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14. ABSTRACT
The overall goal of the project was to develop an animal model of the focal dystonia benign essential blepharospasm. Consistent with the widely held view that dystonia results from an interaction between a predisposing condition and an environmental trigger, we proposed to use 7 Hz deep brain stimulation of the basal ganglia as the predisposing condition and dry eye as the environmental trigger. We hypothesized that the 7 Hz deep brain stimulation would exaggerate the blink adaptations to dry eye into spasms of lid closure characteristic of benign essential blepharospasm (BEB). During this grant, we demonstrated that combining 7 Hz deep brain stimulation with dry eye produced spasms of eyelid closure characteristic of BEB. We also determined that dry eye enhanced the predisposing condition in female, but not male rats. This last result may account for the preponderance of human females exhibiting BEB. Our four publications report these results.

15. SUBJECT TERMS
Dystonia, benign essential blepharospasm, dry eye, motor plasticity, basal ganglia, deep brain stimulation, eyelids, blinking

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1. Introduction

The focal dystonia benign essential blepharospasm (**BEB**), arises from the convergence of a predisposing condition and an environmental trigger. The predisposing condition permits the normal responses to an environmental insult such as dry eye to transform into the spasms of lid closure characteristic of BEB. The overall goal of our project was to develop an animal model of BEB based on the hypothesis that hypersynchronized, 7 Hz neuronal oscillations of the basal ganglia create the predisposing condition and that eye irritation from dry eye can be the environmental trigger. Our demonstration that hypersynchronized oscillations in the basal ganglia produced by 7 Hz deep brain stimulation of the subthalamic nucleus (**STN DBS**) exaggerate neural plasticity in normal male rats (Kaminer et al. 2014) provided a neural mechanism by which hypersynchronized basal ganglia activity could create a predisposing condition. From our studies showing that dry eye initiated neural plasticity in blink circuits to produce compensatory adaptations in blinking (Culoso et al. 2020; Evinger et al. 2002; Kaminer et al. 2011; Peshori et al. 2001; Schicatano et al. 2002), we predicted that combining 7 Hz STN DBS and dry eye would exaggerate neural plasticity and force the normally compensatory adaptive processes in response to dry eye to transform into the characteristics of BEB, *e.g.*, spasms of lid closure, excessive blinking, and trigeminal hyperexcitability.

We proposed two Specific Aims to test our hypothesis. The goal of the first Specific Aim was to show that synchronized theta oscillations in the basal ganglia exaggerated plasticity in the cerebellum and the excitability of trigeminal blink circuits as occurred in BEB patients. The Major Tasks to accomplish Specific Aim 1 were: 1) to investigate effects of synchronized basal ganglia oscillations on activity of the deep cerebellar nucleus neurons; and 2) to investigate the effects of synchronized basal ganglia oscillations on the activity of superior colliculus neurons. The purpose of the second Specific Aim was to demonstrate that synchronized 7 Hz oscillations established in the basal ganglia were sufficient to predispose mammals to develop BEB. The Major Tasks to accomplish Specific Aim 2 were: 1) to determine whether combining synchronized basal ganglia 7 Hz oscillations with corneal irritation was sufficient to develop spasms of lid closure and other characteristics of the focal dystonia BEB; and 2) to perform control experiments to determine that theta frequency was critical in enabling the development of spasms of lid closure.

2. Keywords

Dystonia, benign essential blepharospasm, dry eye, motor plasticity, basal ganglia, deep brain stimulation, eyelids, blinking

3. Accomplishments

Major Goals of the Project

The overarching goal of the investigations was to test our hypothesis that hypersynchronized 7 Hz oscillations in the basal ganglia create a predisposing condition that transforms the normally adaptive modifications initiated by dry eye into the spasms of lid closure, excessive blinking, and trigeminal hyperexcitability characteristic of individuals with the focal dystonia benign essential blepharospasm (**BEB**). To test this hypothesis, the 1st Specific Aim of the project was to demonstrate that synchronized 7 Hz (**theta**) oscillations in the basal ganglia exaggerated plasticity in the cerebellum and excitability of trigeminal blink circuits. Major Task 1 of Specific Aim 1 was to investigate effects of synchronized basal ganglia oscillations on the activity of the deep cerebellar nucleus neurons. Major Task 2 of Specific Aim 1 was to investigate the effects of synchronized basal ganglia oscillations on the activity of superior colliculus neurons. The 2nd Specific Aim of the project was to demonstrate that synchronized theta oscillations established in the basal ganglia were sufficient to predispose mammals to develop blepharospasm. Major Task 1 of Specific Aim 2 was to determine whether combining synchronized basal ganglia theta oscillations combined with corneal irritation supported the development of spasms of lid closure and other characteristics of the focal dystonia BEB. Major Task 2 of Specific Aim 2 was to perform control experiments to determine that theta frequency was critical for the development of spasms of lid closure.

What was accomplished under these goals?

We accomplished the overarching goal of the grant by demonstrating that hypersynchronized 7 Hz oscillations combined with dry eye produced spasms of lid closure, excessive blinking, and trigeminal hyperexcitability characteristic of individuals with BEB (Major Task 1 of Specific Aim 2). We published these results in the Journal of Neuro-Ophthalmology (Evinger 2015; Evinger and Digre 2016) (Appendix).

Specific Aim 2, Major Task 2: This aim was to perform control experiments to demonstrate that dry eye alone did not produce spasms of lid closure, excessive blinking, and trigeminal hyperexcitability characteristic of individuals with BEB. The hypothesis of this grant was that the predisposing condition, 7 Hz STN hypersynchronization increased blink reflex plasticity so that the normally adaptive increases in trigeminal excitability become exaggerated and lead to BEB characteristics. Our previous animal studies of dry eye used only male rats and did not examine blink plasticity specifically (Kaminer et al. 2011; Schicatano et al. 1997). We performed a study that investigated the effect of dry eye on spontaneous blinking, trigeminal excitability, and reflex blink plasticity or modifiability (Culoso et al. 2020) (Appendix). Our data showed that dry eye increased spontaneous blink duration in both males and females, a trend that would produce spasms of eyelid closure if exaggerated. Nevertheless, there were significant differences in the way that dry affected males and females. For males, dry eye increased trigeminal reflex blink excitability at the expense of trigeminal modifiability, whereas trigeminal modifiability increased for females. For neither sex did dry eye alone lead to spasms of lid closure or increased trigeminal excitability typical of our animal model of BEB. The increased modifiability of female trigeminal blink circuity with dry eye, however, may contribute to the preponderance of human females developing BEB (Asgeirsson et al. 2006; Defazio et al. 1999; Defazio et al. 1989).

Specific Aim 1, Major Task 2: The goal of this task was to investigate the effects of STN deep brain stimulation on the activity of superior colliculus neurons. Although data were collected identifying the activity of superior colliculus neurons with reflex and spontaneous blinking, we did not complete this task.

Specific Aim 1, Major Task 1: The goal of this task was to investigate the effects of STN deep brain stimulation on the activity of cerebellar interpositus neurons. The discharge of interpositus neurons determines the duration of lid closure with blinking (Chen and Evinger 2006) so these neurons are critical in producing the spasms of lid closure with BEB. During these experiments, we identified three groups of Purkinje cells that regulated the activity of interpositus neurons during reflex blinking and thereby governed the duration of reflex blinks. We collected sufficient data on these neurons and their interactions with interpositus neurons so that we are preparing a manuscript on these data.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Our study (Evinger 2015; Evinger and Digre 2016) was the first demonstration that hypersynchronized oscillations in the basal ganglia could be responsible for predisposing individuals to develop the focal dystonia benign essential blepharospasm. This information may lead to new approaches to treatment of the disorder. Our study revealing that the modifiability or plasticity of the trigeminal circuits responsible for the development of spasms of lid closure increased significantly more in females than males (Culoso et al. 2020) provided an explanation for why benign essential blepharospasm occurs predominantly in females. These data may lead to new approaches to treatment of the blepharospasm in females.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. Changes/Problems

Changes in approach and reasons for change

There were no changes in approach.

Actual or anticipated problems or delays and actions or plans to resolve them

Just as all investigators experienced, the University shut down caused by the corona virus pandemic seriously disrupted my planned experiments. My laboratory was closed from mid-March until early August so that it was impossible to perform any of the planned experiments. Nevertheless, I spent the time working on the manuscript describing our cerebellar recording data.

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

6. Products

Journal Publications:

Benign Essential Blepharospasm is a Disorder of Neuroplasticity: Lessons from Animal Models." 2015; J. Neuro-ophthalmol. 35:374-379

Evinger C and Digre K, "Role of GABAergic System in Blepharospasm: Response" J Neuro-Ophthalmol 2016; 36: 343-352

Culoso A, Lowe C, Evinger C. "Sex, blinking, and dry eye" J. Neurophysiol. 2020; 123: 831-842

Books or other non-periodical, one time publications

None to report

Other publications, conference papers, and presentations

International:
"Trying to Raise the Window Shades: The Functional Blindness of Benign Essential Blepharospasm"
Invited lecture at Cardiff University, October 28, 2015

Website(s) or other internet site(s)

None to Report

Technologies or techniques

None to Report

Inventions, patent applications, and/or licenses

None to Report

Other Products

None to Report

7. Participants & other collaborating organizations

What individuals have worked on the project over its duration?

Name:	Leslie Craig Evinger	Cynthia Lowe	Ashley Culoso	Donna Schmidt
Project Role:	PI	Technician	Technician	Technician
Research Identifier:	0000-0002-0039-3348			
Nearest Person Month Worked:	48	12	24	18
Contribution:	Experimental design, manuscript preparation, performing experiments	Performing experiments	Performing experiments	Lab manager, histology
Funding Support:	Current grant	Current grant	Current grant	Current grant

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

None to Report

What other organizations were involved as partners?

None to Report

8. Special reporting requirements

Not Applicable

10. Appendices

SOW

Benign Essential Blepharospasm is a Disorder of Neuroplasticity: Lessons from Animal Models." 2015; J. Neuro-ophthalmol. 35:374-379

Evinger C and Digre K, "Role of GABAergic System in Blepharospasm: Response" J Neuro-Ophthalmol 2016; 36: 343-352

Culoso A, Lowe C, Evinger C. "Sex, blinking, and dry eye" J. Neurophysiol. 2020; 123: 831-842

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Culoso A, Lowe C, and Evinger C. Sex, blinking, and dry eye. *J Neurophysiol* 123: 831-842, 2020.

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STATEMENT OF WORK – Month/Day/Year

Site: Stony Brook University
Life Sciences Building
Department of Neurobiology & Behavior
Stony Brook, NY 11794-5230
PI: Leslie Craig Evinger, PhD

Specific Aim 2 (specified in proposal) **Demonstrate that synchronized theta oscillations established in the basal ganglia are sufficient to predispose mammals to develop blepharospasm**	**Timeline**	**Site 1**
Major Task 2: Determine whether combining synchronized basal ganglia theta oscillations with corneal irritation is sufficient to develop spasms of lid closure and other characteristics of the focal dystonia BEB	Months	
Subtask 1: Monitor blink frequency, blink reflex hyperexcitability, and spasms of lid closure in rats during 4 different conditions: 1) No DBS; 2) 7 Hz STN DBS alone; 2) 7 Hz STN DBS and dry eye; and 4) dry eye alone.	1-8	PI
Subtask 2: Data analysis of blink frequency, hyperexcitability, and spasms of lid closure and statistical comparison of data obtained in the 4 conditions.	6-9	PI
Milestone(s) Achieved: Published paper Evinger *J. Neuro-Ophthalmology*, 2015;35:374–379 describing animal model of blepharospasm.	11	PI
Major Task 2: Perform control experiments to determine that theta frequency is critical in enabling the development of spasms of lid closure		
Subtask 1: Repeat Subtask 1 of Major Task 1 using 16 Hz STN DBS or 130 Hz STN DBS.	9-18	PI
Subtask 2: Data analysis of blink frequency, hyperexcitability, and spasms of lid closure and statistical comparison with data obtained in Subtask 1 of Major Task 1.	15-19	PI
Subtask 3: Monitor blink frequency, blink reflex hyperexcitability, and spasms of lid closure in rats during 3 different conditions: 1) No DBS; 2) dry eye alone; and 3) 7 Hz STN DBS and dry eye.	18-24	PI
Subtask 4: Data analysis of blink frequency, hyperexcitability, and spasms of lid closure and statistical comparison with data obtained Subtask 1 of Major Task 1.	22-24	PI
Milestone(s) Achieved: Began work on manuscript describing results of Specific Aim 2 Major Task 2	45-50	PI
Specific Aim 1 **Demonstrate that synchronized theta oscillations in the basal ganglia exaggerate plasticity in the cerebellum and excitability of trigeminal blink circuits**		

Major Task 2: Investigate the effects of synchronized basal ganglia oscillations on activity of the superior colliculus neurons.		
Subtask 1: Record from superior colliculus neurons while rats participate in paired stimulus paradigm and receive 7 Hz, 16 Hz, 130 Hz, or no subthalamic deep brain stimulation.	36-52	PI
Subtask 2: Data analysis of single unit and local field potential data collected in Subtask 1 and correlation of neural activity with blink reflex excitability measures.	40-55	PI
Major Task 1: Investigate effects of synchronized basal ganglia oscillations on activity of the deep cerebellar nucleus neurons.		
Subtask 1: Record from interpositus neurons while rats participate in a blink plasticity paradigm and receive 7 Hz, 16 Hz, 130 Hz, or no subthalamic deep brain stimulation.	48-56	PI
Subtask 2: Data analysis of single unit and local field potential data collected in Subtask 1 and correlation of neural activity with brainstem plasticity measures.	49-57	PI
Milestone(s) Achieved: Submit a manuscript describing data from Specific Aim 1 Major Task 2 and a manuscript reporting data from Specific Aim 1 Major Task 2.	57-60	PI

If human subjects are involved in the proposed study, please provide the projected quarterly enrollment in the following table.

