EYE PAIN: ETIOLOGY AND THERAPEUTIC APPROACHES

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PUBLISHED IN: Frontiers in Pharmacology, Frontiers in Medicine and Frontiers in Neuroscience

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-189-0
DOI 10.3389/978-2-88976-189-0

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EYE PAIN: ETIOLOGY AND THERAPEUTIC APPROACHES

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Citation: Rusciano, D., Bagnoli, P., Galor, A., Gallar, J., eds. (2022). Eye Pain: Etiology and Therapeutic Approaches. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-189-0

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frontiers | Frontiers in Pharmacology

EDITORIAL
published: 27 April 2022
doi: 10.3389/fphar.2022.914809

Editorial: Eye Pain: Etiology and Therapeutic Approaches

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Keywords: eye pain, neuropathic pain, nociceptive pain, dry eye, cornea

Editorial on the Research Topic

Eye Pain: Etiology and Therapeutic Approaches

The eye is heavily innervated by sensory nerve fibers. Corneal sensory nerves sprout from the ophthalmic division of the trigeminal nerve, travel in the nasociliary nerve and its branches, finally entering the cornea through the sclera and the conjunctiva. They form a stromal network supplying different regions of the cornea: midstromal, sub-basal/subepithelial and epithelial. The receptive fields of corneal sensory receptors are large and partially overlapping, thus resulting in poor localization or acuity, but producing a very high level of sensitivity to external stimuli. The central corneal nerve density is approximately 7,000 nerve endings per mm^2, so that cornea sensitivity is 300–600 times higher than skin, and 20–40 times greater than dental pulp. Different types of corneal sensory nerves have been characterized. Approximately 20% belong to the class of mechano-nociceptive receptors responding to mechanical stimuli and responsible for acute sharp pain conducted through thin myelinated fibers. Some 70% are polymodal nociceptors responding to chemical mediators, heat and irritants through slow-conducting, unmyelinated nerve fibers. Finally, 10% are cold receptor fibers activated by cold solutions or cold air, such as it may happen during tear film evaporation. Beside these relevant sensory functions, corneal nerves also regulate reflex tear production and the associated blinking reflex, and contribute to the release of trophic factors, such as substance-P, NGF, KGF, CNTF, PDGF-B, TGF-α and IL1β. In fact, iatrogenic or traumatic damage to corneal sensory fibers may result in neurotrophic keratopathy, characterized by epithelial cell loss and edema. Being so sensitive, the cornea is susceptible to pain. Pain protects tissue from injury. Painful stimuli detected by nociceptors are transmitted *via* action potentials to higher order centers where the pain is perceived.

Pain can be acute, when of high intensity and lasting a short time, or chronic, when its duration reaches and extends over 3 months. Depending on the stimulus triggering pain, it can be differentiated in nociceptive (caused by the physiological response to a noxious event) or neuropathic, when the algic response results from a dysfunction caused by damage of the sensory system (either the peripheral sensory nerves or the central neurons) and hardly treated by topical analgesics when central mechanisms are involved. Chronic pain may have a neuropathic component. Perturbations of the eye surface such as dry-eye, pterygia or conjunctivochalasis, inflammation and infections may be triggers of eye pain. This kind of pain is typically treated by topical antinflammatory agents and ointments, or anesthetics. When eye pain is reported out of proportion to clinical signs, or with no apparent previous insult, neuropathic pain is suspected.

Neuropathic pain is not a reaction to noxious stimuli, rather it is the result from an insult to the nervous system. During regeneration of damaged corneal nerves there is an increase in the expression of ion channels involved in their excitability, which may produce spontaneous activity and a low activation threshold. This altered activity may influence the synaptic

OPEN ACCESS

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *07 April 2022*
Accepted: *11 April 2022*
Published: *27 April 2022*

Citation:
Rusciano D, Bagnoli P, Gallar J and Galor A (2022) Editorial: Eye Pain: Etiology and Therapeutic Approaches. Front. Pharmacol. 13:914809. doi: 10.3389/fphar.2022.914809

connections along the sensory pathway, leading to permanent changes at central levels. To complicate matters, the severity of eye pain, that can be unilateral or bilateral, does not indicate how serious the underlying cause of the discomfort is. Symptoms of neuropathic corneal pain may include sensitivity to light and air, foreign body sensation, burning and severe eye dryness. In fact, symptoms of neuropathic corneal pain may sometimes be confused with dry eye syndrome, although the signs typical of this disease are missing. In fact, patients with neuropathic corneal pain do not respond to usual dry eye treatments. Neuropathic corneal pain can result from eye surgery, chronic dry eye disease generated or aggravated by the use of preservatives in eye drops; extended use of contact lenses; diabetes; neuralgia of the trigeminus. It can be associated with anxiety or depression, headache or migraine, fibromyalgia and autoimmune diseases.

Severe pain sensation and light sensitivity prevent those afflicted with ocular neuropathic pain from performing activities of daily living and is associated with symptoms of anxiety and depression.

While nociceptive pain is targeted through the use of topical therapies, neuropathic pain can be treated with oral agents or adjunctive therapies if a neuropathic component is highly suspected. While many studies have examined treatment outcomes for nociceptive sources of ocular pain, fewer have examined outcomes after treatment of neuropathic ocular pain. Strategies to counteract neuropathic ocular pain include ocular surface treatment, anti-inflammatory compounds, serum containing growth factors which play a crucial role in neuroregeneration and healing, anticonvulsants, opioid agonists and alternative therapies.

To date, however, comprehensive understanding of the mechanisms underlying ocular neuropathic pain is still under exploration and efficient treatment of neuropathic eye pain has yet to be found. The challenge is a better definition of the molecular targets of neuropathic eye pain, and the identification of specific therapeutic agents to be given either as topical or as systemic treatment.

Therefore, given the relatively high frequency of eye pain, the multiplicity of its causes (at least 22 possible causes have been described), and the complexity of neuropathic pain, the aim of this Research Topic has been to collect a series of recent studies focusing on the different aspects of ocular pain, its molecular triggers and innovative treatment strategies.

PAIN MECHANISMS AND PERCEPTION

The thresholds for subjective perception of corneal sensing receptors to different stimuli (cold, mechanical and chemical) applied at increasing intensities is addressed in the manuscript presented by Jayakumar and Simpson, in order to try and dissect patient's processing of the stimulus in the two phases of detection and response.

Among the several stimuli that are known to activate peripheral terminals of trigeminal sensory neurons at the cornea, conjunctiva and sclera, acidic stimuli have been shown to induce the firing of polymodal nociceptors through the activation of specific ion channels. In the paper of Comes et al., ion channels and receptors that are involved in acid sensing are reviewed. Because of the acid environment in the cornea and the conjunctiva, a number of compounds used to treat eye diseases are formulated in acidic solutions to facilitate their solubilization and absorption through the cornea. Despite some of the mechanisms underlying proton sensing in the ocular surface have been elucidated, further studies are needed to clarify the differential role of channels or membrane receptors which might allow to develop specific therapeutic interventions.

A review of Puja et al. describes the recent advances on the role of molecular and cellular mechanisms contributing to peripheral and central pain sensitization of the trigeminal pathway, together with mechanisms underlying corneal sensory transduction and peripheral pain sensitization in the trigeminal spinal nucleus.

The brain networks related to pain processing have been extensively studied with functional neuroimaging over the past 20 years. Based on these observations, supraspinal mechanisms underlying ocular pain are detailed by Pondelis and Moulton, describing the anatomy and the physiology of the different brain regions that receive afferent inputs from the trigeminal system. In the case of nociception, nociceptors' signals traveling through supraspinal centers finally reach the cortex where the pain sensation is generated. On the other hand, neuropathic pain is generated by alterations in the somatosensory nervous system, not necessarily involving peripheral receptors. Clarifying the neural pathways at the origin of neuropathic ocular pain is critical to understanding its mechanisms and ultimately its treatment.

Dry eye disease is often associated with neuropathic ocular pain. Although it is mostly generated by nociceptive stimulation induced by alterations of tear film dynamics, chronic dryness lead to nerve damage and induce morpho-functional changes of corneal nerves. In this context, persistent ocular pain in the absence of detectable signs can be considered a form of neuropathic pain. In the paper by Bereiter et al., the authors try to clarify the basis of ocular hyperalgesia in animal models of dry eye disease by demonstrating that the activation of P2X7R, a purinergic receptor expressed by non-neural cells in the trigeminal nerve pathway, contributes to ocular hyperalgesia and to microglia activation in both male and female animals, an effect that is further amplified by estrogen treatment in females.

Finally, in a preclinical study, Luna et al. using the guinea pig model, provided the first demonstration that a unilateral lesion of the corneal nerves affects the corneal sensitivity in both the ipsilateral and the contralateral eye. This is in line with the clinical finding that some patients with unilateral ocular alteration reported discomfort and pain also in the contralateral eye. Although the mechanisms underlying the contralateral alteration of sensory nerves remains to be determined, available data support the involvement of neuroimmune interactions. These findings imply that in preclinical and clinical studies the contralateral eye cannot be used as a control and that in clinical practice both eyes need to be treated also in the presence of unilateral ocular damage.

OCULAR PAIN HANDLING

In ocular pain handling, preoperative management has been focused to the use of musicotherapy in patients undergoing cataract surgery, the most frequently performed surgical procedure. In a paper by Guerrier et al., a prospective controlled trial including 243 patients has shown that music intervention prior to the surgery can reduce anxiety level and self-reported pain intensity both during cataract surgery under local anesthesia and in the early postoperative period. The underlying mechanisms remain unclear, although molecular mechanisms related to opioid and cytokine metabolism are discussed together with psychophysiological mechanisms bringing to anxiety reduction.

In an opinion article, Santarcangelo and Carli, two experts in pain management, discuss the effectiveness of psychological interventions focused mainly on hypnosis for disease management. In particular, hypnotizability is used as a model to support the view that specific psycho-physiological traits and cognitive strategies can not only reduce pain, but also modulate the pain-related autonomic and immune activity, induce cortical plasticity relevant to pain control, and assist in the choice of the most appropriated treatment.

Topical treatments have been dealt with in four different articles. In a pilot study, Delicado-Miralles et al. investigate the effects of F6H8, an alkane previously shown to alleviate dry-eye associated symptoms, on a healthy ocular surface. Through corneal surface temperature regulation, F6H8 has been shown to increase blinking and tearing thus contributing to alleviate dry eye disease and additional ocular pathologies.

Major efforts are aimed at developing topical therapeutic options to treat neuropathic pain of the cornea. After providing the criteria to distinguish patients with corneal neuropathic pain from those with non-neuropathic ocular discomfort that can be associated with inflammation or dry eye disease, Nortey et al. revise the findings on the efficacy of topical corticosteroids in patients with dry eye and corneal neuropathic pain. In corneal neuropathic pain, serum tears have been described to be of some help in patients experiencing discomfort to light. In addition, topical lacosamide has been shown to exert beneficial effects by decreasing the hyperexcitability of corneal cold-sensitive nerve terminals. Finally, eye drops of naltrexone, an opioid antagonist, have been found to ameliorate corneal neuropathic problems and their efficacy are under active investigation together with topical encephalin modulators as potential pain therapeutics.

Nociceptive pain is targeted through the use of topical therapies, and oral agents or adjunctive therapies can be used if a neuropathic component is highly suspected. Treatment outcomes for nociceptive ocular pain have been more studied than those for neuropathic ocular pain, mostly because most therapies are oriented against nociceptive inflammatory ocular pain, and less have been focused against neuropathic ocular pain. In a retrospective study involving patients with a clinically diagnosed neuropathic ocular surface pain, Patel et al. examine the individual response to different treatments with the aim of studying subjective clinical responses to a number of commonly utilized medications. The individual variability in treatment responses points to the necessity of future research aimed to develop diagnostic tests that can localize nervous system abnormalities together with application of personalized approaches that combine oral, topical or adjuvant medications.

Preclinical evidence about the efficacy of topical gabapentin on neuropathic ocular pain is provided by Cammalleri et al. in a rabbit model system in which eye drops with gabapentin exert analgesic effects coupled to stimulation of tear secretion. Secretagogue efficacy of gabapentin involves both a stimulation of the autonomic nervous system and a direct activation of intracellular signaling cascades, including the PKA/CREB pathway, culminating in the increased expression of aquaporin 5 in the lacrimal gland through mechanisms that remain to be elucidated.

In conclusion, we believe that the collection of papers that are included in this Research Topic represent the state of the art of the present knowledge on corneal pain, and we hope that it can be of inspiration to those scientists who are working on this subject, and to those who are approaching this fascinating research topic.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

OPINION
published: 02 June 2021
doi: 10.3389/fnins.2021.683045

Individual Traits and Pain Treatment: The Case of Hypnotizability

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Keywords: hypnotic susceptibility, cognitive pain control, suggestions, analgesia, imagery, autonomic, immune system, cortical plasticity

INTRODUCTION

Pharmacological, physical and cognitive treatments reduce pain by addressing all pain dimensions. Nonetheless, drugs may be ineffective, and physical activity is not always viable. In contrast, cognitive therapies have usually good outcomes, a wide range of applicability and no side effects. Their efficacy, however, is influenced by cognitive and psychophysiological traits. In this Opinion article hypnotizability is used as a model to support the view that specific psychophysiological traits and cognitive strategies can not only reduce pain, but also modulate the pain-related autonomic and immune activity, induce cortical plasticity relevant to pain control, and assist in the choice of the most appropriate treatment.

Hypnotizability, or hypnotic susceptibility, is a multidimensional trait stable through life (Piccione et al., 1989) and measured by validated scales (Elkins et al., 2015) classifying highly (highs), medium (mediums), and low hypnotizable subjects (lows). It is associated with brain morpho-functional peculiarities (Landry et al., 2017; Picerni et al., 2019) and displays correlates in the sensorimotor (Ibáñez-Marcelo et al., 2019; Santarcangelo and Scattina, 2019), cardiovascular (Jambrik et al., 2004a,b, 2005; Santarcangelo et al., 2012) and cognitive-emotional domain (Diolaiuti et al., 2019). Both highs and lows represent about 15% of the population which consists mainly of mediums (70%).

In healthy subjects the ability to control pain through suggestions for analgesia is linearly correlated with hypnotizability scores (Fidanza et al., 2017). Hypnotic treatments, however, are particularly important for patients with neuropathic and musculo-skeletal pain (Castel et al., 2007; Carli et al., 2008; Jensen et al., 2009a,b; Jensen and Patterson, 2014), which are seldom responsive to pharmacological treatments. They have been found more effective than any other psychological intervention (Jensen et al., 2020), although high hypnotizability predicts better outcomes also in patients, owing to the highs' greater high proneness to modify their bodily condition according to suggestions, and, thus, to relax (De Benedittis et al., 1994), to their peculiar imagery abilities (Ibáñez-Marcelo et al., 2019), and to their attitude to be deeply absorbed in their own mental images (Vanhaudenhuyse et al., 2019).

SUGGESTIONS FOR ANALGESIA

The suggestions for analgesia are requests to imagine that the experienced pain is out of the body or limited to a small part of it, or that a glove prevents one to perceive any nociceptive stimulation.

They are effective on acute/procedural, post-surgery and chronic pain (Jensen and Patterson, 2014; Facco, 2016) and, as most suggestions (Green and Lynn, 2011; Santarcangelo, 2014), can be efficaciously administered in the ordinary state of consciousness, thus not necessarily following the induction of the hypnotic state (Derbyshire et al., 2009; Paoletti et al., 2010; Santarcangelo et al., 2012). In highs, suggestions-induced analgesia, which can be focused on the sensory and/or

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *19 March 2021*
Accepted: *05 May 2021*
Published: *02 June 2021*

Citation:
Santarcangelo EL and Carli G (2021) Individual Traits and Pain Treatment: The Case of Hypnotizability. Front. Neurosci. 15:683045. doi: 10.3389/fnins.2021.683045

emotional dimension of pain, is not accompanied by release of endogenous opiates, but is sustained by the modulation of the activity and connectivity of the pain matrix (Faymonville et al., 2006; Casiglia et al., 2020).

Interestingly, the suggestions for analgesia have been found effective also in healthy mediums undergoing nociceptive stimulation (Fidanza et al., 2017) and in chronic pain patients independently from hypnotizability (Elkins et al., 2007; Jensen, 2011; Jensen and Patterson, 2014; Mazzola et al., 2017; Facco et al., 2018; Sandvik et al., 2020). This can be accounted for by expectation of/motivation to analgesia (Milling et al., 2005; Krystek and Kumar, 2016; Montgomery et al., 2018; Perri et al., 2020) leading to placebo responses (Benedetti, 2013) which can reduce pain and pain-related psychological symptoms in the general population (Liossi et al., 2006; Brugnoli, 2016; Wortzel and Spiegel, 2017; Rousseaux et al., 2020). Thus, suggestions may induce non opioid analgesia in highs, opioid placebo responses in lows and, probably, mixed reactions in mediums. It is particularly interesting, in this respect, that, during hypnotic sessions, oxytocin – the hormone promoting social relationships and acquiescent behavior - is released in the hypnotist and the client and that, in the latter, the lower the hypnotizability score the larger the OXT release. A further contribution to the hypnotist-client relation could be the level of intimacy which has been associated with the polymorphism of the serotonin transporter 5-HTTLPR gene. Its variant associated with greater efficiency is not significantly associated with hypnotizability but may enhance the experience of "rapport" independently from it (Katonai et al., 2017). In brief, suggested analgesia occurs in the general population, although through different mechanisms (Santarcangelo and Consoli, 2018). In addition, in contrast to "constructive imagery" (inducing sensory experiences in the absence of actual stimulations), obstructive suggestions such as analgesia and anesthesia aimed at reducing the perception of actual sensory stimulations can be experienced also by lows if they report mental images as vivid as highs do (Santarcangelo et al., 2010). Thus, in lows, imagery and placebo responses could co-operate in the response to suggestions for analgesia.

NEUROTRANSMITTERS

In the absence of explicit suggestions for analgesia, hypnotizability related differences in pain thresholds (Hilgard, 1967; Agargün et al., 1998; Santarcangelo et al., 2013; Kramer et al., 2014) and perceived pain intensity (Santarcangelo et al., 2010) have been seldom reported. Several studies, however, describe hypnotizability-related differences in genetic polymorphisms and brain neurotransmitters content which may be relevant to pain control in the presence of suggestions and/or to the choice of pain treatments. In fact:

a. highs display the variant of OPMR1 receptors (A118G, rs1799971) characterized by low sensitivity to opiates, high consumption of opioids for post-surgery and cancer pain and low placebo responsiveness more frequently than lows, with mediums displaying intermediate frequencies (Santarcangelo and Consoli, 2018). Thus, opioid treatments are not the most appropriate in highs.
b. the Fatty-Acids- Amino-Hydrolase (FAAH) C385A polymorphism (rs324420) responsible for endocannabinoids (eCBs) degradation is not significantly different between hypnotizability groups but the polymorphism frequencies indicate a trend to higher degradation efficiency from lows to highs (Presciuttini et al., 2020). We may hypothesize that small differences in the eCBs content could be amplified by the eCBs interactions with nor-adrenegic (Scavone et al., 2013) and dopaminergic pathways (Di Filippo et al., 2008). Thus, a contribution of the FAAH polymorphism to the highs' ability to control pain by suggestions for analgesia should not be excluded.
c. oxytocin (OXT), which modulates the sensory and emotional components of pain (Poisbeau et al., 2018), can contribute to the highs' suggestions induced analgesia through activation of the endogenous opioid system and by regulating the eCBs production (Russo et al., 2012). In fact, the polymorphism of the OXT receptor gene associated with high sensitivity (rs53576) is more frequent in highs than in the general population (Bryant et al., 2013).
d. brain nitric oxide (NO) promotes the release of brain dopamine and noradrenaline (Ghasemi et al., 2019), which are involved in pain control. According to post-occlusion flow mediated dilation (FMD), the endothelial NO release at peripheral level is reduced in lows and in the general population, but not in highs (Jambrik et al., 2004a,b; Jambrik et al., 2005). If confirmed at brain level, a continuous release of endothelial NO might amplify the availability of nor-adrenaline and dopamine in highs.

AUTONOMIC AND IMMUNE ACTIVITY

The autonomic and immune activity are strictly related to each other (Pavlov et al., 2018; Walters, 2018; Blake et al., 2019; Elkhatib and Case, 2019; Iovino et al., 2020) in that the former modulates the immune activity (Elenkov et al., 2000; Jänig, 2014; Martelli et al., 2014) and the latter can regulate the function of brain autonomic centers (Elsaafien et al., 2019).

The mechanisms controlling acute inflammation and the associated pain are quite different from those controlling chronic inflammation and chronic pain. In particular, the pro-inflammatory cytokines produced in response to an acute body lesion excite the central nervous system by the activation of vagal afferents and, after penetration through the blood brain barrier, of brain structures which, in turn, generate anti-inflammatory responses. The networks involved in the inflammatory inhibition are: (a) the parasympathetic circuit, limited to vagal afferents and efferents; (b) the parasympathetic-neuroendocrine circuit, which is responsible for the release of corticosteroids; (c) the cytokine-vagal-sympathetic circuit, involving noradrenergic pathways and adrenal epinephrine (Pavlov et al., 2018). In the latter circuit, the mechanisms inhibiting acute inflammation and pain are distinct, triggered by specific contextual/environmental stimuli in animals and by psychological interventions in humans (Bassi et al., 2018).

High hypnotizability is associated with pre-eminent parasympathetic control of heart rate during relaxation in the awake condition with respect to lows (Santarcangelo et al., 2012), with a further shift toward parasympathetic tone after hypnotic induction (De Benedittis et al., 1994), and with greater proneness to reduce sympathetic activation during suggestions of unpleasant experiences associated with instructions for relaxation and well-being (Sebastiani et al., 2007). In contrast, and at variance with cortical and somatic correlates (Santarcangelo and Consoli, 2018), the findings of hypnotizability-related reduction of sympathetic activity associated with suggestion-induced analgesia in healthy subjects are inconsistent (De Pascalis et al., 2001; Paoletti et al., 2010; Santarcangelo et al., 2013). Theoretically, however, the autonomic peculiarities of high hypnotizable individuals – parasympathetic prevalence - should be associated with a more effective immune activity. Hypnotic treatments, in fact, upregulate the expression of immune-related genes in lymphocytes (Kovács et al., 2008), reduce salivary cortisol (Thompson et al., 2011) and immunoglobulin A in surgical patients with breast cancer (Minowa and Koitabashi, 2014), regulate auto-immune disorders (Torem, 2007), human papillomavirus (Barabasz et al., 2010), and pro-inflammatory/anti-inflammatory cytokines in elders (Sari et al., 2017).

CORTICAL PLASTICITY

An ambitious target for chronic pain treatments should be counteracting the disadvantageous cortical plasticity associated with chronic pain, consisting of alteration in the brain gray matter volume (Xiong et al., 2017; McCarberg and Peppin, 2019; Yin et al., 2020) and in long-term potentiation in the anterior cingulate cortex and insular cortex (Zhuo, 2020).

In chronic pain patients Transcranial Magnetic Stimulation (TMS) and electrical direct Transcranial Stimulation (dTCS) are efficaciously used to modulate the activity of pain-related circuits (Klein et al., 2015; Dos Santos et al., 2018; Meeker et al., 2020) together with vagal stimulation (Costa et al., 2019). Theoretically, imaginatively induced analgesia could influence cortical plasticity (Kleim and Jones, 2008) mimicking the effects of TMS and dTCS by suggestions aimed at modulating the activity of the pain matrix (Casiglia et al., 2020) and enhancing the action of descending antinociceptive pathways (Beltran Serrano et al., 2020). The highs' stronger functional equivalence between imagery and perception/action (Ibáñez-Marcelo et al., 2019) and their greater cortical excitability (Spina et al., 2020), in fact, allow them to experience pleasant situations able to buffer the activity of the pain matrix, thus promoting the cognitive re-appraisal of their pain condition. In addition, the activity of the pain matrix itself can be reduced by suggestions (Faymonville et al., 2006; Casiglia et al., 2020) and co-operate to promote long-lasting effects. Suggestive treatment of pain, in fact, induces long-lasting analgesic effects addressing all pain dimensions (Dillworth and Jensen, 2010; Jensen et al., 2014). Of note, cortical long-lasting plasticity is induced also by neutral hypnosis that is the state experienced by highs after hypnotic induction in the absence of specific suggestions (Jiang et al., 2017).

CONCLUSIONS

The pain matrix structure, activity and connectivity (Legrain et al., 2011) are influenced by acute and chronic pain. Our opinion is that that pain experience and physiology are modulated by the physiological correlates of hypnotizability, and that hypnotic assessment may assist in the choice of the most appropriate pharmacological treatments (a); the suggestions for analgesia are effective in both wakefulness and hypnosis and can control pain in a large majority of the general population, although through different mechanisms (b); hypnotizability is an advantageous factor in the control of pain-related autonomic and immune functions (c); hypnotizability-related cortical plasticity may counteract the effects of chronic pain on the structure and function of the pain matrix (d). In conclusion, suggestions for analgesia should be considered for any pain patient and not only after unsuccessful pharmacological treatments.

AUTHOR CONTRIBUTIONS

The authors equally contributed to the paper and agreed on its content.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 07 June 2021
doi: 10.3389/fphar.2021.671238

Effects of Topical Gabapentin on Ocular Pain and Tear Secretion

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Neuropathic ocular pain is a frequent occurrence in medium to severe dry eye disease (DED). Only palliative treatments, such as lubricants and anti-inflammatory drugs, are available to alleviate patients' discomfort. Anesthetic drugs are not indicated, because they may interfere with the neural feedback between the cornea and the lacrimal gland, impairing tear production and lacrimation. Gabapentin (GBT) is a structural analog of gamma-amino butyric acid that has been used by systemic administration to provide pain relief in glaucomatous patients. We have already shown in a rabbit model system that its topic administration as eye drops has anti-inflammatory properties. We now present data on rabbits' eyes showing that indeed GBT given topically as eye drops has analgesic but not anesthetic effects. Therefore, opposite to an anesthetic drug such as oxybuprocaine, GBT does not decrease lacrimation, but–unexpectedly–even stimulates it, apparently through the upregulation of acetylcholine and norepinephrine, and by induction of aquaporin 5 (AQP5) expression in the lacrimal gland. Moreover, data obtained *in vitro* on a primary human corneal epithelial cell line also show direct induction of AQP5 by GBT. This suggests that corneal cells might also contribute to the lacrimal stimulation promoted by GBT and participate with lacrimal glands in the restoration of the tear film, thus reducing friction on the ocular surface, which is a known trigger of ocular pain. In conclusion, GBT is endowed with analgesic, anti-inflammatory and secretagogue properties, all useful to treat neuropathic pain of the ocular surface, especially in case of DED.

Keywords: neuropathic ocular pain, dry eye syndrome, corneal sensitivity, lacrimal gland, autonomous nervous system, aquaporin 5, corneal epithelial cells, PKA/CREB pathway

OPEN ACCESS

Edited by:
Giacinto Bagetta,
University of Calabria, Italy

Reviewed by:
Damiana Scuteri,
University of Calabria, Italy
Carlo Nucci,
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *23 February 2021*
Accepted: *25 May 2021*
Published: *07 June 2021*

Citation:
Cammalleri M, Amato R, Olivieri M, Pezzino S, Bagnoli P, Dal Monte M and Rusciano D (2021) Effects of Topical Gabapentin on Ocular Pain and Tear Secretion. Front. Pharmacol. 12:671238. doi: 10.3389/fphar.2021.671238

INTRODUCTION

Neural regulation plays an integral role in maintaining ocular surface homeostasis by tightly controlling lacrimal gland secretion of tear film containing water, electrolytes and a variety of proteins (Dartt, 2009). Disruption of ocular surface homeostasis as induced by altered activity of the feedback loop between the corneal surface and the lacrimal gland causes disturbing effects of which ocular pain is a major component. The cornea has the densest sensory innervation in the human body and has the potential to be a powerful producer of pain (Galor, 2019). In case of corneal injuries the ciliary nerves innervating the cornea increase their activity, finally resulting in corneal hypersensitivity (Joubert et al., 2019). Stimulation of corneal sensory nerves promotes lacrimal gland secretion by the so-called tear reflex. Sensory afferents from the nerve endings on the corneal surface stimulate efferent sympathetic and parasympathetic fibers of the facial nerve that innervate the lacrimal gland via feedback loops between the ocular surface, lacrimal gland and brain. Activated parasympathetic and sympathetic nerves release acetylcholine (Ach) and norepinephrine (NE),

although other different peptides can also be involved in the autonomic transmission (Russo, 2017). Released neurotransmitters stimulate distinct receptors, thus triggering a cascade of second messenger components activating ion channels and pumps to cause electrolyte, water and protein secretion (Dartt, 2009). Significant involvement of water channels in regulating lacrimal fluid secretion has been demonstrated (Delporte, 2009) with a major role played by aquaporin 5 (AQP5) in both the cornea and the lacrimal gland (Verkman, 2003). A disturbance of the neural feedback loops between the ocular surface and lacrimal glands can contribute to corneal diseases such as dry eye disease (DED), in which both nociceptive and neuroptahic pain may be involved (Galor et al., 2015).

Generally speaking, DED is a multifactorial pathology affecting the ocular surface of the eye, associated with pain that may arise from inflammation, reduction in the volume and/or quality of tears, and damage to the sensitive nerve endings located in the cornea (Galor, 2019). The treatment of DED is palliative, using artificial tears that provide temporary symptomatic relief, but do not address the underlying pathophysiology of the syndrome. Ideally, a complete treatment for neuropathic pain in DED should address tear dysfunction and the associated inflammation, and provide an analgesic effect to soothe the pain without reducing lacrimation.

Different drugs are commonly used to treat ocular pain, including anti-inflammatory, anesthetic and analgesic drugs (Jacobs, 2017). Analgesic drugs include gabapentin (GBT), a structural analog of gamma-amino butyric acid that has been introduced as an adjunctive therapy in epilepsy and is presently widely used to treat several kinds of neuropathic pain. The possibility that GBT may counteract ocular pain has been suggested by several studies (Lichtinger et al., 2011; Pakravan et al., 2012; Wei et al., 2015; Ongun and Ongun, 2019; Yoon et al., 2020), since the first demonstration of systemic GBT efficacy in providing significant pain relief in glaucomatous patients (Kavalieratos and Dimou, 2008). At least some of the clinical effects of GBT are due to high affinity interactions with the α2δ1 auxiliary subunit of presynaptic voltage-gated calcium channels (Taylor and Harris, 2020). However, more work needs to be done to fully understand the mechanisms through which GBT may target ocular tissues, and ameliorate ocular pain. In this respect, the recent finding that eye drops containing GBT exert major anti-inflammatory activity *in vitro* and *in vivo* (Anfuso et al., 2017) suggests the possibility that the analgesic effect of GBT coupled to its anti-inflammatory properties may confer a better ability to an eye drop formulation to treat ocular pain.

