Corneal dry-responsive neurons in the spinal trigeminal nucleus respond to innocuous cooling in the rat

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Kurose M, Meng ID. Corneal dry-responsive neurons in the spinal trigeminal nucleus respond to innocuous cooling in the rat. J Neurophysiol 109: 2517–2522, 2013. First published February 27, 2013; doi:10.1152/jn.00889.2012.—Corneal primary afferent neurons that respond to drying of the ocular surface have been previously characterized and found to respond to innocuous cooling, menthol, and hyperosmotic stimuli. The purpose of the present study was to examine the receptive field properties of second-order neurons in the trigeminal nucleus that respond to drying of the ocular surface. Single-unit electrophysiological recordings were performed in anesthetized rats, and dry-responsive corneal units were isolated in the brain stem at the transition zone between the spinal trigeminal subnucleus caudalis and subnucleus interpolaris. Corneal units were characterized according to their responses to changes in temperature (cooling and heating), hyperosmotic artificial tears, menthol, and low pH. All dry-responsive neurons (n = 18) responded to cooling of the ocular surface. In addition, these neurons responded to hyperosmotic stimuli and menthol application to the cornea. One-half of the neurons were activated by low pH, and these acid-sensitive neurons were also activated by noxious heat. Furthermore, neurons that were activated by low pH had a significantly lower response to cooling and menthol. These results indicate that dry-responsive neurons recorded in the trigeminal nucleus receive input from cold, sensitive primary afferent neurons, with a subset of these neurons receiving input from corneal primary afferent neurons sensitive to acid and noxious heat. It is proposed that acid-insensitive corneal neurons represent a labeled line for laceration in response to evaporation of tears from the ocular surface, whereas acid-sensitive neurons are involved in tearing, elicited by damaging or potentially damaging stimuli.

THE OCULAR SURFACE IS LUBRICATED by a multilayered tear film that provides protection and essential nutrients for the cornea. The regulation of tears involves reflexes initiated by primary afferent neurons innervating the cornea (Acosta et al. 2004; Belmonte and Gallar 2011; Parra et al. 2010; Robbins et al. 2012). Corneal polymodal nociceptors and mechanoreceptors detect potentially damaging stimuli and induce tearing to cleanse the ocular surface (Acosta et al. 2001a, b, 2004; Gallar et al. 1993; Tanelian and Beuerman 1984). Corneal afferents sensitive to drying of the ocular surface appear to increase tearing, as necessitated by evaporation of the tear film (Belmonte and Gallar 2011; Hirata and Meng 2010; Parra et al. 2010; Robbins et al. 2012). Upon further characterization, these dry-responsive corneal primary afferent neurons were shown to be sensitive to innocuous cooling and hyperosmotic stimuli (Hirata and Meng 2010), representing a third class of corneal afferents, defined previously as cold or cool cells (Acosta et al. 2001a; Brock et al. 2001; Gallar et al. 1993). Corneal primary afferent neurons project to and activate neurons in two distinct regions of the spinal trigeminal nucleus (Vsp): the transition region between subnucleus interpolaris and subnucleus caudalis (Vi/Vc) and the more caudally located transition between Vc and the upper cervical spinal cord (Vc/C1) (Marfurt and Del Toro 1987; Meng and Bereiter 1996; Meng et al. 1997; Strassman and Vos 1993). The Vi/Vc transition region is specialized for the central regulation of lacrimation and blinking, and the properties of corneal-sensitive neurons located in this region are consistent with these functions (Hirata et al. 2004). Of particular note, Vi/Vc corneal units project to regions involved in the efferent pathway for lacrimation and blinking, and a portion of Vi/Vc neurons responds to drying of the ocular surface (Henriquez and Evinger 2007; Hirata et al. 2000, 2004; Toth et al. 1999). In comparison, the properties of corneal-sensitive neurons at the Vc/C1 transition region appear similar to nociceptive neurons located in the spinal cord dorsal horn (Meng et al. 1997, 1998).

