Published in final edited form as:

Neuroscience. 2017 May 04; 349: 208–219. doi:10.1016/j.neuroscience.2017.03.003.

Trigeminal brainstem modulation of persistent orbicularis oculi muscle activity in a rat model of dry eye

Mostafaezur Rahman, Kazunari Shiozaki, Keiichiro Okamoto, Randall Thompson, and David A. Bereiter

Department of Diagnostic and Biological Sciences, University of Minnesota School of Dentistry, Moos Tower 18-186, 515 Delaware St. SE, Minneapolis, MN 55455, USA

Abstract

Altered corneal reflex activity is a common feature of dry eye disease (DE). Trigeminal sensory nerves supply the ocular surface and terminate at the trigeminal interpolaris/caudalis (ViVc) transition and spinomedullary (VcC1) regions. Although both regions contribute to corneal reflexes, their role under dry eye conditions is not well defined. This study assessed the influence of local inhibitory and excitatory amino acid neurotransmission at the ViVc transition and VcC1 regions on hypertonic saline (HS) evoked orbicularis oculi muscle activity (OOemg) in urethane-anesthetized male rats after exorbital gland removal (DE). HS increased the magnitude of long duration OOemg activity (OOemgL, >200 ms) in DE compared to sham rats, while short duration OOemg activity (OOemgS, <200 ms) was similar for both groups. Inhibition of the ViVc transition by muscimol, a GABA_A receptor agonist, greatly reduced HS-evoked OOemgL activity in DE rats, whereas injections at the VcC1 region had only minor effects in both groups. Blockade of GABA_A receptors by bicuculline methiodide at the ViVc transition or VcC1 region increased HS-evoked OOemgL activity in DE rats. Blockade of N-methyl-D-aspartate (NMDA) receptors at either region reduced HS-evoked OOemgL activity in DE and sham rats. GABA_Aβ3 receptor density was reduced at the ViVc transition, while NMDA receptor density was increased at both regions in DE rats. Loss of GABAergic inhibition at the ViVc transition coupled with enhanced NMDA excitatory amino acid neurotransmission at the ViVc transition and the VcC1 region likely contribute to altered corneal reflexes under dry eye conditions.

Keywords

Corneal reflex; dry eye; electromyography; orbicularis oculi; GABA receptor; NMDA receptor

Corresponding author; Mostafaezur Rahman, DDS Ph.D., Department of Diagnostic and Biological Sciences, University of Minnesota School of Dentistry, Moos Tower 18-186 Delaware St SE, Minneapolis, MN 55455, USA, Phone; (612) 626-2768; fax (612) 626-2651., mrahman@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Dry eye disease (DE) is defined by an unstable tear film, ocular inflammation and symptoms of discomfort (DEWS Report Definition) (2007). Relief from symptoms, which often include burning or stinging pain and foreign body sensation, is a primary reason that patients seek medical attention for DE (Begley et al. 2002; Rosenthal and Borsook 2015; Velhof et al. 2016). However, peripheral treatments that reduce ocular inflammation (Behrens et al., 2006, Alves et al., 2013) often are inadequate in managing symptoms in moderate to severe DE (Asbell and Spiegel, 2010, Williamson et al., 2014). Furthermore, peripheral signs of tear film instability are not well correlated with symptoms (Johnson, 2009, Sullivan et al., 2014). These findings are consistent with the notion that pain sensation and altered protective reflexes in DE involve persistent changes in the brain; however, little is known about the underlying mechanisms that could account for altered symptoms in DE.

The corneal reflex pathway consists of: i) trigeminal sensory neurons that supply the ocular surface, ii) that terminate on trigeminal brainstem neurons in the subnucleus interpolaris/caudalis (ViVc) transition and the caudalis/upper cervical spinal cord (VcC1) region (Marfurt and Del Toro, 1987, Panneton et al., 2010) that integrate somatosensory signals, and project to iii) orbicularis oculi (OO) motoneurons in the facial motor nucleus that drive eyelid closure (Ongerboer de Visser 1980). Brief electrical or mechanical stimulation of the cornea in humans evokes a single OOemg peak of activity and at long latencies (Accornero et al. 1908; Mukuno et al. 1983; Cruccu et al. 1986). By contrast, brief electrical stimulation of facial skin or supraorbital nerve evokes two peaks of OOemg activity, a short latency short duration peak (R1) and a long latency (R2) long duration peak (Evinger et al. 1991; Pelligrini et al. 1995). Although the corneal reflex and the R2 phase of the eyeblink share similarities in terms of latency and duration, it is likely that elements of the underlying circuitry are different (Berardelli et al. 1985). Evidence from animal studies suggests that second-order neurons at the ViVc transition and VcC1 region contribute to corneal reflexes (Henriquez and Evinger 2005; Henriquez and Evinger 2007; Rahman et al. 2014) and eyeblinks (Pelligrini et al. 1995).

The balance between GABA_A receptor-mediated inhibitory mechanisms and NMDA receptor-mediated excitatory mechanisms in spinal levels is altered after persistent inflammation (Woolf and Salter, 2000, von Hehn et al., 2012). Although corneal-responsive neurons at the ViVc transition and VcC1 regions are greatly influenced by GABA_A and NMDA receptor activation in naïve animals (Bereiter and Bereiter, 1996, Hirata et al., 2003, Hirata et al., 2004), it is not known if these receptor pathways are altered in a model for tear deficient DE. Unlike most previous studies in which transient stimuli were presented, this study used persistent ocular instillation of hypertonic saline (HS) to evoke OOemg activity in sham and DE male rats. The rationale for this approach was that hyperosmolar tears is a common, yet persistent, feature of DE (Sullivan et al. 2014) and that ocular surface conditions influence spontaneous (Acosta et al., 1999) and evoked eyeblink rates in control subjects (Wu et al., 2014) and DE patients (Nakamori et al., 1997, Himebaugh et al., 2009). Previously we reported that DE rats displayed enhanced neural activity and OOemg to HS (Rahman et al. 2015); however, the role of GABA_A and NMDA receptors at the ViVc transition and VcC1 region in mediating enhanced corneal reflexes is not known.

Methods

Animals

A total of 151 male rats (250–350 g, Sprague-Dawley, Harlan, Indianapolis, IN) were used in these experiments. Animals were housed in pairs with free access to food and water. Cages remained in climate and light controlled environment (25 ± 2 °C, 12:12-h light/dark cycle with light on at 7:00 AM). The animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (USA) and conformed to the established guidelines set by The National Institutes of Health guide for the Care and the Use of Laboratory Animals (PHS Law 99–158, revised 2015). All efforts were made to minimize the number of the animals used for experiments.

Exorbital gland excision

Rats were anesthetized with isoflurane (3–5%) and under aseptic conditions, the fur anterior to the ear was shaved, and a small skin incision exposed the masseter muscle and the left exorbital gland. The gland was gently teased off the muscle and excised. The wound margins were treated with 2% xylocaine gel and the incision was closed with absorbable suture. In sham controls, the gland was exposed surgically but was not removed. Ketoprofen (25 mg/kg, ip) was given post-surgically for pain management. Rats survived for 14 days after surgery and prior to testing. Previously, we reported that corneal reflex activity in awake rats was enhanced at 14 days after gland removal consistent with a model for ocular hyperalgesia (Rahman et al., 2015).

