Dry eye disease (DED), affecting over 5% of the general population worldwide, may be caused by insufficient quantity or quality of tears.1 Marked by sensations of corneal dryness, grittiness, irritation, and/or burning pain, DED produces a profound, negative impact on overall quality of life, and imposes a significant economic burden in related health care costs.2 While these sensations often are associated with signs of corneal epithelial cell damage, tear hyperosmolarity, and/or ocular inflammation, this not always is the case, leading to speculation that in some individuals DED represents a form of neuropathic ocular pain.3,4 Regardless of etiology, there is a vital need to improve treatments for ocular pain associated with DED.

Sex steroids appear to have a strong influence over the course of DED. Women on estrogen hormone replacement therapy are 67% more likely to suffer dry eye symptoms.5 However, the combination of estrogen and progesterone increases the prevalence of DED by only 33%, suggesting that progesterone may have a protective role. In preclinical models, progesterone decreased inflammation and neuropathic pain-related behaviors as well as temporomandibular joint evoked pain behaviors.6–10 The mechanism of action may include peripheral and central effects.6,11 The ability of progesterone to alleviate ocular pain has not been examined to our knowledge.

Pain and discomfort from dry eye results from activation of sensory neurons from the ophthalmic branch of the trigeminal nerve.12–14 The cornea is innervated exclusively by small diameter primary afferent neurons that project to two distinct regions within the brainstem spinal trigeminal nucleus (Vsp).15 The caudal projection site is located at the transition zone between the first cervical vertebra (C1) and the spinal trigeminal subnucleus caudalis (Vc), whereas the more rostral projection site is at the transition between Vc and the spinal trigeminal subnucleus interpolaris (Vi).16–18 This dual representation of corneal inputs provides a spatial segregation of two functionally distinct regions.19 The rostral zone is specialized for regulating blinking and tearing reflexes.20–25 In contrast, the caudal zone can be considered a rostral extension of the spinal cord dorsal horn, processing information required for the multitude of nociceptive responses after corneal injury.17,24

The effect of progesterone on corneal sensitivity and nociceptive processing is unknown. We examined the ability of progesterone, applied to the forehead region, to modify corneal mechanical and noxious chemical sensitivities in the
Materials and Methods

Animals

Adult male Sprague Dawley rats (225–275 grams) were obtained from Charles River Laboratories (Cambridge, MA, USA) and housed in an environment with free access to food and water and a controlled 12-hour light/dark cycle. Animals were maintained under standard housing conditions and treated according to the policies and recommendations of the National Institutes of Health (NIH; Bethesda, MD, USA) guidelines for the handling and use of laboratory animals and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved by the committee on animal research at the University of New England. To acclimate the animals to testing conditions, they were handled for 5 minutes per day for 5 days in the experimental room before the experiment.

Surgery

Aqueous tear deficiency was produced by the unilateral excision of the intra- and extraorbital lacrimal glands under isoflurane anesthesia. In sham surgeries, two incisions were made to partially expose the intra- and extraorbital glands. Lidocaine (lidocaine HCl 1% and epinephrine 1:100,000) was injected subcutaneously into the cheek before the incisions to attenuate bleeding. All testing was performed 2 weeks postoperatively.

Corneal Mechanical Thresholds

Corneal mechanical thresholds were assessed using a Cochet-Bonnet esthesiometer (Western Ophthalmics, Lynwood, WA, USA) according to the methods of Wakuta et al. The animal was held gently while a nylon filament was applied to the cornea. A positive response was noted by a clear stimulus-evoked blink and retraction of the eye into the ocular orbit. On some trials, the stimulus also elicited a head withdrawal motion, which occurred in addition to the standard blink and eye retraction. Measurements were made starting with the eye completely closed was recorded over a 3-minute period. After drug application, the total time spent wiping the eye or paw swiping directed toward the eye in which the liquid was applied. Normal facial grooming behavior was not included. In some trials, the stimulus also elicited a head withdrawal motion, which occurred in addition to the standard blink and eye retraction. Measurements were made starting with the eye completely closed was recorded over a 3-minute period. After drug application, the total time spent wiping the eye or paw swiping directed toward the eye in which the liquid was applied. Normal facial grooming behavior was not included.
4.5 mm from obex, a total of 9 sections per animal were counted. The average number of Fos-positive neurons per section was calculated. The experimenter selecting the sections to be quantified and those performing the counting were blind to the treatment of the animals.