The present study stems from the consideration that anesthetics are not best indicated to fight neuropathic ocular pain, especially in case of dry eye, because they are expected to blunt the nervous feedback between the cornea and the lacrimal apparatus, thus inhibiting lacrimation. GBT, on the other hand, being analgesic and not anesthetic, should be devoid of such negative effects on lacrimation. Therefore, we designed *in vivo* experiments in the rabbit, aimed at investigating whether GBT, topically administered, may blunt cornea hypersensitivity induced by formaldehyde and whether its analgesic efficacy interferes with tear secretion regulation. Our unexpected finding that GBT stimulates lacrimation prompted us to address the contribution of the autonomic innervation to GBT-associated regulation of tear production, together with the possibility that GBT might act by regulating AQP5 levels in the lacrimal gland. In these experiments, pretreatment with the anesthetic drug oxybuprocaine (benoxinate: BNX), a topical anesthetic that reduces basal lacrimation (Shiono, 1989) and is mainly used to blunt the activity of corneal nociceptors (Nakamachi et al., 2016), was used to evaluate whether GBT acts indirectly through a modulation of the tear reflex or directly onto the lacrimal gland. In addition, as corneal epithelial cells participate in determining the final tear composition by secreting proteins, electrolytes, and water (Santagati et al., 2005; Meng and Kurose, 2013), further *in vitro* studies with primary human corneal epithelial (HCE-F) cells were carried out to show that GBT - but also BNX - might also directly trigger AQP5 expression through a molecular pathway involving PKA/CREB (Wang and Zheng, 2011).

MATERIALS AND METHODS

Animals

New Zealand albino rabbits (32 males and 37 females, body weight 2.54 ± 0,26 kg, aged 170–190 days) were purchased from a local supplier. Animals were kept at a temperature of 22°C and a relative humidity of 50%. Each rabbit was kept in a single cage and provided with standard rabbit feed and drinking water.

Cultured Cells

Primary HCE-F cells (Cristaldi et al., 2020) were seeded in complete culture medium at a density of 5×10^5 cells/well in a six-well plate and left to adhere overnight in the incubator at 37°C and 5% CO_2. In a first set of experiments, cells were treated in serum-free medium (SFM) with GBT at 0.01, 0.1 or 1 mg/ml for 24 h. In rabbit corneal epithelial cells, GBT at 0.01 and 0.1 mg/ml did not affect cell viability, which was modestly affected by GBT at 1 mg/ml (Anfuso et al., 2017). In a second set of experiments HCE-F cells were treated in SFM with BNX (BP700; Sigma-Aldrich, St. Louis, MO, United States) at 0.03 or 0.15 mg/ml, either alone or in combination with 0.1 mg/ml of GBT for 12 h. Neither GBT nor BNX affected HCE-F cell survival at the tested concentrations. At the end of each incubation period cells were scraped and stored at -80°C until use.

Pharmacokinetics

Thirty microliters of a GBT (sc-201481; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) formulation made 2% in phosphate buffered saline (PBS) were instilled twice at an interval of 2 min in each eye of six New Zealand white rabbits. At 30, 60, and 120 min, two animals per each time point were euthanized, and the four eyes dissected to take separately cornea, conjunctiva and aqueous humor. A fixed amount of acetonitrile and H_2O (50:50) solution was added to the biological samples to extract GBT. After mechanical trituration by Ultra-turrax (cornea and

conjunctiva) and subsequent sonication, samples were centrifuged at 10,000 rpm for 15 min, and GBT contained in the supernatant was quantified by HPLC/MS/MS using the Agilent 6410-A triple quadrupole instrument equipped with a Phenomenex Kinetex C18 column at 25°C, under isocratic conditions using 92% of Buffer A (5 mM ammonium formate pH = 3.0) and 8% of acetonitrile, at a flow rate of 0.1 ml/min. The system is equipped with a positive ionizing mode ESI interface, such that the mass transition for GBT is 172.2 → 154.2 m/z. The operational MS parameters of the instrument were: Gas Temperature 350°C; Gas Flow 8 L/min; Nebulizer 20 psi; Capillary 3500 V; Collision energy 30 V; Dwell-time 70 msec; Fragmentor 50 V. Calibration curves were made in blank tissue extracts (cornea, conjunctiva, aqueous humor) by adding exact amounts (between 2 and 400 ng/ml) of GBT. Values were expressed as ng/mg of tissue.

Corneal Sensitivity

Corneas were sensitized according to a previously published protocol (Lai et al., 2013), with some modifications. Formaldehyde 0.5% (30 µl) was instilled in the right eyes and 1 min after application the eyes were washed with sterile saline to remove residual formaldehyde. Left eyes were left untreated as controls. After formaldehyde application, no evident signs of corneal or conjunctival toxicity including signs of chemical trauma, were observed. The corneal sensitivity was evaluated with a Cochet-Bonnet esthesiometer (nylon thread; 0.12 mm diameter; length variable between 5 and 60 mm; Luneau, Paris, France). The nylon filament of the esthesiometer touched the center of the cornea perpendicularly. A positive response was recorded if > 5 reflexes occurred in 10 consecutive touches. The longest filament length causing a positive result was considered the corneal sensitivity threshold. 5 min after formaldehyde instillation, 30 µl of sterile saline or 2% GBT were instilled in both eyes in the corneal-limbic junction. This route of administration was chosen because it is the intended way of human exposure to the test formulation. At progressing times corneal sensitivity was tested. Whether GBT might act as an anesthetic was evaluated by comparing the effects of 30 µl of either 2% GBT or 0.4% BNX instilled in the eyes of rabbits that did not receive formaldehyde. The number of stimuli necessary to induce a blinking reflex maintaining a fix length of 7.5 mm was counted over time. In any case, the number of mechanical stimuli applied was 10 in order to limit corneal damage.

Schirmer's Tear Test

The secretory effect of the test compounds was evaluated after the instillation of 30 µL of either 2% GBT or 0.4% BNX. In some experiments, BNX was applied 15 min before GBT instillation. Tear secretion was measured using commercially available Schirmer's tear test strips (Contacare Ophthalmics and Diagnostics, Dunstable, United Kingdom), as previously reported (Honkanen et al., 2021). Briefly, in each eye the strip was placed in the mid portion of the lower lid at progressing times and tear production was recorded as the length of moistened strip at 1 min. Then, the heads of the strips were cut and stored at -80°C until use to evaluate tear protein concentration.

Tear Protein Concentration

Tear proteins were extracted from the heads of the Schirmer's tear test strips by elution with 100 µl of 100 mM ammonium bicarbonate containing 0.25% NP-40 with addition of proteinase inhibitors (Roche Applied Science, Indianapolis, IN), as previously described (Yu et al., 2018). Samples were incubated on a rotator overnight at 4°C. Then they were centrifuged, and protein concentration was measured with the Micro BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, United States).

Tissue Harvesting

After the administration of GBT, BNX or BNX before GBT, at progressing times rabbits were narcotized with a mixture of ketamine (40 mg/ml) and xylazine (10 mg/ml) and sacrificed with an overdose of Nembutal (80 mg/kg). Untreated rabbits were sacrificed as well. Lacrimal glands, cornea and conjunctiva were removed without removal of the eye bulb. The brain was removed and hippocampus isolated. All tissues were rinsed in sterile saline and stored at −80°C until use.

ELISA

Before the use, lacrimal glands were cut in smaller pieces, mixed, and randomly divided for ELISA or Western blotting. To determine the content of parasympathetic and sympathetic neurotransmitters, lacrimal glands were homogenized in PBS in the presence of proteinase inhibitors (Roche Applied Science). Homogenates were centrifuged at 5,000 rpm for 5 min and supernatants were immediately used. Protein concentration was measured with the Micro BCA Protein Assay (Thermo Fisher Scientific). Ach and NE content were evaluated by using commercially available ELISA kits, according to the manufacturer instructions (Acetylcholine ELISA kit, OKEH02568, Aviva Systems Biology, San Diego, CA, United States and NA/NE ELISA kit, MBS760375, MyBioSource, San Diego, CA, United States, respectively). Ach and NE levels were calculated by interpolating the absorbance of each sample against the respective calibration curves using lyophilized Ach and NE standards available in the kits.

Quantitative Real Time PCR

To perform quantitative real time PCR (qPCR), total RNA was extracted and purified from rabbit tissues (hippocampus, lacrimal gland, cornea, conjunctiva) and HCE-F cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, United States). First-strand cDNA was generated from 1 µg of total RNA (QuantiTect Reverse Transcription Kit, Qiagen). Real-time PCR amplification was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, United States) on a CFX Connect Real-Time PCR detection system and software CFX manager (Bio-Rad Laboratories). qPCR primer sets were chosen to hybridize to unique regions of the appropriate gene sequence: α2δ1 (Forward: 5′-AGACCC TTCACTGTTGTGGC-3′; Reverse: 5′-ACCCATGGAGAAGCT GGGTA-3′); GAPDH (Forward: 5′-CCGCTTCTTCTCGTG CAGTG-3′; Reverse: 5′-CAATGCGGCCAAATCCGTT-3′). Samples were compared using the relative threshold cycle (Ct Method). The increase or decrease (fold change) was determined

TABLE 1 | List of antibodies used in Western blot.

Antibody	Dilution	Source	Cat. No
Mouse monoclonal anti α2δ1	1:1,000	Thermo Fisher Scientific	MA3-921
Mouse monoclonal anti-AQP5	1:500	Santa Cruz Biotechnology, Inc	sc-514022
Rabbit polyclonal phosphor (Ser/Thr) PKA	1:1,000	Cell Signaling	9621S
Rabbit polyclonal anti-PKA C-alpha	1:1,000	Cell Signaling	4782S
Rabbit polyclonal anti-phospho CREB (Ser133) (87G3)	1:1,000	Cell Signaling	9198S
Mouse monoclonal anti-CREB (86B10)	1:1,000	Cell Signaling	9104S
Mouse monoclonal anti-β-actin	1:2,500	Sigma-Aldrich	A2228
Mouse monoclonal anti-Histone H1 clone AE-4	1:2,000	Sigma-Aldrich	05–457

relative to the hippocampus after normalization to GAPDH, used as the housekeeping gene.

Western Blotting

Samples (hippocampus, lacrimal glands, cornea, conjunctiva, or HCE-F cells) were homogenized in RIPA buffer containing phosphatase and proteinase inhibitor cocktails (Roche Applied Science) or in a nuclear and cytoplasmic extraction buffer (NE-PER Kit, 78,833, Thermo Fisher Scientific). Protein concentration was measured with the Micro BCA Protein Assay (Thermo Fisher Scientific). Samples (30 μg proteins each) were run on 4–20% or 4–12% SDS-PAGE gels and proteins were then transferred on nitrocellulose membranes. Blots were blocked for 1 h with 5% skim milk and incubated overnight at 4°C with the primary antibodies indicated in **Table 1** using β-actin or histone H1 as loading controls. Blots were then incubated for 1 h with HRP-conjugated secondary antibodies (1:5,000) and developed with the Clarity Western enhanced chemiluminescence substrate (Bio-Rad Laboratories). Images were then acquired (ChemiDoc XRS+; Bio-Rad Laboratories). The optical density of the bands was evaluated (Image Lab 3.0 software: Bio-Rad Laboratories). Protein expression level was normalized against β-actin (non-phosphorylated targets) or total non-phosphorylated corresponding protein (phosphorylated targets).

Statistics

Data were analyzed by the Shapiro-Wilk test to verify their normal distribution. Statistical signiflcance was evaluated with Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, United States) using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-test or two-way ANOVA followed by Bonferroni multiple comparison post-test. Data are expressed as means ± SEM of the reported n values. Differences with $p < 0.05$ were considered significant. In compliance with the 3Rs principles for ethical use of animals in scientific research, an a priori power analysis was conducted using the software G*Power 3.0.10 (www.gpower.hhu.de) to determine the minimum number of animals necessary to obtain a statistical power of at least 0.80, with $\alpha = 0.05$, in the presence of a large effect size as expected in these studies.

RESULTS

Gabapentin Pharmacokinetics and Effects on Corneal Sensitivity and Lacrimation

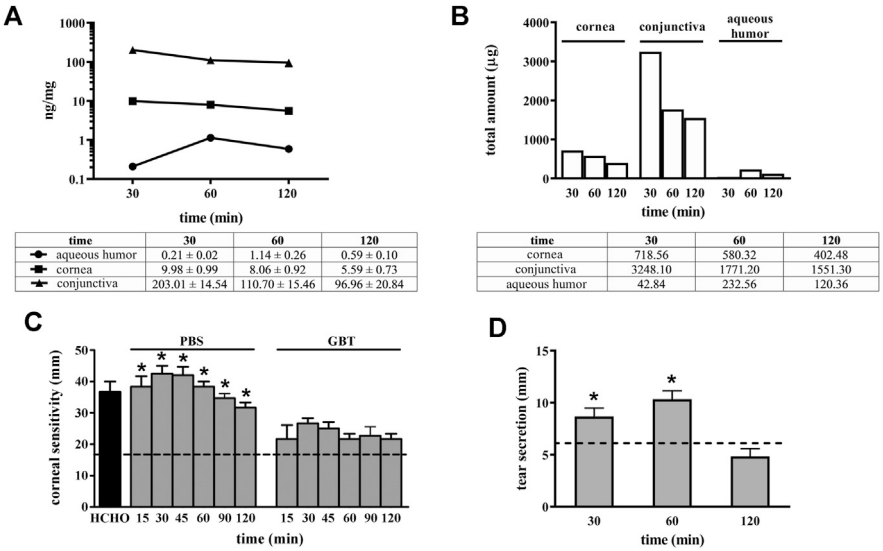
The distribution of GBT eye drops (2% in PBS) has been evaluated in aqueous humor, cornea and conjunctiva. **Figure 1A** shows the GBT concentration per mg of tissue, which results always higher on the ocular surface (conjunctiva and cornea), though slowly decreasing with time (after 120 min there is, respectively, still 50 and 60% of the amount determined at 30 min), while the aqueous humor contains the lowest amount, peaking at 60 min, and then decreasing. **Figure 1B** illustrates the total amount calculated per tissue. To this purpose, we have experimentally estimated the average weight of each tissue in rabbits of 2.5 Kg and 3 months old at 72 mg for cornea, 160 mg for conjunctiva and 204 mg for aqueous humor, which is in line with the expectations (Struble et al., 2014).

It is thus evident that the most part of GBT (97.7% at 30 min, and 96.7% at 120 min) remains in the conjunctiva, from which it can be distributed to neighboring tissues, and the cornea contains most of the remaining amount (2.1% at 30 min, and 2.5% at 120 min).

GBT efficacy on neuropathic ocular pain is shown in **Figure 1C**. Normally, in untreated healthy rabbit eyes a thread length of 18 mm is the higher length able to induce a corneal reflex. After corneal injury with 0.5% formaldehyde, the corneal sensitivity increased by about 2-fold as compared to the basal level, and rabbit eyes responded with a blinking reflex at a thread length of about 40 mm. Control eye drops (PBS alone) did not affect this elevated corneal sensitivity that was instead significantly reduced by 2-fold (a response was elicited at a thread length of about 20 mm, similar to untreated control) after 2% GBT eye drops up to 120 min after their instillation.

In order to confirm that the analgesic effect of GBT eye drops did not decrease lacrimation, as it could be expected from a topical anesthetic, the effects on tear secretion of GBT eye drops was also evaluated. As shown in **Figure 1D**, 2% GBT not only did not decrease lacrimation, but–surprisingly–it significantly increased tear secretion in respect to the basal level at both 30 and 60 min, to lose its effect at 120 min.

Figure 2 shows how corneal sensitivity is modulated by GBT or by the administration of a classical ocular anesthetic (BNX) on naïve corneas. BNX is known to reduce basal lacrimation (Shiono, 1989) and is mainly used to blunt the activity of corneal nociceptors (Nakamachi et al., 2016). In this case, the thread of the esthesiometer was kept at a fixed short length of 7.5 mm, thus eliciting an immediate blinking reflex: one touch was already enough to stimulate a response (**Figure 2A**). The instillation of GBT did not change corneal sensitivity at this rude touch (**Figure 2B**), while the instillation of the anesthetic BNX made the cornea much less responsive to the stimulation with the short



time	30	60	120
aqueous humor	0.21 ± 0.02	1.14 ± 0.26	0.59 ± 0.10
cornea	9.98 ± 0.99	8.06 ± 0.92	5.59 ± 0.73
conjunctiva	203.01 ± 14.54	110.70 ± 15.46	96.96 ± 20.84

time	30	60	120
cornea	718.56	580.32	402.48
conjunctiva	3248.10	1771.20	1551.30
aqueous humor	42.84	232.56	120.36

FIGURE 1 | Pharmacokinetic profile of GBT (2% in PBS) in ocular tissues and GBT analgesic effects. GBT was measured in aqueous humor, cornea and conjunctiva at different times. For each tissue, concentration profiles derived from four samples per treatment are reported in ng/mg of tissue **(A)**, or as total estimated amount in each tissue **(B)**. **(C)** Effects of 2% GBT on corneal pain. Longitudinal evaluation of corneal sensitivity using the Cochet-Bonnet esthesiometry in rabbits treated with PBS or GBT after the instillation of 0.5% formaldehyde. The dotted line indicates the basal value. Differences between groups were tested for statistical significance using one-way ANOVA with Tukey's multiple comparison post-test ($n = 6$). **(D)** Effects of 2% GBT in PBS on tear secretion. The dotted line indicates the basal value. Differences vs. basal secretion were tested for statistical significance using two-way ANOVA with Bonferroni multiple comparison post-test. *$p < 0.05$. All data are plotted as mean ± SEM.

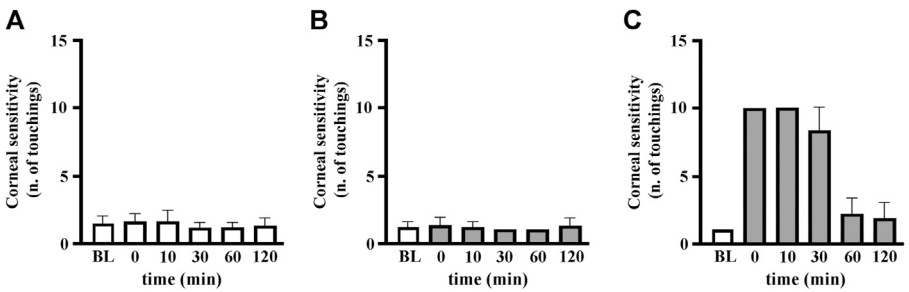


FIGURE 2 | Analgesic and not anesthetic activity of topical GBT. Rabbit eyes ($n = 6$) were instilled with PBS **(A)**, 2% GBT **(B)** or 0.4% BNX **(C)**, and corneal sensitivity was evaluated at different times by the number of touchings at minimal extension (0.75 mm) eliciting a blinking reflex. BL, baseline.

thread, requiring more than five touchings to elicit a blinking reflex (**Figure 2C**).

Gabapentin Effect on Aqueous Tear Secretion and Protein Concentration

To investigate further this unexpected effect of GBT eye drops, a more stringent kinetics of aqueous tear secretion and protein concentration was carried out, in order to better evaluate the effects of 2% GBT at different times after instillation, in comparison to a topical anesthetic (0.4% BNX). GBT produced a significant increase in aqueous tear secretion at 15 min, an effect which was maintained up to 90 min (white columns in **Figure 3A**). This secretagogue activity of GBT resulted in a significant decrease in protein concentration at 15 min, but basal protein levels were restored within 30 min (white columns in **Figure 3B**). In line with previous findings (Shiono, 1989), BNX reduced aqueous secretion by about 50% at 15 min, but basal levels were also recovered within 30 min (black columns in **Figure 3A**). The sudden decrease in the aqueous secretion was accompanied by an increase in protein concentration, which also recovered its basal level within 30 min (black columns in **Figure 3B**). Aqueous tear secretion and protein concentration were also measured following the administration of BNX 15 min before the instillation of GBT. In this protocol, pretreatment with BNX was found to prevent the effect of GBT on both aqueous tear secretion and protein concentration (gray columns in **Figures 3A,B**).

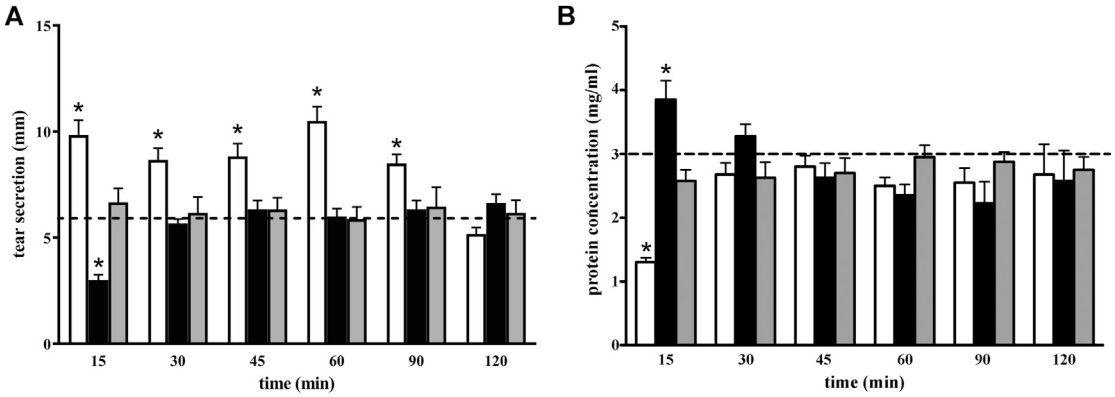


FIGURE 3 | Longitudinal evaluation of aqueous tear secretion **(A)** and total protein concentration **(B)** following 2% GBT (white columns), 0.4% BNX (black columns) or GBT after BNX pretreatment (gray columns). Data are plotted as mean ± SEM. The dotted line indicates the basal value. Differences between groups were tested for statistical significance using two-way ANOVA with Bonferroni multiple comparison post-test. *$p < 0.05$ relative to basal ($n = 6$ eyes).

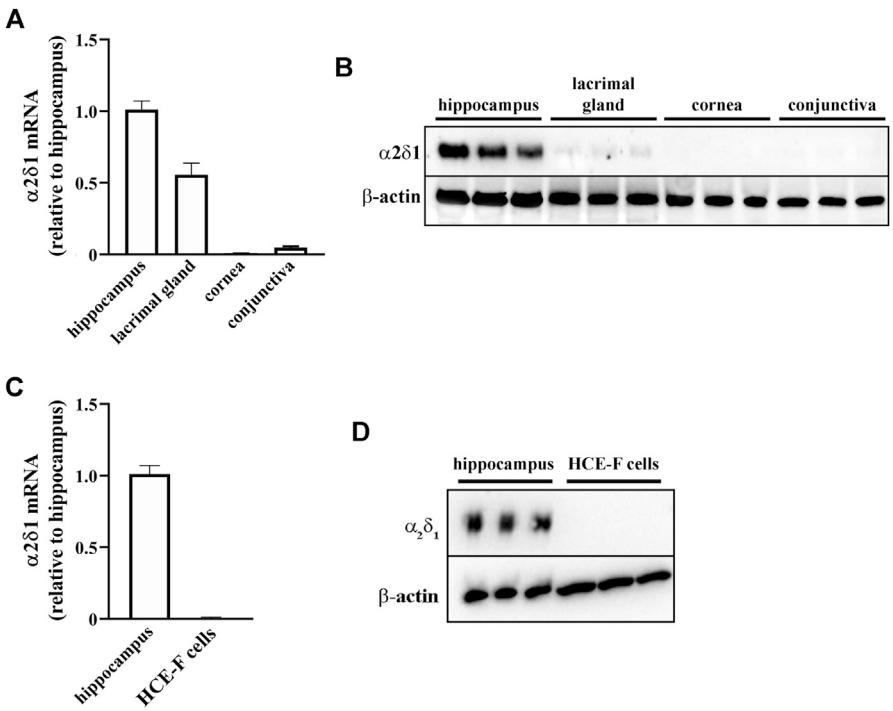


FIGURE 4 | Levels of α2δ1 in rabbit hippocampus, ocular tissues and HCE-F cells. **(A)** Transcript levels of α2δ1 in hippocampus, lacrimal gland, cornea and conjunctiva as determined by qPCR. **(B)** Representative blots depicting levels of α2δ1 in hippocampus, lacrimal gland, cornea and conjunctiva as determined by Western blotting. **(C)** Transcript levels of α2δ1 as determined by qPCR. **(D)** Representative blots depicting levels of α2δ1 in HCE-F as determined by Western blotting. Data are plotted as mean ± SEM ($n = 6$ hippocampus or tissue samples or $n = 5$ HCE-F cells).

To evaluate whether GBT may exert its secretagogue effect through the α2δ1 subunit of the voltage-gated calcium channels, which is a known target of GBT, we evaluated the expression of α2δ1 mRNA and protein in ocular tissues (lacrimal gland, cornea, conjunctiva). As positive control, α2δ1 expression was also evaluated in the hippocampus that is known to express this subunit (Schlick et al., 2010). As shown in **Figure 4**, α2δ1 was largely expressed in the hippocampus at both mRNA (**Figure 4A**) and protein (**Figure 4B**) levels, whereas α2δ1 mRNA was barely detectable in the cornea and conjunctiva (**Figure 4A**), with no evident protein expression (**Figure 4B**). In the lacrimal gland α2δ1 mRNA levels were about 50% lower than in the hippocampus (**Figure 4A**), although a much lower protein expression could be detected (**Figure 4B**). The evaluation of

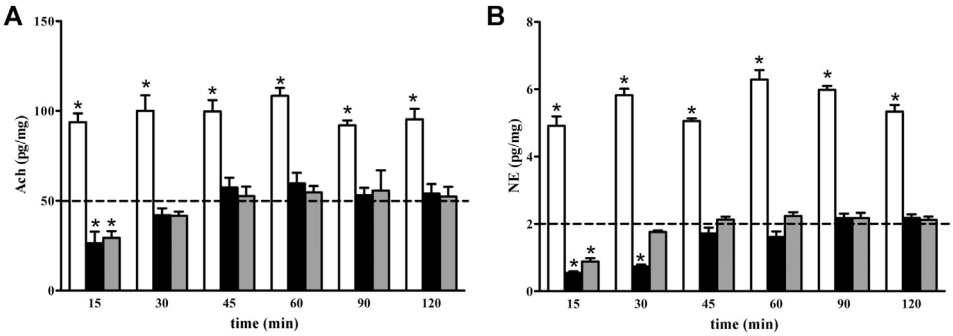


FIGURE 5 | Longitudinal evaluation of Ach **(A)** and NE **(B)** levels following 2% GBT (white columns), 0.4% BNX (black columns) or GBT after BNX pretreatment (gray columns). Data are plotted as mean ± SEM. The dotted line indicates the basal value. Differences between groups were tested for statistical significance using two-ways ANOVA with Bonferroni multiple comparison post-test. *$p < 0.05$ relative to basal, (n = 6 lacrimal glands).

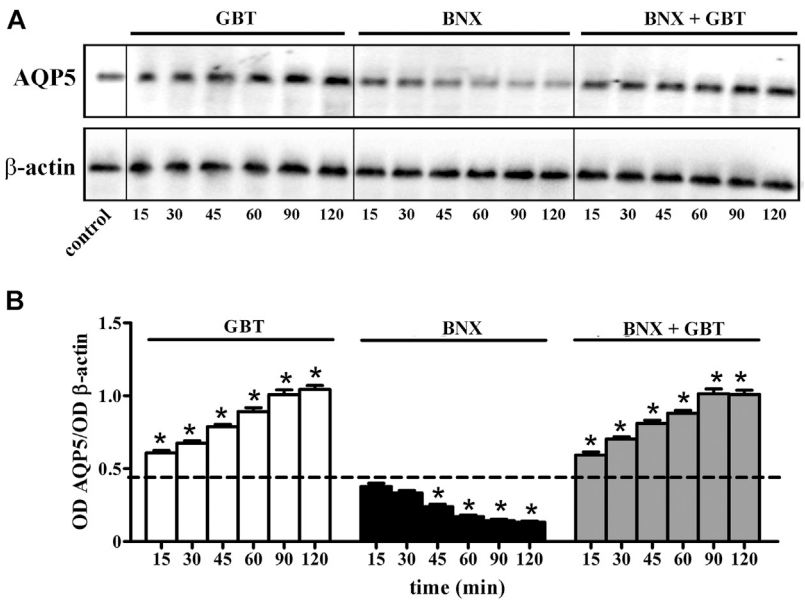


FIGURE 6 | Longitudinal evaluation of AQP5 levels in the lacrimal gland following 2% GBT, 0.4% BNX or GBT after BNX pretreatment. **(A)** Representative blots depicting levels of AQP5 as determined by Western blotting. **(B)** Densitometric analysis of AQP5 levels. The expression of AQP5 was relative to the loading control β-actin. Data are plotted as mean ± SEM. The dotted line indicates the basal value. Differences between groups were tested for statistical significance using one-way ANOVA with Tukey's multiple comparison post-test. *$p < 0.05$ relative to basal (n = 6 lacrimal glands).

mRNA and protein expression of α2δ1 in HCE-F cells demonstrated that α2δ1 is not expressed by human primary corneal epithelial cells (**Figures 4C,D**).

Gabapentin Effect on Acetylcholine and Norepinephrine Levels in the Lacrimal Gland

As shown in **Figure 5**, the levels of both Ach (**Figure 5A**) and NE (**Figure 5B**) were increased 15 min after GBT, an effect that persisted until 120 min (white columns in **Figures 5A,B**). On the contrary, BNX reduced Ach and NE levels with an effect that persisted up to 15 and 30 min, respectively, (black columns in **Figures 5A,B**). BNX pretreatment 15 min before GBT instillation prevented the GBT-induced increase in Ach and NE over the first 15 min after which basal levels were recovered (gray columns in **Figures 5A,B**).

Gabapentin Regulation of Aquaporin 5 Levels in the Lacrimal Gland

We evaluated whether the increased aqueous tear secretion induced by GBT might involve altered expression of AQP5 in the lacrimal gland. As shown in **Figure 6**, GBT progressively

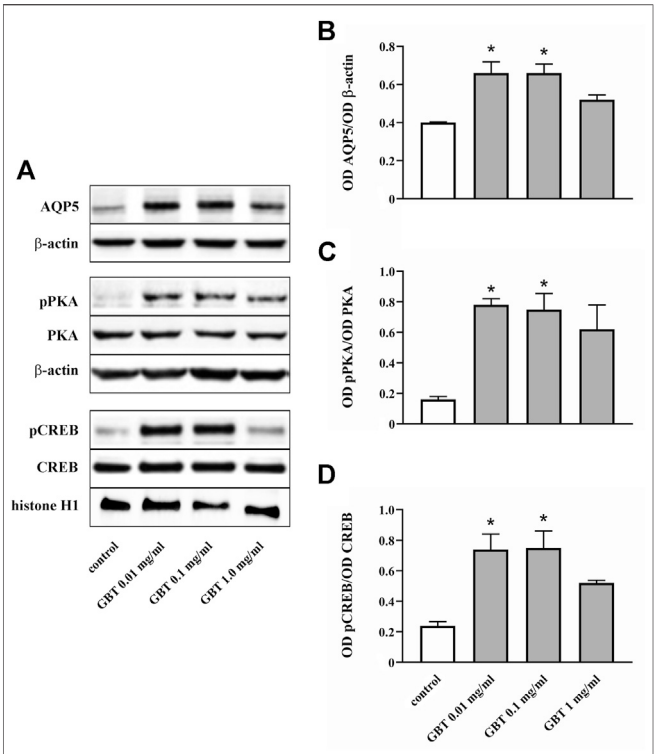


FIGURE 7 | Level of AQP5 and downstream mediators in HCE-F cells treated with GBT at 0.01, 0.1 or 1 mg/ml. **(A)** Representative blots depicting levels of AQP5, pPKA, PKA, pCREB, and CREB after treatment with GBT as determined by Western blotting. **(B-D)** Densitometric analysis of the respective levels. The expression of AQP5 was relative to the loading control β-actin, while the expression of pPKA and pCREB was normalized to the level of PKA and CREB, respectively. Data are plotted as mean ± SEM. Differences between groups were tested for statistical significance using one-way ANOVA with Tukey's multiple comparison post-test. *$p < 0.05$ relative to control ($n = 5$).

increased AQP5 levels to reach about a 2-fold increase within 120 min after instillation. BNX instillation resulted in decreased AQP5 levels that at 120 min were still about 70% lower than the basal level. BNX pretreatment 15 min before GBT did not prevent the GBT-induced increase in AQP5 levels.

