Progesterone Application to the Rat Forehead Produces Corneal Antinociception

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PURPOSE. Ocular pain and discomfort are the most defining symptoms of dry eye disease. We determined the ability of topical progesterone to affect corneal sensitivity and brainstem processing of nociceptive inputs.

METHODS. Progesterone or vehicle gel was applied to the shaved forehead in male Sprague Dawley rats. As a site control, gel also was applied to the cheek on the side contralateral to corneal stimulation. Corneal mechanical thresholds were determined using the Cochet-Bonnet esthesiometer in intact and lacrimal gland excision–induced dry eye animals. Eye wipe behaviors in response to hypertonic saline and capsaicin were examined, and corneal mustard oil-induced c-Fos immunohistochemistry was quantified in the brainstem spinal trigeminal nucleus.

RESULTS. Progesterone gel application to the forehead, but not the contralateral cheek, increased corneal mechanical thresholds in intact and lacrimal gland excision animals beginning <30 minutes after treatment. Subcutaneous injection of the local anesthetic bupivacaine into the forehead region before application of progesterone prevented the increase in corneal mechanical thresholds. Furthermore, progesterone decreased capsaicin-evoked eye wipe behavior in intact animals and hypertonic saline evoked eye wipe behavior in dry eye animals. The number of Fos-positive neurons located in the caudal region of the spinal trigeminal nucleus after corneal mustard oil application was reduced in progesterone-treated animals.

CONCLUSIONS. Results from this study indicate that progesterone, when applied to the forehead, produces analgesia as indicated by increased corneal mechanical thresholds and decreased nociceptive responses to hypertonic saline and capsaicin.

Keywords: progesterone, dry eyes, pain

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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.[20–23] 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

rat. In addition, expression of the immediate early gene *c-Fos* was used to determine the ability of progesterone to alter the activity of neurons located in the rostral and caudal brainstem regions that process corneal inputs.

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 3 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.[25] In sham surgeries, two incisions were made to partially exposed 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 Opthalmics, Lynwood, WA, USA) according to the methods of Wakuta et al.[26] 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 filament fully extended to 60 mm so as to apply the least amount of pressure, and then incrementally shortened in 5-mm steps until a response was obtained. Once the filament elicited a positive response, it was lengthened again and measurements were repeated.

Eye Wipe Behaviors

Animals were acclimated in their holding chamber for 10 minutes before application of 40 μL of either 0.1% capsaicin or 5 M saline into the eye using a micropipette. Animals were tested after mechanical thresholds were assessed 115 minutes after drug application. The total time spent wiping the eye or with the eye completely closed was recorded over a 3-minute period. Eye wipe behavior consisted of either front or hind paw swiping directed toward the eye in which the liquid was applied. Normal facial grooming behavior was not included. In the mouse, studies have differentiated between a pain and itch response based on hind paw and forepaw behaviors elicited by injections into the cheek.[27] In rats, however, capsaicin injected into the cheek evokes hind paw and forepaw behaviors,[28] which is why both were monitored. Additionally, in a previous study, morphine was able to reduce eye wipe behaviors in intact and LGE rats following hypertonic saline application to the eye.[25] Capsaicin was made in a vehicle of 1.5% ethanol and 8.5% Tween 20.

Experimental Drug Application

Progesterone (1.0%) or an aqueous-based vehicle gel was applied to a shaved region on the animal's forehead or, in a subset of animals, to an area of the cheek above the temporomandibular joint contralateral to the eye being tested. Animals were shaved and the area cleaned with an alcohol swab under isoflurane anesthesia 1 day before application.

Local Anesthetic Block

In a subset of experiments, bupivacaine (0.1 mL, 0.5%) or saline (0.05 mL, 0.9%) was injected subcutaneously into the forehead region under light isoflurane anesthesia. Baseline corneal mechanical thresholds were measured 30 minutes after the injection, which was followed by application of progesterone gel to the shaved forehead. Corneal mechanical thresholds were assessed 60 minutes after progesterone application, a time point that assured the anesthetic block had not yet worn off.[29]

Immunohistochemistry

Animals were anesthetized with urethane (2.0 g/kg) and the forehead region was shaved. Progesterone or vehicle was applied to the forehead region and 120 minutes later animals were perfused under deep anesthesia with 0.9% heparinized saline followed by 10% formalin. The 120-minute time point was chosen based on studies showing peak levels of c-fos protein between 90 and 120 minutes from the time of stimulation.[30-32] In some animals, 10% mustard oil (40 μL in mineral oil vehicle) was applied to the left cornea 30 minutes after progesterone or vehicle treatment. In this way, the effects of progesterone application alone on c-Fos expression and the effects of progesterone on mustard oil-evoked c-Fos expression could be determined. Following perfusions, brainstems were removed and post-fixed in 10% formalin for 1 hour and placed in 30% sucrose.