Tear volume measurement

On the day of the experiment rats were anesthetized with urethane (1.2–1.5 g/kg, ip) and spontaneous tear volume was estimated by the increase in wet length of phenol red thread (ZONE-QUICK, Menicon INC., San Mateo, CA). The thread was placed in contact with the cornea/conjunctiva at its inferior-lateral edge and tear volume was measured over 2 min.

Orbicularis oculi muscle electromyography (OOemg)

The left femoral artery was catheterized to monitor arterial blood pressure that was maintained at 90–110 mmHg. Rats were allowed to breathe spontaneously. The wound margins were infiltrated with 2% lidocaine and body temperature was kept at 38°C with a heating blanket. The rat was positioned in a stereotaxic frame and a small portion of the C1 vertebra was removed to expose the dorsal brainstem surface and allow access to the ViVc transition and VcC1 regions. Teflon-coated copper wires (0.12 mm diameter) were implanted in the upper and lower portions of the left OO muscle.

Experimental Design

The recording session began at least 1h after completion of all surgery. Hypertonic saline (HS, 2.5 M NaCl, pH: 7.2, 10 µl) was applied to the ocular surface by a microsyringe (Tashiro et al., 2010) at 20 min intervals to evoke OOemg activity. The HS test solution remained on the eye during the 3 min sampling period and was washed out with artificial tears after each stimulus (total exposure time to each HS stimulus = 3–4 min) to prevent

desensitization or possible damage to the ocular surface. In an initial series, rats received 0.15, 1 and 2.5 M NaCl in sequential order at 20 min intervals to quantify the OOemg responses at different osmolar concentrations. The test stimulus was 2.5 M NaCl, since this dose caused reliable increases in total OOemg that were significantly enhanced in DE versus sham rats (see Fig 6, Rahman et al. 2015). The design for this series was: 1) HS test stimulus, 2) drug injection, 3) HS test at 10 min post-drug, 4) HS test at 30 min post-drug and 5) HS test at 50 min post-drug. Drugs were given without prior knowledge by the experimenter.

Drugs were microinjected slowly into the ViVc transition or VcC1 region, ipsilateral to gland removal, from a glass micropipette (40–80 μ m tip diameter). Pipettes were filled with either phosphate buffered saline (PBS), muscimol, a selective gamma aminobutyric acid subtype A (GABA_A) receptor agonist, bicuculline methiodide, a GABA_A antagonist, or AP5, a selective N-methyl-D-aspartate (NMDA) receptor antagonist. Drugs were dissolved in saline and delivered in a concentration of 0.1 mM or 1 mM in a total volume of 0.2 μ l over 2 min. The drug doses used here were similar or less than those reported to be effective in modulating nociceptive neurons in spinal dorsal horn (Zhang et al., 2001, Sokal and Chapman, 2003, Seagrove et al., 2004). Injection sites at the ViVc transition were approached at 28° off vertical and 45° off midline, and 1.5–2.0 mm below the brainstem surface and positioned at the level of the obex, a surface landmark defined by the caudal end of the fourth ventricle (Yoshida et al., 1991). Sites at the VcC1 region were approached at 43° off vertical, 60° off midline, within 300 μ m of the dorsal brainstem surface and 3–6 mm caudal to the obex. Injection sites were confirmed by histology and were within the targeted areas as shown previously (Hirata et al., 2004).

Data analysis

OOemg activity was sampled at 1000 Hz, amplified (x10k), filtered (bandwidth 10–300 Hz), displayed and stored online for later analyses (AD Instruments, Colorado Springs, CO). Power spectrum analyses indicate that more than 90% of blink energy occurs in frequencies below 200 Hz (Evinger et al. 1991; Van Boxtel 2001) suggesting that the filter and sampling rate parameters used here should adequately capture OOemg signals. Muscle activity was recorded continuously for 6 min beginning 3 min before and until 3 min after each HS stimulus. OOemg activity was rectified and stored as 1 s bins for off-line analyses. Baseline activity was defined as an integrated area under the curve (AUC) for the 3 min epoch (μ V-s per 3 min) sampled immediately prior to each stimulus. For each 6 min sampling period OOemg activity was integrated and assessed as total activity (OOemg minus background). From the raw OOemg signal, integrated long duration activity (OOemgL) was defined as consecutive bins above background lasting more than 200 ms) and short duration activity (OOemgS) was defined as lasting less than 200 ms (Fig 1). A typical response to a 2.5 M NaCl test stimulus in a DE rat is seen for the full 3 min sample period (Fig 1A) and is expanded (red outlined areas) to demonstrate OOemgL (Fig 1B) and OOemgS responses (Fig 1C). Total OOemg activity was calculated as AUC post-stimulus minus baseline AUC. Total OOemg, OOemgL and OOemgS were integrated from the rectified signal over the 3 min post-stimulus period. The response latency (onset) was defined as the first bin at which OOemg exceeded the average baseline AUC.

Total OOemg (OOemgL+OOemgS), OOemgL, OOemgS and response latency evoked by HS were assessed by two-way analysis of variance (ANOVA) corrected for repeated measures on one factor. Significant treatment effects were assessed by Newman-Keuls after ANOVA. The data were presented as mean $\pm$ SEM and the significant level set at $p < 0.05$. Power analyses based on previous studies (Rahman et al., 2015) indicated that a sample size of $n = 5$ per treatment group would provide 80% power at $p < 0.05$. Of the 133 rats used to record OOemg activity, results from 5 experiments could not be included. In two rats, the initial arterial blood pressure fell below 90 mmHg without recovery, while in 3 rats the initial arterial pressure was normal; however, injection of muscimol into the ViVc transition region caused a rapid and sustained drop in blood pressure and respiration rate. This was due to a malfunction of the injector assembly resulting in a large volume injection (>500 nl).

RNA and protein measurements

Total RNA was extracted from tissue samples at the ViVc transition and VcC1 region of sham ($n = 4$) and DE rats ($n = 4$) using Absolutely RNA kit (La Jolla, CA). cDNA was synthesized from 300 ng of each sample using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qPCR was performed in triplicate on 2 μ L cDNA with a Chromo4 DNA engine (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). PCR conditions were as follows: an initial denaturation at 95° C for 3 min, followed by 40 cycles of 95° C for 10 s, 58.5° C for 20 s, and 72° C for 30 s. Data were analyzed using the delta C_T method against two reference genes (GAPDH and UBC). Primer sets were as follows: GAPDH: F: 5'-agacagccgcatcttctgt-3', R: 5'-ctgccgtgggtagatcat-3'; GABA $_A$ β 3: F: 5'-cttgacaatcgagtggctga-3', R: 5'-attccagggtgcagtttg-3'; NR1: F: 5'-cggtcttggaagatacagc-3', R: 5'-gtgggagtgaagtggctgtt-3'. A melting curve was employed to ensure amplicon fidelity.

Sham ($n = 4$) and DE rats ($n = 4$) were perfused with saline and 4% paraformaldehyde and 50 μ m sections were cut transversely on a vibratome. Sections ($n = 3$ per rat) were stained with primary antibody for pNR1(ser896) or GABA $_A$ β 3 (Millipore, Temecula CA) at a dilution of 1:1000, followed by biotinylated anti-rabbit secondary antibody (Millipore). ABC complex was applied followed by DAB color substrate (Vector, Burlingame CA). Sections from each region of interest, centered over the ventrolateral ViVc transition and the lateral portion of the VcC1 region, were imaged at 20x by light microscopy and densitometry was performed using NIH ImageJ software. Data were assessed by ANOVA and presented as mean $\pm$ SD.