	Year 1				Year 2				Year 3
Target Enrollment (per quarter)	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**
Site 1									
Site 2									
Site 3									
Target Enrollment (cumulative)									

Note: The Government reserves the right to request a revised SOW format and/or additional information.

Section Editors: Lynn K. Gordon, MD, PhD
Jonathan Horton, MD, PhD

Benign Essential Blepharospasm is a Disorder of Neuroplasticity: Lessons From Animal Models

Craig Evinger, PhD

Journal of Neuro-Ophthalmology 2015;35:374–379
doi: 10.1097/WNO.0000000000000317

Effectively modeling benign essential blepharospasm (BEB) requires mimicking its root causes. Current evidence points to BEB arising from the confluence of a genetic predisposing condition and an environmental trigger (1). In this "2 hit" hypothesis, the appropriate environmental trigger engenders dystonic behavior because the predisposing condition creates inappropriate brain functioning. Epidemiological studies demonstrate that eye irritation from dry eye, blepharitis, or keratoconjunctivitis is the environmental trigger (1–6). The strength of the association between dry eye and BEB increases in the fifth and sixth decades of life (6) when BEB typically arises (7). Available data strongly support that the predisposing condition is genetic (1,8–12). There is evidence for an autosomal-dominant gene with reduced penetrance contributing to BEB (9,13), but current studies fail to identify any specific genes (8,14). Thus, creating a useful animal model of BEB must involve combining an environmental trigger with a predisposing condition.

Another goal of an animal model is to reproduce the typical symptoms of BEB. The hallmark of BEB is excessive involuntary bilateral lid closure primarily involving the orbicularis oculi muscles (1,15–18). In addition to lid spasms, patients with BEB exhibit trigeminal hyperexcitability (1,15,19–22), an elevated spontaneous blink rate (23), and photophobia (1,24–26). These characteristics are consistent with eye irritation serving as the environmental trigger for BEB because they all appear in patients with dry eye (21,27,28). This relationship between eye irritation and BEB characteristics indicates that eye irritation should be 1 component of an animal model and that the predisposing condition should cause the adaptive changes in eyelid control in response to dry eye to develop into BEB-like characteristics.

Current evidence demonstrates that trigeminal blink circuits undergo plastic, adaptive modifications to compensate for the rapid breakup of the corneal tear film in dry eye (29–32). Dry eye or eye irritation elevates trigeminal blink amplitude and duration to increase meibomian gland secretion and enhance restoration of the tear film (20,32–37). Blink frequency increases to reform the tear film more regularly (20,36–40). The trigeminal reflex blink circuit becomes hyperexcitable to allow tear film breakup to evoke a reflex blink more readily (20,21,32). Finally, the trigeminal reflex blink circuit responds to a single reflex evoking stimulus with multiple blinks to help restore the tear film (20,21,32).

A simple experiment demonstrates that these modifications are part of a compensatory plastic change occurring in the trigeminal complex (32). Within 30 minutes of restraining 1 eyelid to make blinking more difficult, stimulating the supraorbital nerve ipsilateral to the restrained eyelid evokes hyperexcitable reflex blinks and additional blinks in both eyelids. Stimulating the supraorbital nerve contralateral to the restrained eyelid, however, elicits normal blinks in both eyelids. This pattern would occur only if the trigeminal complex receiving signals of corneal irritation from eyelid restraint expressed the plastic changes. Thus, eye irritation initiates plastic compensatory changes in blinking that could be exaggerated in BEB to produce the eyelid abnormalities of this focal dystonia.

We hypothesize that the predisposing condition exaggerates neuroplasticity so that modifications in response to eye irritation become maladaptive and amplify into the characteristics of BEB. There is significant evidence for exaggerated plasticity in dystonia (41,42). With generalized dystonia, homeostatic synaptic plasticity in the striatum is abnormal (43,44). Exaggerated associative plasticity accompanies focal hand dystonia (45–48). Important for our hypothesis, exaggerated plasticity of the trigeminal blink reflex accompanies BEB (49).

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The author reports no conflicts of interest.

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Our initial rodent model of BEB (50) used a small reduction of substantia nigra dopamine neurons to create the predisposing condition and crushing 1 branch of the facial nerve innervating the orbicularis oculi to generate the environmental trigger. The choice of dopamine depletion as a predisposing condition came from observations showing that baboons undergoing poisoning with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) of dopamine neurons exhibited dystonia before developing Parkinsonian movement abnormalities (51) and that there was a disruption of D_2 receptors in patients with BEB (52,53). Thus, changes in dopamine levels or the functioning of specific dopamine receptor subtypes could create the "predisposing condition" for BEB. For an environmental trigger, we created a transient eye irritation by crushing a branch of the facial nerve that provides approximately 30% of the orbicularis oculi innervation. This procedure produced a transient dry eye condition because the weakened eyelid became less effective at restoring the tear film with each blink. The condition was only temporary, however, because regeneration of the crushed nerve branch restored complete lid function within 3 weeks.

In the Schicatano model (50), the BEB-like spasms of lid closure only occurred with the combination of the environmental trigger and the dopaminergic predisposing condition. In the absence of the predisposing condition, the environmental trigger of transient eye irritation slightly increased trigeminal reflex blink excitability and resulted in the development of additional blinks similar to those seen in human dry eye (20,21). Without the environmental trigger, the predisposing condition of a small dopamine neuron loss slightly increased trigeminal reflex blink excitability but did not generate spasms of lid closure. Combining the predisposing condition and the environmental trigger, however, caused long-lasting spasms of lid closure, dramatically elevated trigeminal reflex blink excitability, and increased spontaneous blinking similar to the pattern of blink abnormalities of patients with BEB. These BEB-like characteristics continued after the facial nerve regained full function and eliminated the dry eye. Thus, the BEB-like characteristics of this animal model seemed to result from an exaggeration of the normally compensatory process evoked by eye irritation.

The Schicatano BEB model also was consistent with the important interactions between the cerebellum and basal ganglia that underlie dystonia (54–63). Previous studies demonstrated that the cerebellum was essential for adaptive responses to the eye irritation created by eyelid restraint. Lesions of the cerebellum (30,31) blocked the increases in blink amplitude and duration initiated by eye irritation (20,32–37). Recordings from blink-related neurons in the cerebellar interpositus nucleus revealed the changes in cerebellar activity that accounted for the changes in blink amplitude and duration associated with lid restraint (29). Although the Schicatano model supported the 2 hit hypothesis as the basis of BEB and identified the basal ganglia and cerebellum as key players in this focal dystonia, the model did not explain how the predisposing condition created the exaggerated plasticity that allowed normally adaptive modification to eye irritation to swell into spasms of lid closure.

We hypothesize that the key to the exaggerated plasticity of dystonia is hypersynchronized low-frequency oscillations of basal ganglia activity. Basal ganglia neurons in patients with Parkinson disease and animal models of Parkinson disease exhibit hypersynchronized oscillations in the broad beta band, 10–30 Hz (64–71). In contrast, basal ganglia neurons in dystonic patients exhibit hypersynchronized oscillations in the theta band, 3–10 Hz (71–74). Although the role of these oscillations in modifying voluntary movement is unclear (66,73,75–81), our study in rodents demonstrate that these basal ganglia oscillations modify trigeminal reflex blink plasticity (82).

We directly tested the role of basal ganglia oscillations in blink plasticity by delivering deep brain stimulation to the basal ganglia subthalamic nucleus of normal rats undergoing a blink plasticity paradigm (82). The procedure was a cerebellar-dependent plasticity paradigm that we developed for humans (83) and modified for rodents (84). Other investigators used this paradigm to demonstrate impaired blink plasticity with Parkinson disease (85), but exaggerated blink plasticity with BEB (49). If the frequency of basal ganglia oscillations modulates brainstem plasticity, then beta frequency deep brain stimulation in normal rats should impair trigeminal reflex blink plasticity, whereas theta frequency deep brain stimulation should exaggerate blink plasticity. The Kaminer et al study (82) demonstrated the validity of this postulation. Beta frequency, 16 Hz, deep brain stimulation impaired blink plasticity, whereas theta frequency, 7 Hz, deep brain stimulation exaggerated trigeminal reflex blink plasticity in normal rats. Deep brain stimulation at 130 Hz, a therapeutic frequency for deep brain stimulation in humans (86), however, did not affect blink plasticity in normal rats. Thus, hypersynchronized theta frequency basal ganglia oscillations could create a predisposing condition in which adaptive plasticity initiated by eye irritation exaggerated into spasms of lid closure typical of BEB.

In a preliminary study on 1 rat, we monitored blinking and spasms of lid closure in a normal rat receiving 7 Hz deep brain stimulation of the subthalamic nucleus 4 hours a day combined with mild dry eye produced by exorbital lacrimal gland removal (36). We tested 3 conditions: 1) 7 Hz subthalamic nucleus deep brain stimulation alone (Fig. 1B, gray bars); 2) 7 Hz subthalamic nucleus deep brain stimulation combined with dry eye (Fig. 1B, black bars); and 3) dry eye alone (Fig. 1B, white bars). In Condition 1, the rat received 5 days of 7 Hz subthalamic nucleus deep brain stimulation alone. In Condition 2, combining the predisposing condition and the environmental trigger, we removed the exorbital gland and the rat received 5 days of 7 Hz subthalamic nucleus deep brain stimulation for 4 hours each day. In Condition 3, we discontinued the 7 Hz subthalamic nucleus deep brain stimulation. For all conditions, we monitored blinking (lid closures $<$100 milliseconds) and lid spasms (lid closures $>$100 milliseconds) continuously over a 30-minute period on the last 2 days of each

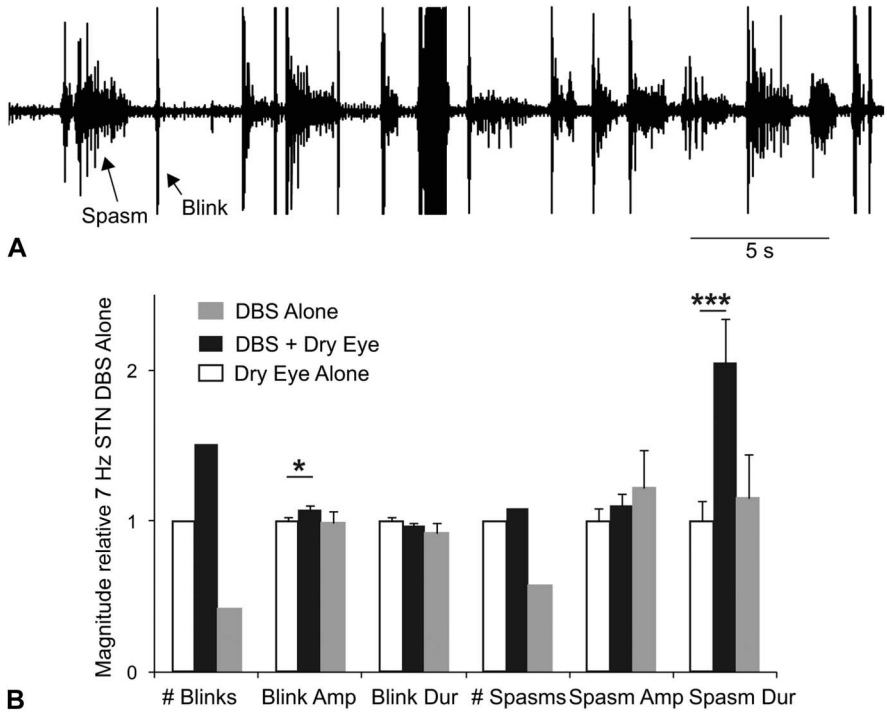

FIG. 1. An animal model of benign essential blepharospasm using 7 Hz deep brain stimulation (DBS) as the predisposing condition. **A**. A recording of spasms of lid closure and excessive blinking by a rat with dry eye receiving 7 Hz subthalamic nucleus (STN) DBS. **B**. Average number of blinks (# Blinks), blink amplitude (Blink amp), blink duration (Blink Dur), number of spasms (# Spasms), amplitude of spasms (Spasm Amp), and duration of spasms (Spasm Dur) relative to 7 Hz STN DBS alone condition. Spasms were lid closures lasting >100 milliseconds. Error bars are SEM. $*P < 0.05$; $***P < 0.001$.