Statistics

Mechanical sensitivity before and after application of drug or vehicle was compared using the sign test, since the data are nonparametric and the observations are paired. Eye wipe comparisons between multiple treatment groups with equal variance and normal distribution were performed using a 2-way ANOVA without repeated measures with a Tukey Kramer post hoc test. Comparisons between two treatment groups with parametric data sets were performed using unpaired t-tests. Data are presented as the mean ± SEM, and significance was defined as P < 0.05.

RESULTS

The effect of progesterone or vehicle gel applied to the forehead on corneal mechanical sensitivity was examined in sham and lacrimal gland excision (LGE) animals (Fig. 1).

Baseline mechanical thresholds were determined before vehicle or progesterone application, and then at 25, 55, or 115 minutes after gel application. Vehicle application in sham animals did not affect mechanical thresholds at any time point tested (Figs. 1A, 1C, 1E; n = 12 animals per time point, P > 0.05, sign test). In contrast, mechanical thresholds after progesterone application increased at all three time points (Figs. 1A, 1C, 1E; n = 12 animals per time point, P < 0.01, sign test), with a maximal threshold of 6.5 g/mm² reached at 115 minutes after progesterone.

In animals tested 2 weeks after LGE, baseline mechanical thresholds were markedly lower than sham-treated animals, ranging from 0.6 to 0.7 g/mm² compared to 1.2 to 3.1 g/mm², as reported previously. Progesterone, but not vehicle, application increased corneal mechanical thresholds in LGE animals at all three time points (Figs. 1B, 1D, 1F; n = 11–12 animals per time point, P < 0.01, sign test). After progesterone, mechanical thresholds increased from 0.6 to 0.7 g/mm² to 0.9 to 1.2 g/mm².

To control for the site of drug application, progesterone or vehicle gel was applied to the skin on the cheek contralateral to the tested cornea (Fig. 2A, inset). In sham and LGE animals, progesterone did not affect corneal mechanical sensitivity when applied to the contralateral cheek (Figs. 2A, 2B; n = 10 animals per treatment group; P > 0.05).

The effect of progesterone on noxious stimulation-evoked eye wipe behavior was examined after corneal application of hypertonic saline and capsaicin (Fig. 3). Analysis of hypertonic saline-evoked eye wipe behavior indicated a significant effect of surgery (sham vs. LGE, F[1,43] = 91.78, P < 0.01, 2-way ANOVA, n = 11–12 animals/treatment group). Tukey post hoc analysis revealed a greater hypertonic saline-evoked eye wipe response in vehicle-treated LGE animals when com-
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**Figure 3.** Progesterone inhibition of noxious stimulation evoked eye wipe behavior. (A) Eye wipe behavior evoked by 5 M saline in sham and LGE-treated animals was examined 120 minutes after vehicle (gray bar) or progesterone (striped bar) application. *N* = 11–12 animals per treatment group. **P* < 0.01 compared to all other treatment groups. (B) Eye wipe behavior after application of capsaicin in naïve, intact animals. *N* = 6/10 vehicle treatment and 12 for progesterone treatment. **P* < 0.01 versus vehicle treatment.

pared to sham animals (Fig. 3A, *P* < 0.01). In addition, a significant effect of drug treatment was found (F[1,43] = 29.44, *P* < 0.01). Post hoc analysis indicated a reduction in hypertonic saline-evoked eye wipe behavior after progesterone treatment to the forehead only in LGE animals (Fig. 3A, *P* < 0.01). The effect of progesterone on capsaicin-evoked eye wipe behavior was evaluated in naïve animals (Fig. 3B). Application of 0.1% capsaicin to the cornea 120 minutes after progesterone produced significantly less eye wipe behaviors compared to the vehicle-treated group (*P* < 0.01, unpaired *t*-test, *n* = 15 for vehicle treatment and 12 for progesterone treatment).