Results

Spontaneous tear volume

DE and sham rats gained weight normally over the 14 day post-surgical period and displayed no obvious changes in general appearance or signs of ocular hyperemia or inflammation. The spontaneous tear volume was measured in most rats and averaged 7.6 ± 0.3 mm²/min ($n = 50$) in the ipsilateral eye of DE rats which was greatly reduced compared to the contralateral eye (19.6 ± 0.4 mm²/min) and compared to the left and right eyes of sham rats ($n = 40$, 19.2 ± 0.3 and 19.4 ± 0.3 mm²/min, respectively, $F_{1,88} = 205$, $p < 0.001$).

NaCl concentration-effect series

Concentrations of 0.15, 1.0 and 2.5 M NaCl were applied to the ocular surface sequentially and total OOemg, OOemgL and OOemgS were measured in sham and DE rats. As seen in Fig 2, total OOemg increased significantly with increased NaCl concentration in sham ($F_{2,18} = 7.6$, $p < 0.005$) and DE rats ($F_{2,18} = 48.9$, $p < 0.001$). Similarly, OOemgL was increased in sham ($F_{2,18} = 4.1$, $p < 0.05$) and DE rats ($F_{2,18} = 40.3$, $p < 0.001$), whereas the integrated OOemgS corresponding to short duration responses did not increase significantly in sham ($F_{2,18} = 2.94$, $p > 0.05$) and DE rats ($F_{2,18} = 2.1$, $p > 0.05$). The experiments reported below used repeated application of 2.5M NaCl as the test stimulus against which drug effects on OOemg activity at the ViVc transition and VcC1 region were assessed. To establish that repeated application of 2.5M NaCl alone did not influence the OOemg response, separate groups of sham ($n = 5$) and DE rats ($n = 5$) received two applications of HS at a 30 min interval. Total OOemg to the initial HS stimulus was not different from that seen 30 min later of sham rats (initial = 2.66 ± 0.6 versus +30 min = $2.82 \pm 0.34 \mu\text{V-s}/3\text{min}$) or DE rats (initial = 6.94 ± 0.53 versus +30 min = $6.66 \pm 0.34 \mu\text{V-s}/3\text{min}$; $F_{1,8} = 0.02$, $p > 0.1$). Similarly, the background OOemg activity was not influenced by repeated applications of 2.5M NaCl ($F_{1,8} = 2.53$, $p > 0.1$).

GABA_A receptors and total OOemg activity

Muscimol injection into ViVc—The background OOemg activity prior to HS stimulation and prior to muscimol injection into the ViVc transition averaged 4.03 ± 0.43 and $3.68 \pm 0.30 \mu\text{V-s}/3 \text{ min}$ for sham ($n = 13$) and DE rats ($n = 15$), respectively ($F_{1,26} = <0.1$, $p > 0.1$). Muscimol injection alone did not affect background OOemg activity ($F_{1,22} = 1.69$, $p > 0.1$). The initial HS (2.5 M)-evoked total OOemg prior to muscimol injection averaged 3.10 ± 0.23 and $7.61 \pm 0.52 \mu\text{V-s}/3 \text{ min}$ for sham and DE rats, respectively ($F_{5,40} = 10.5$, $p < 0.001$). Muscimol injection into the ViVc transition greatly reduced ($F_{3,66} = 39.01$, $p < 0.001$) the total HS-evoked OOemg in sham (Fig 3A) and DE rats (Fig 3B) in a dose-related manner and this inhibition was significantly greater in DE than sham animals ($F_{5,22} = 8.53$, $p < 0.001$). Compared to vehicle-injected rats (mean response latency = $< 2 \text{ s}$), high dose muscimol injection into the ViVc transition caused a transient (at 10 min post-injection) increase in the HS-evoked OOemg response latency in sham ($+30 \pm 9 \text{ s}$, $F_{3,65} = 16.6$, $p < 0.001$) and DE rats ($+160 \pm 15 \text{ s}$, $F_{3,65} = 39.2$, $p < 0.001$).

Muscimol injection into VcC1—The background OOemg activity prior to HS stimulation and prior to muscimol injection into the VcC1 region averaged 3.42 ± 0.19 and $3.43 \pm 0.37 \mu\text{V-s}/3 \text{ min}$ for sham ($n = 16$) and DE rats ($n = 14$), respectively ($F_{1,28} = <0.1$, $p > 0.1$). Muscimol injection alone did not affect background OOemg activity ($F_{1,24} = 1.76$, $p > 0.1$). The initial HS-evoked total OOemg AUC prior to muscimol injection into the VcC1 transition averaged 2.71 ± 0.23 and $8.07 \pm 0.52 \mu\text{V-s}/3 \text{ min}$ for sham and DE rats, respectively ($F_{5,72} = 21.1$, $p < 0.001$). High dose muscimol injection into the VcC1 region had no significant effect on total OOemg activity in sham rats (Fig 4A, $F_{3,72} = 2.47$, $p < 0.1$), whereas both low dose ($F_{3,72} = 6.64$, $p < 0.001$) and high dose muscimol ($F_{3,72} = 24.9$, $p < 0.001$) greatly reduced the HS-evoked total OOemg in DE rats (Fig 4B). Muscimol injection into the VcC1 region did not affect response latency in DE or sham rats ($F_{3,70} = 2.41$, $p < 0.1$).

Muscimol effects on OOemgL and OOemgS—The long duration (OOemgL) response to 2.5 M NaCl accounted for about 52% of the total OOemg AUC prior to muscimol injection in sham rats, whereas it accounted for 87% of the total OOemg response in DE rats (see Fig 1). Injection of low dose muscimol into the ViVc transition had no significant effect on OOemgL ($F_{3,45} = 1.67$, $p > 0.1$), while high dose muscimol caused a marked inhibition (Fig 5A, $F_{3,45} = 17.1$, $p < 0.001$) in DE rats. Muscimol injection into the ViVc transition did not significantly affect the OOemgL or OOemgS responses in sham animals (data not shown). By contrast, muscimol injection into the VcC1 region of DE rats did not significantly reduce the evoked OOemgL response (Fig 5B, $F_{3,45} = 1.56$, $p > 0.1$). HS-evoked OOemgS was not reduced by muscimol injection into either region in sham or DE rats (data not shown).

Bicuculline methiodide (BMI) injection into ViVc—The background OOemg activity prior to BMI injection averaged 5.7 ± 0.73 and 4.1 ± 0.47 $\mu\text{V}\cdot\text{s}/3$ min for sham ($n = 10$) and DE rats ($n = 10$), respectively ($F_{1,18} = 3.61$, $p < 0.1$). BMI injection alone increased the background OOemg activity in DE, but not in sham rats ($F_{3,16} = 6.68$, $p < 0.005$). BMI caused a small but significant increase in the HS-evoked total OOemg response in sham ($F_{3,48} = 2.83$, $p < 0.05$) and a larger increase in DE rats ($F_{3,48} = 8.72$, $p < 0.001$). However, the OOemgL response to HS was markedly increased in DE rats ($F_{3,48} = 6.82$, $p < 0.001$) and marginally increased in sham rats (Fig 6A, $F_{3,48} = 3.61$, $p < 0.025$).