condition and normalized all data to the 7 Hz subthalamic nucleus deep brain stimulation alone condition. In the combined 7 Hz subthalamic nucleus deep brain stimulation and dry eye condition, the rat made more blinks than either the 7 Hz subthalamic nucleus deep brain stimulation alone or dry eye alone conditions (Fig. 1B, # Blinks). In the combined 7 Hz subthalamic nucleus deep brain stimulation and dry eye condition, the rat also exhibited more spasms of lid closure than in the other conditions (Fig. 1B, # Spasms). Moreover, the spasm duration was longer in the combined 7 Hz subthalamic nucleus deep brain stimulation and dry eye condition than in the 7 Hz subthalamic nucleus deep brain stimulation alone or dry eye alone condition (Fig. 1A, B, Spasm Dur). Finally, the rat made significantly larger blinks in the combined 7 Hz subthalamic nucleus deep brain stimulation and dry eye condition than in 7 Hz subthalamic nucleus deep brain stimulation alone condition ($P < 0.05$; Fig. 1B, Blink Amp). Although preliminary, these data indicate that the next rodent model of BEB should be developed by combining theta frequency deep brain stimulation of the subthalamic nucleus and dry eye.

Thus far, animal models of BEB have not been tested for the abnormal sensitivity to light associated with BEB (1,24,87). The neural bases of photophobia in patients with BEB are unknown. Physiological and behavioral studies of photophobia implicate changes in blood flow (88), melanopsin ganglion cell inputs to somatosensory thalamic regions (89), intraocular nociceptors (90), and calcitonin gene-related peptide trigeminal sensitization (91,92). Because all of these mechanisms involve elevated trigeminal excitability, we anticipate that rodent models of BEB will also exhibit exaggerated light sensitivity.

The evidence from animal models indicates that spasms of lid closure and trigeminal hyperexcitability of BEB result from exaggerated neuroplasticity, an amplification of the normally adaptive modifications of blinking initiated by eye irritation. The adaptive plasticity initiated by eye irritation seems to involve the cerebellum (29–31), and the exaggeration of plasticity ensues from abnormal basal ganglia modulation of cerebellar activity (82). These results are consistent with the available data pointing to abnormal cerebellar basal ganglia interactions as a major component of dystonia (62,93–96). Although animal models are not identical to human BEB, they are invaluable for identifying the neural mechanisms and circuits causing BEB.

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Benign Essential Blepharospasm—There Is More to It Than Just Blinking

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Benign essential blepharospasm (BEB) is recognized today as a primary dystonia causing excessive blinking, squeezing, and involuntary contractions of the orbicularis oculi muscles. This involuntary lid closure leads to functional blindness and decreased quality of life. Besides the blinking and squeezing, patients with BEB are known to have trigeminal hyperexcitability as demonstrated by blink reflex testing and photophobia. Patients with BEB frequently use sensory tricks, like touching the side of the eye, humming, or singing that will temporarily improve the spasms. For decades, this led clinicians to consider blepharospasm to be a nonphysiological disorder. However, many studies in the last 60 years have dispelled that belief.

The condition occurs more frequently in women by a ration of almost 3 to 1. Most are white. Although the median age is approximately 53 years, blepharospasm occasionally has been reported in children. Many individuals go years before they are appropriately diagnosed. The most valid findings to make the diagnosis are involuntary eyelid narrowing or closure due to spasms of the orbicularis oculi muscle, bilateral spasms that are synchronous and stereotyped, a sensory trick, and inability to suppress the spasms and blink count voluntarily (1). Many individuals report that there is a family history of dystonia or benign tremor or Parkinson disease. Some predisposing factors are believed to be recent stressful events, a history of dry eye or keratitis, and head trauma (2). BEB has profound effects on visual quality of life and overall quality of life, and there is a tendency to more depression (3). For such a disabling condition, we have limited treatment options. There is a real need for greater understanding of this disorder and better treatments to help our patients.

In the accompanying article, Evinger (4) reviews what animal models teach us about this vexing condition. These models provide hope that if we can model a condition in an animal, we are more likely to be able to understand factors that cause it and create more effective treatments for our patients.

Initially, Evinger reminds us that the etiology of BEB may occur due to a predisposition (e.g., genetic) and an environmental trigger—the so called "2 hit" hypothesis. Although there is no known gene for the condition, frequency of a positive family history suggests that there is a genetic component. But there must also be an environmental trigger. Epidemiological data strongly point to the association of dry eyes and blepharitis as potential environmental triggers.

What dry eye and dry eye symptoms do in predisposed individuals is to exaggerate neuroplasticity by increasing blink frequency and amplitude in an attempt to restore tears. Modifying the trigeminal blink reflex becomes

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K. B. Digre is listed as an inventor on a patent pending for thin-film coatings designed for the treatment of photophobia; she could receive royalties on any commercial sales of these coatings.

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thalamus of both patients with drug-induced blepharospasm and patients with BEB. Notably, glucose hypermetabolism in the thalamus also was found in drug healthy subjects compared to healthy controls, although drug healthy individual had no symptoms of blepharospasm. In the 21 patients with drug-induced blepharospasm, eleven patients successfully lowered the dosage or completely discontinued using the medication, and blepharospasm improved in six of these patients. From these observations, we concluded that benzodiazepines are one of the environmental triggers leading to blepharospasm. We suspect that blepharospasm did not occur in drug healthy subjects because they were not genetically predisposed. Furthermore, we hypothesize that the symptoms of blepharospasm improved in patients with drug-related blepharospasm after benzodiazepines withdrawal because this medication was the environmental trigger. Based on our observations, the drug-induced alteration of the GABAergic inhibition system may be one of the major environmental trigger factors inducing blepharospasm.

Currently, the injection of botulinum toxin A is the most effective treatment for blepharospasm. However, besides dry eyes, blepharitis, and medications, there may exist other environmental triggers, and simply removing of these triggers may be an effective treatment for blepharospasm. PET is a powerful and effective tool to understand blepharospasm pathophysiology; thus, we will continue using PET to research the causes of blepharospasm and identify possible treatment options.

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Role of GABAergic System in Blepharospasm: Response

We thank Dr. Suzuki et al for their response to our articles (1,2) discussing the origins of benign essential blepharospasm. In their response, the investigators agree with the widely accepted "2 hit" hypothesis for the development of focal dystonia in which a genetic predisposing condition creates a neural milieu in which an adaptation to an environmental trigger exaggerates into the development of the focal dystonia (3–6). For blepharospasm, we argue that the effect of this genetic predisposing condition is modification of basal ganglia–thalamo–cortical activity to create a "hyper-motor adaptation" state (7) in which the compensatory adaptations initiated by an environmental trigger such as dry eye or eye irritation (8) exaggerate into benign essential blepharospasm.

Based on their data, Dr. Suzuki et al argue that abnormal GABAergic function is an environmental trigger for the development of blepharospasm. This proposal rests on 3 arguments. First, following withdrawal from benzodiazepine treatment, 6 of 11 patients with drug-induced blepharospasm experienced relief from their spasms of lid closure.

Second, the investigators report that some individuals with long-term drug treatment exhibited hypermetabolism of the thalamus, but did not exhibit drug-induced blepharospasm. Third, the studies by Suzuki et al and others showed that thalamic hypermetabolism occurs in patients with benign essential blepharospasm and in patients exhibiting drug-induced blepharospasm associated with long-term benzodiazepine and thienodiazepine treatment (9–11). The investigators concluded that the individuals on long-term drug treatment who do not exhibit lid spasms lack the genetic predisposing condition to allow blepharospasm to develop. In essence, the authors postulate that thalamic hypermetabolism is the trigger for blepharospasm. We would argue, however, that the authors' data are equally consistent with the hypothesis that thalamic hypermetabolism and reduced GABAergic function are expressions of the genetic predisposing condition rather than the trigger for blepharospasm.

Accepting the argument that focal dystonias require both a predisposing neural milieu and an environmental trigger, removing either the predisposing condition or the trigger should resolve blepharospasm. Thus, the data demonstrating that removing benzodiazepine treatment resolves lid spasms in approximately half of the patients with drug-induced blepharospasm do not distinguish between the GABAergic alterations being a trigger or a predisposing condition. Likewise, the argument that many drug-treated individuals do not develop blepharospasm although they exhibit thalamic hypermetabolism is equally consistent with the proposal that the thalamic hypermetabolism is part of a predisposing condition but that these individuals did not experience a significant environmental trigger of eye irritation to cause blepharospasm. Furthermore, treatment with benzodiazepam, a GABAergic agent, reportedly treats blepharospasm (12). The reduction in GABAergic function that the investigators' studies indicate blepharospasm is also consistent with the "hyper-motor adaptation" identified in focal dystonia (7,13–16). For example, an increase in long-term potentiation accompanies the reduction in GABAergic function in the regions around a cortical lesion (17). Based on these data, we argue that the correlated thalamic hypermetabolism and downregulation of GABA function described by Dr. Suzuki et al are components of the "hyper-motor adaptation" state that allows the development of blepharospasm and other focal dystonias (18).

Our understanding of the genetics of benign essential blepharospasm is in its earliest stages. What we can identify, however, are the modifications of brain function that the genetic predisposing condition creates. We view the investigations reported by Dr. Suzuki et al as adding to the data showing that disrupting GABA function plays a role in creating the "hyper-motor adaptation" that enables brain plasticity to develop unchecked in dystonia.

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An Evaluation of Educational Neurological Eye Movement Disorder Videos Posted on Internet Video Sharing Sites: Comment

We read with great interest the article by Hickman (1) who examined the educational value of eye movement videos on the internet. As medical students with personal experience, we agree with the author that video sharing web sites can be a powerful tool for learning.

With regard to ophthalmology-based teaching, modern undergraduate curricula provide little opportunity for such learning, despite the presence of established international guidelines on core concepts that graduating doctors need to practice safety (2). Coupled with the ever-growing body of knowledge and competencies confronting medical students, one may make the case that ophthalmology teaching will be increasingly reduced. Fan et al (3) found that the mean amount of time spent on ophthalmology lectures across medical schools in Asia and Australia was just one day, whereas in the United Kingdom, 21% of universities have no mandatory clinical training in ophthalmology required in their respective curricula (4). Collectively, these examples demonstrate the paucity of education in both preclinical and clinical pedagogy. As a consequence, it is not surprising that there are shortfalls in the diagnosis and management of eye disease, referral accuracy, and confidence in facing ophthalmic problems (5).

Thus, alternative methods of teaching, such as internet videos, will become increasingly relied upon for education. Azer (6) compared the content of textbooks, eMedicine articles, and YouTube videos and found that YouTube excelled not only on the user interface front but also in terms of content and integration of information across a molecular and clinical level. Videos provide up-to-date, digestible educational resources that also are interactive, while providing the opportunity to ask questions in the comments section. With the increase of mobile technology and smartphone usage, we suggest that this platform should be investigated as a feasible method for formal teaching. However, unsolicited video sharing with a lack of regulation may cause problems and develop misconceptions in knowledge, and we agree with the author's call for further regulation of educational videos.

In his article, Hickman (1) pointed out that videos from educational institutions and medical journals generally contained better content with fewer errors. The peer-review process is an established route to ensuring videos of high educational quality are produced and uploaded. If these videos were incorporated into modern medical curricula, this would direct students to reliable and trustworthy sources of learning. Moreover, medical students could be encouraged to produce their own content, which may be guided and verified by their teachers. Ultimately, we believe that these 2 proposals would help raise the standard of online educational videos and develop safer practitioners and well-rounded graduates.

