, often responding to just approximately 0.1°C changes, hence presumably evoking tears constantly during waking hours (i.e., basal tears).

Acknowledgments

Supported by National Institutes of Health Grants EY023555 (HH) and EY018594 (MIR), and Research to Prevent Blindness Grants to the Department of Ophthalmology, Weill Cornell Medical College.

Disclosure: **H. Hirata**, None; **V. Dallacasagrande**, None; **K. Mizerska**, None; **E. Ivakhnitskaia**, None; **M.I. Rosenblatt**, None

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