The properties of Vi/Vc dry-responsive neurons have yet to be characterized according to their responses to innocuous cooling and hyperosmotic solutions, two stimuli known to activate dry-responsive primary afferent neurons. Furthermore, although a subset of acid-sensitive Vi/Vc neurons responds to corneal drying, it is unknown whether all dry-responsive neurons respond to low pH (Hirata et al. 2004). In the present study, we examined the sensitivity of second-order, dry-responsive neurons at the Vi/Vc region to acidity, thermal stimulation, hyperosmotic solutions, and menthol, an agonist to the transient receptor potential melastatin 8 (TRPM8) channel.

MATERIALS AND METHODS

Animals. Experiments were performed on male Sprague-Dawley rats (300–400 g; Charles River Laboratories International, Wilmington, MA) that were group housed and given free access to food and water. Procedures were approved by the Institutional Animal Care and Use Committee at the University of New England and were treated according to the policies and recommendations of the NIH guidelines for the handling and use of laboratory animals.

Surgery. Animals were anesthetized with 2–3% isoflurane for surgery. The right femoral vein was catheterized for infusion of drugs, and the right femoral artery was catheterized for continuous blood-pressure monitoring. The trachea was cannulated, and immediately afterwards, the animal was placed on a ventilator for artificial ventilation. End-tidal carbon dioxide (CO2) was maintained between 3.5% and 4.5%. Body
temperature was maintained at 37°C with a feedback-controlled heating pad. After positioning the head in a stereotaxic apparatus, the occipital bone was partially removed, and the dorsal brain stem was exposed from 0.5 mm rostral to obex to the C1 vertebral bone. The brain stem was kept moist with warm mineral oil. Electrophysiological recordings commenced at least 45 min after completion of surgery.

**Single-unit recordings.** Extracellular single-unit recordings from corneal-responsive neurons located in the Vl/Vc transition region were performed using tungsten microelectrodes (7–9 MΩ impedance; FHC, Bowdoin, ME). Corneal units were identified by responses to light mechanical stimulation of the cornea or responses that could be evoked by the placement of a cold metal probe (tip diameter ~1 mm) near the cornea. Cutaneous receptive fields were examined by brushing, pressing, and pinching the area around the eye and snout. During recording, artificial tears (AT) were liberally applied to the cornea as necessary to prevent drying of the ocular surface.

The effect of ocular fluid status was examined by recording the rate of discharge during wet and dry conditions. The dry corneal condition was produced by removing excess fluid from the ocular surface. Fluid was wicked away with Kimwipes (Kimberly-Clark, Neenah, WI) by lightly touching the tear meniscus at the lateral canthus for ~10 s, avoiding direct mechanical contact with the cornea. AT were reapplied to the eye, 120 s after tear removal, so that the entire corneal surface was covered with fluid, typically requiring ~10 μl fluid. Only corneal units that increased their rate of discharge during the dry condition were examined further.

Data were acquired by a CED Micro1401, and isolated neurons were analyzed with Spike2 (Cambridge Electronic Design, Cambridge, UK). At the end of each experiment, animals were euthanized with an overdose of sodium pentobarbital (>100 mg/kg iv) and perfused with saline, followed by 10% formalin after lesions of the recording (20 μA; 20 s) site. Frozen sections (30 μm) were cut, mounted, and stained with cresyl violet for histological identification of the recording sites.

**Thermal stimulation.** Controlled thermal stimulation with a 5-mm² contact thermode was used to examine responses to both cooling and heating stimuli (TSA-II; Medoc, Ramat Yishai, Israel). From a holding temperature of 35°C, the heating stimulus consisted of a sequence of five step ramps down to 1°C at 4°C intervals (Hirata and Meng 2010). Each cooling stimulus lasted for a duration of 20 s with a 20-s interval between stimuli. The mean frequency of activity during presentation of the stimulus. The mean frequency of activity during the dry condition was based on the average discharge over the final 30 s of a 120-s dry condition, with the wet condition defined as average activity for 30 s preceding the dry condition. Statistical analyses for the comparison of wet and dry corneal condition and the effect of thermal and chemical stimulation on neuronal discharge were performed with a two-way ANOVA, followed by Fisher least significant difference multiple comparison post hoc tests (SigmaStat 3.5; Systat, Chicago, IL). Data are expressed as means ± SE, and P < 0.05 was considered to be statistically significant.