Gabapentin Regulation of Aquaporin 5 Levels in Human Corneal Epithelial Cells

Whether GBT affects AQP5 expression at the corneal level was evaluated by an *in vitro* model of primary human corneal epithelial cells, the HCE-F model (Cristaldi et al., 2020). As shown in **Figures 7A,B** GBT treatment for 24 h at 0.01 and 0.1 mg/ml significantly increased AQP5 levels by about 55%, while no significant effects were observed after GBT at 1.0 mg/ml. AQP5 expression in epithelial cells is known to involve the PKA/CREB pathway that positively regulates both expression and localization of AQP5 (Yang et al., 2003; Kumari et al., 2012). Whether GBT-induced upregulation of AQP5 might be coupled to the PKA/CREB pathway was then investigated. As shown in **Figures 7A,C,D**, GBT at 0.01 and 0.1 mg/ml increased PKA phosphorylation by about 5-fold, while the phosphorylated form of the nuclear CREB was increased by about 3-fold. GBT at 1.0 mg/ml had no effect. In **Figure 8**, the respective effects of GBT and BNX on AQP5 expression were addressed. BNX administration at 0.03 and 0.15 mg/ml resulted in increased levels of AQP5 – similar to GBT–without significant differences between the two concentrations. Consistent with the increase of AQP5, BNX also elevated the amounts of pPKA and pCREB, to levels similar to those obtained with GBT. The association of GBT (0.1 mg/ml) and BNX (0.03 or 0.15 mg/ml) did not influence the expression of neither AQP5 nor the signaling molecules pPKA and pCREB.

DISCUSSION

We have shown that topical GBT attenuates ocular pain in a rabbit model of corneal injury induced by formaldehyde

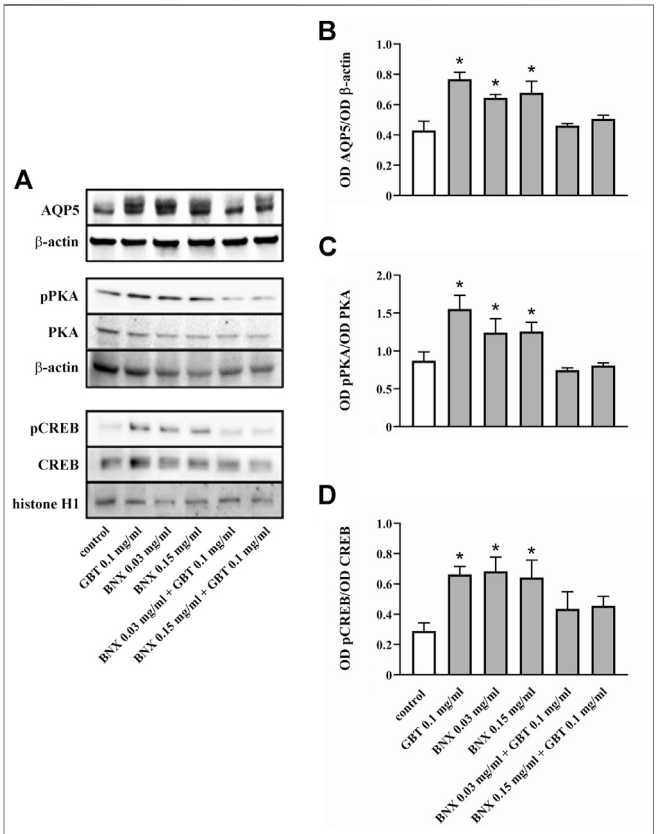


FIGURE 8 | Level of AQP5 and downstream mediators in HCE-F cells treated with 0.1 mg/ml GBT or BNX at 0.03 or 0.15 mg/ml, either alone or in combination. **(A)** Representative blots depicting levels of AQP5, pPKA, PKA, pCREB, and CREB after treatment with GBT, BNX either alone or in combination as determined by Western blotting. **(B–D)** Densitometric analysis of the respective levels. The expression of AQP5 was relative to the loading control β-actin, while the expression of pPKA and pCREB was normalized to the level of PKA and CREB, respectively. Data are plotted as mean ± SEM. Differences between groups were tested for statistical significance using one-way ANOVA with Tukey's multiple comparison post-test. *$p < 0.05$ relative to untreated ($n = 5$).

administration to the cornea through an analgesic but not anesthetic mechanism. However–differently from a topical anesthetic drug such as BNX, which reduces lacrimation–GBT rather stimulates tear secretion by exerting a regulatory role on autonomic neurotransmission and AQP5 expression levels in the lacrimal gland, through a mechanism that seems to be independent from its main receptor α2δ1. Results from *in vitro* experiments using HCE-F cells, a model of human corneal epithelial cells, show that GBT treatment induces AQP5 overexpression by involving the PKA/CREB pathway. This suggests the possibility that, at least at the corneal level, GBT may influence tear secretion by a direct effect on AQP5 expression.

Analgesic and Secretagogue Effects of Gabapentin

GBT systemic administration has therapeutic efficacy for neurological and psychiatric disorders such as epilepsy, anxiety, and migraine, and, together with its derivatives, belongs to the most used drugs for neuropathic pain management (Fornasari, 2017). The main target of GBT is the α2δ1 auxiliary subunit of presynaptic voltage-gated calcium channels through which GBT reduces the release of multiple excitatory neurotransmitters thus decreasing neuropathic pain (Taylor and Harris, 2020). In addition, there is evidence that GBT may play a role in reducing nociceptive pain (Hamidi et al., 2014; Scuteri et al., 2020). However, the exact mechanism through which GBT, by involving multiple players, exerts its analgesic effect is complex and not definitively clarified (Taylor and Harris, 2020). In ocular neuropathies, systemic administration of GBT or its analog pregabalin has been demonstrated to reduce pain (Lichtinger et al., 2011; Pakravan et al., 2012; Wei et al., 2015; Ongun and Ongun, 2019; Yoon et al., 2020), although its efficacy in counteracting ocular pain in patients with DED has been questioned (Ozmen, 2020).

The present finding that protein levels of the α2δ1 subunit of voltage-gated calcium channels are absent in ocular tissues is not unexpected, taking into consideration the effects of GBT observed here. It is indeed known that the α2δ1-mediated effects of GBT result in reduced neurotransmitter release (Taylor and Harris, 2020), while our results demonstrate increased levels of both Ach and NE in the lacrimal gland. This finding suggests that targets different from the α2δ1 subunit are involved in mediating the effects of GBT (on both pain and tear secretion) when topically applied. The additional finding that in the lacrimal gland protein levels of the α2δ1 subunit are almost absent despite the presence of α2δ1 transcripts is in line with similar studies (Gao et al., 2001; Gong et al., 2001; Dolphin, 2013), suggesting that the expression of α2δ genes may be subjected to post-transcriptional regulation. The possibility remains that GBT may act to relieve pain on the terminal endings that densely innervate the corneal surface (Galor et al., 2015) and are likely to be damaged by formaldehyde used here to induce ocular sensibilization. This possibility will be investigated in future work. An attractive hypothesis is that the stimulation of tear secretion by GBT may explain at least in part its efficacy in counteracting ocular pain, as an increase in tear secretion may improve the lubricating effect of tears on the ocular surface. In fact, among the options available to treat patients suffering from DED, tear replacement is widely used to restore the original homeostasis of the ocular surface and to attenuate patient discomfort and pain, for instance by reducing the friction on the ocular surface, as attrition consequent to lubrication deficits has been recognized to impact on ocular pain (van Setten, 2020). In this respect, artificial tears based on hyaluronic acid have been used since the early 1990 to alleviate dry eye signs and symptoms in DED patients thanks to its water retention and lubricant properties (Yang et al., 2021). On the other hand, there is evidence that artificial tears that solely swell and absorb water show poor effects when applied to DED patients affected by neuropathic pain (Galor et al., 2016), suggesting that those patients without neuropathic pain are more likely to benefit from tear replacement. As shown for the first time by the present results, the analgesic effect of GBT is coupled to a secretagogue activity, an effect which is prevented by the administration of the anesthetic BNX, at least soon after its administration. In this respect, more work should be done to clarify the mechanisms behind the results of their interaction, in particular because BNX and GBT express a completely different mode of action, with BNX able to block sodium channels and to prevent synaptic transmission, supporting the involvement of different downstream mechanisms modulated by BNX and GBT.

As also shown by the present findings, at 15 min after GBT instillation there is an increase in tear secretion paralleled by a decrease in tear protein concentration. This may be explained by assuming that the increase in water secretion follows a faster kinetics than the increase in protein secretion. Therefore, at 15 min the GBT-dependent increase in water secretion dilutes tears, an effect that is no more detectable at increasing time due to the GBT-dependent increase in protein secretion that would parallel water secretion thus restoring a normal tear protein concentration.

Mechanisms Underlying Gabapentin-Associated Tear Secretion

The autonomic nervous system acts on both the cornea and the lacrimal gland by regulating tear secretion through the release of its main neurotransmitters of which Ach mostly influences water secretion, while NE regulates protein secretion (Dartt, 2009). As shown by the present results, GBT causes upregulation of both Ach and NE levels in the lacrimal gland suggesting that GBT may act on lacrimation through a modulation of the autonomic neurotransmission. On the other hand, since a crude homogenate of lacrimal glands has been used here, we cannot be sure that NE and Ach are actually released by the autonomic nervous system as their origin may be diverse. However, it is unlikely that they might come, for instance, from the plasma permeating the tissue, in which NE levels in the rabbit are about 1 ng/ml (Yokoyama et al., 1992), because, if their origin is systemic from the blood stream, this would hardly explain the local sudden increase (within 15 min) of both neuromodulators elicited by GBT. If confirmed by further experiments, this would

be the first demonstration of GBT effects on the autonomic innervation at the level of the eye, while some interactions between GBT and the autonomic nervous system had already been shown (Tanabe et al., 2005; Takasu et al., 2006; Hayashida et al., 2007; Hayashida et al., 2008).

The possibility that GBT may exert a secretagogue role is presently unknown. The only mention about a link between GBT and secretagogue activity (however of the inhibition type) can be found in a case report of a patient with aquagenic wrinkling of the palms describing ameliorative effects of GBT treatment presumably through GBT action on sodium retention by epidermal cells thus promoting skin drying (Emiroglu et al., 2017).

Considering our model, the simplest explanation of the secretagogue action of GBT is that autonomic modulation of tear secretion involves water channels among which aquaporins are likely to play an important role. Some information about enhanced aqueous tear secretion and aquaporins dates back to 1997 when increased levels of AQP5 have been determined in the lacrimal glands of mice after parasympathetic stimulation by pilocarpine (Ishida et al., 1997). In the lacrimal gland, AQP5 is mainly localized at acinar cells, although abundant AQP5 also appears to be localized in the lacrimal duct system (Ding et al., 2010). At the functional level, reduced expression of AQP5 has been determined in the lacrimal gland of patients with Sjögren syndrome, an autoimmune pathology characterized by extreme eye dryness (Tsubota et al., 2001). In addition, abnormal levels of AQP5 are present in tears of patients with dry eye indicating that AQP5 leaks into the lacrimal fluid from damaged cells of the lacrimal gland or the cornea (Ohashi et al., 2003). In animal models, reduced levels of AQP5 have been measured in the lacrimal glands of pregnant rabbits characterized by DED (Ding et al., 2011).

Little is known on AQP5 regulation by autonomic innervation in the lacrimal gland. Recently, in models of DED, PACAP has been reported to induce tear secretion by promoting a PKA-mediated upregulation of AQP5 (Nakamachi et al., 2016). Our results suggest that AQP5 expression is a necessary, however not sufficient condition for increased tear secretion. In fact, GBT increases both lacrimation and AQP5 expression, whereas the effects of BNX on lacrimation are uncoupled from AQP5 expression, since the decrease in lacrimation happens and is concluded before AQP5 decrease. Moreover, when BNX is given soon before GBT, the secretagogue effect of GBT disappears despite the progressive increase of AQP5, supporting the involvement of different targets for BNX and GBT.

In addition to the lacrimal gland, AQP5 is also expressed in the corneal epithelium (Raina et al., 1995) where it is responsible for water movement to the ocular surface (Verkman et al., 2008). In particular, deletion of AQP5 reduces corneal water permeability (Thiagarajah and Verkman, 2002) thus causing marked tear film hypertonicity due to its role as a major component of an osmotically-driven water pathway that contributes to maintain tear isotonicity (Thiagarajah and Verkman, 2002; Ruiz-Ederra et al., 2009).

Results obtained by using HCE-F cells as an *in vitro* model of corneal epithelial cells show that GBT upregulates AQP5 expression via a signaling pathway that involves the activation of the PKA/CREB pathway, thus suggesting that GBT may affect tear secretion also by acting at the corneal level. The stimulatory effect of GBT on AQP5 expression and its pathway of activation tends to decrease with the increasing amount of GBT, and peaks at the two lower doses of 0.01 and 0.1 mg/ml, in line with what expected for a ligand showing a hormetic-like biphasic dose response in a ligand-receptor interaction (Calabrese, 2013). Surprisingly, and in contrast to what observed in the lacrimal gland, also BNX stimulates AQP5 expression in corneal epithelial cells through the same pathway activated by GBT (PKA/CREB), and with the same modality (higher dose, lesser effect), suggesting that *in vivo* there is an interplay between the autonomic nervous system and the regulation of lacrimation and AQP5 expression, while in a cell monolayer *in vitro* the effect is mediated only by biochemical interactions, which apparently use the same pathway to regulate AQP5 expression. Moreover, the association of GBT and BNX *in vitro* results in no stimulation on AQP5 expression and pathway activation. Most likely, given the fact that both compounds impinge on the same regulatory pathway (PKA/CREB), the association of the two might result in an overstimulation of the system, like in the case of high doses of GBT or BNX, finally resulting in a lesser response in terms of activation.

In conclusion, the present data demonstrate the analgesic and not anesthetic effect of a topical formulation of GBT as eye drops, and for the first time show a secretagogue effect of GBT, that is likely to involve both a stimulation of the autonomic nervous system and a direct activation of intracellular signaling cascades, including the PKA/CREB pathway, culminating in the increased expression of AQP5. However, whether this effect may also involve a modulation of ion channels remains to be determined as no evaluation of transmembrane ion movement/potential has been performed in the present study. Therefore, future mechanistic investigations will be therefore required to decipher the targets through which GBT acts to increase tear secretion. Overall, the presence in the same molecule of analgesic, anti-inflammatory (Anfuso et al., 2017) and secretagogue effects suggest a useful application of GBT eye drops in the treatment of medium/severe dry eye, in which the algic and inflammatory components accompany tear deficiency.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The experimental protocol was approved by the Commission for

Animal Wellbeing of the University of Pisa (permit number: 350/2018-PR, February 9, 2018).

AUTHOR CONTRIBUTIONS

Participated in research design: MC, PB, MDM, and DR. Conducted experiments: MC, RA, MO, SP, and MDM. Performed data analysis: MC, MDM, and DR. Wrote or contributed to the writing of the manuscript: MC, PB, MDM, and DR.

FUNDING

This work was supported by a grant from Sooft Italia, SpA (Montegiorgio, FM, Italy).

ACKNOWLEDGMENTS

We wish to thank Anat Galor, Bascom Palmer Eye Institute, Miami, FL (United States) for critical reading of the manuscript.

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Conflict of Interest: MDM received a study grant from Sooft Italia SpA. MO, SP, and DR are employees of Sooft Italia SpA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CLINICAL TRIAL
published: 30 September 2021
doi: 10.3389/fphar.2021.748296

Efficacy of Preoperative Music Intervention on Pain and Anxiety in Patients Undergoing Cataract Surgery

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Reviewed by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 27 July 2021
Accepted: 15 September 2021
Published: 30 September 2021

Citation:
Guerrier G, Bernabei F, Lehmann M, Pellegrini M, Giannaccare G and Rothschild P-R (2021) Efficacy of Preoperative Music Intervention on Pain and Anxiety in Patients Undergoing Cataract Surgery. Front. Pharmacol. 12:748296. doi: 10.3389/fphar.2021.748296

The aim of the present study was to investigate the impact of preoperative music exposure on intra- and post-operative pain during cataract surgery. This study was conducted alongside a prospective single-masked randomized controlled trial (ClinicalTrials.gov NCT02892825). Patients undergoing first eye cataract surgery were included and randomly assigned to either the intervention or control group. Patients in the intervention group had a 20-min music session through earphones before surgery, while patients in the control group wore earphones without music. Anxiety level evaluated using the visual analog scale and heart rate were collected before and after music intervention. Pain level was assessed using the Numerical Pain Rating Scale, during the surgical procedure, prior to discharge and 7 days postoperatively. A total of 243 patients were included: 119 in the intervention group and 124 in the control group. No significant differences in baseline characteristics, including age, sex and rate of treated hypertension were found between the 2 groups (all *p-values* > 0.05). In addition, no significantly differences were found in heart rate and anxiety level before music intervention between the 2 groups (all *p-values* > 0.05). Conversely, anxiety level was significantly lower in the music group after the intervention (respectively, 1.3 ± 1.1 vs 3.2 ± 2.2; $p < 0.05$). Patients in the music group reported a lower mean pain level during surgical procedure and before discharge compared with control group (respectively, 1.2 ± 0.5 vs 2.1 ± 1.1, $p = 0.03$ and 0.23 ± 0.4 vs 0.81 ± 0.7, $p = 0.04$). No difference was found in pain level 7 days postoperatively (0.1 ± 0.3 vs 0.2 ± 0.4, $p = 0.1$). A significant correlation was found between anxiety level and intraoperative pain level ($R = 0.64$, $p = 0.02$). In conclusion, music intervention was effective in reducing anxiety level and self-reported pain both during surgery and in the early postoperative period.

Clinical Trial Registration: https://clinicaltrials.gov/ct2/home, identifier NCT02892825.

Keywords: cataract surgery, pain, music intervention, music therapy, anxiety

INTRODUCTION

Cataract surgery represents one of the most commonly performed procedures in the world (Pascolini and Mariotti, 2012). Advancements in anesthesia and surgical techniques made it possible to perform most of the procedures for cataract extraction under topical anesthesia (Pellegrini et al., 2020; Lundström et al., 2021). On the one hand, this achievement has greatly reduced operating times and side effects related with local or general anesthesia (Giannaccare et al., 2021; Lundström et al., 2021). On the other hand, despite sedation (e.g. benzodiazepines and opioids) which could be administered pre- and intra-operatively, patients may experience a state of considerable anxiety along with a certain level of pain and discomfort during the surgical procedure (Rothschild et al., 2013; Shi et al., 2019).

Preoperative music intervention is a non-expensive and easily applicable technique with no side effects that showed significant beneficial effects on patients' anxiety in different surgical populations (Choi et al., 2018; Wan et al., 2020; Kakar et al., 2021). In addition, music intervention has been proved to lead to a better patient's cooperation and a reduced intraoperative blood pressure during surgery under topical anesthesia (Choi et al., 2018; Fu et al., 2020; Muddana et al., 2020).

Although the underlying mechanism of music therapy remains still unclear, it has been demonstrated that it induces molecular changes related to opiates and cytokine processes (Stefano et al., 2004). Different psychophysiological mechanisms have been proposed, and in particular music would be effective in distracting patients from the surgical procedure, mainly when they listen to music of their preference (Gaberson, 1995; Allen et al., 2001; Clements-Cortes and Bartel, 2018).

Previous studies demonstrated the beneficial effect of music intervention on both anxiety and blood pressure in patients undergoing cataract surgery (Leo et al., 2003; Wiwatwongwana et al., 2016; Muddana et al., 2020). However, there is little knowledge regarding the effect of preoperative music therapy on intra- and post-operative subjective pain in this patient population (Choi et al., 2018).

Interestingly, it has been demonstrated that pre-operative anxiety is positively correlated with intra- and post-operative pain level in a variety of surgical populations (Robleda et al., 2014; Bandeira et al., 2017). Therefore, it is possible to speculate that music intervention may result in beneficial effect on pain level in patients undergoing cataract surgery. Thus, the aim of the present study was to investigate the effect of music intervention on self-reported pain intensity during first eye cataract surgery and in the early post-operative period.

MATERIALS AND METHODS

Design and Patients

This study was conducted between February 2017 and July 2018 at the Ophthalmology service, OphtalmoPôle de Paris of the Cochin Hospital (Paris, France), alongside a prospective single-masked randomized controlled trial aiming at evaluating the effect of music intervention on anxiety-induced hypertension during cataract surgery performed under local anesthesia (ClinicalTrials.govIdentifier: NCT02892825). The study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the Comité de Protection des Personnes Paris-Ile-de-France III (N°2016-A00728-43). Written informed consent was obtained from all study subjects. Patients scheduled for first eye cataract surgery under local anesthesia were screened to be enrolled in the study. Exclusion criteria were hearing loss, speech impairment, uncontrolled hypertension, psychiatric disorders, dementia, deprivation of liberty by judicial or administrative decision or under legal protection. Uncontrolled hypertension was defined as a systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg, despite anti-hypertensive therapy, during the pre-anesthesia examination. In addition, patients with complicated cataract and hard nuclear cataracts with nuclear opalescence scores 5 or greater on Lens Opacities Classification System-III system were excluded from the study (Chylack et al., 1993).

Music Intervention and Surgical Procedure

Patients were randomized using a computer-generated, interactive web-response system (Cleanweb®, Telemedecine technologies S.A.S, Boulogne-Billancourt, France) and assigned to the music or the control group. After the randomization, all patients had dedicated headphones (BOSE AE2®) positioned. Patients in the music group were shown how to handle a tablet interface by a trained nurse in order to choose a music program according to their preferences (MUSIC CARE® Paris, France). In the control group, headphones were placed on patient's ears, but no music was played. A sleeping mask concealing patient's eyes was applied to all participants. In both groups, headphones and masks were left in place for 20 min and the headphones were removed before the surgical procedure. Before surgical procedure, 0.03 mg/kg oral midazolam was administered to all patients.

Cataract surgery was performed at one eye of each patient under topical anesthesia by an experienced surgeon (ML, PRR, DM). Topical anesthesia consisted in administration of oxybuprocaine 0.5% drops into the conjunctival sac 3 times in the 15 min preceding surgery. The primary steps of the surgery were a self-sealing temporal limbal 2.2 mm incision, subsequently capsulorhexis, hydrodissection and phacoemulsification in the capsular bag were performed. Finally, a foldable intraocular lens was inserted in the capsular bag. Intracameral 1 mg cefuroxime injection was administered at the end of surgery. Postoperative topical therapy consisted of fluoroquinolone eye drops for 1 week and dexamethasone eye drops for 1 month.

Data Collection

Data regarding age, sex, ocular and medical history including presence of treated hypertension, and duration of surgical procedure were collected.

Anxiety level was measured by the anesthetist using a visual analogue scale for anxiety (VAS-A) before and after music intervention (Facco et al., 2013). The VAS-A scale is comprised of a horizontal line 100 mm long with the indication "no anxiety" to the left and "worst possible anxiety" to the right. In addition heart rate were measured using pulse

TABLE 1 | Baseline characteristics of patients undergoing cataract surgery.

Characteristics	Intervention group (n = 119) mean ± SD or n (%)	Control group (n = 124) mean ± SD or n (%)	p-value
Age (yr)	67.3 ± 10.4	68.5 ± 11.2	0.4
Sex			0.9
Female	63 (52.9)	65 (52.4)	
Male	56 (47.1)	59 (47.6)	
Treated hypertension	11 (9.2)	13 (10.5)	0.8

SD, Standard deviation.

TABLE 2 | Heart rate and anxiety level in the music intervention and in the control group.

Measures	Intervention group (n = 119) mean ± SD	Control group (n = 124) mean ± SD	p-value
Heart rate before intervention (bpm/min)	75.3 ± 7.6	77.1 ± 9.4	0.1
Heart rate after intervention (bpm/min)	67.3 ± 7.6	68.7 ± 13.1	0.3
Anxiety level before intervention (VAS)	3.2 ± 2.2	3.3 ± 2.3	0.8
Anxiety level after intervention (VAS)	1.3 ± 1.1	3.2 ± 2.2	**<0.001**

SD, Standard deviation; VAS, Visual analogue scale.

oximetry (Onyx II 9550, NONIN, Plymouth, MI, United States) before and after music intervention.

Pain level was evaluated by the anesthetist using a verbally administered 0-to-10 numerical pain rating scale (NPRS), where 0 indicates "No pain" and 10 "The worst possible pain": 1) intraoperatively, before the insertion of the foldable intraocular lens; 2) postoperatively, before the discharge, and 3) 7 days after the surgery (Hjermstad et al., 2011).

Statistical Analysis

The SAS 9.4 statistical software (Copyright© 2016 by SAS Institute Inc., Cary, NC, United States) was used for data analysis. Continuous data were presented as mean ± standard deviation, while categorical data were represented by number and percentage.

Unless otherwise specified, categorical variables were compared by a Chi-square test or Fisher's exact test as appropriate, and continuous variables were compared by a Student's t test or Wilcoxon-Mann-Whitney test as appropriate. In addition, the correlation between preoperative anxiety level after music intervention and intraoperative pain level was evaluated with Pearson correlation test. A p value <0.05 was considered statistically significant.

RESULTS

Two hundred forty-three patients were included in the study and were randomized to receive music intervention (intervention group: n = 119) or headphone with no music (control group: n = 124) before cataract surgery. Demographic and baseline characteristics of patients are reported in **Table 1**. There were no statistically significant differences in age, sex distribution, and proportion of patients with treated hypertension between the 2 groups (all $p > 0.05$).

All patients underwent uneventful cataract surgery and no significant complications were registered in the post-operative period. No difference was found in the duration of procedure between music and control group (respectively, 16.1 ± 6.5 vs 16.5 ± 6.2, $p = 0.08$).

Table 2 shows anxiety level and heart rate before and after music intervention in both groups. No significant differences were observed in heart rate before and after music intervention between the music and control group (respectively, 75.3 ± 7.6 vs 77.1 ± 9.4 bpm/min, and 67.3 ± 7.6 vs 68.7 ± 13.1 bpm/min, always $p > 0.05$). In addition, no difference was found in anxiety level between the 2 groups before music intervention (respectively, 3.2 ± 2.2 vs 3.3 ± 2.3, $p = 0.8$). Conversely, anxiety level was significantly lower in the music group compared to control group after music intervention (respectively, 1.3 ± 1.1 vs 3.2 ± 2.2, $p < 0.001$).

Table 3 shows intra- and postoperative mean pain level as well as its classification according to the NPRS values. In particular, pain was stratified in the following categories: 1) NPRS = 0, 2) NPRS 1-5 and 3) NPRS >5, in the 2 groups in the different timepoints (intraoperatively, postoperatively before discharge and 1 week after surgery). Patients in the music group reported a lower mean intra- and postoperative (before discharge) pain level compared with control group (respectively, 1.2 ± 0.5 vs 2.1 ± 1.1, $p = 0.03$ and 0.23 ± 0.4 vs 0.81 ± 0.7, $p = 0.04$). Conversely, no difference was found in pain level 1 week postoperatively (0.1 ± 0.3 vs 0.2 ± 0.4, $p = 0.1$).

A significant correlation was found between preoperative anxiety level after music intervention and intraoperative pain level ($R = 0.64$, $p = 0.02$).

DISCUSSION

The aim of this randomized control trial was to investigate the effect of preoperative music intervention on the level of pain and

TABLE 3 | Pain score evaluated before and following cataract surgery.

	Intervention group (*n* = 119)	Control group (*n* = 124)	*p*-value
Pain score			
Intra-operative			
Mean ± SD	1.2 ± 0.5	2.1 ± 1.1	**0.03**
NPRS 0 [n (%)]	78 (66%)	50 (40%)	
NPRS 1–5 [n (%)]	30 (25%)	54 (44%)	
NPRS >5 [n (%)]	11 (9%)	20 (16%)	
Post-operative before discharge			
Mean ± SD	0.23 ± 0.4	0.81 ± 0.7	**0.04**
NPRS 0 [n (%)]	102 (86%)	79 (64%)	
NPRS 1–5 [n (%)]	17 (14%)	39 (31%)	
NPRS >5 [n (%)]	0	6 (5%)	
One week after surgery			
Mean ± SD	0.1 ± 0.3	0.2 ± 0.4	0.1
NPRS 0 [n (%)]	108 (91%)	107 (86%)	
NPRS 1–5 [n (%)]	11 (9%)	16 (13%)	
NPRS >5 [n (%)]	0	1 (1%)	

NPRS, numerical pain rating scale; SD, Standard deviation; n, number of patients.

anxiety in patients undergoing first eye cataract surgery. Interestingly, patients in the music group presented a lower level of both anxiety and pain compared with controls. In particular, patients who received music intervention experienced a lower level of anxiety when they entered the operating room and, subsequently, presented a lower level of pain, both during surgery and in the immediate postoperative period.

Previous studies have investigated the effect of pre and peri-operative music intervention on anxiety in patients undergoing ophthalmic surgery, and in particular cataract surgery (Cruise et al., 1997; Allen et al., 2001; Wiwatwongwana et al., 2016; Muddana et al., 2020). Cruise and co-authors evaluated the effect of relaxing music in patients undergoing cataract surgery under peribulbar anesthesia, demonstrating an increased satisfaction in patients who received music intervention (Cruise et al., 1997). More recently, 2 studies evaluated the effect of music intervention administrated both, before and during cataract surgery under topical anesthesia (Wiwatwongwana et al., 2016; Muddana et al., 2020). Both showed that music exposure significantly reduces anxiety and blood pressure, resulting in a better patient's experience. The beneficial effect of music on the preoperative anxiety has also been demonstrated for other types of surgery. Interestingly, music has a long-lasting effect and in particular, it was showed that 15 min of music intervention before the surgical procedure are able to lead to an effective reduction of anxiety (McClurkin and Smith, 2016).

Several reports showed that preoperative anxiety is positively correlated with postoperative pain, in different surgical populations (Bayrak et al., 2019; Navarro-Gastón and Munuera-Martínez, 2020). Indeed, it has been shown that the analgesic effect due to the musical intervention can be helpful in reducing different types of pain, particularly in patients who have to undergo orthopedic, urological and general surgery procedures, and in patients who suffer from chronic pain (Hyung, 2016).