BMI injection into VcC1—The background OOemg activity prior to BMI injection into the VcC1 region averaged 4.12 ± 0.34 and 3.81 ± 0.49 $\mu\text{V}\cdot\text{s}/3$ min for sham ($n = 11$) and DE rats ($n = 10$), respectively ($F_{1,19} = 0.28$, $p > 0.1$). BMI injection alone increased background OOemg activity in DE, but not in sham rats ($F_{3,17} = 5.64$, $p < 0.01$). BMI caused a small but significant increase in the HS-evoked total OOemg response in DE rats ($F_{3,51} = 3.98$, $p < 0.025$), but did not affect the total OOemg response in sham rats ($F_{3,51} < 1.0$, $p > 0.1$). The OOemgL response to HS was markedly increased in DE rats ($F_{3,48} = 5.98$, $p < 0.001$), while the OOemgL response was not affected in sham rats (Fig 6B). The OOemgS response to 2.5 M NaCl was not affected by BMI injection into either region nor was the HS-evoked response latency (< 2 s for all groups, data not shown).

NMDA receptor blockade and total OOemg activity

AP5 injection into ViVc—The background OOemg activity prior to AP5 injection into the ViVc transition was similar for sham (3.24 ± 0.32 , $n = 16$) and DE rats (3.23 ± 0.37 $\mu\text{V}\cdot\text{s}/3$ min, $n = 16$). AP5 injection alone did not affect the background OOemg activity in sham or DE rats ($F_{5,26} = 1.30$, $p > 0.1$). The initial HS test stimulus prior to AP5 evoked a greater increase in the total OOemg in DE than sham rats (7.57 ± 0.44 and 2.78 ± 0.18 $\mu\text{V}\cdot\text{s}/3$ min, respectively, $F_{1,30} = 101.8$, $p < 0.001$). AP5 injection into the ViVc transition significantly reduced the HS-evoked total OOemg response in sham (Fig 7A, $F_{3,78} = 6.87$, $p < 0.001$) and DE rats (Fig 7B, $F_{3,78} = 44.4$, $p < 0.001$). Group comparisons revealed a greater effect of AP5 on the HS-evoked total OOemg response in DE than sham rats ($F_{5,26} = 10.7$, $p < 0.001$). Compared to vehicle-injected rats (mean response latency = < 2 s), high dose AP5 injection into the ViVc transition caused an increase in latency at 10 min post-injection in sham ($+95 \pm 37$ s, $F_{3,76} = 5.6$, $p < 0.005$) and DE rats ($+36 \pm 8$ s, $F_{3,76} = 22.1$, $p < 0.001$).

AP5 injection into VcC1—Background OOemg activity prior to AP5 injection was not different between sham (3.37 ± 0.27 , $n = 17$) and DE rats ($3.57 \pm 0.58 \mu\text{V-s/3 min}$, $n = 16$). AP5 injection alone did not affect background OOemg activity in sham or DE rats ($F_{5,26} = 1.35$, $p > 0.1$). High dose AP5 injection into the VcC1 region greatly reduced the HS-evoked total OOemg response in sham (Fig 8A, $F_{3,81} = 6.34$, $p < 0.001$) and DE rats (Fig 8B, $F_{3,78} = 38.5$, $p < 0.001$), while low dose AP5 also caused a lesser and transient reduction in the evoked total OOemg response in both groups. Comparison across animal groups revealed a highly significant treatment effect of AP5 on HS-evoked total OOemg responses in DE versus sham rats ($F_{5,27} = 13.5$, $p < 0.001$). High dose AP5 caused an increase in response latency at 10 min post-injection in sham ($+62 \pm 37$ s, $F_{3,78} = 4.78$, $p < 0.005$) and DE rats ($+63 \pm 37$ s, $F_{3,78} = 4.98$, $p < 0.005$).

AP5 effects on OOemgL and OOemgS—Low dose AP5 injection into the ViVc transition transiently reduced the OOemgL response ($F_{3,54} = 4.6$, $p < 0.01$), while high dose AP5 caused a prolonged inhibition (Fig 9A, $F_{3,54} = 17.7$, $p < 0.001$) in DE rats. As seen in Fig 9B, low dose AP5 injection into the VcC1 region also caused a transition reduction in the OOemgL response ($F_{3,54} = 4.27$, $p < 0.01$), while high dose AP5 had a more persistent effect ($F_{3,54} = 13.6$, $p < 0.001$). AP5 injection into either region did not affect the HS-evoked OOemgL response in sham rats or DE rats (data not shown). HS-evoked OOemgS was not reduced by AP5 injection into either region in sham or DE rats (data not shown).

GABA_Aβ3 and NMDA receptor expression and protein levels

GABA_Aβ3 receptor expression (qPCR) at the ViVc transition ($1:1.35 \pm 0.25$, $n = 4$) and at the VcC1 region ($1:0.61 \pm 0.2$, $n = 4$) were not different for DE rats when normalized to levels in sham rats. However, as seen in Fig 10A, densitometry revealed a significant decrease in GABA_Aβ3 receptor density at the ViVc transition in DE versus sham animals (12.5 ± 4.2 versus 18.5 ± 1.3 % area, respectively, $n = 4$ per group, mean $\pm$ SD, $p < 0.03$), while receptor density at the VcC1 region for DE and sham animals was similar (17.3 ± 3.1 versus 13.8 ± 2.6 % area, respectively, $p > 0.1$). NR1 expression levels at the ViVc transition were not different for DE rats when normalized to sham values ($1:1.75$), while NR1 was increased significantly at the VcC1 region ($1:2.51$, $p < 0.02$). As seen in Fig 10B, densitometry revealed significant increases in percent area for pNR1 (serine 896 phosphorylation site) at the ViVc transition for DE versus sham rats (16.5 ± 2.6 versus 7.0 ± 2.9 % area, respectively, $p < 0.003$) and at the VcC1 region (17.3 ± 2.9 versus 7.0 ± 0.8 % area, DE and sham, respectively, $p < 0.001$).

Discussion

This study recorded OOemg activity from the upper and lower portions of the OO muscle in response to a noxious ocular surface stimulus and assessed central neural mechanisms that could influence corneal reflexes. These results indicated that ocular instillation of hypertonic saline (HS) evoked an increase in OOemgL that accounted for 87% of the total OOemg response tested 14d after exorbital gland removal (DE rats). OOemgS responses remained constant across all treatment groups. The evidence suggested that loss of GABA_A receptor-dependent mechanisms at the ViVc transition enhanced the OOemgL responses in DE rats

compared to sham controls, while GABAergic mechanisms at the VcC1 region had lesser effects. This was further supported by a loss of GABA_Aβ3 receptor density at the ViVc transition in DE versus sham animals. By contrast, NMDA-dependent effects on HS-evoked OOemgL activity were enhanced at the ViVc transition and the VcC1 region and pNR1 receptor density was increased at both regions in DE compared to sham rats. These results suggested that loss of GABAergic inhibition and increased NMDA-dependent excitatory mechanisms contributed to enhance corneal reflexes in DE rats.