Tissue was sectioned on a cryostat at 30 μm and immunohistochemistry for c-Fos protein was performed on alternate free-floating sections according to the methods of Hitomi et al.[33] Sections were incubated for 60 minutes in 3% normal goat serum (NGS) and incubated overnight on an orbital shaker at 4°C in a 1:250 dilution of rabbit polyclonal anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.1 M PBS with 1% NGS and 0.3% Triton X. Sections then were washed in 0.1 M PBS and incubated in a 1:200 dilution of biotinylated goat anti-rabbit secondary (Vector, Burlingame, CA, USA) containing 1% NGS for 60 minutes. After washing (0.1 M PBS), sections were incubated in an avidin-biotin peroxidase (ABC; Vector Laboratories) complex solution for 60 minutes. Sections then were reacted for 3 to 6 minutes with diaminobenzidine (0.7 mg/kg, Sigma-Aldrich Corp., St Louis, MO, USA) and hydrogen peroxide (0.17 mg/mL), intensified with nickel ammonium sulfate (1.25%) and cobalt chloride (1.0%). After a final wash, sections were mounted onto slides and coverslipped.

Fos-positive neurons were quantified in two regions of the Vsp: the rostral zone, located at the transition between Vi and Vc, and the caudal zone, located at the transition between Vc and C1.[16,34] In the rostral zone, located 0.0 to −0.5 mm from obex, Fos-positive neurons were counted in a total of five sections per animal, while in the caudal zone, located −3.0 to
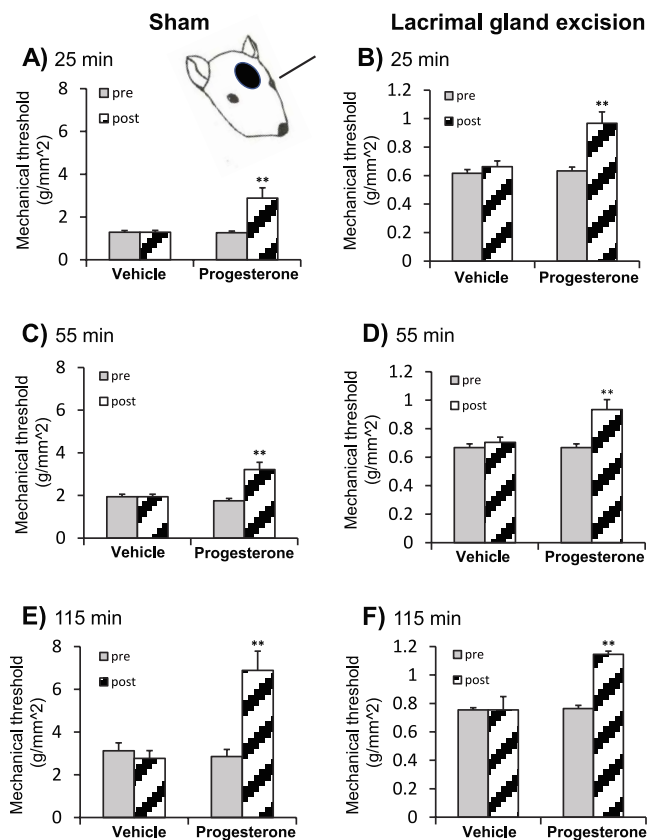
FIGURE 1. Reduction in corneal mechanical thresholds after progesterone gel application to the forehead. *Left*, thresholds measured in animals with sham surgery at (**A**) 25, (**C**) 55, and (**E**) 115 minutes after vehicle or progesterone application. *Right*, thresholds measured in animals with LGE at (**B**) 25, (**D**) 55, and (**F**) 115 minutes after vehicle or progesterone application. In all animals, a baseline mechanical threshold was made before drug or vehicle application (*gray bars*). The *inset* in (**A**) illustrates the location of the topical gel application. Separate groups of animals were run at each time point. $N = 11$–12/group. **$P < 0.01$ versus pre-drug thresholds.