In agreement with this data, we found that intraoperative pain was positively correlated with preoperative anxiety level, and both were reduced in the subjects in the music group. Previously, only one study evaluated the effect of music on pain in this peculiar patient population (Choi et al., 2018). In particular, Choi and co-authors evaluated the effect of Korean traditional music before and during cataract surgery, demonstrating its effectiveness in reducing painful perception (Choi et al., 2018). Our study supports these results and furthermore, we showed that this also applies when the patient chooses the music he/she prefers. Interestingly, a recent study showed that the reduction in pain associated with music exposure is greatest when the subject chooses the music to listen to (Howlin and Rooney, 2021). The study also shows that it is the act of making a choice that determines the greatest effectiveness of the procedure, empathizing the importance of giving patients as much control as possible in music intervention (Howlin and Rooney, 2021).

Unlike previous studies, in the present one, patients were exposed to music only before surgery. This is because, in our opinion, be exposed to music during the procedure could prevent the surgeon from communicating with the patient and vice versa, thus causing a reduction in patient compliance and maybe an increase in anxiety. Cataract surgeons are aware that due to the pain related with the surgical procedure under topical anesthesia, patients can become uncooperative and make abrupt movements that can potentially cause intraoperative complications (Zhu et al., 2021). However, the use of topical anesthesia averts many of the potential systemic and ocular complications associated with regional anesthesia (Maharjan et al., 2021). For these reasons, intraoperative pain control plays a key role for the success of the surgical act. Music intervention is a safe, inexpensive, and easy-to-use procedure that should be strongly encouraged in order to improve the experience and satisfaction of the patient undergoing cataract surgery. We identified some limitations for the present study that deserve mentioning. Firstly, although they are recognized as valid indicators and are widely used in clinical trials, anxiety and pain rating scales remain highly subjective. Secondly, the surgeon's experience was not evaluated, and it must be acknowledged that this information could have helped to better understand the effect of music during

surgery. Thirdly, the cataract grading and ultrasound energy consumption during phacoemulsification were not evaluated in the present study. Although the duration of cataract surgery did not change significantly between the two groups, the evaluation of these two parameters would provide useful information to more accurately assess the effectiveness of music intervention. Further studies evaluating these parameters are warranted to better address this issue. Finally, the use of midazolam may have partially hampered the results. Although the use of the same dose of the drug in all included patients limited the possibility of biasing the results, future sedative-free studies are needed to confirm our findings.

In conclusion, the present study demonstrates that 20 min of music intervention before surgery are effective in reducing anxiety and pain sensation in patients undergoing cataract surgery. Further studies are needed to establish the best approach, in terms of timing, cost and technology, before this intervention can be widely introduced in routine cataract surgery.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comité de Protection des Personnes Paris-Ile-de-France III. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

The nurses and nursing assistants at the study centers ensured the well-being of the participants. The Clinical Research Unit-Clinical Investigation Center, within hospital support structure dedicated to research that helps and support physicians in carrying out their clinical research projects, contributed in the implementation, monitoring and data management of the study.

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frontiers in Medicine

ORIGINAL RESEARCH
published: 26 October 2021
doi: 10.3389/fmed.2021.709712

Deciphering the Action of Perfluorohexyloctane Eye Drops to Reduce Ocular Discomfort and Pain

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Perfluorohexyloctane (F6H8) eyedrops have been recently introduced in Europe as a product to treat dry eye disease, based on its ability to reduce tear film instability in Meibomian gland dysfunction and evaporative dry eye disease, although its mechanism of action is still unknown. In the present pilot study, we evaluated the effects of the ocular instillation of a single drop of commercial F6H8 eyedrops in 20 healthy humans (9 women/11 men), measuring: (a) Corneal surface temperature (CST) from infrared video images; (b) tear volume using phenol red threads; (c) blinking frequency; and (d) ocular surface sensations (cold, dryness, pricking, foreign body, burning, itching, gritty, eye fatigue, watering eyes, and light-evoked discomfort sensations; scored using 10 cm Visual Analog Scales), before and 5–60 min after F6H8 or saline treatment. CST decreased and tearing and blinking frequency increased significantly after F6H8 but not after saline solution. When applied unilaterally, CST decreased only in the F6H8-treated eye. No sensations were evoked after F6H8 or saline. The corneal surface temperature reduction produced by topical F6H8 does not evoke conscious ocular sensations but is sufficient to increase the activity of corneal cold thermoreceptors, leading to an increased reflex lacrimation and blinking that may relieve dry eye condition thus reducing ocular discomfort and pain.

Keywords: ocular discomfort, ocular pain, dry eye, perfluorohexyloctane, blinking, tearing, cold thermoreceptors, corneal surface temperature

OPEN ACCESS

Edited by:
Giulio Ferrari,
San Raffaele Hospital (IRCCS), Italy

Reviewed by:
Melis Palamar,
Ege University, Turkey
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *04 June 2021*
Accepted: *28 September 2021*
Published: *26 October 2021*

Citation:
Delicado-Miralles M, Velasco E, Díaz-Tahoces A, Gallar J, Acosta MC and Aracil-Marco A (2021) Deciphering the Action of Perfluorohexyloctane Eye Drops to Reduce Ocular Discomfort and Pain. Front. Med. 8:709712. doi: 10.3389/fmed.2021.709712

INTRODUCTION

The ocular surface is a unique exposed mucosa that must endure environmental conditions while maintaining its function and integrity (1). Upon their activation by environmental physical and chemical changes acting on their peripheral nerve endings, trigeminal sensory neurons innervating the ocular surface trigger protective responses such as blinking and tearing (2). In particular, there is strong evidence that TRPM8-mediated activation of corneal cold-thermoreceptors constitutes the afferent signal to the CNS for the regulation of tearing and blinking, mechanisms that allow to maintain and distribute moistness of the eye surface (3, 4).

Dry Eye Disease (DED), a condition that affects over 10% of people worldwide (5), is characterized by a loss of the so-called homeostasis of the tear film, that is, the disruption of the equilibrium of the chemical composition and functions of the tear film due to one or more

of the underlying causes of dry eye (6). Due to the multi-etiological origin of DED, no specific treatments are available nowadays, and there is scarce scientific evidence on their effectiveness in the management of the disease. Artificial tears are commonly used by most DED patients (7) although some of them contain preservatives that are known to produce side effects (8).

Perfluorohexyloctane (F6H8) is a semifluorinated alkane liquid that has been used initially in ophthalmology as a long-term vitreous substitute (9). This compound is physically, chemically and physiologically inert, slightly amphiphilic, colorless and laser stable with a density higher than water, and very low surface and interface tensions (10). In addition, as it is a non-aqueous liquid, microbial growth is not possible and therefore, it does not need any preservative (8).

F6H8 applied topically in DED patients reduced their dry-eye associated symptoms in two prospective observational studies (8, 11). As F6H8 increased tear film breakup time and lipid layer thickness in DED patients, it has been proposed that F6H8 could prevent the increased evaporation that causes DED by forming an occlusive layer and reducing shearing forces of the eyelid during blinking (8, 11). This idea is reinforced by the observation that in rabbits, F6H8 improves the quality grade of the tear film lipid layer measured by hand-held interferometry (12). Additionally, in mild to moderate DED patients F6H8 transiently increases tear film thickness 10 min after its application (13). A more recent study showed that topical treatment with F6H8 does not induce changes in corneal endothelium and significantly reduce corneal staining in DED patients, supporting its effectiveness and safety (14).

These results, together with the low surface tension of the compound, led to conclude that the very small drop of F6H8 (about 10 μL) spread uniformly over the ocular surface upon application, forming a protective layer over the tear film and preventing its evaporation. However, the precise mechanisms that would explain the effects of F6H8 in DED are far from being clarified and still need investigation.

In a previous report, we found that F6H8 produces corneal surface temperature changes in tear-deficient guinea pigs (15), suggesting that F6H8 may be more than an inert molecule, forming a non-water mixable thin layer over the tears and reducing tear evaporation. We hypothesized that, in addition to preventing evaporation, F6H8 may facilitate heat exchange between corneal tissue and the environment, thus reducing corneal temperature and activating TRPM8 cold-thermosensitive channels of cold thermoreceptor nerves. In turn, the increased activity of corneal cold nerves will lead to an increase in tearing and blinking rate. To test this hypothesis, we have studied the ocular sensations evoked in a group of volunteers by topical instillation of F6H8, in parallel with its effects on tear production, blinking frequency, and corneal surface temperature measured by infrared thermography. Additionally, we performed a simple experiment to investigate the temperature transmittance of F6H8 as a first approach to understand the mechanism of action of this molecule.

MATERIALS AND METHODS

Subjects

Twenty 20 young healthy volunteers (9 women, 11 men; mean age 24.1 ± 4.4 years, range 19–34 years) participated in this pilot study. After signing an informed consent, volunteers were subjected to a brief anamnesis and filled out an ocular surface discomfort index (OSDI) questionnaire adapted to Spanish-speaking people (16). Individuals with previous eye disease, ocular surgery, OSDI ≥12, as well as daily contact lens users or subjects that were receiving either ocular or systemic drugs were excluded. Participants were instructed to not consume any anti-inflammatory or pain-killer drug in the 48 h previous to the experiment. All experimental procedures were carried out according to the Spanish legal regulations and the Helsinki Declaration, and followed the protocol UMH.INJGa.01.14 approved by the Ethics Committee of the Universidad Miguel Hernández de Elche.

Experimental Protocols

Two different experimental protocols were carried out (**Figure 1**) in the same room, under controlled temperature (24.2 ± 1.5°C, range 21.2–27.9°C) and partial humidity (42.4 ± 7.9%, range 22.6–55.0%). The position of the volunteers and experimenters, as well the distance from the face of the subject to the different objects in the room (video camera, air conditioning outlet, door, windows, etc.) was standardized to avoid any environmental variation along the procedures. As F6H8 does not induce corneal punctate (14), fluorescein corneal staining was considered not necessary. This way, the observed effects of F6H8 were not masked or affected by fluorescein or its excipients.

In experimental protocol 1 (**Figure 1A**), 13 participants received bilaterally a single 10 μL drop of F6H8 (EvoTears™, Brill Pharma S.L., Spain) or saline solution (NaCl 0.9 %, Braun Medical, S.A.) in two different sessions (application order at random). In experimental protocol 2 (**Figure 1B**), corneal surface temperature (CST) was measured in a separate group of 7 subjects (3 women and 4 men) before and after a single 10 μL drop of F6H8 instilled only on the right eye.

Protocol 1: F6H8 or Saline Solution Applied to Both Eyes

Participants in protocol 1 were distributed in two different subsets (**Figure 2A**). In the first subset (protocol 1A) of 7 subjects (3 women and 4 men) CST, blinking frequency and ocular surface sensations were evaluated before and at different times (5, 15, 30, and 60 min) after F6H8 or saline bilateral treatment. In the second subset (protocol 1B) of 6 participants (3 women and 3 men), tearing was measured before and after F6H8 or saline.

Protocol 1A: CST, Blinking Rate and Ocular Sensations

Measurement of corneal surface temperature. Surface temperature of the ocular tissue was measured from video images taken with an infrared thermographic video camera (InfRec R300SR, Nippon Avionics Co. Ltd., Tokyo, Japan). The subject sat quietly with the head in a chin rest, fixing the gaze over the objective of the camera, placed at a fixed distance of 50 cm.

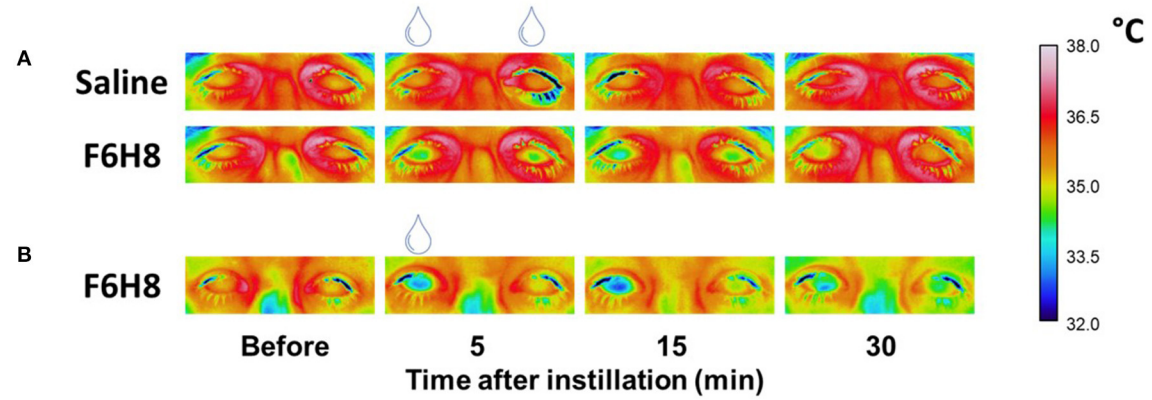

FIGURE 1 | Representative examples of the infrared video images of both eyes taken to define the CST changes after bilateral topical application of saline or F6H8 **(A)** or unilateral (right eye) topical application of F6H8 **(B)**.

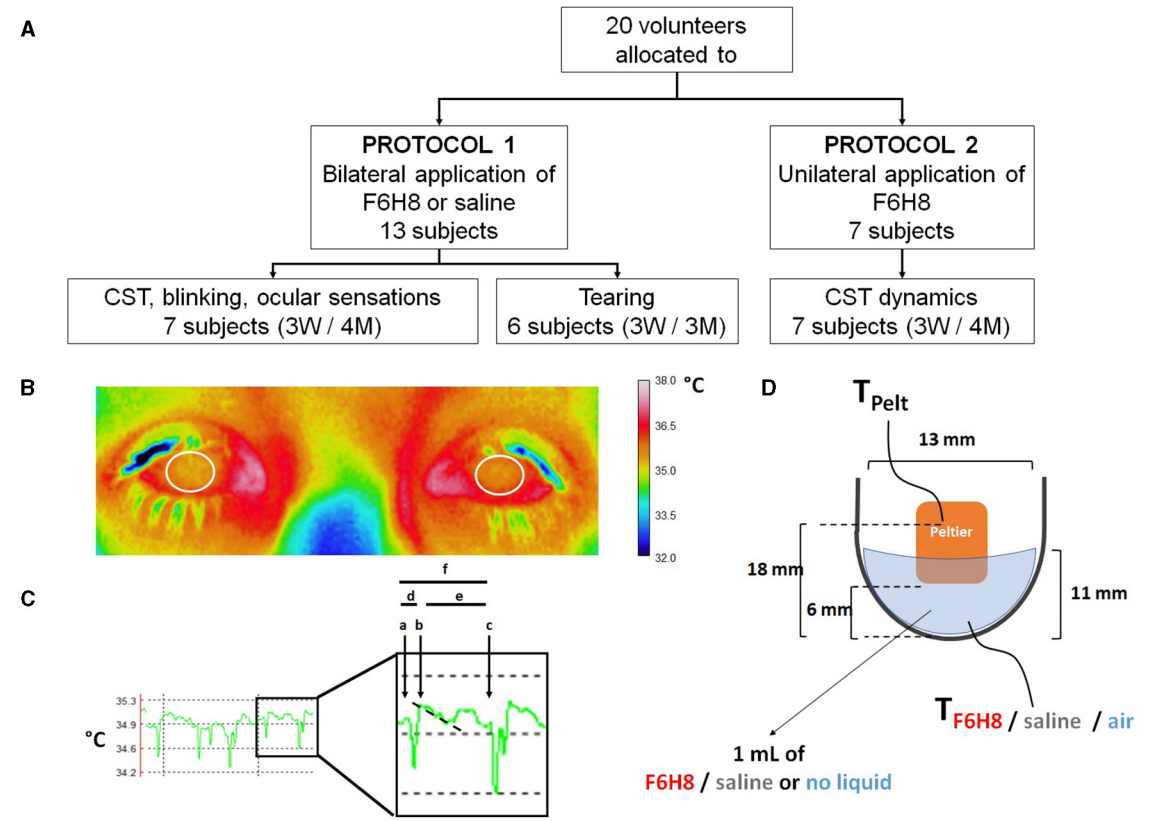

FIGURE 2 | (A) Distribution of volunteer participants among the different experimental protocols of the pilot study. In experimental protocol 1, perfluorohexyloctane (F6H8) or saline solution were applied to both eyes, while in experimental protocol 2, F6H8 was applied only to the right eye. CST: corneal surface temperature. **(B)** CST was calculated from infrared thermographic video images. Temperature values of a 1 cm^2 area of the corneal surface (white circumferences) were averaged to obtain the CST value at a defined time point. **(C)** Parameters measured from infrared thermographic video images of the corneal surface. Six interblink intervals were randomly selected and analyzed to obtain the following variables: (a) CST value immediately before a blink; (b) CST value immediately after blink; (c) CST value immediately before the next blink; (d) CST change during a blink, calculated as b-a; (e) CST change during the interblink interval, calculated as c-b; and (f) CST change between consecutive blinks, calculated as c-a. The time between a and b was considered as the duration of the blink movement. Also, the slope of the temperature decay during the first second of the IBI was calculated (dotted line). **(D)** Schematic representation of the experimental set-up used to measure the dynamics of temperature changes of F6H8 and saline solution during temperature changes induced with a Peltier cell placed inside the liquid in a tube. Temperature was measured with a thermocouple submerged into the liquid contained in a tube. TPelt: temperature of the Peltier cell measured with a PT100 sensor. TF6H8, TSaline and TAir: temperature measured with a thermocouple placed inside F6H8, saline solution, or an empty container, respectively.

Recording parameters (digital 1.6x zoom; 320 x 240 pixels; 60 frames per second; 0.96 emissivity) as well as data extraction (a circular area of 1 cm^2 -range 0.93–1.01 cm^2- specifically located over the cornea) were established using dedicated software (InfRec Analyzer NS9500 Standard, Nippon Avionics Co. Ltd.) (**Figure 2B**). Both eyes were simultaneously recorded for 1 min at different time points: before and 5, 15, 30, and 60 min after the corresponding topical treatment. At the beginning of the 1 min recording, subjects kept their eyes closed for 3 s and blink spontaneously afterwards. CST values calculated by averaging the temperature of 1 cm^2 area of both corneas at the beginning of the last registered interblink period were considered the main parameter to define the effects of F6H8 and saline treatments on ocular surface temperature.

Ocular surface sensations. Immediately after the end of the 1-min IR video recordings performed before and at different times after F6H8 or saline treatment, subjects were asked to use separate 10 cm Visual Analog Scales (VASs; where 0 represents no sensation and 10 is the maximal sensation the subject can imagine) (17) to score the following sensations experienced at the ocular surface: cold, burning, dryness, pricking, foreign body sensation, itching, gritty, eye fatigue, watering eyes, and light-induced discomfort.

Blinking frequency. Immediately afterwards, the number of blinks was manually counted from direct observation of the subjects, who did not know that their blinks were being counted in order to avoid conditioning by the experimental situation (18). Volunteers were asked to read aloud the letters in a LogMar chart placed at 1 m distance, from left to right and from up to down. Blinking frequency (BF) was calculated as the number of blinks during the duration of the task for each subject. The average time needed to perform one complete reading of the chart was 24.6 ± 8.6 s, although depending on the subject it varied between 15 and 60 s. BF while performing this task was measured before, and at 5, 15, 30, and 60 min after the corresponding topical treatment.

Protocol 1B: Tear Volume

Tear volume was assessed before and 5, 15, and 30 min after the corresponding topical treatment using phenol red threads carefully placed during 30 s in the inferior conjunctival sac, near the temporal canthus. Tear volume was expressed as the length of wet thread, measured in mm using a rule.

Protocol 2: F6H8 or Saline Solution Applied Only to the Right Eye

CST was measured in both eyes before and at different time points after F6H8 instillation onto the right eye only, using the infrared thermography analysis described before. To further define the effects of F6H8, the following parameters were analyzed from the IR video recordings performed before, 5 and 60 min after treatment, averaging the values obtained from 6 interblink periods (**Figure 2C**): (a) CST value immediately before one blink; (b) CST value immediately after blink; (c) CST value immediately before next blink. From these values, (d) CST change during blink, (e) CST change during the interblink interval (IBI), and (f) CST change between consecutive blinks were calculated. Also, the slope of the temperature decay during the first second of the IBI was calculated. Additionally, the temperature of 1 cm^2 of the eyelid skin was measured at the different time points before and after eyedrop treatment.

Adaptation of F6H8 and Saline Solution to Temperature Changes

An ultrafine flexible temperature thermocouple (IT-23, Physitemp Instruments LLC, Clifton, NJ, USA) was placed at the bottom of an Eppendorf tube filled with 1 ml of F6H8 or saline solution, or empty of liquid ($n = 4$ observations per condition). Temperature was continuously recorded with a digital thermometer (BAT-12 Microprobe Thermometer, Physitemp Instruments LLC) (**Figure 2D**). Increases and decreases of temperature inside the tube were produced by a home-made temperature controller device whose Peltier cell was placed inside the tube. This device allows changing temperature between 15° and 50°C although only the temperature range close to the normal ocular surface temperature values were explored. From a resting Peltier temperature ($T_{Peltier}$) around 34°C, temperature was increased by 3°C in a single step at an approximate rate of $0.030°C{\cdot}s^{-1}$. After 8 min at 37°C, a 3°C cooling step was induced with the Peltier at a similar cooling rate. $T_{Peltier}$ and temperature of the solution (T_{F6H8}, T_{saline}, or T_{Air}) were recorded simultaneously and stored in a computer using a micro1401 CED interface and Spike2 software (both from Cambridge Electronic Devices, Ltd., Milton, Cambridge, UK) for further off-line analysis. As in the case of human measurements, experiments were made at a room temperature of 23–24°C and a partial humidity around 40%.

Data Analysis

Power analysis for paired comparison analysis (matched pairs) was performed using Gpower*3.1 (19), considering an effect size of 1.5 (Cohen's d), a power of 0.8 and an α-error of 0.05. The minimum number of observations was established in $n = 6$, so that the sample size of participants in each subset of the experimental protocol ($n = 6$ or 7) was enough to achieve statistical significance.

Statistical analyses were performed using IBM SPSS Statistics for Windows (Version 25.0). Descriptive analysis was performed to detect possible outliers. Data distribution was studied with the Kolmogorov-Smirnoff test. Variances were compared using the Levene's test for Equality of Variances, when necessary. Normally distributed variables were compared with the paired Student's *t*-test, ANOVA or Repeated measurements ANOVA. Non-normally distributed parameters were compared with the Wilcoxon's test. Categorical variables were compared with the χ^2 test. Unless otherwise indicated, data are presented as mean ± standard deviation (median ± interquartile range if non-normal). Statistical differences were accepted for $p < 0.05$. Graphs were made with SigmaPlot software v11.0 (Systat Software Inc., San Jose, CA, USA).

TABLE 1 | Ocular surface sensations reported 5 min after bilateral topical treatment with a 10 μL drop of F6H8 or saline solution.

Sensations	F6H8		Saline	
	Sensation intensity	Responding subjects	Sensation intensity	Responding subjects
Cold	0 (0.0)	1/7	0 (0.0)	1/7
Dryness	0 (0.0)	0/7	0 (0.0)	1/7
Burning	0 (0.0)	1/7	0 (3.2)	2/7
Pricking	0 (0.0)	0/7	0 (0.0)	0/7
Foreign body	0 (6.0)	2/7	0 (7.1)	1/7
Itching	0 (0.0)	1/7	0 (6.8)	3/7
Gritty	0 (4.8)	1/7	0 (0.0)	1/7
Eye fatigue	0 (0.0)	1/7	0 (0.0)	0/7
Tearing	0 (0.0)	0/7	0 (8.4)	2/7
Light-evoked discomfort	0 (0.0)	0/7	0 (1.3)	1/7

Data shown on each column are: median (IQR) of VAS units; number of responding/total number of explored subjects. No significant differences were found between F6H8 and saline-treated groups for any sensation (Wilcoxon Signed Rank test and chi square test).

RESULTS

Effects of Topical F6H8 and Saline Solution Applied to Both Eyes

We first studied the effects of bilateral topical instillation of a 10 μL drop of F6H8 on corneal surface temperature (CST), blinking rate and ocular surface sensations measured at different time points after treatment in 7 seven healthy young volunteers. Results were also compared with those obtained after bilateral instillation of an aqueous solution (saline solution).

Ocular Surface Sensations

Sensations of cold, dryness, burning, pricking, foreign body sensation, itching, gritty, eye fatigue, watering eyes, and light-induced discomfort experienced by the volunteers were evaluated before and at different times after the corresponding treatment. Overall, no conscious ocular sensations were evoked by F6H8 or saline treatment at any of the studied time points, being 0 the median of the scored values. As an example, **Table 1** shows the VAS values of the ocular sensations scored 5 min after bilateral topical treatment with F6H8 or saline. In addition, no differences in the proportion of subjects reporting any sensation were found between F6H8 and saline treatments (**Table 1**), although 5 out of the 7 subjects were able to differentiate F6H8 from saline. Two subjects also reported blurry vision for a few seconds after F6H8 application.

Corneal Surface Temperature

At different times after bilateral topical treatment (**Figure 1A**), CST values were calculated by averaging the temperature of 1 cm^2 of corneal surface (**Figure 2B**) in infrared thermographic images taken immediately after eye opening (**Figure 2C,** parameter b, see methods). CST was significantly decreased after F6H8 ($p = 0.001$, Repeated Measurements ANOVA; $p = 0.001$, 0.008, 0012 at 5, 15 and 60 min, respectively, in comparison with the value before treatment, *post hoc* Dunnett's test; $n = 7$) (**Figure 3A**). In contrast, bilateral instillation of saline solution did not modify CST at any of the different time point after treatment (**Figures 1A**, **3A**, inset).

Tear Volume

No significant changes in the volume of tears collected with phenol red threads were found after saline treatment ($p = 0.640$, Repeated measures ANOVA; $n = 6$) (**Figure 3B**). Tear volume was slightly increased only at 5 min after F6H8, although the change was not statistically significant ($p = 0.151$, Repeated Measurements ANOVA; $n = 6$).

Blinking Frequency

Bilateral application of saline did not affect blink frequency at any studied time point (**Figure 3C** inset). In contrast F6H8 significantly increased BF ($p = 0.004$, Repeated Measurements ANOVA; $p = 0.015$ and 0.008 at 5 and 15 min, respectively, in comparison with the value before treatment, *post hoc* Dunnett's test; $n = 7$) (**Figure 3C**).

Effects of Unilateral Administration of F6H8 on CST

This set of experiments was performed to further describe the cooling effect of F6H8. In a separate group of volunteers ($n = 7$, see Methods), CST was measured before and after a single drop of F6H8 applied only onto the right eye in order to compare the dynamics of the temperature change during the interblink intervals (IBIs) in the treated eye, in comparison with the untreated, fellow eye. For that purpose, the evolution of CST values along the interblink interval was analyzed (See Methods and **Figure 2C** for details).

As expected, CST values at the beginning of the IBI were reduced after F6H8 ($p = 0.001$, repeated measures ANOVA; $p = 0.002$ and 0.007 for the values obtained at 5 and 15 min after treatment, respectively, compared with pre-treatment values, *post hoc* Dunnett's test; $n = 7$) with a maximal effect 5 min after treatment (**Figure 4A**). On the contrary, in untreated eyes, no significant changes of the CST values at the beginning of the IBI were found at any explored time point (**Figure 4A**). The CST values obtained at the beginning of the IBI were significantly lower in eyes receiving F6H8 than in untreated eyes at 5 and 15 min after treatment ($p = 0.001$ and 0.023, respectively, paired *t*-test; $n = 7$) (**Figure 4A**). To define if the cooling effect was restricted to the eye surface, we also measured the temperature of the eyelid skin, finding that it was not significantly modified neither in the F6H8-treated (35.01 ± 0.61°C vs. 35.3 ± 0.44°C, before and 5 min after, respectively; $p = 0.072$, paired *t*-test) or untreated eye (34.98 ± 0.57°C vs. 35.37 ± 0.53°C; $p = 0.052$).

To describe in more detail the effects of F6H8 on ocular surface temperature, the dynamics of CST change along an IBI was studied from the infrared video images taken before and 5 min after administration (when the maximal temperature reduction was obtained), as well as 60 min after F6H8 treatment (**Figures 4B–D**). In addition to starting from a lower temperature at the beginning of the IBI (**Figure 4A**), the decay of temperature during IBI was more prominent in the F6H8-treated eyes at 5 min after treatment, and recovered basal values afterwards

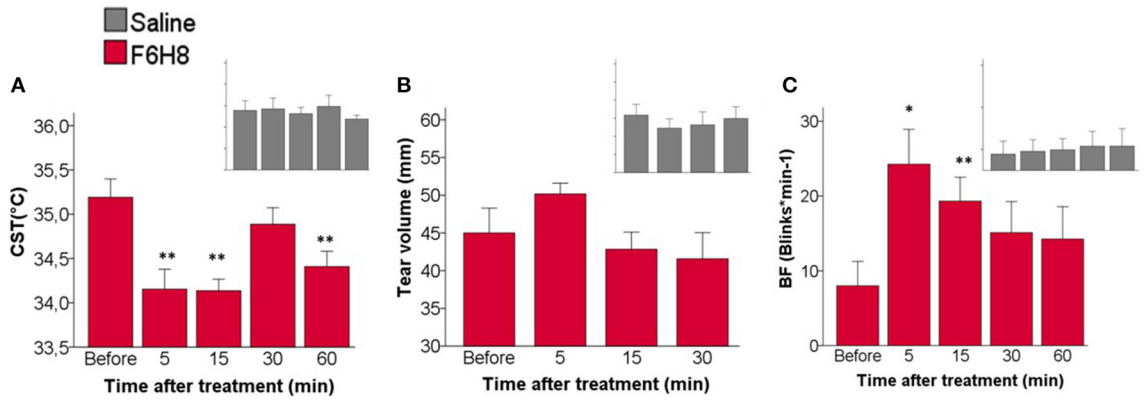

FIGURE 3 | Effects of topical instillation of F6H8 (red) or saline solution (insets in gray) on both eyes. **(A)** CST at the beginning of the last interblink interval measured from infrared thermographic images, $p = 0.001$, Repeated Measurements ANOVA; $^{**}p < 0.01$ Dunnett's test $n = 7$. **(B)** Tear volume measured with phenol red threads, no significant differences, Repeated Measurements ANOVA, $n = 6$. **(C)** Blinking frequency (BF), $p < 0.005$, Repeated Measurements ANOVA; $^{*}p < 0.05$, $^{**}p < 0.01$, Dunnett's test, $n = 7$.

(**Figure 4B**, red bars). This effect was not present in the untreated eye (**Figure 4B**, empty bars). During the IBI, eyes treated with F6H8 cooled faster than untreated eyes, as reflected by the faster slope of the temperature decay during the first second (-0.078 ± 0.16°C/s and -0.165 ± 0.82°C/s, before and 5 min after F6H8, respectively; $p < 0.01$, paired t-test). On the contrary, the slope of temperature decay during IBI did not change significantly in the untreated eye (-0.061 ± 0.145°C/s and -0.082 ± 0.101°C/s, before and 5 min, respectively; $p = 0.437$).