Loss of GABAergic inhibition is well established as a central neural mechanism that facilitates spinal nociception (Woolf and Salter 2000; Zeilhofer et al. 2012). In the trigeminal system, GABAergic pathways contribute to nociception in acute pain models (Tashiro et al., 2014), while loss of GABAergic inhibition in chronic craniofacial pain models enhance nociceptive responses (Martin et al., 2010, Wei et al., 2013, Okada-Ogawa et al., 2015). Previously, we reported that local injection of muscimol inhibited the activity of cornea-responsive neurons at the ViVc transition and the VcC1 region and contributed to intersubnuclear communication between these subnuclei in naïve rats (Hirata et al., 2003). GABAergic interneurons were densely distributed at the ventral ViVc transition and in superficial laminae of the VcC1 region (Ginestal and Matute, 1993, Avendano et al., 2005), where they could directly influence second-order neurons that project to the facial motor nucleus. Alternatively, GABAergic interneurons could influence corneal reflexes via intersubnuclear communication pathways between the ViVc transition and the VcC1 region (Hirata et al. 2003). The present results suggested that GABAergic mechanisms at the ViVc transition played a more critical role in corneal reflexes than those at the VcC1 region, since muscimol injection into the ViVc transition was significantly more effective in reducing the HS-evoked OOemgL in DE than sham rats, while injections into the VcC1 region had only minor effects. Furthermore, GABA_Aβ3 receptor density at the ViVc transition was reduced at the ViVc transition, but not at the VcC1 region, consistent with a loss of GABAergic inhibition. The greater response to muscimol but a reduction in GABA_Aβ3 receptor density may be explained by the possibility that GABA_A receptors composed of subunits other than GABA_Aβ3 were increased in DE rats but were not measured in this study. A higher priority for the ViVc transition in corneal reflexes also agreed with an earlier study in which GABA_A mechanisms acting at the ViVc transition, and not at the VcC1 region, significantly reduced cornea-evoked lacrimation in naïve rats (Hirata et al. 2004). Injection of BMI into the ViVc transition or the VcC1 region enhanced the OOemgL response in DE rats; however, this may have been due to the recruitment of additional GABAergic pathways since BMI injections into the VcC1 region also enhanced the OOemgL response in sham rats. Although descending GABAergic projectors from the rostral ventromedial medulla (RVM) terminate in the dorsal horn (Antal et al. 1996) and corneal stimulation excited a high percentage of RVM neurons (Khasabov et al., 2015), it was not likely that this pathway could account for the differential effects of GABAergic drugs on ViVc transition neurons since direct stimulation of the RVM had a similar inhibitory effect on cornea-responsive neurons at the ViVc transition and VcC1 region (Meng et al., 2000).

NMDA receptor activation is critical for the development of inflammatory and neuropathic pain states (Woolf and Salter, 2000, von Hehn et al., 2012). The present study revealed that NMDA receptor blockade greatly reduced HS-evoked OOemgL activity in DE compared to

sham rats. However, in contrast to the effect of GABAergic mechanisms acting preferentially at the ViVc transition, blockade of NMDA receptors by AP5 at the ViVc transition or the VcC1 region caused a similar reduction in the HS-evoked OOemgL response. This was consistent with findings that receptor density for pNR1, an obligatory subunit of the NMDA receptor, also was markedly increased at the ViVc transition and VcC1 region.

Phosphorylation of NR1 is necessary for receptor activation (Tingley et al., 1997) and behavioral hyperalgesia (Guo et al., 2002, Ultenius et al., 2006). Also, central administration of a NMDA receptor antagonist reduced the number of Fos-positive neurons produced at the ViVc transition and VcC1 region after inflammation of the ocular surface (Bereiter and Bereiter, 1996). Although AP5 may have acted directly on corneal-responsive neurons at the ViVc transition and VcC1 region that project to the facial motor nucleus, it cannot be excluded that glutamatergic intersubnuclear connections between these regions also contributed to the reduction in HS-evoked OOemgL activity. Intersubnuclear pathways have been identified in an in vitro slice preparation (Han et al. 2008), while NMDA-dependent intersubnuclear communication contributed to neuronal hyperexcitability in a model of dental pain (Park et al., 2001).

HS was used to evoke corneal reflexes to ensure that a nociceptive reflex was recruited in anesthetized rats in response to a more natural stimulus. Although the HS concentrations used here were higher than normally found in awake DE subjects, the osmotic threshold necessary to excite corneal afferent fibers in the mouse was reported to be at or above 600 mOsm (Parra et al. 2014). In awake humans, the ocular threshold to evoke stinging pain was ~500 mOsm, while osmotic concentrations in tears have been estimated at nearly 900 mOsm after forced eye opening (Liu et al. 2009). A limitation to this approach, compared to the use of electrical stimuli (Henriquez and Evinger 2005; 2007), was that precise measurement of stimulus latency was lost, although relative latencies across treatment groups were retained. A second limitation of this approach was that actual lid closure could not be measured in anesthetized rats. Thus, functional interpretation of evoked changes in OOemg activity relative to lid movement was based on parallel studies in awake animals or humans. Reflex eyeblinks can range from a transient lowering of the upper eyelid in less than 1s to full closure involving the upper and lower eyelids lasting several seconds (Evinger et al. 1991; Evinger 1995). Eyelid closure in response to ocular stimuli that cause discomfort such as sudden exposure to bright light is often referred to as squint or dazzle reflex and can be estimated by OOemg recording (Stringham et al. 2003; Planis et al. 2006). Videographic measurement of eyelid closure in awake rats by HS applied to the ocular surface revealed a near maximum closure at 900 mOsm, a response that was prevented by prior treatment with BCTC (Yorek et al. 2016), a mixed TRPV1/TRPM8 receptor antagonist (Pomonis et al. 2003). Collectively, these data suggested that the OOemgL activity recorded in the current study under anesthesia was consistent with that seen as eyelid squint in awake subjects evoked by adverse ocular stimuli.

An unresolved issue is whether the effects of muscimol, BMI and AP5 on corneal reflexes have similar effects on innocuous eyeblink reflexes. Comparison of OOemg responses to cornea and supraorbital nerve stimulation activity suggested different central neural pathways; however, only electrical stimuli were applied (Berardelli et al. 1985). The present study revealed that short duration OOemgS activity was not significantly affected by HS;

however, power spectral analyses were not determined leaving open the possibility that drug effects could have altered select frequencies. In studies of voluntary squinting in humans, eyeblink reflexes were reduced in proportion to squinting (Sheedy et al. 2005). Thus, it is possible that high levels of OOemgL activity evoked by HS could have limited OOemgS activity. A second unresolved issue is whether upper and lower eyelid activity were driven from one source or were controlled separately. It is assumed that measurement of upper or lower OOemg activity will be similar in eyeblink (Evinger et al. 1991); however, this has not been adequately tested for corneal reflexes. Neurons that supply the upper and lower eyelids likely derive from separate populations of cells in the facial motor nucleus (Porter et al. 1989; VanderWerf et al. 1998; Gong et al. 2003). Also, the trigeminal ganglion neurons that supply the upper and lower eyelids derive from separate branches of the trigeminal nerve, are differentially distributed in the ganglion and display different terminal densities (May and Porter 1998). It has been reported that under certain pathological conditions upper and lower OOemg activity may be different (Aramideh et al. 1995; Goiato et al. 2015).