−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).
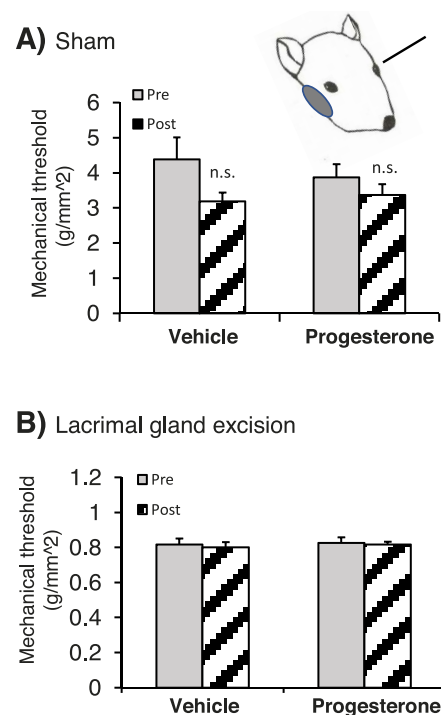

FIGURE 2. Progesterone gel application to the contralateral cheek did not affect corneal mechanical sensitivity in (**A**) sham or (**B**) LGE animals. Mechanical thresholds were determined before (pre) and 60 minutes after (post) application of the topical gel. *Inset*, illustration depicting the location of the gel application relative to the eye that was tested. $N = 10$/group; n.s., no significance.

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/mm^2 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/mm^2 compared to 1.2 to 3.1 g/mm^2, as reported previously.[25] 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/mm^2 to 0.9 to 1.2 g/mm^2.

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 thresholds 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-
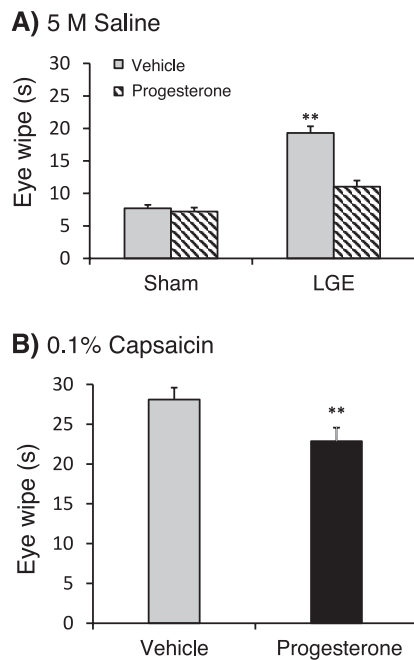
A) 5 M Saline

B) 0.1% Capsaicin

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 = 15 for vehicle treatment and 12 for progesterone treatment. **P < 0.01 versus vehicle treatment.
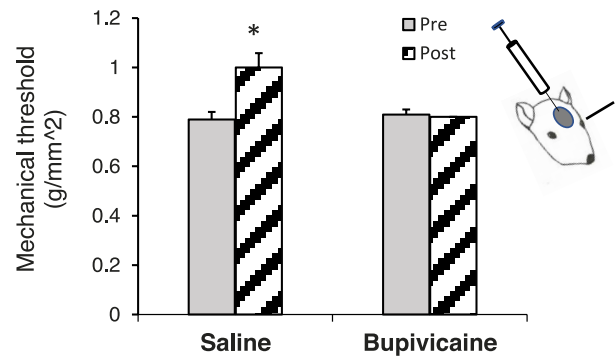

FIGURE 4. Progesterone-induced increase in corneal mechanical thresholds blocked by subcutaneous injection of a local anesthetic. Corneal mechanical thresholds increased in animals that received saline injections, but not bupivacaine injections 30 minutes before the application of progesterone. *Inset*, depiction of the subcutaneous injection site, topical gel application, and corneal mechanical filament. *Gray bars*, mechanical thresholds 30 minutes after injection of saline or bupivacaine; *striped bars*, mechanical thresholds 60 minutes after progesterone application. N = 9 for saline and 11 for bupivacaine treatment. *P < 0.05 versus saline pre.