The reduction of CST induced by F6H8 was present immediately after its application, although the magnitude of the cooling effect was increasing with time during the first 15 min after treatment (**Figures 1B**, **4A**). The increasing cooling during this time was evidenced by the significant differences obtained when comparing CST values of consecutive blinks (**Figure 4C**).

We then compared CST values obtained immediately before and after a blink to measure the magnitude of warming of the ocular surface that occurred during the time when the eyes were closed. This CST increase produced during blink was significantly larger in the eyes receiving F6H8 than in the contralateral, untreated eyes (**Figure 4D**). As this warming of the ocular surface is produced by heat transference between the vascularized palpebral conjunctiva and the avascular corneal tissue, we speculate whether the increased warming during blink was due to a longer duration of the eye closure in F6H8-treated eyes. We then used the IR video recordings to measure blink duration, finding that the duration of eye closure during blink was not modified after F6H8 (0.59 ± 0.14 ms vs. 0.65 ± 0.18 ms, before and 5 min after F6H8, respectively; $p = 0.128$, paired t-test).

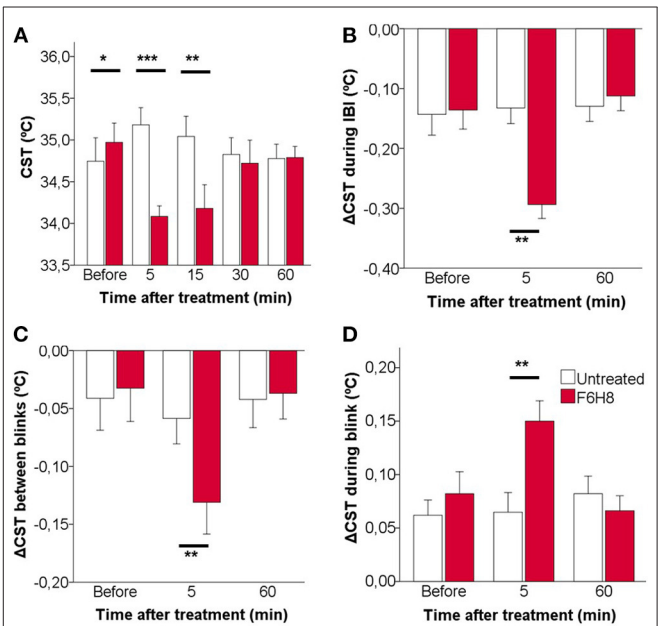

FIGURE 4 | Effects of topical instillation of F6H8 only on the right eye in comparison to the untreated left eye, on CST values measured from infrared thermographic images. **(A)** CST measured immediately after blink, that is, at the beginning of the interblink interval. **(B)** CST change during the IBI. **(C)** CST change between consecutive blinks. **(D)** CST change during blink. Data are mean ± standard deviation, $n = 7$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, paired t-test.

Thermal Adaptation of F6H8 and Saline Solution to Temperature Changes

Measurements done with a thermoprobe using the experimental setup described in **Figure 2D** showed that for a sustained Peltier cell temperature ($T_{Peltier}$) around 33–34°C and a room temperature around 23°C, temperature of the saline solution (T_{saline}) placed in a tube was close to 32°C, that is, around 1°C lower than $T_{Peltier}$ (**Figure 5A**). Temperature of F6H8 (T_{F6H8}) in this condition was around 29°C, that is, around 4–5°C lower than $T_{Peltier}$ (**Figure 5A**). In this regard, T_{F6H8} behaved similarly to the temperature inside the tube measured without

any liquid (T_{air}), about 4°C lower than $T_{Peltier}$ (**Figure 5A**). When changing the Peltier temperature, the profiles of T_{saline}, T_{F6H8}, and T_{air} followed the changes of $T_{Peltier}$ (**Figure 5A**), although maintaining the difference described above. Comparison of T_{saline} and T_{F6H8} during ascending and descending temperature steps showed two different hysteresis patterns (**Figure 5B**). T_{saline} exhibited a slower rate of either increasing or decreasing temperature in comparison with T_{F6H8}. Furthermore, the warming rate and cooling rate of each substance showed that F6H8 tended to cool down faster than saline. Moreover, under our experimental conditions, T_{F6H8} cooling rate was higher around 34°C (**Figure 5B**).

DISCUSSION

During the last 5 years, perfluorohexyloctane has been used as an alternative treatment of DED, particularly for its evaporative form due to Meibomian gland dysfunction (MGD) (8, 11, 13, 20). After 4–8 weeks of treatment with F6H8, MGD patients show an increase of the tear film and the lipid layer thickness (13), and Schirmer I test and Tear Film Break-Up Time (TFBUT) values, as well as a reduction of OSDI scores (8, 11). These data supported the idea that F6H8 is effective to treat evaporative forms of DED by improving the lipid layer of the tear film, and subsequently reducing tear evaporation and increasing tear film stability. This is a conceivable mechanism of action, because dry eye patients show an increased tear evaporation rate (21) and, due to its low surface tension, the F6H8 liquid state can act as a surfactant, forming monolayers at the water/air interphase (22, 23). F6H8 incorporation into artificial lipid systems mimicking the lipid layer of the tear film does not affect the tear film interface properties and restores the fluidity of these artificial lipid layers (24). Moreover, when applied onto healthy rabbit corneas, F6H8 spreads over larger areas than saline with lower viscosity (12). Therefore, F6H8 may contribute to restore, at least partially, the altered tear film lipid layer in evaporative DED patient.

To the best of our knowledge our observations are the first demonstration that, in addition, topical application of F6H8 onto the human eye decreases for several minutes the corneal surface temperature. Five min after administration of F6H8, CST decreased about −0.7°C in all the studied subjects. This ocular surface cooling occurs in parallel to an increase in tearing and blinking frequency that cannot be associated to the activation of the nociceptive corneal nerve fibers responsible to reflex blinking and tearing (25, 26) because any conscious sensation was evoked by F6H8.

The biophysical mechanism explaining the cooling effect of F6H8 is unknown. Cooling of the ocular surface after eye opening has been related to tear evaporation rate. Thus, the possibility exists that the compound would increase it. However, to the best of our knowledge tear evaporation after topical F6H8 administration has not been measured neither in evaporative DED patients, nor in healthy eyes. Only in an experimental model in healthy rabbit eyes *in vivo*, Agarwal et al. have described an acute biphasic effect of F6H8 in the percentage of change of tear evaporation from baseline (12). They noticed that although tear evaporation rate slightly and transiently increased by 5 min after F6H8 instillation, it tended to decrease 60–90 min afterwards, reaching values even below baseline. As the increase of tear evaporation occurred also after application of saline, these authors attributed this finding to the increased tear volume and, possibly, to the transient alteration of the tear film structure due to the instillation itself. An alternative mechanism could be the evaporation of the molecule itself, although F6H8 exhibits a low evaporation rate compared with other semifluorinated alkane molecules. When tested *in vitro*, <1.5% evaporated within 1 h, and more than 50% of the initial volume remained unevaporated after 24 h, both at 35°C (27). However, we cannot exclude that an increased fluid evaporation rate would explain, at least in part, the cooling effect found after F6H8 treatment.

According to Fourier's law of heat conduction, heat flow is inversely proportional to the thickness of the material and directly proportional to (a) the heat diffusion area; (b) the temperature gradient; and (c) the specific thermal conductivity constant of materials. In our experiments we assumed that the temperature gradient among the inner parts of the eye globe, the exposed area of the corneal tissue and the room environment keep constant, and that if F6H8 would cause an increase of the tear film thickness, this would represent a negligible increase of the total distance among the inner parts of the eye and the environmental air. Therefore, it can be hypothesized that a mechanism for the cooling effect of F6H8 would be an increase of the tear film thermal conductivity after the incorporation of F6H8 to the outermost tear film layer. In the presence of F6H8, we observed an increase of the ocular surface warming produced with blink (during the time that the eye is closed) and a faster decay of CST during the IBI. Interestingly, when measuring the thermal adaptation of F6H8 to temperature changes in a quite naïve experiment, we observed that the temperature measured inside liquid F6H8 tends to be between the temperature imposed by the Peltier cell and that of the environmental room air. Despite the absence of experimental data on the heat transmissivity properties of the molecule, it can be speculated that an increase of thermal conductivity and the subsequent increase of heat loss from the ocular surface to the environment is produced after topical administration of F6H8. However, an effect on radiative cooling cannot be ruled out with the present set of experiments.

In the present experiments we have not studied the time course of F6H8 removal from the ocular surface. Despite that, it seems reasonable that, at least partially, the compound could be drained continuously together with tear fluid, thus explaining the attenuation of the cooling effect of F6H8 with time. Previous studies estimated that the basal turnover of the tear film lipid layer occurs at an approximated rate of 1% per min in healthy humans (28). Therefore, if F6H8 is homogeneously distributed along the lipid layer and both are drained together, 30 min after F6H8 application it would be expected that 30% of the compound have been removed from the front of the eye. This value could be even higher, given that the molecule has a specific-gravity greater than water (9, 29). Thus, when the head is in vertical position -as in our experiments- F6H8 would tend to be accumulated in the lower part of tear film and the inferior tear meniscus, which could accelerate its draining. Despite deeper studies on

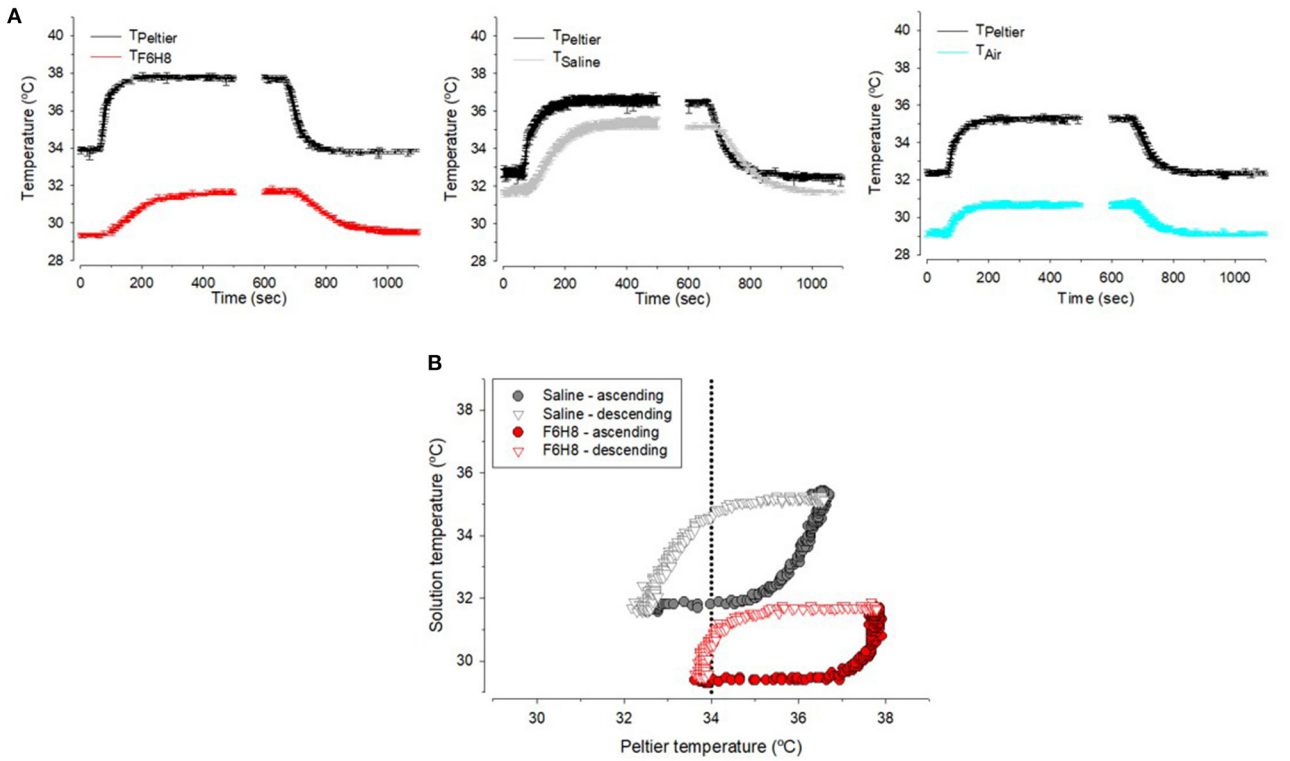

FIGURE 5 | (A) Sample recordings of the temperature measured inside the tube filled with F6H8, saline or empty (air) during experimental warming or cooling in the conditions explained in method and **Figure 1D**. **(B)** Hysteresis curves during warming (closed symbols) or cooling (open symbols) inside saline (gray) and F6H8 (red).

the spatiotemporal dynamics of F6H8 and its distribution and removal from the ocular surface would be welcomed, existing literature on the precorneal residence of a F6H8 drop in an *ex vivo* model of porcine eye shows a rapid drop of 36% of the substance in the precorneal space during the first 10 min after its application. Interestingly, from that moment on, F6H8 elimination slowed down and 56% is still in the precorneal space 1 h after application (27), and even tends to accumulate in the corneal epithelium (27, 30).

The F6H8-induced cooling of the ocular surface was produced in parallel with transient increases in tearing and blinking. Considering the role of corneal cold-thermoreceptors on basal tear production and blinking (3, 4), it seems conceivable that the changes in the activity of this population of trigeminal sensory neurons would be signaling the F6H8-induced CST reduction, thus inducing reflex changes in tearing and blinking. Since the classical observations by Mapstone (31), both blinking and tearing are considered as physiologic reflex responses that counteract the ocular surface cooling produced during eye opening. In our experiments we found prominent and long-lasting effects of F6H8 in blinking frequency. We also confirmed that along a blinking cycle, the eye is closed about 6% of the time and open about 94% of the time, a long period when the cornea is losing heat to the environment. Blinking may counteract CST cooling by three mechanisms: (a) passive prevention of heat loss; (b) heat transfer from the eyelids to the ocular surface during blink; and, (c) re-layering of the warm tear film over the cornea (31). F6H8 increased blinking rate and reduced IBI duration therefore reducing the time that the ocular surface is losing heat to the environment due to the cornea-air temperature gradient. In the same direction, a slight increase of the eye-closure time was observed, therefore favoring the heat exchange from the lids to the cornea. However, both processes were not enough to counteract the net cooling effect that is possibly related to the spreading of the compound over the whole tear film with each blink and the subsequent F6H8 evaporation. Increased blink and tearing rate are due to an increase in the TRPM8-dependent activity of cold thermoreceptor neurons, whose central axon projections have synapses with second order neurons of the trigeminal brainstem complex (32). The corneal nerve endings of these thermosensitive cold neurons are activated by cooling and tear hyperosmolarity (33), and also by the continuous oscillatory changes of temperature and wetness produced in the front of the eyes while they are open. Psychophysical experiments showed that a corneal cooling between 1 and 2°C is needed to evoke conscious sensations of cooling, while reducing the corneal temperature beyond these values elicits sensations of irritation (17, 25). The increased neural activity evoked in cold thermoreceptor neurons, especially in those belonging to the high background-low threshold subtype (HB-LT), by the small temperature and/or osmolarity changes produced during the interblink interval (expected to be around

0.5°C) is sufficient to evoke blink reflex (34), while more intense corneal cooling recruit the low background-high threshold (LB-HT) cold thermoreceptor endings, whose activation, together with that of nociceptive nerve endings, is claimed to evoke irritation and pain sensations (35). In the present experiments we found that F6H8 reduces the corneal temperature ∼1°C and also induces a fast and intense further cooling of the corneal surface during the interblink interval, two times larger in F6H8-treated eyes than in untreated or saline-treated eyes. We propose that this corneal temperature drop produced by F6H8 increases the firing of HB-LT cold thermoreceptor nerve endings to a level enough to reflexively increase blinking rate and tear production, although not enough to evoke cooling sensations arising from the ocular surface. The absence of conscious sensations after F6H8 may be also explained by the higher tear film stability produced by the molecule (8, 11). The F6H8 layer formed over the tear film would reduce aqueous tear evaporation and prevents the local production of tear hyperosmolarity and drying spots that are leading to the activation of corneal nerve endings and development of ocular sensations of dryness and irritation (36).

In summary, we described here the unknown long-lasting cooling effect of F6H8 when applied topically onto the healthy ocular surface. This effect was paralleled by thermal homeostatic responses to protect the avascular ocular surface, such as the increase of tearing and blinking, both reflex responses driven by the TRPM8-mediated activation of corneal cold-thermoreceptors in response to ocular surface cooling. Besides this temperature reduction, F6H8 increases tear film stability and thickness, which limits the production of the local osmolarity changes underlying the genesis of ocular sensations. As a concluding remark, F6H8 instilled onto the eye reduces corneal surface temperature enough to increase tearing and blinking rate but not to evoke conscious sensations of ocular discomfort. The increased tear volume more frequently redistributed over the ocular surface helps to prevent corneal dryness and contributes to the clinical benefits of F6H8 in DED and other ocular surface pathologies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Órgano Evaluador de Proyectos de la Universidad Miguel Hernández de Elche. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MD-M and EV acquired and interpreted the data. EV and JG conceived and designed the work. AD-T and AA-M contributed to design the experiments. MCA and AA-M equally contributed to supervise the work. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Spanish Agencia Estatal de Investigación and the European Regional Development Fund grants SAF2017-83674-C2-1-R and SAF2017-83674-C2-2-R, the Generalitat Valenciana Excellence Program grant PROMETEO/2018/114, Predoctoral fellowships ACIF/2019/054 from GV (MD-M) and FPU16/00283 from Spanish Ministry of Universities (EV), and PID2020-115934RB-I00/AEI/10.13039/501100011033.

ACKNOWLEDGMENTS

Authors are grateful to Carolina Luna for her expert technical assistance.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 15 November 2021
doi: 10.3389/fmed.2021.767967

Unilateral Corneal Insult Also Alters Sensory Nerve Activity in the Contralateral Eye

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After the unilateral inflammation or nerve lesion of the ocular surface, the ipsilateral corneal sensory nerve activity is activated and sensitized, evoking ocular discomfort, irritation, and pain referred to the affected eye. Nonetheless, some patients with unilateral ocular inflammation, infection, or surgery also reported discomfort and pain in the contralateral eye. We explored the possibility that such altered sensations in the non-affected eye are due to the changes in their corneal sensory nerve activity in the contralateral, not directly affected eye. To test that hypothesis, we recorded the impulse activity of the corneal mechano- and polymodal nociceptor and cold thermoreceptor nerve terminals in both eyes of guinea pigs, subjected unilaterally to three different experimental conditions (UV-induced photokeratitis, microkeratome corneal surgery, and chronic tear deficiency caused by removal of the main lacrimal gland), and in eyes of naïve animals *ex vivo*. Overall, after unilateral eye damage, the corneal sensory nerve activity appeared to be also altered in the contralateral eye. Compared with the naïve guinea pigs, animals with unilateral UV-induced mild corneal inflammation, showed on both eyes an inhibition of the spontaneous and stimulus-evoked activity of cold thermoreceptors, and increased activity in nociceptors affecting both the ipsilateral and the contralateral eye. Unilateral microkeratome surgery affected the activity of nociceptors mostly, inducing sensitization in both eyes. The removal of the main lacrimal gland reduced tear volume and increased the cold thermoreceptor activity in both eyes. This is the first direct demonstration that unilateral corneal nerve lesion, especially ocular surface inflammation, functionally affects the activity of the different types of corneal sensory nerves in both the ipsilateral and contralateral eyes. The mechanisms underlying the contralateral affectation of sensory nerves remain to be determined, although available data support the involvement of neuroimmune interactions. The parallel alteration of nerve activity in contralateral eyes has two main implications: a) in the experimental design of both preclinical and clinical studies, where the contralateral eyes cannot be considered as a control; and, b) in the clinical practice, where clinicians must consider the convenience of treating both eyes of patients with unilateral ocular conditions to avoid pain and secondary undesirable effects in the fellow eye.

Keywords: dry eye, sensory nerve activity, contralateral effects, corneal inflammation, corneal lesion

OPEN ACCESS

Edited by:
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San Raffaele Hospital (IRCCS), Italy

Reviewed by:
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Takefumi Yamaguchi,
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *31 August 2021*
Accepted: *11 October 2021*
Published: *15 November 2021*

Citation:
Luna C, Quirce S, Aracil-Marco A, Belmonte C, Gallar J and Acosta MC (2021) Unilateral Corneal Insult Also Alters Sensory Nerve Activity in the Contralateral Eye. Front. Med. 8:767967. doi: 10.3389/fmed.2021.767967

INTRODUCTION

The ocular surface (OS) is innervated by different functional types of sensory neurons that not only evoke conscious sensations but also contribute to corneal tissue tropism and initiate protective motor and autonomic reflexes such as blinking and tearing (1–7). There are two types of nociceptor fibers innervating the cornea, namely, mechanonociceptors, which express Piezo2 channels and respond only to mechanical forces (8, 9), and polymodal nociceptors, which express a diversity of ion channels, such as TRPA1, TRPV1, ASIC, and Piezo2 that allow them to respond to a variety of stimuli applied on their receptive field, including mechanical forces, heating, and several irritant chemical substances (9–13). In the conjunctiva, low-threshold mechanoreceptors that evoke touch when stimulated have been also described (14, 15). The selective stimulation of these two populations of nociceptors in humans evokes sensations of irritation and pain, although with different qualities of the sensation (6). In humans, the selective activation of polymodal nociceptors has been described as the sensory arm to evoke the aforementioned protective tearing and blinking reflexes (1, 7). Additionally, cold thermoreceptors have been described in the cornea (10, 11, 16–19). Cold thermoreceptors express TRPM8 transducing channels and typically respond to decreases in temperature with different thresholds: canonical cold thermoreceptors with high background activity at normal corneal temperature and high sensitivity to cooling, and presumed cold nociceptors, that exhibit a low background activity and requires more intense cooling to increase its firing rate. Their selective stimulation in humans evokes sensations described as freshness/cold or dryness/pain, respectively, depending on the amplitude of temperature decrease (6). In addition, to evoke conscious sensations, the activity of cold thermoreceptors expressing TRPM8 channels contribute to the control of basal tearing and spontaneous blinking (3–5, 20).

We have previously shown that after unilateral OS inflammation or lesion, corneal sensory nerve activity and sensations are altered in the ipsilateral eye (21–26). As in other tissues, corneal nociceptors (specially polymodal nociceptors) are sensitized after lesion or during ocular surface inflammation (22–27). Nociceptor sensitization is characterized by an increase in spontaneous activity, reduction of the response threshold, and/or increased response to stimulation with stimuli of the same intensity (28). Additionally, corneal nociceptors also contribute to the inflammatory processes of the OS (a process known as neurogenic inflammation) (29, 30) by their release of pro-inflammatory neuropeptides as substance P and Calcitonin Gene-Related Peptide (CGRP) (31–34). The activity of cold thermoreceptors is decreased under inflammation (23, 24) through the inhibition of the TRPM8 channels by inflammatory mediators (35), and increased in chronic tear deficiency through changes in the Na+ and K+ channel expression and/or activity (25).

However, no previous studies have defined the changes in the sensory nerve activity at the contralateral eye nerves after unilateral nerve lesion or inflammation and if the putative change in their neural activity affects sensations only or also affects ocular tropism and protective reflexes. This is of special relevance because previous studies suggest bilateral changes in corneal sensitivity in patients with unilateral infectious keratitis (36), as well as bilateral changes in corneal nerve morphology and density in humans (37–42) and animals (43, 44).

In the present study, we recorded the impulse activity of mechano- and polymodal nociceptor and cold thermoreceptor nerves in guinea pigs, innervating the cornea of both eyes, under different experimental inflammatory or injury conditions (UV-induced photokeratitis, microkeratome surgery, and chronic tear deficiency) affecting only one eye, to answer the question of whether unilateral eye surface damage also modifies the nerve activity and sensitivity of the contralateral fellow eye. This information is important for the design of research protocols in the future, in which the use of contralateral eyes as controls should be excluded, and also to determine the convenience of applying bilateral treatment when unilateral ocular inflammation, infection, or injury affect one of the eyes.

MATERIALS AND METHODS

Animals

Guinea pigs of both sexes, weighing 200–400 g at the beginning of the experiment, were used. The study was performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, the European Union Directive (2010/63/EU), and the Spanish regulations on the protection of animals used for research, following protocols approved by the Ethics Committee of the Universidad Miguel Hernández de Elche. The animals were kept in individual cages under a controlled day–night cycle with free access to food and water.

Experimental Groups

The animals were distributed into four groups: (a) Control: A group of 42 animals without any experimental manipulation; (b) Unilateral UV-irradiation: A group of 9 animals subjected to unilateral UV irradiation of the OS; (c) Unilateral microkeratome corneal lesion: A group of 6 animals in which unilateral corneal nerve lesion was caused mechanically; (d) Unilateral main lacrimal gland excision: A group of 6 animals subjected to unilateral excision of the main lacrimal gland.

After the different experimental interventions, the animals were allowed to recover postoperatively and then housed individually under standard conditions in a certified animal facility. They were inspected daily for ocular inflammation, corneal epithelial defects or infections, as well as for abnormal behavior, and were treated accordingly. Before euthanizing the animal for the *ex vivo* electrophysiological recording of the electrical activity of corneal sensory nerves (see below), the ocular surface of both eyes was evaluated with a pocket slit lamp and the tear volume was measured.

Unilateral UV Irradiation

Under deep anesthesia (80 mg/kg ketamine and 4 mg/kg xylazine, i.p.), 254 nm UV-C radiation (1,000 mJ/cm^2) was delivered for 49 min to one eye of the animal with a UV lamp (VL-4.C 230V 50/60 Hz; Vilber Lourmat, Marne-la-Vallée, France) placed at a distance of 17 cm from the eye. The animals were euthanized 48 h after the UV irradiation and both eyes were excised for electrophysiological recording. In a previous work, we showed that after the exposure to this intensity of UV radiation, mild clinical signs of inflammation and significant changes of the spontaneous and stimulus-evoked activity of the corneal sensory receptors were developed in the ipsilateral eye, maximal of 48 h after UV (24).

Unilateral Microkeratome Corneal Lesion

In the anesthetized guinea pigs (ketamine 50 mg/kg and xylazine 5 mg/kg, i.p.; topical 0.1% tetracaine, and 0.4% oxybuprocaine), a corneal flap of 4 mm diameter was cut at the mid-stromal depth in one eye using a custom-made microkeratome (Deriva Global, SL, Valencia, Spain) designed for the guinea pig eye (26). As the more prominent effects on the nerve activity in the lesioned eye were seen 24–48 h after the corneal lesion, in the present work, the animals were euthanized 24–48 h after the corneal lesion to study the corneal nerve activity in both eyes.

Unilateral Main Lacrimal Gland Excision

The animals were anesthetized with ketamine (90 mg/kg i.p.) and xylazine (5 mg/kg i.p.) for the unilateral removal of the main lacrimal gland. After performing an 8 mm skin incision on the temporal side, posterior to the lateral canthus, the fibrous capsule of the exorbital lacrimal gland was exposed and dissected, and the lacrimal gland was carefully excised (25). A drop of antibiotic (3 mg/ml tobramycin) was applied onto the surgical area before suturing the skin incision using a 6.0 braided silk suture. Before euthanasia and *ex vivo* recording, animals were housed for 4–11 weeks after surgery to fully develop corneal nerve alterations consecutive to chronic tear deficiency (25).

Electrophysiological Recording and Analysis

The animals were killed with an i.p. overdose of sodium pentobarbitone, and their eyes, together with the bulbar and tarsal conjunctiva, were enucleated. The whole eye or the excised cornea (see below) was placed in a custom recording chamber superfused with the following physiological solution (in mM): 133.4 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgCl_2$, 16.3 $NaHCO_3$, 1.3 NaH_2PO_4, and 7.8 glucose, gassed with carbogen to a pH = 7.4. The temperature of the perfusion solution was maintained at a basal temperature of 34°C with a homemade feedback-controlled Peltier device. Two types of preparations were used for the *ex vivo* recording of the corneal nerve activity, namely, the "whole eye" and the "isolated cornea" preparations (23, 24, 45). The "whole eye" preparation was particularly suitable for recording the polymodal and mechanosensory nociceptive units in the ciliary nerves, whereas, in the "isolated cornea" preparation, the activity of cold-sensitive units could be more easily identified [see (23) for a detailed schema of the preparations].

Recording Preparations

Whole Eye Preparation

The connective tissue and extraocular muscles in the back of the eye were carefully removed to expose and isolate the ciliary nerves traveling around the optic nerve. The eye was then placed in a recording chamber divided into two compartments by an elastomer-coated plastic wall (Sylgard 184; Dow Corning, Midland, Michigan, United States). The front of the eye including the cornea and the conjunctiva was introduced into a round perforation made in the center of the dividing wall to which the bulbar conjunctiva was pinned, thereby isolating the anterior segment from the back of the eye and the ciliary nerves, and preventing the direct exposure of the ciliary nerves to the solutions applied onto the corneal surface. The anterior compartment was continuously bathed with saline solution at 34°C and the back compartment was filled with warm mineral oil. The thin nerve filaments were teased apart from the ciliary nerve trunks and placed on an Ag-AgCl electrode for a monopolar recording of the unitary impulse activity of the axons innervating the cornea. Electrical signals from the recording electrode were fed to a 50-Hz noise eliminator (HumBug, Digitimer; Welwyn, United Kingdom) and then amplified and filtered (DAM50 amplifier; WPI, Sarasota, Florida, United States). Then, the signals passed through an analog-digital converter (CED Micro-1401; Cambridge Electronic Design, Cambridge, United Kingdom) and were stored in a personal computer (PC) with Spike2 software (v8.0; Cambridge Electronic Design) for the offline analysis.

Isolated Cornea Preparation

The corneas were excised around the limbus and then pinned to the bottom of a recording chamber continuously superfused with a physiological solution maintained at 34°C with a homemade Peltier device. To record the nerve terminal impulse (NTI) activity, a 50-μm-diameter glass micropipette filled with the physiological saline solution was applied gently to the corneal surface using a micromanipulator and then attached to the cornea by slight suction with a syringe. The electrical signals with respect to an Ag/AgCl pellet placed in the chamber were passed through a 50 Hz noise eliminator, amplified (AC preamplifier NL 103; Digitimer, Welwyn, United Kingdom), filtered (high pass 150 Hz, low pass 5 kHz; filter module NL 125; Digitimer), and then transferred to a PC with a Cambridge Electronic Design (CED) micro-1401 acquisition system and dedicated software, to be stored until the offline analysis.

Experimental Protocols

To study the electrophysiological activity of the corneal polymodal nociceptors, mechanonociceptors, and cold thermoreceptors, the following experimental protocols were performed:

Polymodal Nociceptors and Mechanonociceptors

After recording the electrical activity for 1 min to determine the spontaneous activity of the unit, the receptive field (RF) of the nociceptor fiber was located and mapped by mechanical stimulation with a fine paintbrush and a suprathreshold von

Frey hair. Then, the mechanical threshold was determined with calibrated von Frey hairs of increasing force (range, 0.078–4 mN; Bioseb, Vitrolles, France). To ascertain its polymodality, the chemical sensitivity was tested with a low-flow jet of 98.5% carbon dioxide (CO_2) and 1.5% air applied onto the RF for 30 s (CO_2 pulse). The response to the temperature changes was eventually tested by changing the temperature of the perfusion solution for 30 s from 34°C up to 45°C (heating ramp) or down to 20°C (cooling ramp). At least 3 min were allowed between the different stimuli.