**RESULTS**

A total of 18 dry-responsive neurons were recorded from 14 animals. Recording sites were identified from electrolytic lesions in 10 animals (Fig. 1A). The location of these sites was in the ventral pole of Vsp at the transition between Vl and Vc. Receptive fields typically included the entire cornea, and no excitatory cutaneous receptive fields were found. Whereas all neurons responded to mechanical stimulation and drying of the ocular surface, two distinct populations of neurons could be identified based on their response to acid. Nine of the 18 units responded to low pH with an increase in discharge, defined as a positive Rmag value, whereas the remaining neurons were unresponsive to acid. Both groups of neurons were located in similar areas of the ventral pole of Vsp (Fig. 1A).

Group comparisons between acid-sensitive and -insensitive neurons revealed a significant difference in their response to drying [Fig. 1B; F(1, 32) = 37.31, P < 0.001]. Both groups of neurons responded to drying with an increase in discharge; however, the response of acid-insensitive neurons to the dry condition was greater than acid-sensitive neurons (P < 0.05). There was no difference between acid-sensitive and -insensitive neurons in ongoing activity during the wet condition [Fig. 1B; P > 0.05].

Acid-sensitive and -insensitive neurons increased their discharge in response to hyperosmotic AT, demonstrating a statistically significant increase in Rmag, 5–20 s after application of the hyperosmotic solution to the cornea [Fig. 1C; F(2, 48) = 21.69, P < 0.001]. The difference in magnitude between acid-insensitive and -sensitive neurons to hyperosmotic AT did not reach statistical significance. By 105–120 s after application of the hyperosmotic AT, the response had attenuated to a Rmag of 11.57 ± 7.56 in acid-sensitive neurons and 28.05 ± 15.68 in acid-insensitive neurons, values that were no longer different from vehicle (AT) controls (P > 0.05).

Both acid-sensitive and -insensitive neurons responded to cooling of the ocular surface (Fig. 1D). Statistical analysis revealed a significant effect of temperature [F(5, 96) = 11.94, P < 0.001] and neuronal cell group [F(1, 96) = 12.92, P < 0.001] on the magnitude of the response. For acid-insensitive neurons, increases in cold-evoked responses were observed beginning at 31°C, whereas increases in cold-evoked activity in acid-sensitive neurons did not reach significance until 23°C (Fig. 1D). In addition to the difference in threshold for cold-evoked activity, the overall cold-evoked discharge in acid-insensitive neurons was consistently higher compared with acid-sensitive neurons at all cooling temperatures. In contrast to the cooling responses, noxious heat increased neuronal discharge only in the acid-sensitive neurons, with a significant effect of temperature [F(1, 29) = 11.26, P < 0.01] and neuronal cell group [F(1, 29) = 13.07, P = 0.001]. The Rmag response to noxious heat was 148.34 ± 45.13 in the eight
acid-sensitive neurons tested compared with $-23.03 \pm 18.00$ in the seven acid-insensitive neurons tested (Fig. 1D).

Typical response patterns for acid-sensitive and -insensitive neurons are shown in Fig. 2. In addition to the distinction based on acid sensitivity, the most prominent difference between the two categories of neurons was in their response to noxious thermal stimulation. Whereas acid-sensitive neurons increased their discharge in response to noxious heat, acid-insensitive neurons did not demonstrate an increase in activity to this stimulus. Instead, acid-insensitive neurons were often inhibited by noxious heat, which was then followed by a strong rebound discharge observed following the decrease in temperature from the plateau of 52°C down to the holding temperature of 35°C (Fig. 2).

Finally, in addition to applying thermal stimuli with a contact thermode, the effect of the cooling agent menthol was examined in seven acid-sensitive and nine acid-insensitive neurons. Both cell groups responded to menthol with an increase in discharge (Fig. 3, A and B). However, whereas the overall discharge was comparable at early time periods, with a Rmag of 239.91 ± 79.75 in acid-sensitive neurons and 190.72 ± 35.72 in acid-insensitive neurons at 5–20 s postmenthol, acid-sensitive neurons demonstrated a considerably faster decay in their response (Fig. 3C). A statistical comparison of the Rmags at the early and late time periods revealed a significant difference between acid-sensitive and -insensitive neurons at 105–120 s following menthol application [F(1,32) = 12.10, $P < 0.001$]. At this later time point, the Rmag of acid-insensitive neurons remained elevated (116.75 ± 48.75), whereas the Rmag in acid-sensitive neurons returned to control levels (12.91 ± 11.05).