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.
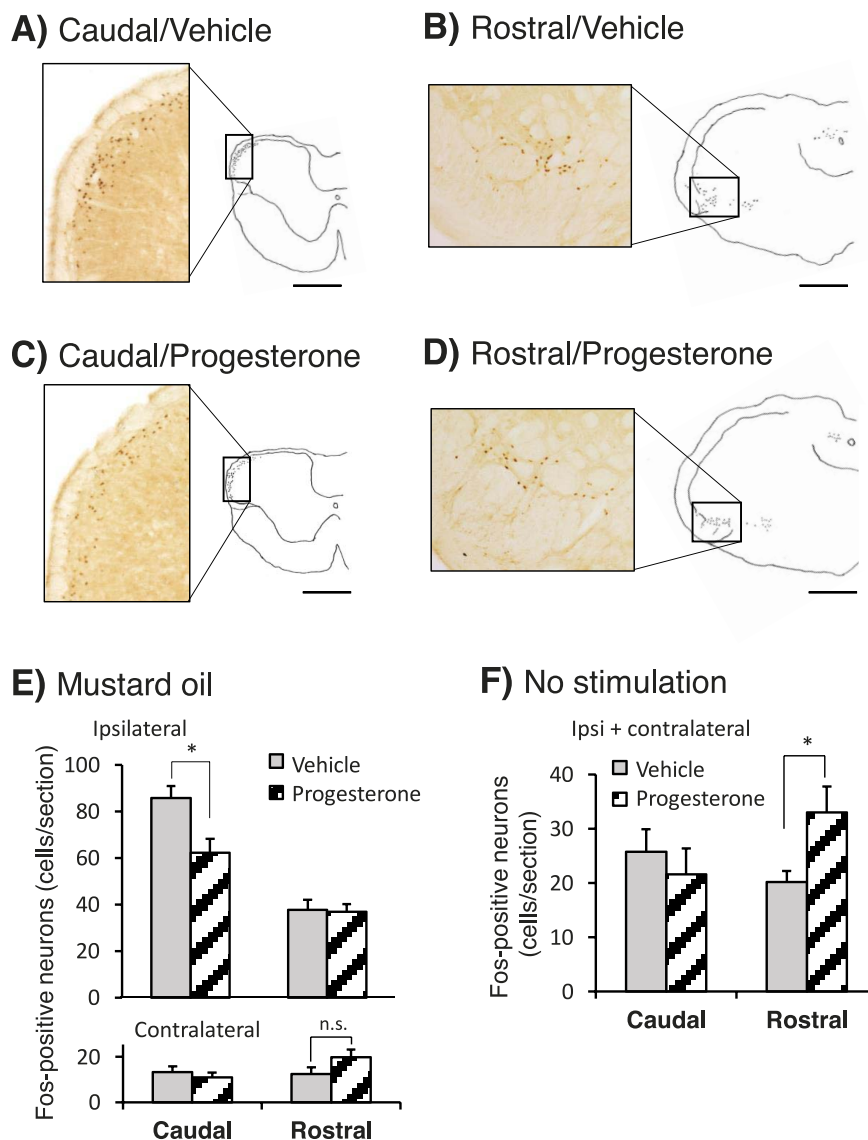
FIGURE 5. Progesterone decreased mustard oil-induced Fos-positive neurons in the caudal region of the Vsp. Representative cameral lucida drawings of caudal sections (**A**, **C**) and rostral sections (**B**, **D**) of Vsp with photomicrographs showing c-Fos immunoreactivity after mustard oil application to the cornea in vehicle-treated (**A**, **B**) and progesterone-treated (**C**, **D**) animals. (**E**) Fos-positive neurons in the rostral and caudal regions of the Vsp ipsilateral (*top*) and contralateral (*bottom*) to the side of mustard oil application to the cornea. $N = 7$/treatment group. (**F**) Number of Fos-positive neurons in the rostral and caudal Vsp in animals receiving no corneal stimulation. $N = 5$–6/treatment group. *$P < 0.05$; n.s., no significance.

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.[37] Sensory neurons make up the majority of skin innervation, with additional innervation provided by the autonomic nervous system.[38] 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.[39,40] 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-

ing.[20-23] 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 as 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.[22,41]

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.[42,43] 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.[44-46]

The decrease in mustard oil-induced Fos-positive neurons in the caudal zone of Vsp after progesterone treatment is consistent with its antinociceptive 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.[16,18,34] In the caudal zone, the number of Fos-positive neurons correlates directly with the intensity of noxious stimulation.[16] Furthermore, analgesic drugs, such as morphine, reduce the number of noxious stimulation–induced Fos-positive neurons in a dose-dependent manner.[35] 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.[47] 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. These nuclear receptors are found in skin keratinocytes and basal cells.[48] Expression in specific populations of primary afferent neurons has not been described. In addition to the classical nuclear receptors, progesterone actions also are conveyed through nonclassical membrane-associated progestin receptors.[49-53] These membrane-associated receptors modify cell functions through the rapid activation of G-protein mediated intracellular cell signaling pathways. In our study, nongenomic actions such as these are likely responsible for progesterone-induced antinociception, given its ability to increase corneal mechanical thresholds within 25 minutes after application.

Consistent with previous reports, LGE increased corneal mechanical sensitivity and eye wipe responses to hypertonic saline.[25,54,55] Importantly, progesterone was effective in reducing these signs of ocular hypersensitivity, demonstrating its potential use in treating ocular irritation and pain from dry

eye and other conditions that involve sensitization of corneal nociceptors. The effectiveness of progesterone in relieving pain due to nerve injury and inflammation is in line with previous reports demonstrating antinociceptive actions of progesterone in preclinical inflammatory and neuropathic pain models.[6-10,36] Of note, baseline mechanical thresholds also were lower in animals that received a subcutaneous injection of saline or bupivacaine before progesterone application. This lower threshold is likely due to the isoflurane anesthesia that was used before the injection, as isoflurane at low concentrations can increase motor excitability.[56,57]

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 antinociceptive 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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