The enhanced OOemg activity of DE rats was consistent with reported symptoms of ocular irritation and pain in DE patients (Begley et al. 2002; Vehof et al. 2013) and the increased responsiveness of cornea-responsive neurons at the ViVc transition and the VcC1 region in DE rats (Rahman et al. 2015). The preferential contributions of GABAergic mechanisms at the ViVc transition compared to the VcC1 region to affect OOemg activity also was consistent with previous studies suggesting that the pathway from trigeminal sensory nerves to the ViVc transition and then to OO motoneurons in facial motor nucleus was more direct than pathways that pass via the VcC1 region (Pellegrini et al., 1995, Henriquez and Evinger, 2007).

The present study suggested that persistent reduction in tear volume was sufficient to cause significant structural and functional changes in trigeminal brainstem circuits closely associated with the integration of ocular pain signals. These results further suggested a dominant role for GABAergic mechanisms at the ViVc transition and a lesser role for GABAergic influence at the VcC1 region. In addition to loss of GABAergic inhibition, DE rats also displayed evidence of enhanced NMDA receptor activity at the ViVc transition and the VcC1 region. Altered amino acid neurotransmission in trigeminal brainstem may be novel therapeutic targets for management of adverse symptoms in DE.

Acknowledgments

This study was supported in part by a grant from the National Eye Institute (EY 021447).

Abbreviations

AP5	2-amino-5-phosphonopentonic acid
AUC	area under the curve
BMI	bicuculline methiodide
DE	dry eye

GABA	gamma-aminobutyric acid
HS	hypertonic saline
MAP	mean arterial pressure
NMDA	N-methyl-D-aspartate
OOemg	orbicularis oculi muscle electromyography
OOemgL	long duration OOemg
OOemgS	short duration OOemg
ViVc	trigeminal subnucleus interpolaris/caudalis transition
VcC1	trigeminal subnucleus caudalis/upper cervical spinal cord junction

References

1. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop. *Ocul Surf.* 2007; 5:75–92. [PubMed: 17508116]
2. Accornero N, Berardelli A, Bini G, Cruccu G, Manfredi M. Corneal reflex elicited by electrical stimulation of the human cornea. *Neurology.* 1980; 30:782–785. [PubMed: 7190247]
3. Acosta MC, Gallar J, Belmonte C. The influence of eye solutions on blinking and ocular comfort at rest and during work at video display terminals. *Exp Eye Res.* 1999; 68:663–669. [PubMed: 10375429]
4. Alves M, Fonseca EC, Alves MF, Malki LT, Arruda GV, Reinach PS, Rocha EM. Dry eye disease treatment: a systematic review of published trials and a critical appraisal of therapeutic strategies. *Ocul Surf.* 2013; 11:181–192. [PubMed: 23838019]
5. Antal M, Petko M, Polgar E, Heizmann CW, Storm-Mathisen J. Direct evidence of an extensive GABAergic innervation of the spinal dorsal horn by fibres descending from the rostral ventromedial medulla. *Neuroscience.* 1996; 73:509–518. [PubMed: 8783266]
6. Aramideh M, Ongerboer de Visser BW, Koelman JH, Speelman JD. Motor persistence of orbicularis oculi muscle in eyelid-opening disorders. *Neurology.* 1995; 45:897–902. [PubMed: 7746403]
7. Asbell PA, Spiegel S. Ophthalmologist perceptions regarding treatment of moderate-to-severe dry eye: results of a physician survey. *Eye Contact Lens.* 2010; 36:33–38. [PubMed: 20009946]
8. Avendano C, Machin R, Bermejo PE, Lagares A. Neuron numbers in the sensory trigeminal nuclei of the rat: A GABA- and glycine-immunocytochemical and stereological analysis. *J Comp Neurol.* 2005; 493:538–553. [PubMed: 16304625]
9. Begley CG, Caffery B, Chalmers RL, Mitchell GL. Use of the dry eye questionnaire to measure symptoms of ocular irritation in patients with aqueous tear deficient dry eye. *Cornea.* 2002; 21:664–670. [PubMed: 12352083]
10. Behrens A, Doyle JJ, Stern L, Chuck RS, McDonnell PJ, Azar DT, Dua HS, Hom M, Karpecki PM, Laibson PR, Lemp MA, Meisler DM, Del Castillo JM, O'Brien TP, Pflugfelder SC, Rolando M, Schein OD, Seitz B, Tseng SC, van Setten G, Wilson SE, Yiu SC. Dysfunctional tear syndrome: a Delphi approach to treatment recommendations. *Cornea.* 2006; 25:900–907. [PubMed: 17102664]
11. Berardelli A, Cruccu G, Manfredi M, Rothwell JC, Day BL, Marsden CD. The corneal reflex and the R2 component of the blink reflex. *Neurology.* 1985; 35:797–801. [PubMed: 4000481]
12. Bereiter DA, Bereiter DF. N-methyl-D-aspartate and non-N-methyl-D-aspartate receptor antagonism reduces Fos-like immunoreactivity in central trigeminal neurons after corneal stimulation in the rat. *Neuroscience.* 1996; 73:249–258. [PubMed: 8783246]