DISCUSSION

Dry-responsive corneal units at the Vi/Vc transition region were characterized for responses to thermal, osmotic, and chemical stimuli, with the prediction that dry-responsive Vi/Vc neurons would respond to cooling and hyperosmolarity in a manner consistent with the properties of dry-responsive primary afferent neurons (Hirata and Meng 2010). As expected, dry-responsive neurons responded to cooling and hyperosmotic solutions applied to the cornea. Upon further analysis, two types of corneal dry-responsive neurons could be distinguished based on their sensitivity to acetic acid: acid-sensitive and -insensitive neurons. Acid-insensitive neurons had a greater response cooling and the TRPMS agonist menthol compared with acid-sensitive neurons. Furthermore, in contrast to acid-sensitive neurons, acid-insensitive neurons were unresponsive to noxious heat. These results indicate that at least two categories of second-order, dry-responsive corneal units exist, each likely receiving input from different types of corneal primary afferent neurons.

We focused exclusively on corneal-responsive neurons located at the Vi/Vc transition region based on previous work that discovered dry-responsive neurons in this region (Hirata et al. 2004). In this previous study, only CO$_2$-responsive (acid-
sensitive) corneal units were tested for responses to drying of the ocular surface, which would have missed sampling acid-insensitive corneal units (Hirata et al. 2004). In addition, this earlier study did not examine the effect of thermal and hyperosmotic stimulation on dry-responsive corneal units, two stimuli that are likely to participate in producing the response to corneal drying (Hirata and Meng 2010; Robbins et al. 2012).

Functionally, it has been proposed that corneal units located at the Vi/Vc transition region are involved in the regulation of tearing, blinking, and other homeostatic functions unique to the

Fig. 2. Example of an acid-sensitive (left) and acid-insensitive (right) neuron. A: drying of the ocular surface increased the ongoing activity in the acid-sensitive and -insensitive neuron. AT were removed from the ocular surface with Kimwipes positioned near the posterior canthus to avoid mechanical stimulation of the cornea. B: application of an acidic solution (pH 2.5) to the corneal surface evoked a robust response in the acid-sensitive neuron, whereas activity remained unaffected in the acid-insensitive neuron. C: the application of 5% mannitol in AT caused an increase in activity in acid-sensitive and -insensitive neurons. Although in this example, the response in the acid-sensitive neuron adapted over the course of the stimulus, this was not reflected in the overall group means (see text for details). D: a series of cooling ramps elicited increased activity in the acid-sensitive and -insensitive neuron. Noxious heat increased the activity of the acid-sensitive neuron yet did not affect the activity in the acid-insensitive neuron.

Fig. 3. Menthol responses in acid-sensitive and -insensitive neurons. A: neuronal discharge following menthol application to the cornea while recording from an acid-insensitive neuron. B: discharge recorded from an acid-sensitive neuron following menthol application. C: group average discharge following menthol application indicated a more sustained response in acid-insensitive neurons (black line) compared with acid-sensitive neurons (gray line).
cornea, whereas corneal units at the Vc/C1 region are important in nociceptive responses common to all craniofacial tissues (Hirata et al. 2004). In support of this hypothesis, inactivation of the Vi/Vc region, but not the Vc/C1 region, with microinjections of the GABA_A agonist muscimol, blocked CO₂-induced lacrimation (Hirata et al. 2004). These results point to the importance of the Vi/Vc region in noxious stimulation-evoked tearing, since CO₂ stimulation of the cornea produces pain by lowering the pH of the ocular surface (Acosta et al. 2001a, b; Belmonte et al. 2004; Chen et al. 1995). The finding that all dry-responsive neurons are activated by mild cooling indicates that this region may also represent a critical relay in basal tearing, which is tearing elicited by simple evaporation of the tear film (Parra et al. 2010; Robbins et al. 2012).