Cold Thermoreceptors

Nerve impulses originating at the single cold-sensitive nerve terminals were identified by their usually regular ongoing discharge at the basal temperature, which increased with cooling and decreased with warming. After recording the spontaneous activity at the basal temperature for at least 1 min, a cooling ramp from 34 to 20 °C was performed, followed by rewarming to 34 °C for 3 min. Then, a heating ramp to 45 °C was applied for 30 s before returning to the basal temperature.

Analysis of Sensory Nerve Activity

The characteristics of the spontaneous and stimulus-evoked impulse activity of the sensory nerves recorded in the control eyes, inflamed/lesioned/tear deficient eyes, and in contralateral eyes were analyzed offline using the Spike2 software. In the present work, the following parameters were calculated to compare the differences between the control, insulted, and contralateral eyes:

Polymodal Nociceptors

(a) The spontaneous activity was measured for 1 min at the beginning of the recording, before any intended stimulation (in impulses/s). (b) The mechanical threshold (in mN). (c) The latency of the impulse discharge evoked by the CO_2 pulse, measured as the time (in s) elapsed between the onset of the gas pulse and the beginning of the impulse response. (d) The mean discharge rate of the response evoked by the CO_2 pulse (in impulses/s). (e) Postdischarge, the mean discharge rate (in impulses/s) for 30 s after the end of the CO_2 pulse.

Mechanonociceptors

(a) Spontaneous activity at the beginning of the recording, before any intended stimulation (in impulses/s). (b) Mechanical threshold (in mN).

Cold Thermoreceptors

(a) Ongoing activity measured for 1 min at a basal temperature of 34°C at the beginning of the recording (in impulses/s). (b) Cooling threshold, calculated as the decrease in temperature (in °C) during the cooling ramp from 34 to 20°C required to increase the mean frequency of discharge by 25% for 20 s before the ramp. (c) Peak frequency, the maximal value of the firing frequency (in impulses/s) reached during the cooling ramp. (d) Temperature change needed for the peak frequency, as the temperature change (in °C) is required to reach the peak frequency value during the cooling ramp.

Tear Volume Measurement

Tearing was measured in both eyes under stable environmental conditions (23°C temperature; 55% humidity) using commercial phenol red threads (Zone-Quick; Menicon, Tokyo, Japan) without topical anesthesia (23–25), before and after inducing the corneal insults (48 h after UV radiation, 24–48 h after the microkeratome lesion and 4 weeks after removal of the main lacrimal gland). The lower lid was gently pulled down, the folded 2 mm end of the thread was gently placed on the nasal palpebral conjunctiva, and the lid was then released. After a period of 30 s, the lower lid was, again, pulled down and the thread was gently removed. The entire length of the red-stained portion of the thread (in mm) was measured with a ruler under a stereomicroscope with an accuracy of 0.5 mm. The length of the red thread reflects both the tear volume in the conjunctival sac and the tear secretion over the 30 s of measurement.

Statistical Analysis of Data

The data were collected and processed for statistical analysis using the SigmaPlot software (SigmaPlot 11.0; Systat Software Inc, Point Richmond, California, United States). Unless otherwise stated, the data are expressed as mean ± SEM, with n being the number of explored units or eyes, as appropriate. The differences between the data from the different experimental groups were explored using a *t*-test or Mann-Whitney as needed. The differences between more than two groups were tested using one-way ANOVA or ANOVA on ranks, as needed. A *P*-value of 0.05 or less was considered significant.

RESULTS

Unilateral UV Irradiation-Induced Mild Ipsilateral Inflammation, and the Sensitization of Nociceptors and Cold Thermoreceptor Inhibition in Both the Ipsilateral and Contralateral Eyes

Effects on the Ipsilateral, UV-Irradiated Eye

Forty-eight hours after the unilateral ocular exposure to 1,000 mJ/cm^2 UV radiation, mild inflammation of the ocular surface (especially mild conjunctival hyperemia) could be observed only in the ipsilateral eye. The corneal nociceptors recorded in the ipsilateral eyes were sensitized, as reflected by the development of spontaneous activity (present in 3.1% of the 259 mechanonociceptor units from the control eyes and 18.5% of the 27 mechanonociceptor units recorded in the UV-irradiated eyes; $p < 0.05$, *Z*-test) and the significant decrease of the mechanical threshold of mechanonociceptors (0.64 ± 0.04 mN vs. 0.32 ± 0.03 mN, control vs. UV-irradiated eyes; $p < 0.01$, Mann-Whitney test). The spontaneous activity of polymodal nociceptors was significantly increased (6.6% in the control vs. 22% in the UV-irradiated eyes; $n = 152$ and 41, respectively; $p < 0.01$, Z-test), and also the discharge rate evoked by chemical stimulation was significantly higher (1.9 ± 0.2 imp/s vs. 3.4 ± 0.5 imp/s, control vs. UV-irradiated; $n = 110$ and 45 units, respectively, $p < 0.01$, Mann-Whitney test), suggesting the development of sensitization also in the polymodal

TABLE 1 | The spontaneous activity and stimulus-evoked responses of corneal nociceptors recorded in the control eyes and the eyes contralateral to UV irradiation, microkeratome lesion, or lacrimal gland removal (tear deficiency).

	Control eyes	Contralateral eyes		
		Uv radiation	Microkeratome lesion	Tear deficiency
Mechanonociceptors				
Mechanical threshold (mN)	0.64 ± 0.04	0.41 ± 0.12	0.42 ± 0.06*	0.58 ± 0.18
Spontaneous activity				
Present in (%)	3,1%	0%	0%	0%
Spontaneous activity (imp/s)	0.7 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
n	275	11	42	8
Polymodal nociceptors				
Mechanical threshold (mN)	0.35 ± 0.03	0.35 ± 0.04	0.32 ± 0.03	0.27 ± 0.14
Spontaneous activity				
Present in (%)	6.6%	25%*	12.5%	0%
Spontaneous activity (imp/s)	0.6 ± 0.3	1.1 ± 0.9	0.8 ± 0.3	0 ± 0
Response to CO_2 pulse				
Latency (s)	13.01 ± 0.77	10.68 ± 1.78	9.42 ± 1.85	16.80 ± 2.65
Mean discharge rate (imp/s)	1.94 ± 0.16	2.59 ± 0.47	2.12 ± 0.65	1.28 ± 0.51
Postdischarge (imp/s)	2.13 ± 0.50	2.86 ± 1.49	0.46 ± 0.15*	0.76 ± 0.39
n	167	19	17	6

*$p < 0.05$, *t-test or Mann-Whitney or Z-test (%), differences with control eyes.*

TABLE 2 | The spontaneous and stimulus-evoked activity of the corneal cold thermoreceptors recorded in the control eyes and the eyes contralateral to UV irradiation, microkeratome lesion, or lacrimal gland removal (tear deficiency).

	Control eyes	Contralateral eyes		
		Uv radiation	Microkeratome lesion	Tear deficiency
Cold thermoreceptors				
Ongoing activity at 34°C				
Present in (%)	100%	100%	100%	100%
Ongoing activity (imp/s)	9.0 ± 0.5	8.6 ± 2.2	6.8 ± 0.8*	12.5 ± 2.8*
Response to cooling ramp from 34 to 20 °C				
Threshold (Δ°C)	−2.7 ± 0.2	−1.6 ± 0.1*	−2.3 ± 0.2	−1.9 ± 0.3
Peak frequency (imp/s)	30.6 ± 1.3	24.7 ± 4.1	29.6 ± 2.2	35.4 ± 5.5
Temp. change to peak frequency (Δ°C)	−6.1 ± 0.4	−4.0 ± 0.5*	−7.7 ± 0.9	−5.1 ± 0.6
n	67	14	24	10

*$p < 0.05$ *t-test, differences with control eyes.*

nociceptor units recorded in the UV irradiated corneas. The spontaneous and cold-evoked activity of cold thermoreceptors was reduced in the UV-irradiated eyes (30.6 ± 1.3 imp/s vs. 18.9 ± 1.8 imp/s, control vs. UV-irradiated; $n = 67$ and 19, respectively; $p < 0.001$, *t*-test), while the cooling threshold and temperature to reach the peak frequency were not modified (data not shown).

Effects on the Contralateral, Non-irradiated Eye

In the contralateral eyes, no clinical signs of inflammation were found 48 h after UV irradiation. On the contrary, the activity of the different types of corneal nerves showed changes similar to those observed in the ipsilateral eye, although overall to a lesser degree. The mechanical threshold of mechanonociceptors was slightly reduced, although the difference was not statistically significant (**Table 1**). Similarly, the spontaneous activity of the mechanonociceptors and polymodal nociceptors did not change (**Table 1**). Twenty-five percent of the polymodal nociceptors exhibited spontaneous activity, which means the frequency was significantly higher than in the control eyes (**Table 1**), although no changes were observed in their response to CO_2 pulses (**Table 1**).

The ongoing activity at the basal temperature and peak response to the cooling ramps of cold thermoreceptors were not significantly modified in the contralateral eyes (**Table 2**), although the cooling threshold and the temperature change to reach the peak frequency were significantly reduced in comparison with the control eyes (**Table 2**).

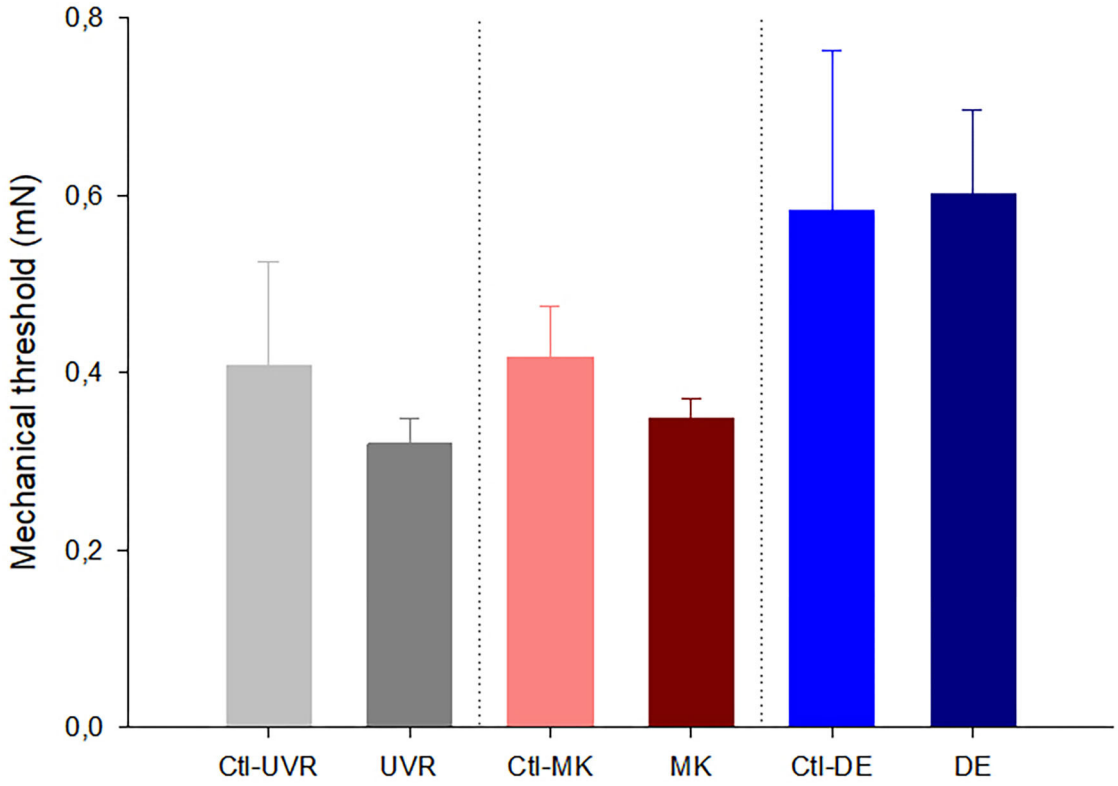

FIGURE 1 | Effects of unilateral UV irradiation, microkeratome lesion, and main lacrimal gland removal on the mechanical threshold of the mechanonociceptor units recorded in the ipsilateral and contralateral (denoted by "Ctl" label) eyes. The data are presented as mean ± SEM. No significant differences were found between the data from the ipsilateral and contralateral eyes inside each group, Mann-Whitney test.

Differences Between Nerve Activity and Tear Volume of UV-Irradiated and Contralateral Eyes

When comparing the activity of the mechanonociceptors (**Figure 1**), polymodal nociceptors (**Figure 2**), and cold thermoreceptors (**Figure 3**) from the UV-irradiated and contralateral eyes, no significant differences were found. The tear volume was not significantly affected in neither the irradiated nor in the contralateral eyes when compared with the control (10.6 ± 0.7m, $n = 42$; 12.7 ± 1.9 mm, $n = 9$; 11.3 ± 1.3 mm, $n = 9$; control, UV-irradiated eyes and contralateral, respectively; $p > 0.05$, one-way ANOVA).

Unilateral Corneal Microkeratome Lesion-Induced Ipsilateral Inflammation and the Sensitization of Nociceptors in Both the Ipsilateral and Contralateral Eyes

Effects on the Ipsilateral, Microkeratome Lesioned Eye

Twenty-four to 48 h after the unilateral corneal lesion with the microkeratome, a mild inflammation of the ocular surface (especially mild conjunctival hyperemia) was developed in the ipsilateral eye. The corneal surgical lesion with a microkeratome induced the sensitization of corneal nociceptors. The mechanical threshold of mechanonociceptors was significantly reduced (0.64 ± 0.04 vs. 0.35 ± 0.0.2 mN, control vs. lesioned eyes; $n = 275$ and 48, respectively; $p < 0.05$, Mann-Whitney test), and the response to the CO_2 pulses of polymodal nociceptors was significantly increased (1.9 ± 0.2 vs. 3.4 ± 0.5 imp/s, control vs. lesioned; $n = 110$ and 33, respectively; $p < 0.01$, Mann-Whitney test). The spontaneous and stimulus-evoked activity of the cold thermoreceptors of the contralateral eyes presented values similar to that of the control eyes (data not shown).

Effects on the Contralateral, Non-lesioned Eye

The mechano-nociceptors of the contralateral eyes presented a significantly lower mechanical threshold, although they did not develop spontaneous activity (**Table 1**). The spontaneous and stimulus-evoked activity of the polymodal nociceptors was not significantly affected or slightly reduced in the contralateral eyes (**Table 1**). Similarly, the spontaneous and cold-evoked activity of the cold thermoreceptor of the eyes contralateral to the microkeratome lesion was similar to the control or slightly reduced (**Table 2**).

Differences Between Nerve Activity and Tear Volume of Microkeratome Lesioned and Contralateral Eyes

No differences were noticed in the mechanical threshold of the mechano-nociceptors recorded in the lesioned and contralateral (**Figure 1**). Regarding polymodal nociceptors, the spontaneous

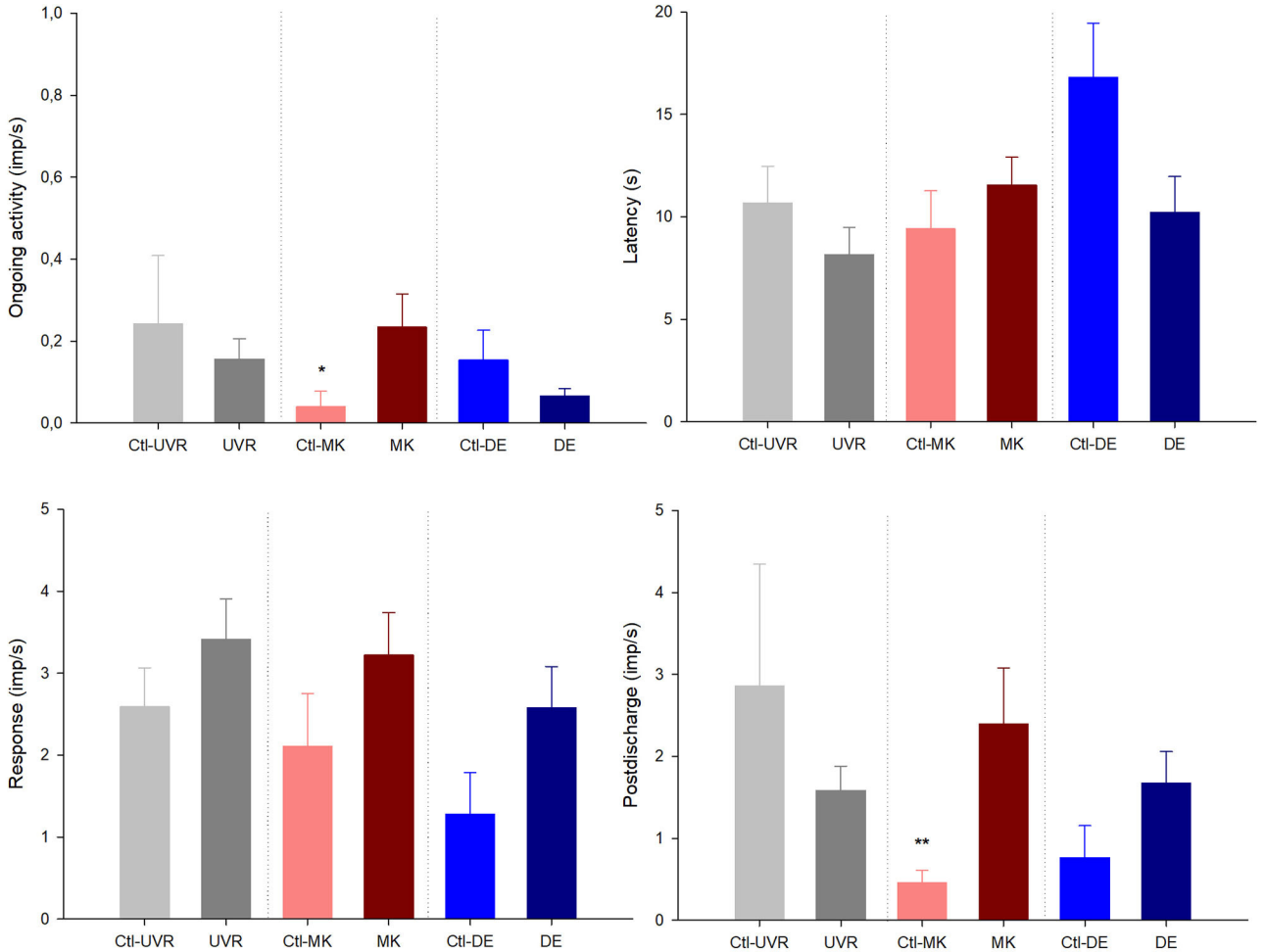

FIGURE 2 | The effects of unilateral UV irradiation, microkeratome lesion, and main lacrimal gland removal on the ongoing activity and the response to the chemical stimulation with carbon dioxide (CO_2) of the polymodal nociceptor units recorded in the ipsilateral and contralateral (denoted by "Ctl" label) eyes. The data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, *t*-test or Mann-Whitney, as needed, the differences between the data from the ipsilateral and contralateral eyes.

activity and postdischarge to CO_2 stimulation were statistically lower in the contralateral than the lesioned eyes (**Figure 2**). No differences were found when comparing the spontaneous and stimulus-evoked activity of cold thermoreceptors (**Figure 3**).

No changes in tear volume were found in the lesioned and contralateral eyes compared with the control (10.6 ± 0.7 mm, $n = 42$; 7.5 ± 0.9 mm, $n = 6$; 10.8 ± 2 mm, $n = 6$; control, lesioned and contralateral eyes, respectively; $p > 0.05$, one-way ANOVA).

Unilateral Lacrimal Gland Removal Decreased Tear Volume and Sensitized Corneal Cold Thermoreceptors in Both the Ipsilateral and Contralateral Eyes

Effects on the Ipsilateral Eye

Four weeks of tear deficiency induced by the unilateral removal of the main lacrimal gland induced signs of mild OS inflammation only in the operated side, with mild conjunctival hyperemia in all the operated guinea pigs and occasional mild corneal punctate after fluorescein staining. The tear volume was significantly reduced in the operated side and also in the contralateral eye, although to a lesser extent (see below Differences between the nerve activity and tear volume of the ipsilateral and contralateral eyes for details). The mechanical threshold of the mechanonociceptors was not modified 4 weeks after the lacrimal gland ablation (0.64 ± 0.04 vs.0.6 ± 0.09 mN, control vs. tear-deficient eyes; $n = 167$ and 27, respectively; $p > 0.05$, Mann-Whitney test). The spontaneous activity did not change (0.6 ± 0.3 imp/s vs.0.6 ± 0.4 imp/s, control vs. tear-deficient eyes; $n = 259$ and 27, respectively) and the response to CO_2 pulses (1.9 ± 0.2 vs. 2.6 ± 0.5 imp/s, control vs. lesioned; $n = 110$ and 14, respectively) of polymodal nociceptors was slightly increased, although the differences were not statistically significant. On the other hand, the spontaneous activity at the basal temperature (9 ± 0.5 vs. 14 ± 2.8 imp/s, control vs. tear-deficient eyes; $n = 67$ and 8, respectively; $p < 0.01$, *t*-test) and stimulus-evoked

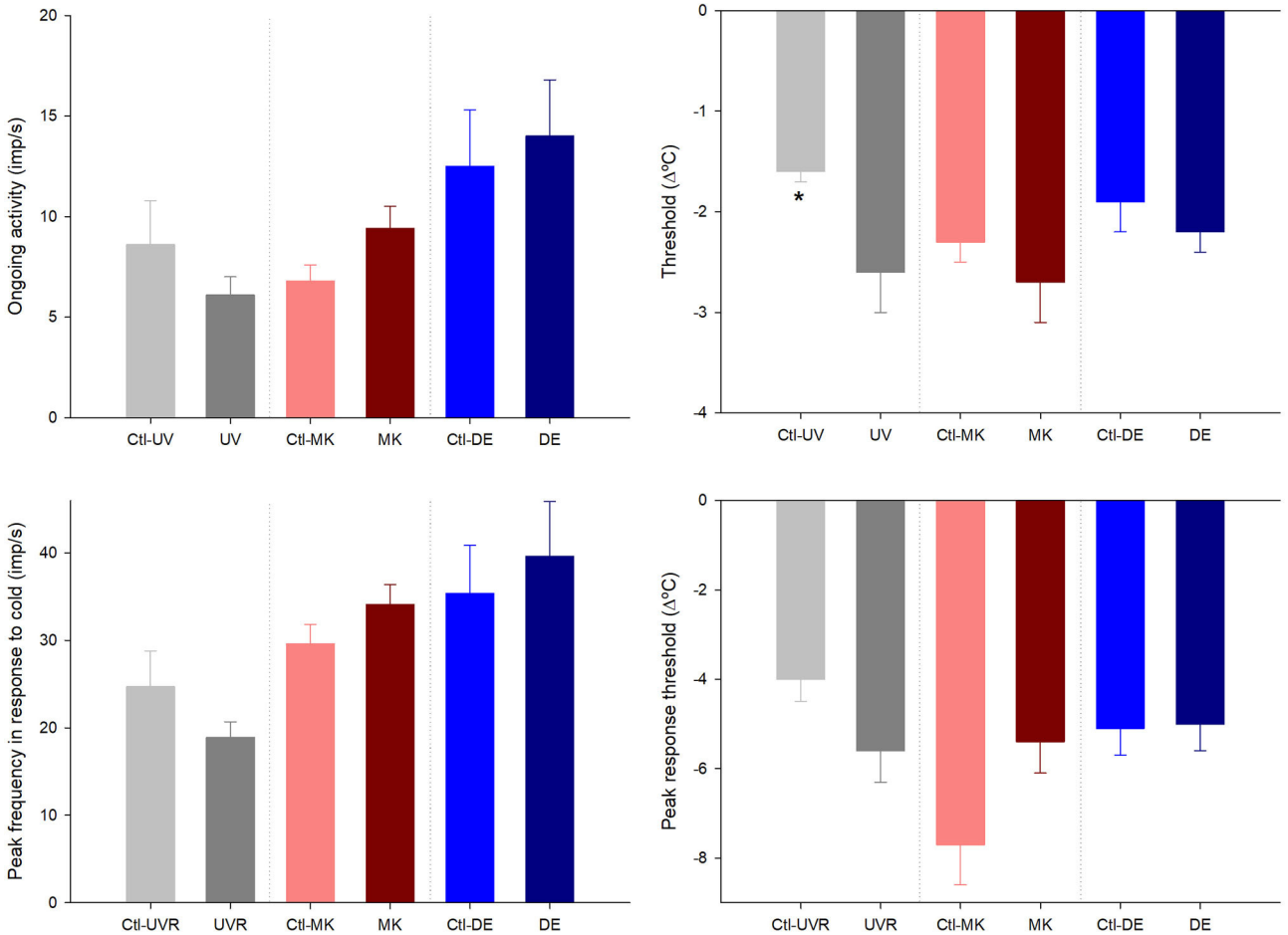

FIGURE 3 | Effects of unilateral UV radiation, microkeratome lesion, and main lacrimal gland removal on the ongoing activity and the response to cooling ramps of the cold thermoreceptor units recorded in the ipsilateral and contralateral (denoted by "Ctl" label) eyes. The data are presented as mean ± SEM. *$p < 0.05$, *t*-test or Mann-Whitney, the differences between the ipsilateral and contralateral data inside each group.

activity (peak frequency to cooling ramps: 30.6 ± 1.2 vs. 39.6 ± 6.3 imp/s, control vs. tear-deficient; $p < 0.05$, *t*-test) of the cold thermoreceptors of the operated side was significantly higher than in the control eyes, with no changes in the cold response thresholds (data not shown).

Effects on the Contralateral Eye

Similar to the ipsilateral eyes, the activity of the mechanonociceptors and polymodal nociceptors was not significantly modified in the eyes contralateral to lacrimal gland removal (**Table 1**), except for the small but significant higher spontaneous activity of the polymodal nociceptors compared with those of the naïve, control eyes (**Table 1**). Similarly, a higher value of spontaneous activity was the only significant difference found in the activity of the cold thermoreceptors recorded in the contralateral eyes (**Table 2**).

Differences Between the Nerve Activity and Tear Volume of the Ipsilateral and Contralateral Eyes

No significant differences were found when comparing the spontaneous and stimulus-evoked activity of the mechanonociceptors (**Figure 1**), polymodal nociceptors (**Figure 2**), and cold thermoreceptors (**Figure 3**) recorded in the eyes contralateral and ipsilateral to lacrimal gland removal.

As expected, according to previous work (25), 11 weeks after the unilateral lacrimal gland excision, tear volume was significantly decreased in both eyes (10.6 ± 0.7, 1.3 ± 0.4, 4.0 ± 1.0 mm; control, ipsilateral and contralateral eyes, respectively, $n = 6$; $p < 0.001$, ANOVA on ranks, with Dunn's *post hoc* test). The tear volume reduction was significantly larger on the ipsilateral side than on the contralateral side ($p < 0.05$, Mann-Whitney test).

DISCUSSION

For the first time, the present results show the development of the same changes in the sensory nerve activity in both eyes after the experimental unilateral inflammation or lesion of the OS in only one eye. Corneal nerve activity is altered in the corneas of the contralateral side, although the magnitude of those changes was usually smaller than in the ipsilateral side and does not always achieve a statistically significant level. Mild corneal

inflammation produces a sensitization of nociceptors and an inhibition of the activity of the cold thermoreceptors (23, 24), and the surgical lesion of corneal nerves produces a sensitization of nociceptors (26) and only small changes in the activity of cold thermoreceptors. Chronic tear deficiency is characterized by the inflammation of the OS and is known to produce nerve damage that can lead to corneal neuropathy (46). This condition also induces the sensitization of corneal sensory nerves, being the cold thermoreceptors the type of corneal fibers more affected by tear deficiency (25). The present results show that even when only one eye is primarily affected by inflammation or injury, the corneal sensory nerves of the contralateral side do not behave like those of the naïve eyes, as their spontaneous and/or stimulus-evoked activity is altered in the same way as in the lesioned side, although sometimes to a lesser degree.

In the present work, three different types of insults were performed to only one eye of the experimental animals. Among these three types of OS damage, the UV-induced corneal inflammation and chronic tear deficiency induced by lacrimal gland removal were the models that produced more significant changes in the activity of the corneal sensory receptors in the contralateral eye, while the corneal nerve damage produced by the unilateral corneal surgery presented fewer effects on the contralateral sensory nerve activity. The level of contralateral effects seems to depend on the degree of the inflammation induced in the ipsilateral eye, such that the greater the inflammation in the affected eye, the greater the contralateral effects.

The results support the idea that the contralateral effects are mostly due to an interaction between the nervous and the immune system as some authors have pointed out and as we will discuss here. One possible explanation for the contralateral effects would be the existence of innervation from the contralateral trigeminal ganglion, the trigeminal nuclei at the brainstem, or even at the superior levels of the central nervous system. It was described that there are a small number of ocular nerve fibers that travel to the contralateral trigeminal ganglion (47) and also that the central projections of some trigeminal ganglion neurons project to both the ipsilateral and contralateral trigeminal brainstem nuclei (48). These information crossing would explain, at least in part, how the altered sensory nerve activity arising at the damaged eye would affect the activity in the neurons processing the sensory information from the contralateral side.

Another possible explanation is that the contralateral effects are mediated by the existence of neuro-immune interactions. Some authors found evidence of a sympathetic inflammatory response in the contralateral side after unilateral inflammation or lesion, whose results support an interaction between the nervous and the immune system in the ocular tissues. In experimentally induced unilateral glaucoma, there is an activation of the macro- and micro-glia in both retinas, explained by an immune response after the breakdown of the blood-retinal barrier of the experimental eye (49–52).

Numerous previous works have studied the morphology and density of corneal nerves contralateral to a unilateral infection or injury. Almost all of them show that after unilateral damage, there is not only a decrease in the density of the subbasal nerves in the ipsilateral but also in the contralateral side. In patients with unilateral herpes simplex keratitis or herpes zoster ophthalmicus, there is a decrease in the corneal subbasal plexus nerve density in the affected eye and also in the contralateral eye (39, 40, 42), which explains the reduction of the contralateral corneal sensitivity (36). Moreover, there is also a bilateral increase in the dendritic cell density, which correlates with the decrease of the corneal subbasal nerves (37, 38, 40, 41), as well as in the levels of the pro-inflammatory cytokines in tears in patients with unilateral bacterial keratitis (43, 53). Several authors have also observed an increase of chemokines like MCP-1 in the aqueous humor in the contralateral eye after unilateral cataract surgery (54), although it has been suggested that the increase of MCP-1 is produced only in diabetic patients (55). In mice, after a unilateral surgical axotomy of the ciliary nerves or penetrating keratoplasty, there is also a decrease in the contralateral subbasal nerves (43, 56). After surgery, there is a bilateral release of substance P that has been proposed to abolish the immune privilege of both eyes (56) through disabled T regulatory cells (57). It has been also described that after corneal alkali burn, pro-inflammatory cytokines also increase Substance P and the expression of its receptor in the contralateral trigeminal ganglion, which supports the idea that substance P seems to be involved in the contralateral propagation of inflammation through corneal sensory nerves (30, 58).