13. Cruccu G, Ferracuti S, Leardi MG, Fabbri A, Manfredi M. Nociceptive quality of the orbicularis oculi reflexes as evaluated by distinct opiate- and benzodiazepine-induced changes in man. *Brain Res.* 1991; 556:209–217. [PubMed: 1933356]
14. Evinger C. A brain stem reflex in the blink of an eye. *News Physiol Sci.* 1995; 10:147–153.
15. Evinger C, Manning KA, Sibony PA. Eyelid movements. Mechanisms and normal data. *Invest Ophthalmol Vis Sci.* 1991; 32:387–400. [PubMed: 1993591]
16. Ginestal E, Matute C. Gamma-aminobutyric acid-immunoreactive neurons in the rat trigeminal nuclei. *Histochemistry.* 1993; 99:49–55. [PubMed: 8468194]
17. Goiato MC, Santos MR, Monteiro BC, Moreno A, Bannwart LC, Filho AJ, Guiotti AM, Haddad MF, Pesqueira AA, Dos Santos DM. Electrical activity of the orbicularis muscles before and after installation of ocular prostheses. *Int J Oral Maxillofac Surg.* 2015; 44:127–131. [PubMed: 25457831]
18. Gong S, Zhou Q, LeDoux MS. Blink-related sensorimotor anatomy in the rat. *Anat Embryol.* 2003; 207:193–208. [PubMed: 14551765]
19. Guo W, Zou S, Guan Y, Ikeda T, Tal M, Dubner R, Ren K. Tyrosine phosphorylation of the NR2B subunit of the NMDA receptor in the spinal cord during the development and maintenance of inflammatory hyperalgesia. *J Neurosci.* 2002; 22:6208–6217. [PubMed: 12122079]
20. Han SM, Ahn DK, Youn DH. Pharmacological analysis of excitatory and inhibitory synaptic transmission in horizontal brainstem slices preserving three subnuclei of spinal trigeminal nucleus. *J Neurosci Methods.* 2008; 167:221–228. [PubMed: 17900704]
21. Henriquez VM, Evinger C. Modification of cornea-evoked reflex blinks in rats. *Exp Brain Res.* 2005; 163:445–456. [PubMed: 15785953]
22. Henriquez VM, Evinger C. The three-neuron corneal reflex circuit and modulation of second-order corneal responsive neurons. *Exp Brain Res.* 2007; 179:691–702. [PubMed: 17216149]
23. Himebaugh NL, Begley CG, Bradley A, Wilkinson JA. Blinking and tear break-up during four visual tasks. *Optom Vis Sci.* 2009; 86:E106–114. [PubMed: 19156014]
24. Hirata H, Okamoto K, Bereiter DA. GABA_A receptor activation modulates corneal unit activity in rostral and caudal portions of trigeminal subnucleus caudalis. *J Neurophysiol.* 2003; 90:2837–2849. [PubMed: 12890797]
25. Hirata H, Okamoto K, Tashiro A, Bereiter DA. A novel class of neurons at the trigeminal subnucleus interpolaris/caudalis transition region monitors ocular surface fluid status and modulates tear production. *J Neurosci.* 2004; 24:4224–4232. [PubMed: 15115818]
26. Hirata H, Takeshita S, Hu JW, Bereiter DA. Cornea-responsive medullary dorsal horn neurons: modulation by local opioid agonists and projections to thalamus and brainstem. *J Neurophysiol.* 2000; 84:1050–1061. [PubMed: 10938327]
27. Johnson ME. The association between symptoms of discomfort and signs in dry eye. *Ocul Surf.* 2009; 7:199–211. [PubMed: 19948103]
28. Khasabov SG, Malecha P, Noack J, Tabakov J, Okamoto K, Bereiter DA, Simone DA. Activation of rostral ventromedial medulla neurons by noxious stimulation of cutaneous and deep craniofacial tissues. *J Neurophysiol.* 2015; 113:14–22. [PubMed: 25185804]
29. Liu H, Begley C, Chen M, Bradley A, Bonanno J, McNamara NA, Nelson JD, Simpson T. A link between tear instability and hyperosmolarity in dry eye. *Invest Ophthalmol Vis Sci.* 2009; 50:3671–3679. [PubMed: 19324847]
30. Marfurt CF, Del Toro DR. Corneal sensory pathway in the rat: a horseradish peroxidase tracing study. *J Comp Neurol.* 1987; 261:450–459. [PubMed: 3112189]
31. Martin YB, Malmierca E, Avendano C, Nunez A. Neuronal disinhibition in the trigeminal nucleus caudalis in a model of chronic neuropathic pain. *Eur J Neurosci.* 2010; 32:399–408. [PubMed: 20704591]
32. May PJ, Porter JD. The distribution of primary afferent terminals from the eyelids of macaque monkeys. *Exp Brain Res.* 1998; 123:368–381. [PubMed: 9870597]
33. Meng ID, JWH, Bereiter DA. Parabrachial area and nucleus raphe magnus inhibition of corneal units in rostral and caudal portions of trigeminal subnucleus caudalis in the rat. *Pain.* 2000; 87:241–251. [PubMed: 10963904]

34. Mukuno K, Aoki S, Ishikawa S, Tachibana S, Harada H, Hozumi G, Saito E. Three types of blink reflex evoked by supraorbital nerve, light flash and corneal stimulations. *Jpn J Ophthalmol.* 1983; 27:261–270. [PubMed: 6855018]
35. Nakamori K, Odawara M, Nakajima T, Mizutani T, Tsubota K. Blinking is controlled primarily by ocular surface conditions. *Am J Ophthalmol.* 1997; 124:24–30. [PubMed: 9222228]
36. Okada-Ogawa A, Nakaya Y, Imamura Y, Kobayashi M, Shinoda M, Kita K, Sessle BJ, Iwata K. Involvement of medullary GABAergic system in extraterritorial neuropathic pain mechanisms associated with inferior alveolar nerve transection. *Exp Neurol.* 2015; 267:42–52. [PubMed: 25736265]
37. Ongerboer de Visser BW. The corneal reflex: electrophysiological and anatomical data in man. *Progr Neurobiol.* 1980; 15:71–83.
38. Panneton WM, Hsu H, Gan Q. Distinct central representations for sensory fibers innervating either the conjunctiva or cornea of the rat. *Exp Eye Res.* 2010; 90:388–396. [PubMed: 20004193]
39. Park SJ, Chiang CY, Hu JW, Sessle BJ. Neuroplasticity induced by tooth pulp stimulation in trigeminal subnucleus oralis involves NMDA receptor mechanisms. *J Neurophysiol.* 2001; 85:1836–1846. [PubMed: 11353000]
40. Parra A, Gonzalez-Gonzalez O, Gallar J, Belmonte C. Tear fluid hyperosmolality increases nerve impulse activity of cold thermoreceptor endings of the cornea. *Pain.* 2014; 155:1481–1491. [PubMed: 24785271]
41. Pellegrini JJ, Horn AK, Evinger C. The trigeminally evoked blink reflex. I. Neuronal circuits. *Exp Brain Res.* 1995; 107:166–180. [PubMed: 8773237]
42. Plainis S, Murray IJ, Carden D. The dazzle reflex: electrophysiological signals from ocular muscles reveal strong binocular summation effects. *Ophthalmic Physiol Opt.* 2006; 26:318–325. [PubMed: 16684158]
43. Pomonis JD, Harrison JE, Mark L, Bristol DR, Valenzano KJ, Walker K. N-(4-Tertiarybutylphenyl)-4-(3-cholorophyridin-2-yl)tetrahydropyrazine -1(2H)-carbox-amide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: II. in vivo characterization in rat models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther.* 2003; 306:387–393. [PubMed: 12721336]
44. Porter JD, Burns LA, May PJ. Morphological substrate for eyelid movements: innervation and structure of primate levator palpebrae superioris and orbicularis oculi muscles. *J Comp Neurol.* 1989; 287:64–81. [PubMed: 2477400]
45. Rahman M, Okamoto K, Thompson R, Bereiter DA. Trigeminal pathways for hypertonic saline- and light-evoked corneal reflexes. *Neuroscience.* 2014; 277:716–723. [PubMed: 25086311]
46. Rahman M, Okamoto K, Thompson R, Katagiri A, Bereiter DA. Sensitization of trigeminal brainstem pathways in a model for tear deficient dry eye. *Pain.* 2015; 156:942–950. [PubMed: 25734990]
47. Rosenthal P, Borsook D. Ocular neuropathic pain. *Br J Ophthalmol.* 2015
48. Seagrove LC, Suzuki R, Dickenson AH. Electrophysiological characterisations of rat lamina I dorsal horn neurones and the involvement of excitatory amino acid receptors. *Pain.* 2004; 108:76–87. [PubMed: 15109510]
49. Sheedy JE, Gowrisankaran S, Hayes JR. Blink rate decreases with eyelid squint. *Optom Vis Sci.* 2005; 82:905–911. [PubMed: 16276323]
50. Sokal DM, Chapman V. Effects of spinal administration of muscimol on C- and A-fibre evoked neuronal responses of spinal dorsal horn neurones in control and nerve injured rats. *Brain Res.* 2003; 962:213–220. [PubMed: 12543472]
51. Stringham JM, Fuld K, Wenzel AJ. Action spectrum for photophobia. *J Opt Soc Am A Opt Image Sci Vis.* 2003; 20:1852–1858. [PubMed: 14570098]
52. Sullivan BD, Crews LA, Messmer EM, Foulks GN, Nichols KK, Baenninger P, Geerling G, Figueiredo F, Lemp MA. Correlations between commonly used objective signs and symptoms for the diagnosis of dry eye disease: clinical implications. *Acta Ophthalmol.* 2014; 92:161–166. [PubMed: 23279964]