It appears that the function of at least three types of Vi/Vc corneal units can be inferred based on their receptive field properties. The first type has low, ongoing activity and may be involved primarily in the regulation of blinking, a category of neuron that seems not to have been sampled in the current study (Henriquez and Evinger 2007). The second type, which encompasses the acid-sensitive, dry-responsive neurons, has higher ongoing activity and is likely involved in tearing and potentially blinking produced by low pH, hyperosmotic, cooling, and noxious heat stimulation (Hirata et al. 2004). Finally, a third type of neuron, comprised of acid-insensitive, dry-responsive neurons and first described in this study, responds to mild cooling and hyperosmotic stimuli, consistent with an involvement in the replenishment of tears lost through evaporation.

Implicit from results in this study is information on the properties of primary afferent neurons projecting to the two classes of dry-responsive Vi/Vc neurons. Second-order, acid-insensitive neurons appear to receive input exclusively from primary afferent cool cells, due to the close resemblance of their response characteristics (Cabanes et al. 2003; Carr et al. 2003; de la Pena et al. 2005; Gallar et al. 1993; Hirata and Meng 2010; Parra et al. 2010). Primary afferent cool cells are also activated by cooling, menthol, and hyperosmotic stimuli (Gallar et al. 1993; Hirata and Meng 2010; Parra et al. 2010). In contrast, acid-sensitive, dry-responsive neurons likely receive input from polymodal neurons and cool cells, since they have robust responses to heat and acid, as well as cooling (Gallar et al. 1993). Alternatively, it is possible that acid-sensitive neurons do not receive input from cool cells, since a significant percentage of polymodal corneal afferents responds to cold, only with a lower (colder) threshold than the typical cool cell (Acosta et al. 2001a).

The response of both acid-sensitive and -insensitive neurons to menthol indicates that at least a portion of the cooling response is due to TRPM8 channels (Bautista et al. 2007; de la Pena et al. 2005; Madrid et al. 2006; McCoy et al. 2011; McKemy et al. 2002; Nealen et al. 2003; Parra et al. 2010; Peier et al. 2002; Thut et al. 2003; Zanotto et al. 2007). The TRPM8 channel plays an important role in the menthol and cold-evoked activation of corneal primary afferent neurons, demonstrated by the lack of responses of these stimuli in TRPM8 knockout animals (Parra et al. 2010). In addition, lacrimation induced by application of menthol to the cornea was blocked in animals lacking TRPM8 (Robbins et al. 2012).

Whereas there is strong evidence for the involvement of TRPM8 in the cooling response, less is known regarding the channels responsible for activating corneal afferent neurons in response to hyperosmotic stimuli. Regardless, neurons at the Vi/Vc region appear to be particularly sensitive to changes in osmolarity on the ocular surface. A comparison of neuronal discharge in response to hypertonic saline applied to the cornea found an approximately threefold-greater response in Vi/Vc neurons compared with corneal units recorded at the Vc/C1 transition region (Tashiro et al. 2010).

Conclusions. In summary, the characterization of dry-responsive neurons at the Vi/Vc transition region has revealed two distinct populations of neurons. Acid-insensitive neurons were activated at warmer temperatures and had an overall greater response to cooling and menthol compared with acid-sensitive neurons, and it is proposed that these acid-insensitive neurons represent a labeled line for lacrimation in response to evaporation of tears from the ocular surface. In contrast, acid-sensitive neurons are likely involved in the production of tears elicited to protect the eye from potential tissue-damaging stimuli, evidenced by their robust response to low pH and noxious thermal stimulation.

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DISCLOSURES
The authors have no financial or other relationships that might lead to a conflict of interest.

AUTHOR CONTRIBUTIONS
Author contributions: M.K. and I.D.M. conception and design of research; M.K. performed experiments; M.K. and I.D.M. analyzed data; M.K. and I.D.M. interpreted results of experiments; M.K. and I.D.M. prepared figures; I.D.M. drafted manuscript; M.K. and I.D.M. edited and revised manuscript; M.K. and I.D.M. approved final version of manuscript.

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