Further supporting this, Guzman et al. (44) showed that unilateral corneal injury affects the contralateral ocular surface mucosa. After an injury, the sensory nerves of the OS are damaged, thus producing a neurogenic inflammatory reflex that is initiated in the injured eye through the activation of polymodal nociceptive sensory nerves expressing the TRPV1 channel. Subsequently, a neurogenic reflex is produced in both eyes, including the release of substance P that is the effector of the contralateral inflammatory response (44). Epithelial cells, DCs, and T cells express functional substance P receptors, and this neuropeptide exerts numerous proinflammatory functions (59) playing an important role in the ocular surface epithelial barrier function and DC pathophysiology (60).

In summary, the activation and sensitization of ipsilateral polymodal nociceptors after unilateral corneal nerve lesion or inflammation would produce the bilateral release of the substance P. The increase of substance P and proinflammatory substances in the contralateral ocular surface will produce an inflammation-like condition in the contralateral eye that would explain the changes of nerve activity that we have found in the present work, that is, the sensitization of corneal nociceptors and the reduced activity of cold thermoreceptors (23, 24). In the case of the unilateral ablation of the main lacrimal gland, which produces mainly an increase in the activity of cold thermoreceptors and has fewer effects on corneal nociceptors (25), it should be noticed that despite removing the gland of only one side, there is also a significant decrease in the tear volume in the contralateral eye, which could contribute to the effects observed in the activity of the contralateral cold thermoreceptors. However, we can only speculate if there is an immune-mediated effect on the contralateral nerves first and

consequently, a decrease in tear volume or vice versa, that is, the chronic reduction of tearing in the contralateral eye led to changes in nerve activity. Although guinea pigs show delayed epithelial wound healing in both the ipsilateral and contralateral eyes after lacrimal gland removal in only one side (61), we only can speculate whether it is due to the chronic decrease in tear secretion, as no delay was observed in the microkeratome lesioned corneas (unpublished data), where there are no changes in tear secretion. Also, in the inflammatory and corneal lesion models used in the present work, there were no changes in the tear volume of either eye, although there were significant effects on the nerve activity in the contralateral eye, which are most probably mediated by neuro-immune interactions. Although Fakih et al. (62) did not study the effects on the contralateral side, in their mice models of tear deficiency, there is also an increase in pro-inflammatory markers and immune cells in the ipsilateral trigeminal ganglion and trigeminal brainstem nuclei 21 days after the surgery, indicating neuronal and microglial markers in the trigeminal brainstem and indicating how the effects of this pathology develop and maintains.

In conclusion, the ocular surface lesion and, especially, inflammation affect the activity of the unilateral corneal sensory receptors and also produces similar effects, although to a lesser degree, in the contralateral eye. The development of changes in the corneal nerve activity in the contralateral eye explains the development of ocular discomfort and pain sensation in the contralateral eye, which may not present any clinical sign. This has to be considered not only in experimental science, because the contralateral eye cannot be considered as a control, but also in the clinic. Even when only one eye has been affected by inflammation, infection, or injury, the pertinence of treating both eyes must be considered to avoid pain and other unwanted effects on the fellow eye.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Universidad Miguel Hernández de Elche.

AUTHOR CONTRIBUTIONS

CL and SQ did the experiments and analyzed the data. CL, SQ, JG, and MA interpreted the data. JG and MA conceived, designed, and supervised the work. MA wrote the manuscript. All authors reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Spanish Agencia Estatal de Investigación and the European Regional Development Fund Grants SAF2017-83674-C2-1-R, SAF2017-83674-C2-2-R, and PID2020-115934RB-I00 funded by MICIN/AEI/1013039/5011100011033; the Generalitat Valenciana Excellence Program grant PROMETEO/2018/114 and Predoctoral Fellowship BES-2015-072638 from AEI (SQ). The APC was funded in part by Universidad Miguel Hernández de Elche.

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frontiers in Pharmacology

REVIEW
published: 24 November 2021
doi: 10.3389/fphar.2021.773871

Proton Sensing on the Ocular Surface: Implications in Eye Pain

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Protons reaching the eyeball from exogenous acidic substances or released from damaged cells during inflammation, immune cells, after tissue injury or during chronic ophthalmic conditions, activate or modulate ion channels present in sensory nerve fibers that innervate the ocular anterior surface. Their identification as well as their role during disease is critical for the understanding of sensory ocular pathophysiology. They are likely to mediate some of the discomfort sensations accompanying several ophthalmic formulations and may represent novel targets for the development of new therapeutics for ocular pathologies. Among the ion channels expressed in trigeminal nociceptors innervating the anterior surface of the eye (cornea and conjunctiva) and annex ocular structures (eyelids), members of the TRP and ASIC families play a critical role in ocular acidic pain. Low pH (pH 6) activates TRPV1, a polymodal ion channel also activated by heat, capsaicin and hyperosmolar conditions. ASIC1, ASIC3 and heteromeric ASIC1/ASIC3 channels present in ocular nerve terminals are activated at pH 7.2–6.5, inducing pain by moderate acidifications of the ocular surface. These channels, together with TRPA1, are involved in acute ocular pain, as well as in painful sensations during allergic keratoconjunctivitis or other ophthalmic conditions, as blocking or reducing channel expression ameliorates ocular pain. TRPV1, TRPA1 and other ion channels are also present in corneal and conjunctival cells, promoting inflammation of the ocular surface after injury. In addition to the above-mentioned ion channels, members of the K_{2P} and P2X ion channel families are also expressed in trigeminal neurons, however, their role in ocular pain remains unclear to date. In this report, these and other ion channels and receptors involved in acid sensing during ocular pathologies and pain are reviewed.

Keywords: ocular surface, pain, ion channels, protons, ocular disease

OPEN ACCESS

Edited by:
Dario Rusciano,
Sooft Italia SpA, Italy

Reviewed by:
Ernest Jennings,
James Cook University, Australia
Philippe Séguéla,
McGill University, Canada

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *10 September 2021*
Accepted: *09 November 2021*
Published: *24 November 2021*

Citation:
Comes NA, Gasull X and Callejo G (2021) Proton Sensing on the Ocular Surface: Implications in Eye Pain. Front. Pharmacol. 12:773871. doi: 10.3389/fphar.2021.773871

1 INTRODUCTION

Physical and chemical stimuli from the environment are sensed by sensory nerve terminals present in the cornea and conjunctiva. Whereas the cornea lies in front of the iris and pupil, the conjunctiva covers the posterior part of the eyelids (palpebral conjunctiva) towards the conjunctival fornix and continues with the anterior part of the sclera until the corneoscleral limbus (bulbar conjunctiva). Both structures are the first line of defense against potential damaging stimuli of the inner eye structures and are covered by a tear film that moistures and lubricates the anterior ocular surface avoiding damage of the corneal epithelium. Acidic insults can reach the ocular surface when we are in contact with exogenous acidic substances. Besides, different infections, allergic or inflammatory conditions can promote an acidic environment in the cornea or the conjunctiva. Moreover, many

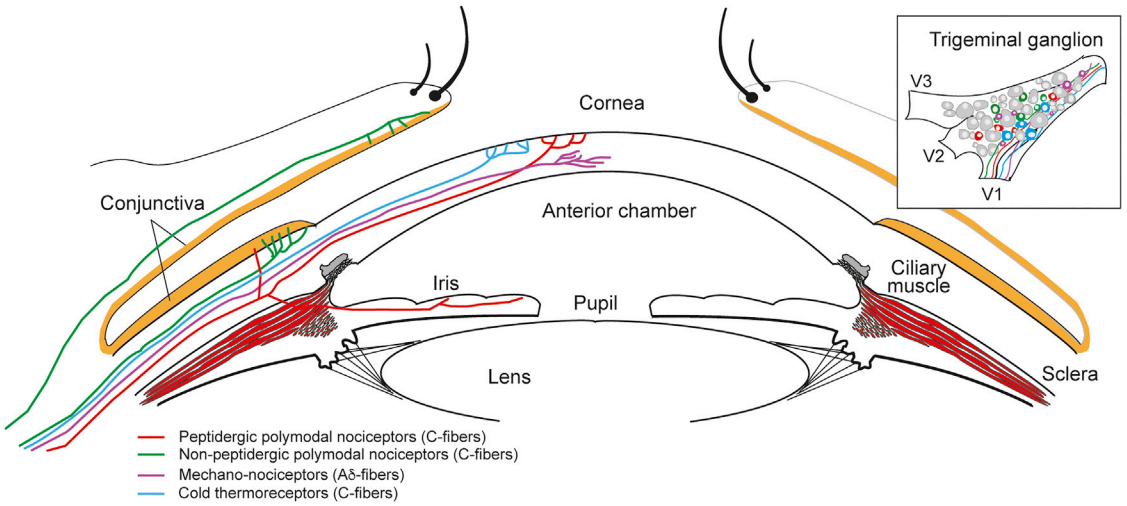

FIGURE 1 | Sensory innervation of the ocular anterior segment. Diagram of the anterior part of the eye showing the different types of nerve fibers innervating the different structures and the ocular surface. Sensory innervation is provided by the ophthalmic nerve arising from the V1 branch of the trigeminal ganglion (upper right inset).

ophthalmic drugs used as eyedrops are formulated in acidic solutions to be able to solubilize or stabilize the active compound. All these acidic stimuli activate different mechanisms in the ocular surface, mostly ion channels activated by protons in peripheral sensory nerves, which detect and transduce these stimuli to higher brain areas to evoke painful sensations and to induce protective responses. Acidic conditions can also activate ion channels in corneal epithelial, endothelial or conjunctival cells, thus promoting inflammatory states. The mechanisms involved in proton sensing in these ocular structures are reviewed in this report.

2 THE OCULAR SURFACE

2.1 Ocular Innervation

The trigeminal ganglion, through the ophthalmic nerve, provides non-visual sensory innervation to the entire eyeball. Peripheral axons of trigeminal neurons innervate the anterior ocular surface, namely the cornea and conjunctiva, but also the uvea (**Figure 1**), where they have a critical role in ocular inflammation (Mintenig et al., 1995; Belmonte et al., 1997). Most of the sensory neurons innervating the eye detect mechanical, thermal and chemical stimuli in the noxious range to protect the eyeball, evoking responses to minimize damage and to promote tissue repair. Besides, Edinger-Westphal nucleus localized in the brainstem supplies autonomic parasympathetic innervation of the eye through the oculomotor nerve (ten Tusscher et al., 1994; Reiner et al., 1983). The iris, the ciliary body/ciliary muscle and parts of the iridocorneal angle (uveal trabecular meshwork and scleral spur) are innervated by parasympathetic nerve fibers that synapse in the ciliary ganglion, entering the ocular globe through the short ciliary nerves. In addition, some parasympathetic fibers arrive from the pons through the geniculate ganglion (Petrosal). Later, they synapse in the pterygopalatine ganglion before entering the eye (Ruskell, 1970). Furthermore, sympathetic nerve fibers from the superior cervical ganglion innervate the eyeball through both the long and short ciliary nerves. They innervate the ciliary body (central stroma and stroma of the ciliary processes), the iris and parts of the iridocorneal angle (**Figure 1**). Contrary, the cornea is innervated almost exclusively by sensory fibers, lacking autonomic innervation.

As mentioned, the ocular surface is densely innervated by trigeminal sensory neurons (Belmonte et al., 2004; Belmonte et al., 2011; Belmonte, 2019), most of them nociceptors (pain sensory neurons; **Figure 1**). Two main types of nociceptors are present: about 70% are polymodal nociceptors (C-fibers) that respond to mechanical stimulation, extreme temperatures, exogenous chemical irritants and endogenous molecules released by tissue injury. Between 15 and 20% of the nerve fibers are mechano-nociceptors (Aδ-fibers), activated by noxious mechanical forces. Finally, cold thermoreceptors constitute the third population of fibers that innervate the cornea (10–15%), which detect changes in temperature in the non-noxious cold range and regulate basal tearing rate among other functions (Belmonte et al., 2004; Belmonte, 2019). Several ion channels present in the peripheral terminals of these neurons have been characterized and play significant roles in ocular pain (acute, inflammatory or of neuropathic origin), including the sensitivity to protons, as well as in other ocular sensations, such as ocular dryness (**Figure 2**). A description of some of these ion channels and receptors is detailed in the following sections.

Recent studies using next-generation sequencing techniques have defined 11 subtypes of Dorsal Root Ganglia (DRG) sensory neurons according to different membrane receptors, ion channels, transcription factors and neuropeptides characteristically and similarly expressed (Chiu et al., 2014;

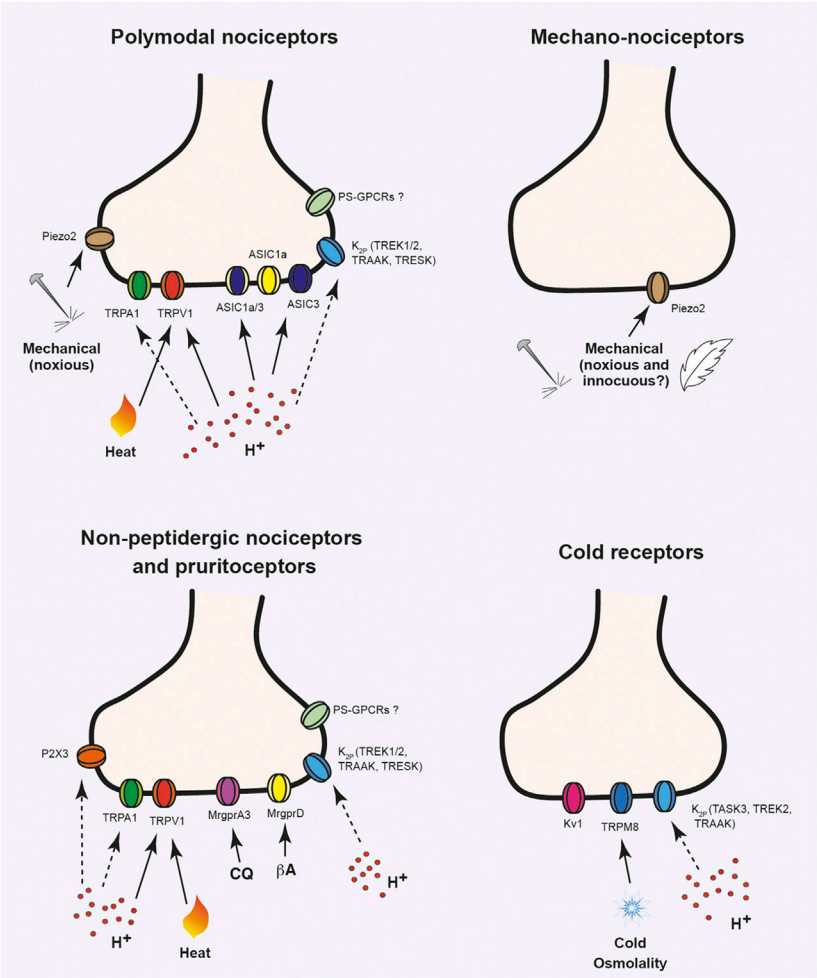

FIGURE 2 | Ion channels activated or modulated by acidic stimuli in ocular sensory neurons. Ion channels expressed in trigeminal sensory neuron terminals innervating the cornea, sclera and conjunctiva. Different types of nociceptive fibers are shown: mechano-nociceptors that respond to high threshold mechanical stimulation; polymodal nociceptors, which can be activated by chemical, mechanical and thermal (noxious heat or cold) stimulation; cold thermoreceptors that express TRPM8 and respond to non-noxious cold stimuli and non-peptidergic sensory neurons, involved in nociception and itch sensitivity. Solid lines indicate direct activation by stimulus. Dashed lines indicate modulation of ion channel activity by protons. CQ, chloroquine; βA, β-alanine.

Usoskin et al., 2015; Zeisel et al., 2018). The classification of these neurons according to their gene expression patterns and the known roles of these genes on sensory transduction and neuronal excitability, has permitted to define different subgroups of neurons according to their putative function. Therefore, we can differentiate populations of low-threshold mechanoreceptors (touch) and proprioceptors, cold thermoreceptors, heat thermoreceptors as well as peptidergic and non-peptidergic nociceptors activated by high threshold mechanical, thermal or chemical stimuli (Usoskin et al., 2015; Nguyen et al., 2017; Zeisel et al., 2018). Also, specific subpopulations of nociceptors have been described to respond specifically to pruritogens and can be classified as itch receptors or pruritoceptors.

Despite most of the studies have been done in DRG neurons, a few transcriptomic studies in the whole trigeminal ganglia have been done (Flegel et al., 2015; Nguyen et al., 2017; LaPaglia et al., 2018). Trigeminal neurons show a similar distribution of sensory neuron subtypes according to their gene subpopulation markers, which, in general, are similar to those found in the DRG, but specific differences in gene expression are present (Flegel et al., 2015; Nguyen et al., 2017; LaPaglia et al., 2018). A specific transcriptomic study on ocular sensory neurons is still lacking but evidence from different transgenic animal models or from functional studies indicate that some of these neuronal subpopulations of neurons identified in the DRGs or in the TGs neurons specifically innervate different parts of the ocular surface. Specifically, the larger population of sensory neurons innervating the cornea are peptidergic polymodal nociceptors (for review see Belmonte et al. (2011), Belmonte (2019)). At the transcriptomic level, these neurons express distinctive nociceptive markers such as the capsaicin and heat sensitive ion channel TRPV1 (Transient Receptor Potential cation channel subfamily V member 1), the ion channel TRPA1

(Transient Receptor Potential cation channel subfamily A member 1) activated by irritant substances as well as the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (Nguyen et al., 2017). Functional studies have shown that corneal neurons respond to acid, hot temperatures, mechanical stimuli and capsaicin (Chen et al., 1997; López de Armentia et al., 2000; Cabanes et al., 2002; González-González et al., 2017), which is in agreement with their receptor's expression. Thus, upon stimulation, peripheral terminals of sensory neurons release CGRP and substance P, which are proinflammatory neuropeptides and contribute to neurogenic inflammation and nociceptor sensitization. Although it has not been clearly demonstrated, it is possible that a population of Aδ-fibers constituting "silent" nociceptors innervates the cornea and annex ocular structures. It is thought that these sensory terminals are only activated after local inflammation occurs and the fibers are somehow sensitized. The cellular correlate of these fibers has not been yet established.

Besides polymodal nociceptors, mechanonociceptor neurons (15–20%) only respond to high threshold mechanical stimuli and they express the mechanosensitive channel Piezo2 but do not express neuropeptides (e.g., CGRP) or the cold-sensitive ion channel TRPM8 (Transient Receptor Potential cation channel subfamily M member 8), thus constituting a different subpopulation of corneal neurons besides polymodal nociceptors or cold thermoreceptors (Bron et al., 2014). The last population of sensory neurons innervating the cornea are cold thermoreceptors that express TRPM8. They provide profound innervation of the corneal epithelium and respond to moderate cold stimuli and to changes in osmolarity (Parra et al., 2010; Parra et al., 2014; Quallo et al., 2015). Importantly, these neurons are activated in ocular dryness conditions, when temperature slightly decreases and osmolarity increases due to tear evaporation. Their increase in firing activates a brainstem neuronal loop that regulates basal tearing and blinking rate (Parra et al., 2010; Parra et al., 2014; Quallo et al., 2015). At the molecular and functional levels two populations of TRPM8 have been identified. One presents a high expression pattern of TRPM8 and a lower threshold for activation by cold (González-González et al., 2017; Nguyen et al., 2017), whereas the second one shows a lower expression of TRMP8 and a higher threshold for activation (activation at lower temperatures). This population might co-express other channels like TRPV1 and could be functionally similar to polymodal nociceptors (González-González et al., 2017).

Interestingly, the conjunctiva, presents a different pattern of innervation compared with the cornea. Two neuronal populations innervate this structure but not the cornea: non-peptidergic MAS-related G protein-coupled receptor member D positive neurons ($MrgprD^+$) and $MrgprA3^+$ sensory neurons (Huang et al., 2018). $MrgprD^+$ neurons also express lysophosphatidic acid receptors LPAR3 and LPAR5 and are involved in mechanical pain and skin itch mediated by β-alanine. In the eye, $MrgprD^+$ fibers mainly innervate the marginal conjunctiva, a region that contacts with the eye surface during blinking (lid wiper) (Huang et al., 2018). Conversely, $MrgprA3^+$ fibers are enriched in medial and lateral conjunctival areas (corners of the eye). These fibers are also activated by histamine, serotonin, chloroquine (an MrgprA3 agonist) and NPFF (that activates MrgprC11), constituting a common pathway for ocular itch (Huang et al., 2016; Huang et al., 2018).

2.2 Ion Channels in Ocular Sensory Neurons

The different types of sensory neurons innervating the ocular surface express multitude of ion channels that detect and transduce different physical, thermal or chemical stimuli or that participate in the electrical activity of these neurons (**Figure 2**). As mentioned earlier, different members of the Transient Receptor Potential (TRP) family of ion channels are present ocular sensory neurons. Particularly, cold thermoreceptors express TRPM8, activated by moderate cold stimuli and menthol (Belmonte and Gallar, 2011). Polymodal nociceptors express TRPV1 and TRPA1 (Belmonte et al., 1991; González-González et al., 2017), purinergic P2X receptors as well as members of the Acid-Sensing Ion Channels, since ASIC1 and ASIC3 currents have been detected in corneal sensory neurons (Callejo et al., 2015). The mechanotransducer channel Piezo2 is present in about 30% of corneal afferent neurons (Bron et al., 2014). Although some expression might exist in some polymodal nociceptors, Piezo2 seems to be mostly restricted to medium- to large-sized sensory neurons positive for neurofilament 200 (NF200) and negative for TRPV1 and CGRP, which suggests its expression in pure mechanonociceptors conducting in the range of Aδ-fibers rather than corneal polymodal nociceptors (Bron et al., 2014; Fernández-Trillo et al., 2020). Moreover, sensory-specific ablation of Piezo2 reduces the percentage of corneal mechanosensitive neurons *in vitro* (Fernández-Trillo et al., 2020). Conjunctival $MrgprD^+$ and $MrgprA3^+$ afferent neurons also contain TRPV1 and TRPA1 channels that participate in itch stimuli transduction (Huang et al., 2016). These neuronal subpopulations also present a characteristic expression of different ion channels involved in the generation and propagation of action potentials (APs) and in the control of neuronal excitability such as voltage-gated sodium ($Na_v1.7$, 1.8, and 1.9), calcium (Cacna1/2/3), potassium (Kcns1, Kcnip4) channels and members of the K_{2P} ion channel family.

Several of these channels are directly activated by protons or modulated by them, thus constituting the transducers for acidic stimuli in the ocular surface (**Figure 2**). A detailed role of each channel is provided below.

3 PROTON-SENSING IN THE OCULAR SURFACE

3.1 TRPV1

The vanilloid receptor TRPV1 is one of the best characterized members of the subfamily of the thermosensitive channels TRP. It is a non-selective cation channel permeable to Na^+ and Ca^{2+} and its activation depolarizes nociceptive sensory neurons (Basbaum et al., 2009). In fact, it has been mainly detected in nociceptors of the trigeminal and the dorsal root ganglia (Clapham, 2003) although they have been found in different

brain regions (Tóth et al., 2005). As mentioned before, TRPV1 is a molecular transducer of thermal and chemical painful stimuli, playing a significant role in nociception (Caterina et al., 1997). It is activated by noxious heat, with a thermal threshold activation of >42°C, low pH, voltage and capsaicin, the pungent component responsible for the spiciness of chili peppers (Caterina et al., 1997; Tominaga et al., 1998). TRPV1-mediated thermal sensitivity can be modulated by a variety of components of the inflammatory soup (Tominaga et al., 1998; Pethő and Reeh, 2012). Thus, a variety of endogenous bioactive lipids act as positive allosteric regulators of TRPV1 while proinflammatory agents such as cytokines, prostaglandins, bradykinin and neurotrophins act on their specific receptors modulating TRPV1 through intracellular signaling pathways. TRPV1 activity is essential in the cellular mechanisms by which tissue damage and nociceptor persistent activation can cause acute sensitivity to noxious heat stimuli, thermal hyperalgesia and neurogenic inflammation (Caterina et al., 2000; Szolcsányi and Sándor, 2012). TRPV1 is widely used as a molecular marker for a specific subset of polymodal nociceptors, the small-diameter peptidergic C-fibers characterized by being unmyelinated, slow-conducting and, in most cases, expressing substance P and CGRP (Tominaga et al., 1998).

As mentioned in the previous section, in mammals, the cornea is mainly innervated by three types of afferent sensory neurons: polymodal nociceptors, mechano-nociceptors and cold-sensitive neurons. In this regard, the presence of TRPV1 in the cornea was initially identified in heat-sensitive polymodal nociceptors by topical application of hypertonic saline, acetic acid and capsaicin (Belmonte and Giraldez, 1981; Belmonte et al., 1988; Belmonte et al., 1991; Gallar et al., 1993). Specifically, about 50% of corneal polymodal C-fibers are stimulated by capsaicin in cats (Belmonte et al., 1991; Chen et al., 1997). Subsequently, TRPV1 channel has been detected almost exclusively in small-diameter C axons colocalizing with CGRP and SCGII (secretogranin II) (Kobayashi et al., 2005; Schecterson et al., 2020).

After reporting the dense innervation of the cornea by the ophthalmic branch of the trigeminal nerve (Arvidson, 1977), the molecular profile of corneal trigeminal neurons expressing TRPV1 has been determined by neuronal retrograde tracing. An important percentage of corneal afferent nerve fibers express TRPV1 in different tested animals (González-González et al., 2017). In guinea pig, 45% of corneal sensory fibers are positive for TRPV1 (the molecular marker of polymodal nociceptors), 28% are positive for Piezo2 (the putative marker of mechano-nociceptors) whereas 8% of them express TRPM8 (a marker of cold-sensitive neurons). In addition, no co-expression between TRPV1 and Piezo2 has been detected in this class of nerve fibers but 6% of TRPV1-immunoreactive neurons also expressed TRPM8. The same study has reported that more than 90% of $TRPV1^+$ corneal afferents are probably polymodal nociceptors (Alamri et al., 2015). In rat trigeminal ganglion, 37% of corneal afferent sensory neurons has been found to express TRPV1 while around one third of the $TRPV1^+$ afferents express substance P and three quarters of these co-expressed CGRP. Therefore, TRPV1 could act in conjunction with substance P and/or CGRP to transduce nociception in corneal sensory neurons (Murata and Masuko, 2006). In contrast, a slightly lower proportion (23%) of rat corneal afferents has been reported to express TRPV1 in another study (Nakamura et al., 2007). In addition to the different animal models used in the studies, this variability in the proportion of sensory neurons expressing TRPV1 could be explained because cholera toxin subunit B, used in most of the mentioned studies, preferentially labels neurons with large cell bodies whereas the retrograde tracer Fluorogold used in the study led by Nakamura is known to label small and large neurons. In addition to neuronal retrograde tracing, immunohistochemistry and double label *in situ* hybridization, the presence of TRPV1 in corneal polymodal nociceptors has been functionally demonstrated with the strong tearing and blinking response evoked by ocular application of capsaicin (Gonzalez et al., 1993; González-González et al., 2017).

The functional effect of capsaicin on TRPV1 in sensory nerve fibers and its capacity to elicit a burning sensation in the eye allowed to suggest that the action of capsaicin could be involved in the perception of painful thermal stimuli *in vivo* (Caterina et al., 1997). Besides, protons can positively modulate the activation of capsaicin-sensitive sensory neurons, enhancing the capsaicin effect (Petersen and LaMotte, 1993). Experimentally, the stimulation of corneal polymodal nociceptors by extracellular protons can be achieved by the application of acidic solution (such as acid acetic solutions) (Chen et al., 1995) or by pulses of a gas mixture with CO_2 applied to the corneal surface (Belmonte et al., 1999). In the latter case, CO_2 combines with H_2O in the tear film covering the cornea resulting in carbonic acid formation which effectively decreases the pH despite the buffering capacity of bicarbonate-containing tears. In parallel, corneal pain has been quantified in humans as a response to the same stimuli with CO_2 demonstrating that activation of nociceptors expressing TRPV1 by acidic pH could be a cause of pain following tissue injury (Chen et al., 1995). Human subjects identify burning pain and irritation sensation experimentally caused by acidic stimulation (CO_2 pulses) on the corneal surface, that it is known to recruit polymodal sensory afferents in the cat's cornea (Acosta et al., 2001a).

Interestingly, TRPV1 can be a target to manage pain perception and it could be useful after an injury or at postoperative level (Weyer-Menkhoff and Lötsch, 2018). Resiniferatoxin (RTX), a potent TRPV1 agonist, strongly activates TRPV1 generating cellular toxicity resulting from an excessive influx of calcium (Olah et al., 2001). When RTX is administered peripherally, it produces reversible analgesia due to the inactivation of nociceptors expressing TRPV1 (Neubert et al., 2003). Subsequent studies have shown that RTX directly infused into the trigeminal ganglion eliminates pain perception as well as neurogenic inflammation. In the rat cornea, a single topical application of RTX reduces capsaicin sensitivity producing transient analgesia for up to 5 days with no adverse side effects observed in histological studies (**Table 1**; Bates et al., 2010). Therefore, RTX could have the potential to manage acute pain caused by ophthalmic surgeries, and corneal conditions such as abrasions or ulcers. For chronic pain, it remains to be

TABLE 1 | Proton-sensing ion channels involved in ocular surface pathologies.