The mechanism of action for progesterone’s effect on corneal sensitivity may involve the modulation of neurons innervating skin. To test this hypothesis, the local anesthetic bupivacaine (*n* = 11) or isotonic saline (*n* = 9) was injected subcutaneously under the region of progesterone application (Fig. 4). Corneal mechanical thresholds were determined just before and 60 minutes after progesterone application. In saline-injected animals, progesterone application produced a significant increase in corneal mechanical thresholds (Fig. 4, *P* < 0.05, sign test). In contrast, injection of bupivacaine completely blocked the increase in mechanical thresholds normally produced by progesterone (Fig. 4, *P* > 0.05, sign test).

The effect of progesterone on the central processing of corneal inputs was examined using c-Fos immunohistochemistry induced by mustard oil application to the cornea. Mustard oil application to the cornea has been used extensively to evoke c-Fos in the Vsp, and drugs used to treat pain also reduce the number of corneal mustard oil-evoked Fos-positive neurons.16,34,35 Fos-positive neurons are located in two primary regions within the Vsp after noxious corneal stimulation with mustard oil.16 In the caudal zone, at the transition between Vc and C1, Fos-positive neurons were located primarily in the superficial laminae of the dorsal horn ipsilateral to the side of mustard oil application (Figs. 5A, 5C). In the rostral zone, Fos-positive neurons were located in the ventrolateral portion of the transition between Vc and Vi (Figs. 5B, 5D). Application of progesterone 30 minutes before mustard oil stimulation of the cornea reduced the number of Fos-positive neurons in the caudal zone compared to vehicle-treated animals (Fig. 5E, top, *P* < 0.05, unpaired *t*-test, *n* = 7 animals/treatment group). No difference in the number of Fos-positive neurons was found in the rostral zone (Fig. 5E, top, *P* > 0.05, unpaired *t*-test). Furthermore, progesterone did not affect the number of Fos-positive neurons quantified on the side contralateral to mustard oil application (Fig. 5E, bottom); although Fos-positive neurons in the rostral region were numerically greater in progesterone-treated animals compared to vehicle-treated animals (19.8 ± 3.4 vs. 12.4 ± 3.1, respectively), this difference did not reach significance (*P* = 0.13, unpaired *t*-test). In animals that did not receive any corneal stimulation, progesterone did not affect the number of Fos-positive neurons in the caudal zone (Fig. 5F). However, in the rostral zone, progesterone caused a significant increase in the number of Fos-positive neurons compared to vehicle treatment (Fig. 5F; *n* = 5 and 6 for vehicle and progesterone treatment, respectively, *P* < 0.05, unpaired *t*-test). No difference was found in the caudal zone between progesterone- and vehicle-treated unstimulated animals.

**Discussion**

In previous studies, progesterone has been shown to reduce inflammation and neuropathic pain-related behaviors in preclinical models.6–10,36 Our results indicated that progesterone, applied to the forehead region in rats, reduces corneal mechanical sensitivity and capsaicin-evoked eye wipe behaviors, and after LGE-induced dry eye also decreases mechanical hypersensitivity and hypertonic saline-evoked eye wipes. Furthermore, the increase in corneal mechanical thresholds was prevented by the subcutaneous injection of the local anesthetic bupivacaine.
The site of topical application appears to be an important factor in the ability of progesterone to affect corneal sensitivity. Progesterone had no effect on corneal mechanical thresholds when applied to the side of the face contralateral to the tested eye. This result, along with the rapid onset of analgesia (<30 minutes), suggested a direct action on neurons innervating the skin rather than a systemic effect subsequent to absorption. Further support for a localized action is provided by the ability of bupivacaine to block the effect of progesterone when applied to the forehead.