53. Tashiro A, Bereiter DA, Thompson R, Nishida Y. GABAergic influence on temporomandibular joint-responsive spinomedullary neurons depends on estrogen status. *Neuroscience*. 2014; 259:53–62. [PubMed: 24316475]
54. Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, Huganir RL. Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *J Biol Chem*. 1997; 272:5157–5166. [PubMed: 9030583]
55. Ultenius C, Linderöth B, Meyerson BA, Wallin J. Spinal NMDA receptor phosphorylation correlates with the presence of neuropathic signs following peripheral nerve injury in the rat. *Neurosci Lett*. 2006; 399:85–90. [PubMed: 16469445]
56. van Boxtel A. Optimal signal bandwidth for the recording of surface EMG activity of facial, jaw, oral, and neck muscles. *Psychophysiology*. 2001; 38:22–34. [PubMed: 11321618]
57. VanderWerf F, Aramideh M, Otto JA, Ongerboer de Visser BW. Retrograde tracing studies of subdivisions of the orbicularis oculi muscle in the rhesus monkey. *Exp Brain Res*. 1998; 121:433–441. [PubMed: 9746150]
58. Vehof J, Kozareva D, Hysi PG, Harris J, Nessa A, Williams FK, Bennett DL, McMahon SB, Fahy SJ, Direk K, Spector TD, Hammond CJ. Relationship Between Dry Eye Symptoms and Pain Sensitivity. *JAMA Ophthalmol*. 2013
59. von Hehn CA, Baron R, Woolf CJ. Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron*. 2012; 73:638–652. [PubMed: 22365541]
60. Wei B, Kumada T, Furukawa T, Inoue K, Watanabe M, Sato K, Fukuda A. Pre- and post-synaptic switches of GABA actions associated with Cl⁻ homeostatic changes are induced in the spinal nucleus of the trigeminal nerve in a rat model of trigeminal neuropathic pain. *Neuroscience*. 2013; 228:334–348. [PubMed: 23103796]
61. Williamson JF, Huynh K, Weaver MA, Davis RM. Perceptions of dry eye disease management in current clinical practice. *Eye Contact Lens*. 2014; 40:111–115. [PubMed: 24508770]
62. Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science*. 2000; 288:1765–1768. [PubMed: 10846153]
63. Wu Z, Begley CG, Situ P, Simpson T. The effects of increasing ocular surface stimulation on blinking and sensation. *Invest Ophthalmol Vis Sci*. 2014; 55:1555–1563. [PubMed: 24557346]
64. Yorek MS, Davidson EP, Poolman P, Coppey LJ, Obrosova A, Holmes A, Kardon RH, Yorek MA. Corneal Sensitivity to Hyperosmolar Eye Drops: A Novel Behavioral Assay to Assess Diabetic Peripheral Neuropathy. *Invest Ophthalmol Vis Sci*. 2016; 57:2412–2419. [PubMed: 27145474]
65. Yoshida A, Dostrovsky JO, Sessle BJ, Chiang CY. Trigeminal projections to the nucleus submedialis of the thalamus in the rat. *J Comp Neurol*. 1991; 307:609–625. [PubMed: 1714465]
66. Zeilhofer HU, Benke D, Yevenes GE. Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control. *Annu Rev Pharmacol Toxicol*. 2012; 52:111–133. [PubMed: 21854227]
67. Zhang Z, Hefferan MP, Loomis CW. Topical bicuculline to the rat spinal cord induces highly localized allodynia that is mediated by spinal prostaglandins. *Pain*. 2001; 92:351–361. [PubMed: 11376908]

Highlight points

- Muscimol injection into ViVc transition caused a greater inhibition of evoked OOemg activity in DE than sham rats.
- Muscimol injection into VcC1 caused a smaller decrease in evoked OOemg activity in DE and sham rats.
- BMI injection into ViVc transition or VcC1 enhanced evoked OOemg activity in DE rats.
- AP5 injection into ViVc transition or VcC1 region greatly reduced evoked OOemg activity in DE and sham rats.
- GABA α 3 receptor density was reduced at ViVc, while pNR1 was increased at ViVc and VcC1 in DE versus sham rats.

Figure 1.

Examples of hypertonic saline (HS) - evoked OOemg activity in a male rat 14 days after exorbital gland removal. A. Total OOemg sampled over 3 min. Boxed areas indicate expanded displays in B and C. B. Long duration OOemg (OOemgL) response (>200ms). C. Short duration OOemg (OOemgS) response (<200ms). Arrow = HS stimulus (2.5 M NaCl). Calibration: A = 200μV and 30 s; B and C = 200μV and 1 s.

Figure 2. Effect of different concentrations of NaCl solutions on integrated values for total OOemg, OOemgL and OOemgS activity in sham (upper panel) and DE rats (lower panel). *p < 0.05, **p < 0.01 versus 0.15 M; b = p < 0.01 sham versus DE groups. n = 5–6 rats per group.

Figure 3. Effect of the GABA_A receptor agonist, muscimol, injected at the ViVc transition on HS-evoked integrated total OOemg activity in sham (upper panel) and DE rats (lower panel). *p < 0.05, **p < 0.01 versus pre-drug; b = p < 0.01 versus vehicle. n = 4–5 rats per group.

Figure 4.

Effect of muscimol injection into the VcC1 region on HS-evoked integrated total OOemg activity of sham (upper panel) and DE rats (lower panel). **p < 0.01 versus pre-drug; a = p < 0.05 versus vehicle. n = 4–6 rats per group.

Figure 5. Effect of muscimol injection into the ViVc transition and VcC1 region on HS-evoked integrated OOemgL activity (>200ms duration) in DE rats. ** $p < 0.01$ versus pre-drug; a = $p < 0.05$, b = $p < 0.01$ low versus high dose. n = 4–6 rats per group.

Figure 6.

Effect of the GABA_A receptor antagonist, BMI, injection into the ViVc transition and VcC1 region on HS-evoked integrated OOemgL activity of sham and DE rats. * $p < 0.05$, ** $p < 0.01$ versus pre-drug. $n = 5-6$ rats per group.

Figure 7.

Effect of NMDA receptor antagonist, AP5, injection into the ViVc transition on HS-evoked integrated total OOemg activity of sham (upper panel) and DE rats (lower panel). *p < 0.05, **p < 0.01 versus pre-drug; a = p < 0.05, b = p < 0.01 versus vehicle. n = 5 rats per group.

Figure 8.

Effect of AP5 injection into the VcC1 region on HS-evoked integrated total OOemg activity of sham (upper panel) and DE rats (lower panel). ** $p < 0.01$ versus pre-drug; a = $p < 0.05$, b = $p < 0.01$ versus vehicle. n = 5–6 rats per group.

Figure 9. Effect of AP5 injection into the ViVc transition and VcC1 region on HS-evoked integrated OOemgL activity in DE rats. ** $p < 0.01$ versus pre-drug. $n = 5-6$ rats per group.

Figure 10.

Densitometry for GABA_Aβ3 and pNR1 protein sampled at the ViVc transition and VcC1 region of sham and DE rats. A. GABA_Aβ3. B. pNR1. *p < 0.05, **p < 0.01 versus sham. n = 4 rats per group and 3 sections sampled per rat.