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RESEARCH ARTICLE | *Control of Movement*

Sex, blinking, and dry eye

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Culoso A, Lowe C, Evinger C. Sex, blinking, and dry eye. *J Neurophysiol* 123: 831–842, 2020. First published January 15, 2020; doi:10.1152/jn.00635.2019.—Blinking sustains the corneal tear film generated by sexually dimorphic lacrimal and meibomian glands. Our study examines whether trigeminal control of blinking is also sexually dimorphic by investigating trigeminal reflex blinking, associative blink modification, and spontaneous blinking in male and female rats before and after unilateral dry eye caused by exorbital gland removal. Before gland removal, female rats exhibited a lower threshold for evoking trigeminal reflex blinks, a weaker effect of associative blink modification, and longer-duration spontaneous blinks than males. Spontaneous blink rate, reflex blink excitability, and occurrence of blink oscillations did not differ between the sexes. Reanalysis of previous data showed that humans showed the same blink sexual dimorphisms as rats. During the first 2 wk of dry eye, trigeminal blink circuit excitability and blink oscillations steadily rose in male rats, whereas excitability and blink oscillations did not change in females. Following dry eye, spontaneous blink duration increased for both males and females, whereas spontaneous blink rate remained constant for males but decreased for females. The associative modification treatment to depress trigeminal blink amplitude initially produced blink depression in males that converted to blink potentiation as trigeminal excitability rose, whereas females exhibited progressively more blink depression. These data indicated that dry eye increased excitability in male trigeminal reflex blink circuits at the expense of circuit modifiability, whereas trigeminal modifiability increased in females. This increased modifiability of female trigeminal blink circuits with dry eye may contribute to the preponderance of females developing the focal dystonia, benign essential blepharospasm.

NEW & NOTEWORTHY All the elements controlling the corneal tear film are sexually dimorphic. Blinking, which smooths and maintains the tear film, also exhibits sex differences. Dry eye increases the sexual dimorphisms of blinking, including increased exaggeration of excitability in males and enhanced modifiability of the female trigeminal complex. This increased modifiability may explain female predominance in the development of the focal dystonia, benign essential blepharospasm.

blepharospasm; blink; dry eye; sex; trigeminal

INTRODUCTION

Dry eye presents a unique challenge to the trigeminal system. To compensate for corneal drying, the trigeminal system must identify the abnormally rapid breakup of the corneal tear film and compensate by regulating the aqueous and lipid tear components released by the lacrimal and meibomian glands, respectively, and altering blinking (Belmonte et al. 2017; Truong et al. 2014). Neurons within the spinal trigeminal complex border region between the interpolaris and caudalis subnuclei (Vi/Vc) recognize corneal drying and increase the aqueous tear component by activating the lacrimal glands via the superior salivatory nucleus (Hirata et al. 2004; Katagiri et al. 2015; Okamoto et al. 2012). Because each blink recreates and smooths the tear film (Begley et al. 2006; Himebaugh et al. 2009; Korb et al. 1994; Owens and Phillips 2001), it is not surprising that the Vi/Vc region involved in lacrimal regulation also drives reflex blinking (Gong et al. 2003; Henriquez and Evinger 2007; Hirata et al. 2000; Pellegrini et al. 1995) and perhaps modulates spontaneous blinking (Kaminer et al. 2011). The neural adaptations of the trigeminal complex that compensate for the rapid tear film breakup of dry eye also express themselves as changes in blinking. With dry eye or eye irritation, trigeminal reflex blink excitability increases such that a single trigeminal blink-evoking stimulus elicits multiple blinks (Evinger et al. 2002; Peshori et al. 2001; Schicatano et al. 2002). The present study investigates these blink modifications in a rat model of dry eye produced by removal of the exorbital gland, a rodent lacrimal gland (Lorber 1993; Walcott et al. 2005). To probe the properties of trigeminal neural adaptation, we examine how dry eye affects the system response of the trigeminal blink to an associative learning paradigm that depresses blink amplitude (Mao and Evinger 2001; Ryan et al. 2014).

Most of the components that maintain the human corneal tear film are sexually dimorphic. The cornea (Gupta et al. 2005; Wang et al. 2012), lacrimal glands (Albietz 2000; Marcozzi et al. 2003), and meibomian glands (Den et al. 2006; Sullivan et al. 2017) all exhibit sexual dimorphisms. Likewise, rodent exorbital and meibomian glands exhibit similar sex-specific differences (Ferrara et al. 2004; Richards et al. 2006; Sashima et al. 1989; Schirra et al. 2006; Sullivan et al. 2017). Although little evidence exists concerning sexual dimorphisms of Vi/Vc neural circuits involved in controlling the lacrimal and meibomian glands and blinking, studies of trigeminal pain from migraine and temporomandibular joint disorder show sexual dimorphisms in humans and rodents (Bereiter 2001; Bereiter et al. 2002; Chai et al. 2014; Flake et al. 2005; Gazerani et al. 2005; Okamoto et al. 2005; Steiner et al. 2003; Tashiro et al. 2014), suggesting that blinking might also exhibit sexual dimorphisms. The present study investigates sex differences in blink patterns created by dry eye. These differences may explain why the focal dystonia, benign essential blepharo-

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spasm (BEB), which can be triggered by dry eye (Defazio et al. 1999, 2011; Hallett et al. 2008; Jinnah and Hallett 2011), occurs predominantly in females (Defazio et al. 2017; Hallett et al. 2008).

METHODS AND MATERIALS

Experiments were performed on four male and five female Sprague Dawley rats (175–550 g) maintained on a reversed 12-h:12-h light/dark cycle and fed ad libitum. Data were collected in a darkened room during the rats' subjective night. All experiments received approval by the Stony Brook University Institutional Animal Care and Use Committee and complied with all federal, state, and university regulations regarding the use of animals in research.

Surgery. Under general anesthesia (ketamine, 90 mg/kg and xylazine, 10 mg/kg), rats were prepared for chronic recording of the orbicularis oculi EMG (OOemg) and stimulation of the supraorbital branch of the trigeminal nerve (SO) (Dauvergne and Evinger 2007; Evinger et al. 1993; Ryan et al. 2014). To record the OOemg, a pair of Teflon-coated stainless steel wires (0.003-inch diameter bare, 0.0055-inch diameter coated; number 791000; A-M Systems, Everett, WA) with ~1 mm exposed at the tip were implanted into the orbicularis oculi (OO) muscle, one wire placed below the lateral canthus and the other in the lower lid below the pupil. To stimulate the SO, a nerve cuff containing a pair of stainless steel wires with the insulation removed (0.003-inch diameter bare, 0.0055-inch diameter coated; A-M Systems number 791000) encased in Teflon tubing (1-mm diameter; number 163300; Small Parts, Miami, FL) was placed around the SO branch of the trigeminal nerve. Wires were led subcutaneously to a connector embedded in a dental acrylic platform on the skull. The platform was attached to the skull by four stainless steel screws. A silver wire connected to one of the stainless steel screws served as the ground. Rats received an analgesic (ketorolac, 7 mg/kg) for at least 24 h after the surgery. Rats were alert and eating within 24 h of the surgery. The experiments began at least 1 wk postsurgery.

After collection of control data, the exorbital gland was removed unilaterally. Rats were anesthetized (ketamine, 90 mg/kg and xylazine, 10 mg/kg), and the subcutaneous exorbital lacrimal gland, which is located just anterior and slightly below the ear (Lorber 1993; Walcott et al. 2005) ipsilateral to the OOemg electrodes, was removed. Exorbital gland removal created an ~50% reduction in tearing in the ipsilateral eye (Bereiter et al. 2018; Katagiri et al. 2015; Meng et al. 2015; Rahman et al. 2015b, 2017). This mild dry eye did not produce corneal ulcerations because rats have two additional glands that contribute to the aqueous portion of the tear film (Williams 2002). Rats received a postoperative analgesic (ketorolac, 7 mg/kg) for at least 24 h after the surgery. Dry eye data collection began 2 days after the exorbital gland surgery.

Paradigms. In all experiments, the SO stimulus was relative to threshold (T), the minimum current at which a unipolar, 100-μs stimulus reliably elicited the R1 component of the reflex blink. For each rat, this current was determined at the beginning of each day and held constant throughout that day's experiment. Across all subjects and days tested, threshold currents ranged from 100 to 900 μA, with a median of 212.5 μA. For each rat, threshold varied little across days. Across all rats, the threshold coefficient of variation for current ranged between 0.1 and 0.22, with a mean of 0.17 $\pm$ 0.02. All data were collected at twice threshold (2T), a stimulus intensity that evokes a strong R1 response and a small, inconsistent R2 component in rats (Basso et al. 1993; Dauvergne and Evinger 2007; Evinger et al. 1993; Ryan et al. 2014).

Reflex and spontaneous blinks were monitored as the rats moved freely in their home cage in a darkened room during their subjective night. Data collection from each day consisted of five blocks: *1*) pretreatment, *2*) treatment: high-frequency SO stimulation (HFS), *3*) immediately posttreatment, *4*) 30 min posttreatment, and *5*) 60 min posttreatment. In the pretreatment block, rats received 30 trials of paired 2T SO stimuli with an interstimulus interval of 100 ms. The three posttreatment blocks were identical to the pretreatment blocks except that there were 20 rather than 30 trials. Thus two blinks were evoked in each nontreatment trial. The first evoked blink was called the Condition blink and the second blink termed the Test blink. The intertrial interval varied pseudorandomly over the range of 20 $\pm$ 5 s. The HFS treatment used in these experiments was a blink motor learning paradigm to depress blink amplitude (Mao and Evinger 2001; Ryan et al. 2014). Each HFS treatment trial consisted of a single SO stimulus at 2T to evoke a reflex blink followed by five, 2T SO stimuli delivered at 400 Hz during the R1 but before the onset of the R2 component of the OOemg activity. The latency between first SO stimulus and HFS was adjusted for each rat based on their average R1 latency. Rats received 60 of these HFS trials during the treatment block. The intertrial interval for HFS trials varied pseudorandomly over the range of 20 $\pm$ 5 s. Determination of reflex blink excitability and blink oscillations were made on data collected in the pretreatment block to avoid contamination by changes induced by HFS treatment (Ryan et al. 2014). On each day, spontaneous blinking data were collected after completing collection of trigeminal reflex blink data. Spontaneous blinking was recorded for 15 to 30 min while rats moved freely in their home cage (Kaminer et al. 2011).

Data collection and analysis. Data were collected for 1 wk before and 2 wk after unilateral removal of the exorbital gland. Control data were those collected in the week before exorbital gland removal, and dry eye data were those collected in the 2 wk following exorbital gland removal except for the first 2 days after gland removal when the rats were not tested while they received the ketorolac analgesic.

OOemg signals were amplified (A-M Systems; model 1700; 4-channel differential amplifier), filtered at 0.3–5 kHz, collected at 4 kHz per channel (DT 2831; Data Translation, Marlboro, MA; 12-bit analog-to-digital resolution) and stored for later offline analysis on laboratory-developed software. Blink amplitude was determined by integrating the rectified OOemg activity between the beginning and end of each blink component (Dauvergne and Evinger 2007; Evinger et al. 1991; Kaminer et al. 2011; Pellegrini and Evinger 1995; Pellegrini et al. 1995; Ryan et al. 2014).

Because SO stimulus intensity was constant within a day, changes in SO-evoked reflex blink amplitude post-HFS were changes in trigeminal reflex blink gain. We normalized within-day treatment-induced modifications of blink amplitude by dividing both the average pre- and the average post-HFS R1 OOemg amplitude by the median pre-HFS R1 OOemg amplitude for that day. As there were no consistent differences in data among post-HFS blocks (Ryan et al. 2014), we combined the three post-HFS blocks for analysis. We quantified the effect of the HFS treatment by subtracting the averaged normalized pre-HFS blink amplitude from the averaged normalized post-HFS blink amplitude and termed this measure gain change. In this procedure, a negative value signified a decrease in blink gain following HFS treatment, blink depression, whereas a positive value indicated an increased blink gain, blink potentiation.

For spontaneous blink data, we analyzed the blink rate, blinks/min, and interblink interval (IBI) before and after exorbital gland removal. To prevent the change from SO stimulation to no stimulation from affecting spontaneous blink patterns, data from the first 5 min of spontaneous blink data collection were discarded, and only the remaining 10 to 25 min of data collection were analyzed. Spontaneous blink amplitude was determined by integrating the rectified OOemg activity between the beginning and end of each blink. Blink duration was measured as the time between the start and end of the blink OOemg activity.