The effectiveness of bupivacaine in preventing progesterone-induced increases in corneal mechanical thresholds indicates that progesterone, either directly or indirectly, works through the activation of neurons innervating the forehead. Bupivacaine provides a relatively long duration (>4 hours) of anesthesia, greatly outlasting the period of sensory testing. Sensory neurons make up the majority of skin innervation, with additional innervation provided by the autonomic nervous system. Sensory innervation of the cornea and forehead is through the ophthalmic branch of the trigeminal nerve, whereas the cheek region is innervated by the maxillary and mandibular branches. Progesterone application to the forehead in the absence of corneal stimulation increased Fos-positive neurons located in the rostral zone of the Vsp, providing evidence for progesterone-induced increases in brainstem neuronal activity. Although progesterone did not produce a significant increase in Fos-positive neurons in the rostral region in mustard oil-treated animals, this is likely attributable to the large increase in Fos-positive neurons produced by the mustard oil application, thereby masking the effect of progesterone. Of note, on the contralateral side, progesterone treatment showed a trend toward elevated levels of Fos-positive neurons.

The rostral zone of Vsp contains neurons with corneal receptive fields and has been demonstrated to regulate homeostatic ocular reflexes, such as tearing and blink-
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The elevated levels of Fos-positive neurons in this region following progesterone application raises the possibility that progesterone may improve the tear film through an increase in secretions. The response properties of neurons activated by progesterone treatment are unknown; however, cold cells are a potential candidate. The rostral zone of Vsp contains a large number of cold-sensitive neurons that regulate secretions and blinking in response to drying of the ocular surface.

While the effect of progesterone on primary afferent cold cells has not been explored, progesterone produces changes in core body temperature indicative of increased cold cell activity. Systemic progesterone increases body temperature, which has been linked to actions on thermal regulating neurons in the preoptic area of the hypothalamus. However, hypothalamic projections of thermoreceptive neurons in Vsp also could have a role in progesterone regulation of body temperature. Through this pathway, an increase in cold cell activity would produce hyperthermia by activating thermo-effector regions involved in raising body temperature.

The decrease in mustard oil-induced Fos-positive neurons in the caudal zone of Vsp after progesterone treatment is consistent with its antinoceptive properties in the eye wipe test. The caudal zone of Vsp processes nociceptive inputs in a manner similar to the spinal cord dorsal horn and has a primary role in corneal nociception. Quantification of protein for the immediate early gene c-fos has been used extensively as a marker for neuronal activity following corneal stimulation. In the caudal zone, the number of Fos-positive neurons correlates directly with the intensity of noxious stimulation. Furthermore, analgesic drugs, such as morphine, reduce the number of noxious stimulation-induced Fos-positive neurons in a dose-dependent manner. In our study, while progesterone decreased Fos-positive neurons in the caudal zone following mustard oil, Fos-positive neurons in the rostral zone remained unaffected, indicating a selective reduction in nociceptive transmission. A role for the rostral zone of Vsp in nociceptive processing is less clear, although the rostral zone can interact with and modify the processing of nociceptive inputs in more caudal regions of Vsp.

In fact, inhibition of neurons in the rostral zone led to the activation of corneal-responsive neurons in the caudal zone. Such inter-subnuclear communication could be the basis for progesterone-induced reduction in Fos-positive neurons in the caudal zone of Vsp.

Progesterone appears to produce antinociception through the modulation of nerve fibers in the ophthalmic branch of the trigeminal nerve, yet the specific mechanisms by which progesterone affects these neurons are unknown. Classical progesterone actions are on intracellular receptors that, upon binding, translocate to the nucleus and regulate gene expression. A role for the rostral zone of Vsp in nociceptive processing is less clear, although the rostral zone can interact with and modify the processing of nociceptive inputs in more caudal regions of Vsp.

In conclusion, our results provide evidence that progesterone application to the ophthalmic region reduces corneal sensitivity in the uninjured and injured states. In addition, the antinoceptive effect of progesterone appears to work through local rather than systemic actions. Further testing in human subjects is needed to confirm these findings and determine whether progesterone application to the forehead region is an effective treatment for ocular irritation and pain.

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