Statistical tests of significance ($P < 0.05$) were performed with SPSS software (SPSS, Chicago, IL). Data were presented as the means $\pm$ SE.

RESULTS

Trigeminal reflex blinking: excitability, amplitude, and blink oscillations. The human trigeminal blink system responds to corneal discomfort of dry eye (Rosenthal and Borsook 2012) with increased trigeminal reflex blink excitability and the production of blink oscillations (Evinger et al. 2002). Likewise, rats exhibit an increase in trigeminal reflex blink excitability (Fig. 1) and blink oscillations (Fig. 2) following exorbital gland removal.

Trigeminal reflex blink excitability was quantified by dividing the R1 amplitude of the second, Test blink, by the R1 amplitude of the first, Condition blink (Fig. 1, *A* and *B*). Before dry eye, the amplitude of the second, Test, blink was smaller than that of the first, Condition, blink for both a male (Fig. 1*A*, Pre) and a female rat (Fig. 1*B*, Pre). In these examples, the excitability ratio was 0.5 for the male and 0.75 for the female. Averaged over males and females, there was no significant difference in trigeminal blink excitability between the sexes (male = 0.95 ± 0.14, female = 1.15 ± 0,08; $t_{(30)} = -1.32$, $P > 0.05$). As expected from human studies (Evinger et al. 2002; Peshori et al. 2001; Schicatano et al. 2002), normalized trigeminal reflex blink excitability increased significantly in the 2 wk following exorbital gland removal relative to before dry eye averaged over all rats [$t_{(79)} = -2.08$, $P < 0.05$; Fig. 1*C*, All]. Separating the excitability data based on sex, however, revealed striking differences between males and females. Following exorbital gland removal, Test blink amplitude became larger than Condition blink amplitude in male rats (Fig. 1*A*, Post). In this example, the Test/Condition ratio measurement of excitability was 1.19. Over all male rats, the normalized excitability ratio increased by 79% in males following exorbital gland removal [$t_{(33)} = -3.39$, $P < 0.01$, Fig. 1*C*, Male]. In contrast to the males, dry eye did not change the ratio between Condition and Test amplitude in females (Fig. 1*B*, Post). In this example, the excitability ratio was 0.65. Averaged over all females, normalized excitability decreased slightly but was not significant [$t_{(43)} = 1.6$, $P > 0.05$, Fig. 1*C*, Female], so the increased excitability of male, but not female, rats following gland removal is responsible for the significant difference in their excitability [male = 1.55 ± 0.15, female = 0.94 ± 0.04; $t_{(47)} = 4.31$, $P < 0.001$].

One possible explanation for the changes in excitability associated with dry eye was that the SO current needed to evoke a reflex blink changed following exorbital gland removal. SO threshold stimulus intensity, however, did not change significantly following exorbital gland removal for either males [$t_{(31)} = 0.64$, $P > 0.05$; Fig. 1*D*, Male] or females [$t_{(45)} = 0.72$, $P > 0.05$; Fig. 1*D*, Female]. Nevertheless, the threshold current needed to evoke a blink differed significantly between males and females both before [$t_{(31)} = 4.35$, $P < 0.001$] and after [$t_{(47)} = 4.72$, $P < 0.001$] gland removal. Thus, in both normal and dry eye conditions, eliciting a trigeminal reflex blink required a lower current stimulus for females than for males.

Because the excitability measure is a ratio of the amplitude of the second, Test, blink divided by the amplitude of the first,

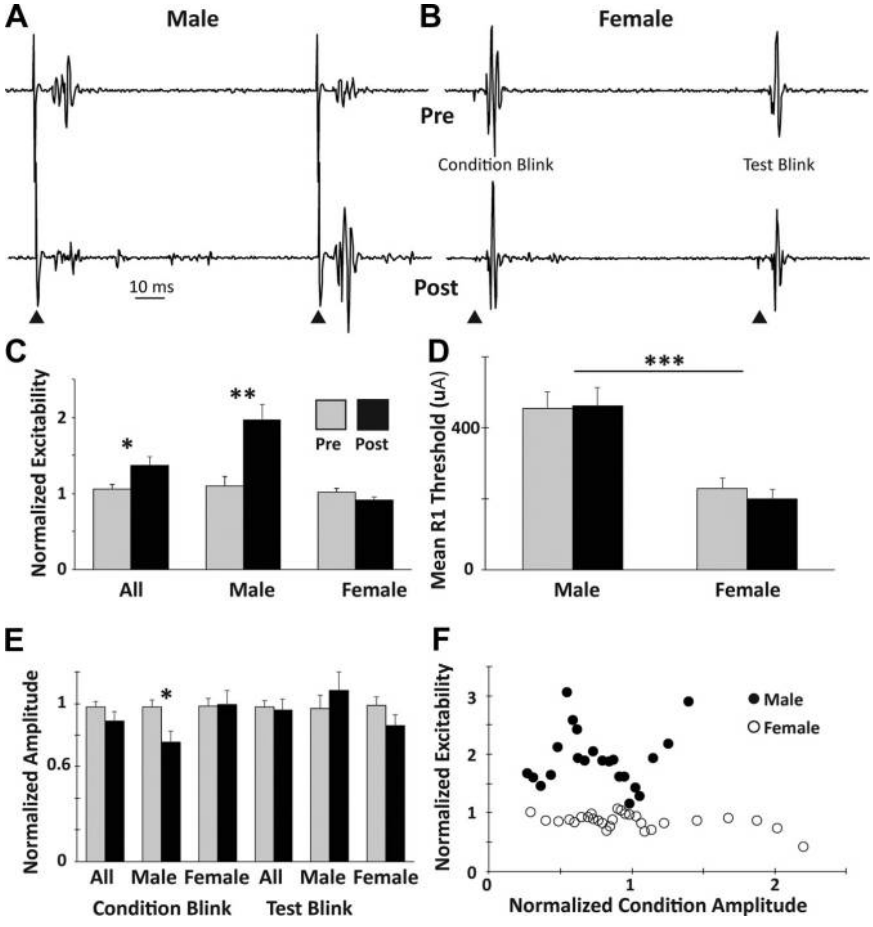

Fig. 1. Trigeminal reflex blink excitability before and after exorbital gland removal. *A*: single trial showing orbicularis oculi EMG (OOemg) activity of a Condition and Test blink evoked by identical supraorbital branch of the trigeminal nerve (SO) nerve stimuli (▲) delivered 100 ms apart before (Pre) and after (Post) exorbital gland removal from a male rat. *B*: single trial showing OOemg activity of a Condition and a Test blink evoked by identical SO nerve stimuli (▲) delivered 100 ms apart before (Pre) and after (Post) exorbital gland removal from a female rat. *C*: mean reflex blink excitability normalized to the median blink excitability before exorbital gland removal for all rats ($n = 9$), male rats ($n = 4$), and female rats ($n = 5$) before exorbital gland removal (shaded bars) and after gland removal (solid bars). *D*: mean threshold for evoking an SO-evoked blink for male ($n = 4$) and female ($n = 5$) rats before (shaded bars) and after exorbital gland removal (solid bars). *E*: mean blink amplitude normalized to the median blink amplitude before exorbital gland removal for all rats ($n = 9$), male rats ($n = 4$), and female rats ($n = 5$) for Condition and Test blink components before (shaded bars) and after gland removal (solid bars). *F*: normalized excitability plotted as a function of normalized condition blink amplitude after exorbital gland removal for males (●) and females (○). Each data point is the mean value of all trials for a single day for an individual rat. Error bars are SE of the mean; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

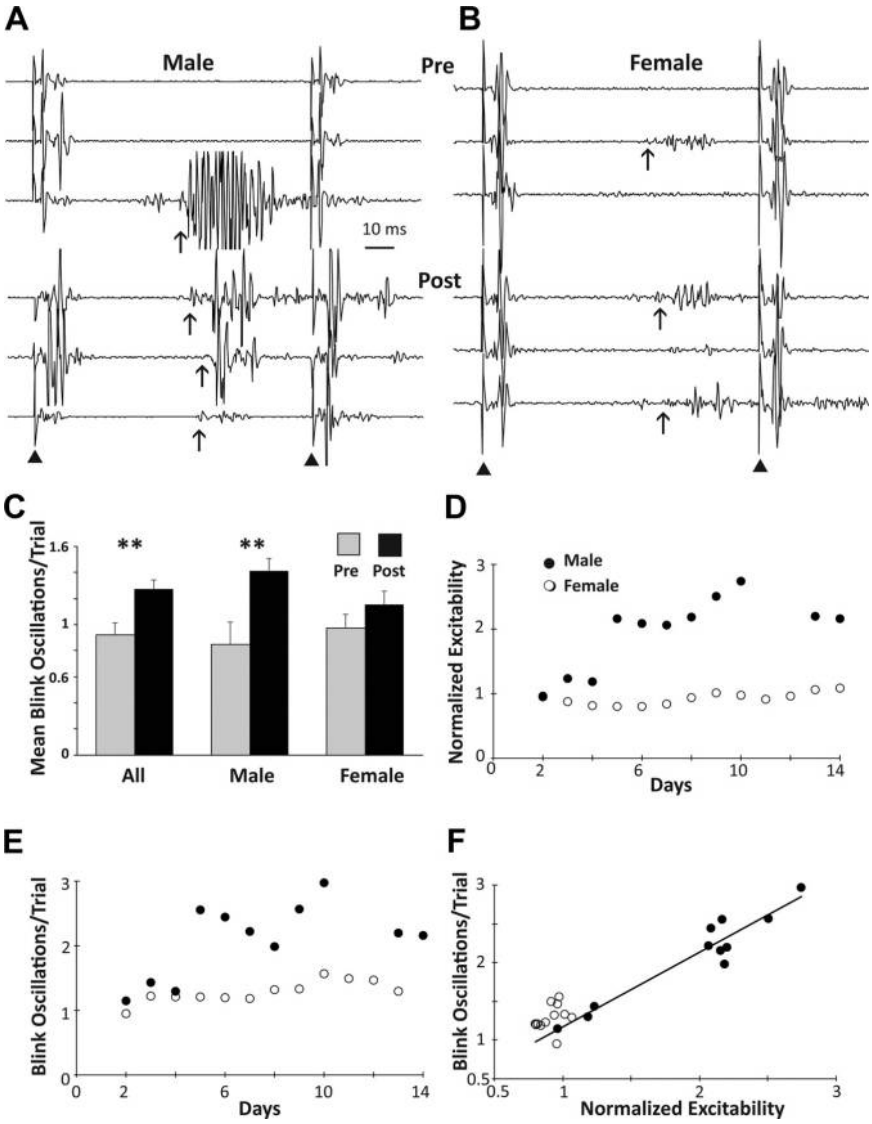

Fig. 2. Blink oscillations before and after exorbital gland removal. *A*: 3 consecutive trials of orbicularis oculi EMG (OOemg) activity showing a Condition and a Test blink evoked by identical supraorbital branch of the trigeminal nerve (SO) nerve stimuli (▲), as well as blink oscillations (↑) occurring on some trials before (Pre) and all trials after (Post) exorbital gland removal for a male rat. *B*: 3 consecutive trials of OOemg activity showing a Condition and a Test blink evoked by identical SO nerve stimuli (▲), as well as blink oscillations (↑) occurring on some trials before (Pre) and after (Post) exorbital gland removal for a female rat. *C*: mean number of blink oscillations per trial for all ($n = 9$), male ($n = 4$), and female ($n = 5$) rats before (shaded bars) and after exorbital gland removal (solid bars). *D*: normalized excitability as a function of days after exorbital gland removal for males (●) and females (○). Each data point is the mean value of all rats for a single day normalized to the median excitability of each rat before exorbital gland removal. *E*: normalized oscillations/trial as a function of days after exorbital gland removal for males (●) and females (○). Each data point is the mean value of all rats for a single day normalized to the median oscillations/trial for each rat before exorbital gland removal. *F*: normalized blink oscillations/trial plotted as a function of normalized blink excitability after exorbital gland removal for males (●) and females (○). Each data point is the mean value of all rats for a single day. Error bars are SE of the mean; $^{**}P < 0.01$.

Condition, blink, the increased blink excitability in male rats following exorbital gland removal could result from a reduction in Condition blink amplitude, an increase in Test blink amplitude, or both. In example record of Fig. 1*A*, the amplitude of the Condition blink with dry eye (Post) was smaller than that before gland removal (Pre). Averaged over all male rats, the amplitude of Condition blinks decreased by 22% after gland removal [$t_{(33)} = 2.43$, $P < 0.05$; Fig. 1*E*, Condition Blink, Male]. Female rats, however, did not show a significant change in Condition blink amplitudes pre- and post- exorbital gland removal [$t_{(44)} = -0.07$, $P > 0.05$; Fig. 1*B* and *E*, Condition Blink, Female]. The small increase in the mean Test blink amplitude of male rats was insignificant [$t_{(33)} = -0.71$, $P > 0.05$; Fig. 1*E*, Test Blink, Male], as was the small decrease in mean Test blink amplitude of female rats [$t_{(43)} = 1.48$, $P > 0.05$; Fig. 1*E*, Test Blink, Female]. Thus a decrease in Condition blink amplitude contributed to the elevated trigeminal reflex blink excitability of males following gland removal.

To explore whether changes in Condition blink amplitude alone were responsible for the increased trigeminal reflex blink excitability following gland removal in males, normalized excitability was plotted as a function of the normalized Condition blink amplitude for each day after gland removal for each rat (Fig. 1*F*). For this analysis, Condition blink amplitude and excitability after gland removal each day were normalized to the median blink amplitude and excitability, respectively, for each animal before gland removal. If changes in Condition blink amplitude alone established blink excitability (Test/Condition), then blink excitability should increase as Condition blink amplitude decreased. This pattern, however, did not occur. For females (Fig. 1*F*, ○), blink excitability remained constant even though normalized Condition blink amplitude varied twofold. Although the male blink excitability ratio was elevated relative to females, male excitability did not change consistently with Condition blink amplitude (Fig. 1*F*, ●). Thus Condition amplitude alone did not determine reflex blink excitability following gland removal. Rather dry eye produced a fundamental difference in trigeminal blink system excitability between male and female rats.

In normal humans over 40, a single SO stimulus often evokes a reflex blink followed by one or more additional blinks, termed blink oscillations because they occur with comparable IBIs (Peshori et al. 2001). Dry eye and eye irritation increase the probability of blink oscillation occurrence (Ev-

inger et al. 2002; Schicatano et al. 2002). Normal rats also exhibited blink oscillations following SO stimulation (Fig. 2, *A* and *B*, ↑, Pre). As shown in three consecutive trials for a male (Fig. 2*A*, Pre) and a female rat (Fig. 2*B*, Pre), there could be no blink in between the Condition and Test SO-evoked blinks (Fig. 2*A*, Pre top 2 traces; Fig. 2*B*, Pre top and bottom traces), or an additional blink, a blink oscillation, that occurred after the Condition blink (Fig. 2*A*, Pre bottom trace; Fig. 2*B*, Pre middle trace). Before gland removal, males averaged 0.85 ± 0.18 blink oscillations/trial (Fig. 2*C*, Male, shaded bar), and females averaged 0.97 ± 0.11 blink oscillations/trial (Fig. 2*C*, Female, shaded bar) with 0.92 ± 0.1 blink oscillations/trial averaged over all rats (Fig. 2*C*, All, shaded bar). There was no significant difference between the occurrence of oscillations/trial before gland removal between males and females [$t_{(30)} = -0.66$, $P > 0.05$], nor were there differences in the latency of blink oscillation onset relative to the SO stimulus between males and females [males: 67.9 ± 5.6 ms, females: 70.3 ± 4.3; $t(28) = -0.35$, $P > 0.05$]. As expected from dry eye in humans (Evinger et al. 2002; Peshori et al. 2001; Schicatano et al. 2002), exorbital gland removal significantly increased the number of blink oscillations/trial overall [1.27 ± 0.08; $t_{(79)} = -2.89$, $P < 0.01$; Fig. 2*C*, All, solid bar]. This rise in the occurrence of blink oscillations with dry eye, however, was driven primarily by the male rats. In the examples of three consecutive trials following gland removal for a male (Fig. 2*A*, Post) and a female (Fig. 2*B*, Post), the SO stimulus evoked a blink oscillation in each trial for the male (Fig. 2*A*, Post ↑) but in only two of the three trials for the female (Fig. 2*B*, Post ↑). Overall, blink oscillations/trial increased significantly for males [1.41 ± 0.1; $t_{(34)} = -3$, $P < 0.01$; Fig. 2*C*, Male, solid bar] but not for females [1.15 ± 0.11; $t_{(43)} = -1.13$, $P > 0.05$; Fig. 2*C*, Female, solid bar].

In humans with dry eye, the frequency of blink oscillations is a function of trigeminal reflex blink excitability (Evinger et al. 2002). If reflex blink excitability controls the frequency of blink oscillations in rats, then blink oscillation frequency should covary with trigeminal blink excitability over days after exorbital gland removal. To test this premise, we normalized the blink excitability for each day after exorbital gland removal to the median blink excitability for all the data before gland removal for each rat. Plotting the averaged, normalized blink excitability for each day showed that normalized male excitability increased after gland removal for *days 4* through *10* and then appeared to stabilize (Fig. 2*D*, ●). As expected from the absence of increased excitability with dry eye in females (Fig. 1*C*), normalized excitability remained constant for females across days following gland removal (Fig. 2*D*, ○). Consistent with a linkage between blink excitability and blink oscillation frequency, averaged, normalized blink oscillations/trial exhibited similar changes as excitability following gland removal (Fig. 2*E*). We normalized the blink oscillation frequency for each day after exorbital gland removal to the median blink oscillations/trial for all the normal data for each rat. Plotting the averaged, normalized blink oscillations/trial for each day showed that oscillation frequency remained constant for females (Fig. 2*E*, ○), whereas normalized blink oscillations/trial increased for *days 4* through *10* and then appeared to stabilize for males (Fig. 2*E*, ●). To establish that blink oscillation frequency covaried with trigeminal reflex blink excitability, we plotted averaged, normalized blink oscillations/trial each day as a function of averaged, normalized reflex blink excitability for that day for male and female rats with dry eye (Fig. 2*F*). Blink oscillations/trial increased linearly with blink excitability for male rats (Fig. 2*F*, ●; df = 10, $r^2 = 0.91$, $P < 0.01$). In contrast, there was no relationship between excitability and blink oscillations/trial for females (Fig. 2*F*, ○; df = 11, $r^2 = 0.12$, $P > 0.05$). Thus in rats (Fig. 2*F*) and humans (Evinger et al. 2002) there was an apparent linkage between trigeminal blink excitability and the likelihood of additional blinks evoked by SO stimulation. Because dry eye elevates reflex blink excitability in male, but not female, rats (Fig. 1, *C* and *F*), blink oscillations/trial significantly increase in male, but not in female, rats with dry eye.

Trigeminal reflex blink plasticity. Exorbital gland removal modified the excitability of trigeminal blink circuits in male rats (Figs. 1 and 2). To explore trigeminal complex modifiability initiated by dry eye further, we employed an associative learning paradigm of HFS of the SO nerve that depresses reflex blink amplitude (Mao and Evinger 2001; Ryan et al. 2014). Because the SO current used to evoke the trigeminal reflex blinks did not change within or across days (Fig. 1*D*), a change in the amplitude of reflex blinks following HFS revealed a modification of the efficacy of the trigeminal system in generating reflex blinks, a change in trigeminal reflex blink gain. In the examples illustrated in Fig. 3, *A* and *B*, separating the data by sex revealed that R1 amplitude 60 min after HFS was 58% smaller (Fig. 3*A*, T60) than before HFS (Fig. 3*A*, Pre HFS) in a male rat. In contrast, R1 amplitude 60 min after HFS (Fig. 3*B*, T60) was only 30% smaller than R1 amplitude before HFS in a female rat (Fig. 3*B*, Pre HFS). When averaged over all rats regardless of sex [$t_{(32)} = 2.92$, $P < 0.01$] (Fig. 3*E*, All, shaded bar), daily HFS treatment 1 wk before exorbital gland removal showed that HFS treatment reduced the R1 blink reflex gain by 18.3 ± 6.2%. Although significant, this depression was less than that reported in our previous study using only male rats (Ryan et al. 2014). Separating the present data by sex, however, revealed that male rats exhibited a 35.1 ± 5.1% [$t_{(13)} = 6.86$, $P < 0.001$] decrease in blink gain (Fig. 3*E*, Male, shaded bar), whereas the average gain decrease for female rats was an insignificant 5.9 ± 9.3% [$t_{(18)} = 0.64$, $P > 0.05$; Fig. 3*E*, Female, shaded bar].

Exorbital gland removal dramatically altered the response to HFS treatment of both males and females (Fig. 3, *A–E*). Example records from the second week after gland removal (Fig. 3, *C* and *D*) showed that, 60 min after HFS, R1 amplitude (Fig. 3*C*, T60) was 340% larger than R1 amplitude before HFS for a male rat (Fig. 3*C*, Pre HFS). For the female rat, however, R1 amplitude was 56% smaller than R1 amplitude 60 min after HFS (Fig. 3*D*, T60) than before HFS (Fig. 3*D*, Pre HFS). Averaged over the 2 wk following exorbital gland removal, males showed an 11.3 ± 12.3% [$t_{(20)} = -0.86$, $P > 0.05$] potentiation of blink reflex gain (Fig. 3*E*, Male, solid bar), whereas females exhibited a −12.5 ± 7.4% depression of blink gain [$t_{(27)} = 1.7$, $P > 0.05$; Fig. 3*E*, Female, solid bar]. Averaging over the entire 2-wk post-gland removal period, however, obscured any changes in trigeminal modifiability that developed over time with dry eye. To determine whether the effect of HFS on reflex blink gain changed over time, the blink reflex gain data for each day were averaged with the preceding and subsequent day for each sex (Fig. 3*F*). For males, HFS

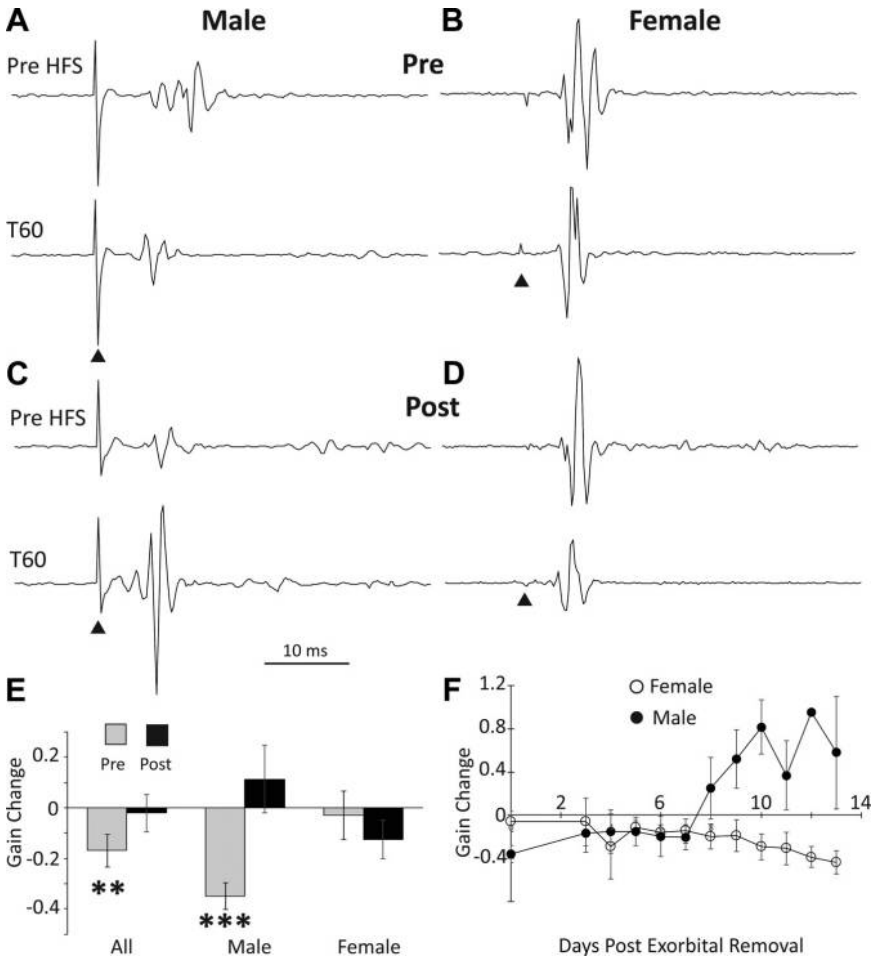

Fig. 3. Modifiability of trigeminal reflex blink gain before and after exorbital gland removal. *A*: for a male rat before exorbital gland removal (Pre), a single trial of a blink evoked by supraorbital branch of the trigeminal nerve (SO) nerve stimulation (▲) before high-frequency stimulation (HFS) treatment (Pre-HFS) and 60 min after HFS treatment (T60). *B*: for a female rat before exorbital gland removal (Pre), a single trial of a blink evoked by SO nerve stimulation (▲) before HFS treatment (Pre-HFS) and 60 min after HFS treatment (T60). *C*: for a male after exorbital gland removal (Post), a single trial of a blink evoked by SO nerve stimulation (▲) before HFS treatment (Pre-HFS) and 60 min after HFS treatment (T60). *D*: for a female rat exorbital gland removal (Post), a single trial of a blink evoked by SO nerve stimulation (▲) before HFS treatment (Pre-HFS) and 60 min after HFS treatment (T60). *E*: mean gain change following HFS treatment for all ($n = 9$), male ($n = 4$), and female ($n = 5$) rats before (shaded bars) and after (solid bars) exorbital gland removal. Negative values indicate depression, and positive values indicate potentiation of blink amplitude produced by the HFS treatment. *F*: mean gain change per day averaged over 3-day intervals following exorbital gland removal for male (●) and female (○) rats. The points at 0 are the mean gain change over the 7 days before exorbital gland removal. Error bars are SE of the mean; $^{**}P < 0.01$, $^{***}P < 0.001$.

treatment initially produced weak depression that converted to blink potentiation over *days 9–14*, following dry eye (Fig. 3*F*, ●). During this period, HFS significantly potentiated the blink reflex gain by 0.74 ± 0.17 [$t_{(5)} = -5.2$, $P < 0.01$]. For females, however, blink depression increased over the 2 wk following exorbital gland removal (Fig. 3*F*, ○). This depression became most pronounced between *days 9–14* when it reached -0.27 ± 0.09 [$t_{(8)} = 3.17$, $P < 0.05$]. Thus dry eye altered trigeminal complex modifiability in a sex-specific manner, particularly in the second week following exorbital gland removal.

Spontaneous blinking. Although spontaneous blinks occur independently of an external stimulus or a conscious decision to blink, there is evidence that the trigeminal blink circuits play a role in modulating spontaneous blinking (Kaminer et al. 2011). Given the changes in trigeminal blink circuits with dry eye, we examined spontaneous blinking before and after exorbital gland removal. Averaged over all rats, the mean blink rate was 4.8 ± 0.3 blinks/min before exorbital gland removal (Fig. 4*A*, All, shaded bar) with a mean IBI of 13.9 ± 0.8 s. The mean blink rate was not significantly different [$t_{(30)} = 1.16$, $P > 0.05$] between males (5.3 ± 0.6; Fig. 4*A*, Male, shaded bar) and females (4.5 ± 0.4) blinks/min (Fig. 4*A*, Female, shaded bar). Likewise, the mean IBI was not significantly different between males (13.6 ± 1.4 s) and females [14.1 ± 0.9 s; $t_{(30)} = -0.27$, $P > 0.05$] before gland removal. Despite the lack of differences in spontaneous blink rate between males and females before exorbital gland removal, the OOemg characteristics of spontaneous blinks exhibited sexual dimorphisms (Fig. 4, *B–D*). The duration of male spontaneous blink OOemg activity was less than that of females. In these examples, the duration of a male rat's spontaneous blink OOemg activity was 44.5 ms (Fig. 4*B*, Pre) compared with 75.5 ms for a female rat (Fig. 4*C*, Pre). Before dry eye, the mean duration of spontaneous blink OOemg activity differed significantly between males and females [$t_{(30)} = -3.04$, $P < 0.01$; Fig. 4*D*, Duration, shaded bars]. Averaged over all males, OOemg duration was 74.4 ± 5.6 ms (Fig. 4*D*, Duration, Male, shaded bar), whereas the mean female OOemg duration was 115.8 ± 10.5 ms (Fig. 4*D* Duration, Female, shaded bar). In addition to dissimilar durations, the time at which OOemg activity peaked occurred differed significantly between males and females [$t_{(30)} = 3.03$, $P < 0.01$]. In the examples of Fig. 4, *B* and *C*, the peak OOemg activity occurred 16 ms after the onset of OOemg activity in the male (Fig. 4*B*, Pre ↓), and the OOemg activity peaked 22 ms after the onset of OOemg activity in the female (Fig. 4*C*, Pre ↓). Averaged across male rats, the peak OOemg activity occurred 25.2 ± 2.4 ms into a 74.4 ± 5.6 ms duration lid closure (Fig. 4*D*, Time to Peak, Male, shaded bar), so that the OOemg activity peaked 33.9% into the OOemg activity. For females, peak OOemg activity occurred 48.9 ± 6.2 ms into the 115.8-ms duration OOemg activity (Fig. 4*D*, Time to Peak, Female, shaded bar), so that the OOemg activity peaked 42.2% into the OOemg activity.

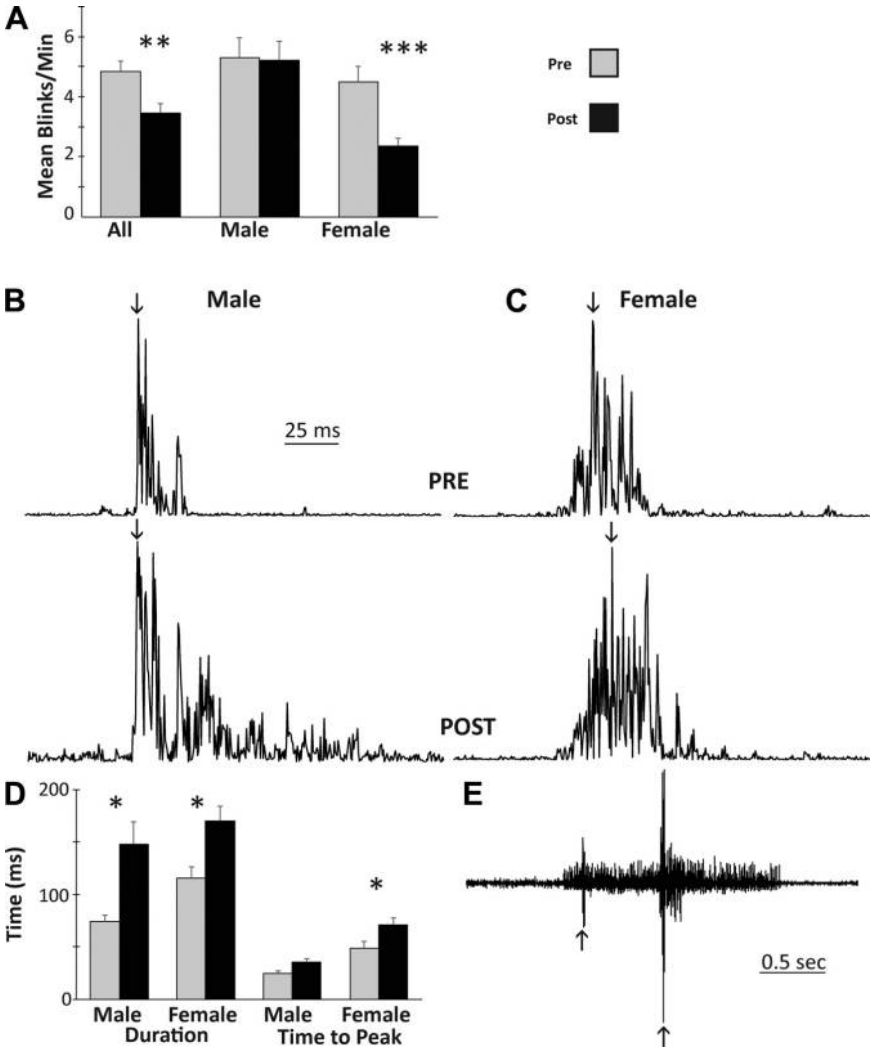

Fig. 4. Effect of exorbital gland removal on spontaneous blinking. *A*: mean blinks/min for all ($n = 9$), male ($n = 4$), and female ($n = 5$) rats before (shaded bars) and after (solid bars) exorbital gland removal. *B*: rectified orbicularis oculi EMG (OOemg) recording of a single spontaneous blink before (Pre) and after (Post) exorbital gland removal from a male rat. ↓ Indicates peak OOemg activity. *C*: rectified OOemg recording of a single spontaneous blink before (Pre) and after (Post) exorbital gland removal from a female rat. ↓ Indicates peak OOemg activity. *D*: mean spontaneous blink duration and mean time to peak OOemg activity for male and female rats before (shaded bars) and after (solid bars) exorbital gland removal. *E*: unrectified OOemg activity showing squinting with superimposed blinks (↑) from a male rat. Error bars are SE of the mean; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Exorbital gland removal significantly reduced the female spontaneous blink rate but did not affect the male blink rate. For females, the mean spontaneous blink rate decreased significantly from 4.5 ± 0.5 to 2.3 ± 0.5 blinks/min [$t_{(52)} = 5.61$, $P < 0.001$; Fig. 4*A*, Female, solid bar] and the mean IBI more than doubled to 30.7 ± 3.0 s [$t_{(52)} = -4.19$, $P < 0.001$]. Following exorbital gland removal, neither the mean blink rate (5.2 ± 0.5; Fig. 4*A*, Male, solid bar) nor the mean IBI (12.4 ± 1.25 s) changed significantly for males [$t_{(33)} = 0.12$, $P > 0.05$; $t_{(33)} = 0.7$, $P > 0.05$]. Nevertheless, exorbital gland removal significantly increased the duration of both male and female spontaneous blinks (Fig. 4, *B–D*). In the example records, the male rat's OOemg duration was 117.5 ms (Fig. 4*B*, Post), and the female rat's OOemg duration was 100.5 ms (Fig. 4*C*, Post). Averaged over all rats, the mean duration of spontaneous blink OOemg activity rose from 74.4 ± 5.6 ms to 147.5 ± 21.5 ms for males [$t_{(33)} = -2.6$, $P < 0.05$; Fig. 4*D*, Duration, Male, solid bar] and from 115.8 ± 10.5 to 169.9 ± 14.3 for females [$t_{(52)} = -2.6$, $P < 0.05$; Fig. 4*D*, Duration, Female, solid bar]. In the example records, the time to peak OOemg was 10 ms for the male (Fig. 4*B*, Post ↓) and 27 ms for the female rat (Fig. 4*C*, Post ↓). Averaged over all rats, the time to peak OOemg activity increased significantly from 48.9 ± 6.2 ms to 70.7 ± 6.8 ms following gland removal for females [$t_{(52)} = -2.12$, $P < 0.05$]. The increased time to peak OOemg activity, however, matched the increase in spontaneous blink duration, such that the peak OOemg occurred 41.6% into the OOemg activity, nearly identical to the value before dry eye. Thus dry eye in females stretched the duration of the blink without altering its overall shape (Fig. 4*C*). For males, however, peak OOemg activity occurred an average of 35.52 ± 3.6 ms into the 147.5 ± 21.5 ms duration blink (Fig. 4*D*), so that the OOemg activity peaked at 23.9% of the time into OOemg activity. The difference in time to peak OOemg before and after exorbital gland removal was not significant for males [$t_{(33)} = -2.0$, $P > 0.05$]. The lengthened duration of male spontaneous blink OOemg activity occurred primarily by increasing OOemg activity after the peak OOemg so that the pattern of OOemg activity differed before and after dry eye (Fig. 4*B*). Another adaptation to dry eye seen in both sexes was the appearance of squinting, a prolonged low level of OOemg activity that only partially closed the eye (Fig. 4*E*). Blinks that fully closed the lid could be superimposed on these periods of squinting (Fig. 4*E*, ↑).

DISCUSSION

Sexual dimorphisms in reflex and spontaneous blinking. The present study identified three sexual dimorphisms of trigeminal

reflex and spontaneous blinking in normal rodents. First, the threshold current for evoking a trigeminal reflex blink was higher in males than in females (Fig. 1*D*). Second, the HFS paradigm for depressing trigeminal reflex blink gain (Mao and Evinger 2001; Ryan et al. 2014) produced a significantly larger gain reduction in male than in female rats (Fig. 3*E*). Third, the duration of spontaneous blink OOemg activity was longer in females than in males (Fig. 4*D*). To determine whether similar sexual dimorphisms occurred in humans, we reanalyzed data from our previous studies of subjects without blink abnormalities. Consistent with the rodent data (Fig. 1*D*), the threshold current necessary to evoke the R2 trigeminal blink component was significantly larger for males than for females [males 3.1 mA $\pm$ 0.19 (n = 13); females 2.3 mA $\pm$ 0.18 (n = 17); $t_{(28)} = 3.02$, $P < 0.01$; Fig. 5*A*] (Peshori et al. 2001; Schade Powers et al. 2010; Schicatano et al. 2002). Our data using HFS to depress trigeminal reflex blink amplitude in human subjects (Mao and Evinger 2001) also suggested the same sex difference. Although the number of male and female subjects was too small to analyze statistically, the data trend was as observed in rodents (Fig. 3*E*). The average human gain decrease for the two males was -0.27, whereas the three females exhibited a 0.04 gain increase. Human spontaneous blinking also showed the same sexual dimorphism in blink duration as did rats. The duration of lid closing correlates with the duration of OOemg activity in humans and rodents (Evinger et al. 1991; Pellegrini et al. 1995). Using eyelid-closing data from a previous study of human spontaneous blinking (Kaminer et al. 2011), we examined whether human females exhibited longer-duration lid closing during spontaneous blinks than did males. Plotting lid-closing duration as a function of spontaneous blink amplitude showed that blink duration increased as a power function for females ($y = 29.85x^{0.46}$, $r^2 = 0.86$), whereas male blink duration exhibited only small changes as spontaneous blink amplitude increased (Fig. 5*B*). For spontaneous blinks over 30°, lid closure duration was significantly longer in females than in males [$t_{(5)} = -15.8$, $P < 0.00001$]. Thus rodents and humans share similar sexual dimorphisms in trigeminal reflex and spontaneous blinking.

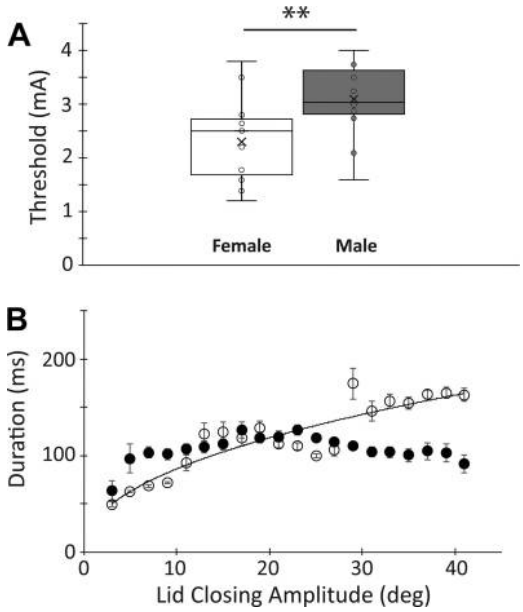

Fig. 5. Sex differences in human blinks. *A*: threshold current required to evoke the R2 response in male and female subjects. Data from Peshori et al. 2001; Schade Powers et al. 2010; Schicatano et al. 2002. *B*: mean spontaneous blink duration plotted as a function of blink amplitude for male (●) and female (○) subjects. Error bars are SE of the mean. Data from Kaminer et al. 2011. $^{**}P < 0.01$.

These sexual dimorphisms of blinking might be influenced by hormonal changes. As with the present study in rodents, our previous studies in humans did not obtain menstrual cycle information. Nevertheless, the estrous cycle did not appear to affect our rodent results. Female rats typically exhibit a 4- to 5-day estrous cycle (McClintock 1984), and we acquired data independently of the cycle during the week preceding exorbital gland removal. If data were sampled randomly during the female cycle and the cycle stage modulated blink sexual dimorphisms, then female rat data should show more variability across the week than males. In our data, however, there was no difference in the coefficient of variation between these sexually dimorphic characteristics. For blink threshold current, the mean coefficient of variation was 0.1 $\pm$ 0.04 (n = 4) for males and 0.07 $\pm$ 0.02 (n = 5) for females [$t_{(7)} = 0.83$, $P > 0.05$]. Likewise, there was no difference in the mean coefficient of variation for spontaneous blink OOemg duration between males (0.18 $\pm$ 0.05, n = 4) and females [0.19 $\pm$ 0.08, n = 5; $t_{(7)} = -0.1$, $P > 0.05$] in the week before exorbital gland removal. In contrast to blink threshold and duration, female rats exhibited a more variable coefficient of variation for gain change than males (females = 2.9 $\pm$ 2.4, n = 5; males = -0.46 ± 0.09, n = 4) although this difference did not achieve statistical significance [$t_{(7)} = -1.21$, $P > 0.05$]. Consistent with our data, a previous metastudy on rats reported that the estrous cycle did not cause more variability in female than in male neural traits, e.g., histology and neurochemistry, although behavioral traits exhibited more variability than other traits in both males and females (Becker et al. 2016).

Females exhibit a lower threshold for trigeminal reflexes in the lower face, as well as in the supraorbital region (Figs. 1*D* and 5*A*). As with the blink reflex, the threshold for the masseter exteroceptive reflex is lower in women than in men (Komiyama et al. 2005). In addition, the perception threshold for nonnoxious thermal and tactile stimuli to the cheek is lower in females than in males (da Silva et al. 2014; Komiyama et al. 2009; Matos et al. 2011; Yekta et al. 2010), and mild electrical shocks to the lip evoke larger potentials at shorter latencies in women than in men (Polich et al. 1995). The presence of these sexual dimorphisms in both humans and rats suggests that there is an evolutionary advantage to increased trigeminal sensitivity or that a neural adaptation to sex differences expressed in the periphery has occurred.

Blink adaptations in response to exorbital gland removal: sexual dimorphisms. Exorbital gland removal produces several trigeminal blink system adaptations. For males, condition blink amplitude decreases, and blink oscillations/trial increase concomitantly with trigeminal reflex blink excitability (Figs. 1, *A*, *C*, and *E*, and 2, *C–F*), whereas none of these parameters change significantly for females (Figs. 1, *B*, *C*, and *E*, and 2, *C–F*). Although decreased condition blink amplitude is a plausible explanation for the increased trigeminal excitability of males, the observation that blink excitability was unrelated to variations in condition blink amplitude in either males or females (Fig. 1*F*) argues that dry eye increases excitatory trigeminal drive in males but not females. In males, the

elevated blink excitability caused by dry eye appears responsible for their increased frequency of blink oscillations (Fig. 2*F*). With no increase in blink excitability (Fig. 1*C*), female blink oscillation frequency does not change with dry eye (Fig. 2, *E* and *F*). It is possible that the lack of increased excitability and blink oscillations in female rats results from decreased corneal sensitivity, such as that seen in human females with dry eye (Benítez-Del-Castillo et al. 2007). This explanation is unlikely, however, because human females with dry eye or aging exhibit elevated trigeminal blink excitability and blink oscillations (Evinger et al. 2002; Peshori et al. 2001; Schicatano et al. 2002).

As with humans without dry eye (Bentivoglio et al. 1997; Tsubota et al. 1996; Yolton et al. 1994), no consistent difference exists between control male and female rat spontaneous blink rates (Fig. 4*A*) or mean IBI. In humans, dry eye significantly increases the spontaneous blink rate and decreases the mean IBI (Harrison et al. 2008; Himebaugh et al. 2009; Johnston et al. 2013; Ousler et al. 2014; Tsubota et al. 1996). Unlike the increased blink rate of dry eye humans, dry eye produced by exorbital gland removal in rats did not significantly increase the spontaneous blink rate of males and significantly decreased the spontaneous blink rate of females (Fig. 4*A*). The mean IBI of male rats decreased 8.8% in the present study and 29% in our previous study (Kaminer et al. 2011). In contrast to these modest drops in mean IBI of male rats, dry eye in humans caused much larger reductions in mean IBI of 63% (Tsubota et al. 1996) and 57% (Johnston et al. 2013). Dry eye significantly decreases the female rat spontaneous blink rate (Fig. 4*A*) and increases IBI by 54.1%. Differences between rodent and human tear film may account for the disparities in rodent and human spontaneous blinking. Human tear film thickness is ~3 μm, whereas the rat tear film is ~11.3 μm (King-Smith et al. 2004). Given that the rate of human spontaneous blinking correlates inversely with the time to tear film break up (Al-Abdulmunem 1999; Himebaugh et al. 2009; Nakamori et al. 1997; Rahman et al. 2015a; Yap 1991), a thicker tear film might be slower to break up and thereby support a lower spontaneous blink rate. A longer tear breakup time for rats can account for the lower blink rate of normal rats, 4.8 blinks/min, compared with the normal blink rate of 17.6 blinks/min in humans (Kaminer et al. 2011). If one response to exorbital gland removal is to thicken the meibum content of tear film to lengthen the tear film breakup time, then spontaneous blinking might not change or might even decrease following gland removal in rats. Thickening the meibum layer may be an adaptation to dry eye shared by humans and rodents. Humans with dry eye appear to release meibum continuously at a higher rate than individuals with normal tear function (Cho et al. 2019). In addition, as larger and stronger blinks increase the thickness of the lipid tear film layer (Korb et al. 1994), the increased duration of human spontaneous lid closures (Ousler et al. 2014; Rodriguez et al. 2013; Tsubota et al. 1996) and rat blinks (Fig. 4, *B–D*) following dry eye may aid tear film stabilization.

The HFS paradigm revealed a strong effect of exorbital gland removal on trigeminal modifiability (Fig. 3). Over the 2 wk following gland removal, males exhibited a progression from the expected HFS-induced depression to potentiation (Fig. 3*F*) that paralleled the increase in trigeminal excitability over the same period (Fig. 2*D*). In contrast to males, the weak depression caused by HFS in females before gland removal became a significant depression over the 2 wk following dry eye (Fig. 3*F*). HFS-induced modifications are known to occur in the trigeminal complex (Mao and Evinger 2001; Ryan et al. 2014), and the present study showed that dry eye altered spinal trigeminal system modifiability in a sex-specific manner. The conversion of depression to potentiation in males (Fig. 3) indicated that the increased spinal trigeminal blink excitability (Fig. 1, *C* and *F*; Fig. 2, *D–F*) overwhelmed the HFS-associative mechanisms that normally produced reflex blink depression. Females, however, did not show a significant increase in excitability (Fig. 1, *C* and *F*) or blink oscillations (Fig. 2, *C*, *E*, and *F*) following exorbital gland removal; rather females exhibited enhanced trigeminal modifiability demonstrated by the increasingly effective HFS paradigm (Fig. 3*F*).

The enhanced spinal trigeminal modifiability present in females with dry eye (Fig. 3*F*) may offer an explanation for the female predominance of the focal dystonia, BEB (Asgeirsson et al. 2006; Defazio et al. 2001; Hallett et al. 2008), that is characterized by excessive blinking and involuntary spasms of lid closure (Adams et al. 2006; Berardelli et al. 1985; Defazio et al. 2017; Hallett et al. 2008; Tolosa and Martí 1988). The chief hypothesis for the origin of BEB is that it develops from the convergence of a predisposing condition and an environmental trigger (Defazio et al. 2011; Hallett et al. 2008; Jinnah and Hallett 2011). Genetic factors appear to be the best candidate for the predisposing condition (Clarimon et al. 2007; Defazio et al. 1993, 2003, 2011, 2017; Dhaenens et al. 2005; Hallett et al. 2008; Hammer et al. 2019; Misbahuddin et al. 2002; Xiao et al. 2016). Epidemiological studies showing the presence of dry eye or eye irritation before the development of BEB indicate that dry eye or eye irritation is an environmental trigger initiating BEB (Defazio et al. 2011, 2012, 2017; Elston et al. 1988). In this "two-hit" model of BEB, the predisposing condition exaggerates the modifiability of the trigeminal system and other neural network elements so that adaptations to dry eye become exaggerated and morph into spasms of lid closure. In support of this BEB hypothesis, exorbital gland removal combined with a predisposing condition can create blepharospasm-like lid spasms in male rats (Evinger 2013; Schicatano et al. 1997). Also consistent with this hypothesis, the HFS paradigm reveals that patients with BEB exhibit exaggerated trigeminal blink modifiability (Quartarone et al. 2006). If dry eye increases the modifiability of trigeminal blink circuits in human females, as occurs in female rats (Fig. 3*F*), then the predisposing condition would be more likely to exaggerate trigeminal adaptations in females with dry eye than in males, thus leading to a female preponderance in developing BEB.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.C., C.L., and C.E. performed experiments; A.C., C.L., and C.E. analyzed data; A.C., C.L., and C.E. interpreted results of experiments; A.C., C.L., and C.E. edited and revised manuscript; A.C., C.L., and C.E. approved final version of manuscript; C.E. conceived and designed research; C.E. prepared figures; C.E. drafted manuscript.

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