Channel	Disease	Treatment	Effects	Behavioral response	Animal model	References
TRPV1	Ocular pain	Resiniferatoxin (RTX, agonist)	Ca^{2+}-induced cytotoxicity	Reduces capsaicin-induced eye wiping	Rat	Neubert et al. (2003)
	Allergic keratoconjuntivitis	Capsazepine (antagonist)	Abolishes nerve fiber spontaneous activity; reduces firing response	Attenuates eye blinking and tearing	Guinea pig	Bates et al. (2010)
	Dry eye disease	siRNA Tivanisiran (SYL1001)	Not tested	Improves tear quality and hyperemia; reliefs ocular discomfort and pain, avoid damage to the ocular surface	Rat	Moreno-Montañés et al. (2018), Fakih et al. (2021)
	Dry eye disease	A784168 (antagonist)	Not tested	Reduces increased eye blinking induced by lacrimal gland excision	Guinea pig	Benitez-Del-Castillo et al. (2016)
	Photokeratitis	Capsaicin (agonist)	Not tested	Increased blinking	Guinea pig	Acosta et al. (2013)
TRPA1	Allergic keratoconjuntivitis	HC-030031 (antagonist)	Reduces mechanical threshold; attenuates responsiveness to CO_2	Attenuates eye blinking	Guinea pig	Bates et al. (2010)
	Corneal injury and inflammation	$TRPA1^{-/-}$ Knockout mice	Decrease macrophage infiltration; stromal neovascularization and fibrosis	Not tested	Mouse	Katagiri et al. (2015)
ASIC3	Allergic keratoconjuntivitis	APETx2 toxin (antagonist)	Not tested	Reduces allergen-induced blinking	Rat	Callejo et al. (2015)
	Dry eye disease	APETx2 toxin (antagonist)	Not tested	No effect on acid-induced blinking	Rat	Callejo et al. (2015)

determined whether reapplication of RTX generate longer lasting corneal analgesia. Likewise, treatment with the TRPV1 antagonist capsazepine, prior to allergic challenge, abolishes spontaneous activity and sensitivity to heat of polymodal nociceptors and reduces their firing response to a CO_2-mediated acidic stimulus (**Table 1**). It also attenuates the increased blinking rate found in a model for allergic keratoconjunctivitis in guinea pig (Acosta et al., 2013). Because the augmented blinking is considered a nocifensive response to the eye discomfort caused by the high chemical activation of polymodal nociceptors, TRPV1 has been associated to ocular irritation during allergic episodes (Acosta et al., 2013). In a similar way, an increased blinking rate to topical capsaicin has been described in a guinea pig model of keratitis performed by eye exposure to UV radiation (photokeratitis; **Table 1**). Hence, the discomfort sensation reported by humans after UV irradiation may be the result of sensitization of polymodal nociceptors by the local release of inflammatory mediators and TRPV1 activation (Acosta et al., 2014). In addition to allergic keratoconjunctivitis and photokeratitis, polymodal nociceptors may play a role in other pathologies that affect the cornea like herpes simplex virus keratitis (Gallar et al., 2010) and corneal sensitivity associated to diabetes mellitus (Neira-Zalentein et al., 2011).

A significant number of TRPM8-expressing sensory neurons also express TRPV1 and this channel could be involved in cold nociception of the cornea enhancing excitability of $TRPM8^{+}$ cells (Li et al., 2019). TRPV1 could also act as a pharmacological target for the treatment of dry eye disease (DED), characterized by tear instability, ocular dryness, irritation, itch, pain and visual disturbances, and commonly linked with ocular surface inflammation (Messmer, 2015). Co-expression between TRPV1 and TRPM8 channels is increased in corneal cool cells of the experimental rat model for DED performed by lacrimal gland excision (LGE), that shows enhanced sensitivity to capsaicin (Hatta et al., 2019). Besides, different studies propose that TRPV1 could intervene in the increased nocifensive response associated with DED in rats treated with the LGE procedure (Bereiter et al., 2018). In this sense, mRNA expression of TRPV1, TRPA1, ASIC1, and ASIC3 is upregulated in the ophthalmic division of the trigeminal ganglion of a mice model of chronic DED (Fakih et al., 2021). Interestingly, instillation of capsazepine not only inhibits the aforementioned genetic upregulation but also reliefs corneal neurosensory symptoms and reduces anxiety-linked behaviors that characterize severe DED. Similarly, TRPV1 protein levels have been found increased in the trigeminal ganglion of a rat model of DED, in which the channel is involved in the enhanced nocifensive responses (Bereiter et al., 2018). Taken together, TRPV1 antagonists could be potential analgesics for DED treatment (Fakih et al., 2021). Likewise, topical administration of tivanisiran (formerly named SYL1001), a siRNA designed to silence the expression of TRPV1, improves tear quality, hyperemia, ocular pain and discomfort characteristic of DED (Moreno-Montañés et al., 2018). Thus, Phase I and II clinical trials have already determined the most effective doses for its therapeutic use to alleviate DED symptoms (Benitez-Del-Castillo et al., 2016). In the same sense, topical application of A784168, a potent antagonist of TRPV1, diminishes the blink rate in a model of chronic tear deficiency in guinea pig (Masuoka et al., 2020).

3.2 TRPA1

TRPA1, the only member of the subfamily of ankyrin TRP channels described, is a voltage-dependent, non-selective channel permeable to Ca^{2+}, Na^{+}, and K^{+}. TRPA1 is expressed

in sensory neurons that innervate skin, intestinal and pulmonary epithelium, inner ear hair cells and olfactory epithelium, among others (Basbaum et al., 2009). It has a similar main structure as TRPV1 with some specific features such as the presence of a long-terminal ankyrin domain including the regions that confer the thermal and chemical sensitivity (Cordero-Morales et al., 2011) and a TRP-like domain after the S6 transmembrane segment instead of the standard TRP motif. TRPA1 channel contributes to the perception of a great variety of chemical substances that causes pain manifested as burning, skin and eye irritation as well as thermal and mechanical hypersensitivity (Bandell et al., 2004). Reactive chemicals that activate TRPA1 include allyl isothiocyanate (AITC) (the pungent compound found in horseradish, mustard oil and wasabi), cinnamaldehyde (the organic compound responsible for the characteristic taste and smell of cinnamon), allicin (from garlic extract) and diallyl disulfide (from onion) (Logashina et al., 2019). TRPA1 is also activated in response to noxious cold temperatures (<17°C) and endogenous agents such as reactive oxygen species (Logashina et al., 2019). Moreover, it senses environmental irritants such as tear gas, acrolein from air pollution and tobacco smoke and endogenous proalgesic and proinflammatory agents (Bautista et al., 2006; Lindsay et al., 2014). In addition, TRPA1-deficient mice show a significant reduction in painful responses to formaldehyde and 4-hydroxynonenal, which are aldehydes that activate TRPA1 (Macpherson et al., 2007). Furthermore, TRPA1 has been associated to chronic itch (Wilson et al., 2013) and hypersensitivity in different experimental models of persistent inflammatory pain (Dai et al., 2007; Lennertz et al., 2012). Thus, TRPA1 could have a great potential as analgesic and anti-inflammatory target to treat pathologies such as different types of dermatitis (Liu et al., 2013; Oh et al., 2013) and migraine (Materazzi et al., 2013).

TRPA1 is expressed in approximately 35% of the sensory neurons of the trigeminal ganglion (Jordt et al., 2004). In the mouse cornea, TRPA1 channel is mainly expressed in medium-diameter myelinated Aδ-fibers where it colocalizes with neurofilament protein NF200 and secretogranin 3 (SCG3) although it is also present in C-fiber nociceptive sensory neurons (**Figure 2**; Kobayashi et al., 2005; Schecterson et al., 2020). Similar to TRPV1, TRPA1-related mechanisms play a key role in the persistent tear reduction and symptoms of ocular discomfort observed in the rat model of DED (Katagiri et al., 2015). In addition to this, pretreatment with a TRPA1 antagonist (HC-030031) before the allergic challenge reduces the mechanical threshold of polymodal nociceptors, tend to attenuate the enhanced response de CO_2 and reverse the enhanced blinking in an animal model of allergic keratoconjunctivitis (**Table 1**; Acosta et al., 2013). With HC-030031 treatment, as well as in TRPA1$^{-/-}$ knockout mice, it has been reported a decrease in macrophage infiltration, stromal neovascularization and corneal fibrosis in a mouse model of corneal injury. Inhibition of the TGF-β1 signaling pathway in fibroblasts with the loss or blockade of TRPA1 would explain, in part, its role in corneal repair. Therefore, TRPA1 represents a potential therapeutic target for corneal lesions and ocular infections associated to inflammatory fibrosis that can lead to vision loss if not treated properly (Okada et al., 2015).

3.3 Acid-Sensing Ion Channels

The Acid-Sensing Ion Channel (ASIC) family belong to the ENaC/Degenerin (DEG) ion channel superfamily which in rodents is composed by at least six different subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) encoded by four genes (accn1-4) (Kellenberger and Schild, 2015). In humans, this ion channel family is expanded by the expression of three and two splice variants for ASIC3 and ASIC4, respectively. However, expression of ASIC1b mRNA has never been identified. Evidence using x-ray crystallography (Jasti et al., 2007) and atomic force microscopy (Carnally et al., 2008) have revealed that functional ASIC channels are arranged as homo- and heterotrimeric channels where specific subunit composition confer different biophysical and pharmacological properties to an ASIC trimer (Hesselager et al., 2004). They are voltage-independent, ligand-gated cation channels mainly permeable to Na^+ (although ASIC1a homomers are also permeable to Ca^{2+} (Waldmann et al., 1997) activated by extracellular protons and different nonproton ligands (Kellenberger and Schild, 2015; Vullo and Kellenberger, 2019). Not all ASIC subunits can form proton-gated functional channels, for instance, ASIC2b and ASIC4 do not form proton-sensitive homomeric channels but the can interact with other ASIC subunits modulating their ion channel properties and kinetics in response to extracellular acidosis (Hesselager et al., 2004). All ASIC subunits have been detected in DRG and TG neurons from mouse and human tissues (although ASIC4 shows a weak expression) (Zeisel et al., 2018; Nguyen et al., 2017; Flegel et al., 2015; Hockley et al., 2019; Schuhmacher and Smith, 2016), including neurons that innervate the cornea in mice (**Figure 2**; Callejo et al., 2015). Due to their ability to detect increasing proton concentrations in the extracellular environment, they have been involved in many physiological and pathological processes such as synaptic plasticity, learning and memory, fear conditioning, pain, migraine, epileptic seizures and ischemic stroke (Wemmie et al., 2013; Deval and Lingueglia, 2015; Dussor, 2015; Kellenberger and Schild, 2015; Lee and Chen, 2018; Vullo and Kellenberger, 2019).

As mentioned above, a decrease in pH in the tear film covering the anterior ocular surface induces a nociceptive response in animal models and irritation and burning sensation, and to a lesser extent stinging pain, in humans (Chen et al., 1997; Belmonte et al., 1999; Acosta et al., 2001b; Feng and Simpson, 2003; Callejo et al., 2015). These sensations are evoked by the activation of polymodal nociceptive fibers innervating the cornea and conjunctiva (Chen et al., 1995; Belmonte et al., 1999). Initial studies performed to characterize the molecular identity of acid sensors in the cornea demonstrated that after CO_2-mediated acidic stimulation with CO_2 to the cat cornea, 50% of polymodal fibers responding to acid also responded to the TRPV1 agonist capsaicin. These fibers were also blocked by the TRPV1 antagonist capsazepine (Chen et al., 1997), indicating the role of this ion channel in the detection of proton ion concentrations in the cornea. However, the existence of acid-sensitive polymodal fibers that do not respond to capsaicin, suggests the functional expression of others ion channels/receptors capable of detecting extracellular

acidosis in the anterior ocular surface. Besides TRPV1, ASICs have been identified as detectors of acidic stimuli in sensory neurons innervating the cornea (**Figure 2**; Callejo et al., 2015). TRPV1 and ASIC channels differ in their pH sensitivities, whereas TRPV1 is activated at lower pH values (pH 6.4 or below), ASIC channels can detect moderate changes in pH (between pH 7.4 and 6). Moderate acidic stimulation (pH = 6.6) induces depolarization and action potential (AP) firing in a subpopulation of corneal neurons in culture (Callejo et al., 2015). This pH-evoked neuronal firing is abolished by the pretreatment with specific ASIC antagonists such as toxins PcTx1 and APETx2, which inhibit homomeric ASIC1a channels and ASIC3-containing channels, respectively. It is worth mentioning that only 14% of neurons that respond to acid are blocked by PcTx1, whereas APETx2 abolishes the AP firing of the remaining acid responders (86%). This difference could be explained by the expression of different ASIC subunits in the same corneal neuron and a wider range of inhibition of APETx2, which blocks all ASIC3-containing channels. Accordingly, voltage-clamp recordings of corneal neurons in response to moderate acidic pH showed currents with biophysical and pharmacological characteristics of homomeric ASIC1a, homomeric ASIC3 and/or heteromeric ASIC1/3 channels. Moreover, the application of moderate acidic solutions in the rat cornea induces nocifensive behaviors (blinking and scratching) that can be partially prevented with selective and non-selective ASIC antagonists (Callejo et al., 2015). In contrast, the application of a specific ASIC3 agonist (2-guanidine-4-methylquinazoline, GMQ), that can activate ASIC3 at physiological pH, enhances the AP firing rate of corneal sensory fibers and increases the blinking and tearing rate in guinea pigs (Callejo et al., 2015). Altogether, these data suggest that ASIC channels are important proton sensors in the ocular surface, where they are functionally expressed, and crucially participate in the detection of moderate acidifications applied to the cornea and the consequent transduction to painful sensation.

ASIC channel inhibition has been proven effective to ameliorate pain in different animal models of inflammation, where specific inhibition leads to a reduced nocifensive behavior in animals after mechanical and chemical stimulation of the inflamed tissues (Deval et al., 2008; Karczewski et al., 2010; Walder et al., 2010; Deval et al., 2011). Particularly in the eye, ASICs have been involved in a model of allergic keratoconjunctivitis (**Table 1**; Callejo et al., 2015). In this model, the inhibition of ASIC3-containing channels by APETx2 did not prevent the nocifensive behaviors triggered by the application of an acidic solution (pH 5) on the ocular surface, however, APETx2 treatment reduced the blinking rate of animals exposed to the allergen when a solution at physiological pH was applied. Moreover, whole-cell recordings of labelled TG corneal neurons derived from these animals showed an increase in ASIC current density that was partially inhibited by APETx2 (Callejo et al., 2015). Taking together, these results indicate that ASIC channels play an important role in the development of ocular inflammation and sensitization after an allergic challenge.

3.4 Two-Pore Domain Potassium Channels (K_{2P}s)

The family of K_{2P} K^+ channels was the last one identified and described, which has 15 members grouped into six subfamilies (TWIK, TREK, TASK, TALK, THIK, and TRESK) based on sequence and functional similarities (Enyedi and Czirják, 2010). The first K_{2P} channel identified was TWIK1, for Tandem of pore domains in a Weak Inward-rectifying K^+ channel. Now, this subfamily also contains TWIK2 and KCNK7 (K_{2P}7.1). The TREK (TWIK-RElated K^+ channel) subfamily contains TREK1, TREK2, and TRAAK (TWIK-Related Arachidonic acid Activated K^+) channels. Members of this subfamily are activated by arachidonic acid, polyunsaturated fatty acids (PUFAs), volatile anesthetics, and pain-related stimuli. The TASK (TWIK-related Acid-Sensitive K^+ channel) subfamily contains TASK1, TASK3, and TASK5 (K_{2P}15.1, KCNK15), and these channels have the common property of being inhibited by extracellular acidification. The TALK (TWIK-related ALkaline pH-activated channel) subfamily includes TALK1, TALK2 (K_{2P}17.1, KCNK17) and TASK2 and they have an important role in sensing extracellular alkaline pH. THIK1 and THIK2 conform the THIK (Tandem pore domain Halothane-Inhibited K^+) channel subfamily and both channels are inhibited by halothane. The last subfamily identified was TRESK (TWIK-RElated Spinal cord K^+) that has only one member, TRESK (K_{2P}18, KCNK18), with the lowest structural and functional similarity to other K_{2P} channels. This channel is the only one in the family being regulated by the intracellular Ca^{2+} concentration through calcineurin-mediated dephosphorylation.

The main role attributed to K_{2P} channels in most cell types is the regulation of membrane potential, as they constitute the leak of potassium through the plasma membrane. Therefore, they are commonly refereed as leak or background potassium channels and their function, together with the Na^+/K^+ pump, helps to set the resting membrane potential. K_{2P} channels are the main sustained K^+ conductance that establish the resting membrane potential in neurons, influencing neuronal excitability over a wide range of membrane potentials, especially between resting and action potential threshold, and shaping the duration, frequency and amplitude of the action potential. Basic biophysical properties of this family of channels, regulation, and interaction with other proteins are reviewed elsewhere, including some comprehensive and extensive reviews (Enyedi and Czirják, 2010; Busserolles et al., 2019).

Almost all K_{2P} channels are expressed in DRG and TG neurons but the relative expression of each channel varies between different neuronal populations and species. In humans, the most prevalent channels in DRG and TG are THIK-2, TASK1 and TWIK1, followed by TREK1, TASK2 and TRESK (Flegel et al., 2015). Other studies found TRESK as the most expressed channel in human TG (Medhurst et al., 2001; LaPaglia et al., 2018). In mouse and rat sensory neurons, TRESK, TRAAK, TREK2, TREK1, TWIK1, and TWIK2 are the most highly expressed channels although relative expression may vary between studies. Interestingly, mutations in TRESK have been involved in pain derived from familial migraine with aura

(Lafrenière et al., 2010). This effect is thought to be mediated by non-functional homomeric TRESK channels and heteromeric TRESK/TREK1 or TRESK/TREK2 channels, which enhances trigeminal nociceptors excitability and triggers migraine pain (Royal et al., 2019).

As mentioned, it is known that sensory neurons in the trigeminal ganglia express some of these channels but, at the ocular level, no detailed characterization of the channel types expressed in sensory neurons exist to date. Nevertheless, transcriptomic data indicates that TRESK is enriched in TG compared to DRGs and the presence of TREK1 and TREK2 has also been shown in the TG (Yamamoto et al., 2009; Nguyen et al., 2017; LaPaglia et al., 2018). In particular, TREK1, TREK2 and TRAAK are expressed in small-medium diameter trigeminal neurons (likely nociceptors) which show a significant overlap with TRPV1 expression (Yamamoto et al., 2009). In contrast, poor colocalization of these channels is shown with TRPV2 or TRPM8 in trigeminal neurons, with only small colocalization of TREK1 and TRPM8 in some cells (Yamamoto et al., 2009). In this sense, transcriptomic studies have shown that TRPM8-positive trigeminal neurons express TASK3 and, to a lesser extent, TREK1 (Morenilla-Palao et al., 2014). Therefore, it is likely that nociceptive and thermoreceptive sensory neurons specifically innervating the ocular surface present K_{2P} expression. The members of the TREK subfamily of channels are modulated by changes in pH. The most studied channel, TREK1, in addition to its activation by arachidonic acid and mechanical stimuli, it is also modulated by changes in pH. Intracellular acidification activates the channel and, in addition, enhances channel activity in response to other stimuli, such as membrane stretch (Maingret et al., 1999). Also, extracellular stimuli that induce a decrease in intracellular pH such as bicarbonate (HCO_3^-) or CO_2, produce the same effects (Chen et al., 1997; Lee and Chen, 2018). Whether CO_2 application to the ocular surface produces an intracellular acidification in sensory nerve terminals it is not known. Nevertheless, if this occurs, TREK1 activation would hyperpolarize the terminal, thus preventing action potential firing. Nevertheless, extracellular acidification strongly inhibits TREK1 and activates TREK2, another closely related channel (Sandoz et al., 2009). These effects are due to histidines H126 and H151 in the extracellular loop of TREK1 and TREK2, respectively, that act as proton sensors. This extracellular regulation of TREK1 would depolarize the cell by inhibiting its potassium current. This might occur in the ocular surface when acidic sensitivity is tested by application of low pH solutions or acetic acid (Belmonte et al., 1991; Callejo et al., 2015). TRESK is also regulated by pH and both extracellular and intracellular acidification inhibit the channel current while alkalinization slightly enhances the channel activity (Sano et al., 2003; Callejo et al., 2013). Despite no clear demonstration is available to date, it is possible that combined inhibition of TREK1 and TRESK by extracellular acidification of the ocular surface reduces channels activity and promotes nociceptor terminals depolarization and firing or, at least, facilitates their activation by other stimuli.

K_{2P} channels TASK3, TREK1, and TASK2 have been also found in cold-sensitive $TRPM8^+$ sensory neurons (Morenilla-Palao et al., 2014). The ocular surface, particularly the cornea, present a high innervation by these neurons which, in addition to detect changes in temperature in the cold range, are involved in the regulation of basal tearing rate and the detection of osmolarity changes and ocular dryness (Parra et al., 2010; Belmonte and Gallar, 2011; Quallo et al., 2015). As mentioned before, two subclasses can be distinguished in the population of corneal cold thermoreceptor neurons: a larger population of low-threshold cold thermoreceptors (high TRMP8 expression) and a smaller population of high-threshold cold thermoreceptors (low TRPM8 expression). This last population comprises about 30% of the ocular cold-thermoreceptors and are silent until strong cooling activates them, probably acting as cold nociceptors (Belmonte, 2019). TASK3 is highly expressed in about 30% of these neurons and its activity is highly sensitive to acidification (Kim et al., 2000). The sensitivity of TRPM8 sensory neurons to cold or to TRPM8 agonists (e.g., menthol) is enhanced in the absence of TASK3 or by inhibiting the channel with an acidic solution (pH 6). Interestingly, deletion of TASK3 in mice eliminates the population of high-threshold cold thermoreceptors, indicating that the channel plays a significant role in setting the temperature threshold of these neurons. Therefore, TASK3, together with Kv1, can be acting as a brake in excitability, dampening the sensitivity to cold temperatures of high-threshold, cold-sensitive nociceptive neurons (Madrid et al., 2009; Morenilla-Palao et al., 2014). Whether a simple pH change, without the temperature drop, can activate these neurons has not been tested.

3.5 Other Receptors

3.5.1 Proton-Sensing G Protein-Coupled Receptors

On top of the proton-activated ion channels described above, the sensitivity to acid has been described for other ion channels and receptors. Several G protein-coupled receptors (GPCRs) engage heteromeric G proteins in response to acidic stimuli and were termed accordingly as proton-sensing GPCRs (PS-GPCRs). The group of PS-GPCRs is formed by six receptors; the initially described GPR4, GPR65 (TDAG8, T-cell death-associated gene 8), GPR68 (OGR1, Ovarian cancer G protein-coupled receptor 1), and GPR132 (G2A) (Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004), together with the recently identified GPR31 and GPR151 (Mashiko et al., 2019). Conserved histidines localized in their extracellular domain confer them the ability to be activated by acidic stimuli in the pH range of 7.6–5.6 (Ludwig et al., 2003; Ishii et al., 2005), however, they also can be modulated by other endogenous and exogenous molecules such as lipids (Murakami et al., 2004; Wang et al., 2004) and synthetic ligands (Ludwig et al., 2003). Upon activation they engage different intracellular signaling pathways linked to the function of different G proteins, including stimulation of inositol phosphate or cAMP production. Due to their ability to sense extracellular acidification, different studies have investigated their role in inflammatory mouse models. The expression of GPR4, GPR65, and GPR132 is upregulated in several mouse models of inflammatory pain (Chen et al., 2009; Dai et al., 2017). Several studies have shown that ablating the function of these receptors, by pharmacological or transgenic approaches, reduces persistent

pathological pain from inflammatory and neuropathic origin (Dai et al., 2017; Hsieh et al., 2017; Miltz et al., 2017). However, although their role as acid sensors and their involvement in inflammatory conditions have been demonstrated, their expression in sensory fibers innervating the ocular surface has never been determined, and therefore, their role in the detection of acidic insults and inflammatory conditions affecting the cornea and conjunctiva remains to be studied.

3.5.2 Purinergic P2X Receptors

Purinergic P2X receptors are another group of ion channels gated by ATP or other purinergic derivatives, but protons act as allosteric modulators modulating the activation and function of these receptors (Coddou et al., 2011). The potency of activation of $P2X_2$ by specific agonists is enhanced 5- to 10-fold by acidification and even small changes in extracellular pH (7.1–7.2), enhance the response of $P2X_{2/3}$ heteromeric receptors. In contrast, acidification inhibits most of the homomeric P2X receptors. In particular, in some studies, $P2X_3$ is slightly inhibited by acidification but in other P2X subtypes, acidic pH exerts a dual effect, shifting the concentration-response curve to the right but increasing the current amplitude and activation time constant. Almost all P2X receptors have been detected in the trigeminal ganglia of both human and rodent species (Manteniotis et al., 2013; Flegel et al., 2015). $P2X_3$ receptor is mainly expressed in sensory ganglia and mRNA and protein is found in the cell bodies of both small and large trigeminal sensory neurons but has the highest level of expression among smaller neurons, specially, in non-peptidergic $IB4^+$ neurons (Staikopoulos et al., 2007). $P2X_4$, $P2X_5$, and $P2X_6$ also show significant levels of expression in the trigeminal ganglia and lower levels are found for $P2X_1$, $P2X_2$ (Flegel et al., 2015). Despite the studies in trigeminal ganglion neurons, and like PS-GPCRs, there is a lack of specific studies on purinergic receptors in the sensory nerve endings innervating the anterior part of the eye (cornea, sclera and conjunctiva) thus the role of protons modulating purinergic signaling remains to be properly studied.

3.6 Ion Channels in Ocular Non-Neuronal Cells

In addition to the proton-sensing ion channels expressed in peripheral nerves innervating the cornea or the conjunctiva, cells from the corneal epithelium, endothelium or the conjunctiva also express ion channels with important functions for ocular physiology. Members of the TRP family have also been identified in corneal cells, including TRPV1-4, TRPM8, TRPA1, or TRPC4 (Mergler et al., 2014). TRPV1 has been found in the epithelium, stroma and endothelium of the cornea (Yang et al., 2013a; Mergler et al., 2014). In the epithelium, TRPV1 activation leads to an increase in intracellular calcium that induces inflammatory cytokine release through MAPK (mitogen-activated protein kinase) signaling (Zhang et al., 2007), thus it appears that TRPV1 has a significant role in infiltration of inflammatory mediators in the corneal epithelium and stroma. The channel has been also involved in cell migration and proliferation, thus promoting corneal epithelial wound healing response. Because TRPV1 is sensitive to protons, it is likely that acidification contributes to ocular surface inflammation though this channel, promoting the release of interleukins and other inflammatory mediators.

4 ACIDIC SUBSTANCES AND COMMERCIAL DRUGS

A chemical injury with an acidic substance on the ocular surface is a medical emergency that must be evaluated and treated immediately. The treatment is usually based on reestablishing corneal clarity, recovering the ocular surface and avoiding increased intraocular pressure and damage to the optic nerve to prevent visual impairment. In contact with the cornea, acidic substances (pH < 4) denature and precipitate proteins, and their coagulation produces the opacity of the cornea that characterizes severe acid burns. After the acidic injury, the recovery phase begins in which the corneal epithelium and the stroma are restored, inflammatory mechanisms become evident on the ocular surface and there is stromal ulceration and corneal scarring (Singh et al., 2013). The main early signs of an acid burn in the eye include ocular pain and irritation, increased tear secretion, swollen eyelids and blurred vision. TRPV1 and ASICs, activated by low pH, are the main channels that mediate eye pain after acid injury. Hence, decreased expression or blocking of TRPV1 reduces pain caused by chemical injuries at the cornea (Moreno-Montañés et al., 2018; Hatta et al., 2019). In addition to mediate pain responses, TRPV1 is involved in the release of proinflammatory cytokines after an injury of corneal epithelial cells. Therefore, it is a good candidate to control eye pain in corneal injuries and inflammatory responses in the wound healing process (Yang et al., 2013b). As mentioned above, TRPA1 has also been associated with corneal regeneration after chemical injuries (Okada et al., 2015).

A number of compounds used to treat eye diseases are formulated in acidic solutions to facilitate their solubilization and absorption through the cornea. Ocular topical application of these drugs can cause adverse side effects associated with irritation and toxicity of the corneal surface (Zhang et al., 2019). Dorzolamide hydrochloride (Trusopt® as tradename), which is a carbonic anhydrase inhibitor indicated to treat ocular hypertension and primary open-angle glaucoma, has a pH value of 5.6 and usually causes ocular irritation in patients (Konowal et al., 1999; Gordon et al., 2008). Other compounds with low pH values used as commercial ophthalmic eye drops to treat glaucoma are the non-selective β-blocker levobunolol hydrochloride (Betagan®; pH 6.5) and the prostaglandin analog latanoprost (Xalatan®, Mylan®, Travatan®, Saflutan®, Cosopt®; pH values between 5.6 and 6.7). It is widely known that they cause temporary burning, redness, itching and blurred vision (Thygesen, 2018). Consistent with this, pH neutralization in some ophthalmic compounds abolishes ocular irritation associated with their use (Loftsson et al., 2012). In this sense, previous studies have shown that ASICs participate in the nociceptive responses produced by Mylan® and Betagan®, and

probably also by Trusopt® (Callejo et al., 2015). The low pH of this last compound, as well as its lower osmolarity and high viscosity might involve the activation of TRPV1 and other mechanisms, thus ASIC blockers are not sufficient to decrease its irritative effect (Callejo et al., 2015). At this point, identification and characterization of ion channels and the molecular mechanisms mediating ocular discomfort caused by ophthalmic drugs is essential to try to avoid undesirable side effects.

5 SUMMARY

The ocular surface is a particular structure of the body greatly exposed to external environment and to many irritative and painful stimuli. This is probably the reason why the cornea presents the highest sensory innervation of the body, which allows to detect potentially damaging stimuli and to respond accordingly with protective behaviors such as blinking or tearing. Several mechanical, chemical or thermal stimuli are known to activate corneal, conjunctival and scleral peripheral terminals of trigeminal sensory neurons (particularly, nociceptors). Among them, acidic stimuli are known induce firing of polymodal nociceptors through activation of specific ion channels in these neurons. Part of these responses are mediated by members of the ASIC family, as about 2/3 of corneal sensory neurons present ASIC-like currents. Specifically, homomeric ASIC1a, ASIC3 and heteromeric ASIC1/3 channels have been identified (Callejo et al., 2015). These channels are activated by moderate acidifications (pH 7.2–6.6) that can occur in the ocular surface during inflammation or allergic conditions, in addition to insults from external acidic solutions. In this sense, ASIC also contribute to nociceptor sensitization and pain during allergic keratoconjunctivitis, as blockade of ASIC3 channels diminish nocifensive behavior in rodent models (Callejo et al., 2015). Interestingly, in addition to protons, other compounds such as GMQ is able to activate ASIC3 in the ocular surface, inducing nocifensive behaviors (blinking and tearing), as well as firing of ocular sensory nerve fibers. Other members of the family such as ASIC1b, ASIC2a and ASIC2b are also probably present in ocular nociceptors, as expression has been found in the trigeminal ganglion (Callejo et al., 2015). Nevertheless, no clear identification in ocular sensory neurons has been provided to date.

Another channel long involved in acid sensing is TRPV1, as this channel is directly activated by protons (Tominaga et al., 1998). Despite moderate acidifications (pH 6–7) enhance the responses to capsaicin and heat, the channel needs stronger acidifications (pH < 6) to be directly gated by protons (Tominaga et al., 1998). These biophysical properties seem to indicate that it is unlikely that acidification occurring during ocular inflammation or allergy can directly activate the channel but, certainly, can enhance its response to heat or other compounds, thus intensifying painful sensations. In fact, blocking TRPV1 or TRPA1 with specific antagonists or siRNAs has been demonstrated to reduce polymodal nociceptors activity and ocular surface irritation by exogenous compounds or during allergic keratoconjunctivitis (Luna et al., 2007; Acosta et al., 2013). Besides, ASICs seem more prone to mediate the acidic responses to moderate acidifications. Nevertheless, important exogenous acidic insults are likely to activate both types of channels, thus a major activation of sensory nerve terminals will be achieved.

The contribution of other channels and receptors that are likely expressed in corneal or conjunctival nerve fibers are poorly studied to date. Some of these, such as K_{2P} channels can have a significant influence in the excitability of ocular sensory fibers, modulating their excitability and, in consequence, pain sensitivity. Because these potassium channels are polymodal integrators, like TRPV1 and TRPA1, different stimuli including protons, can modulate their activity to enhance or diminish nociceptive input.

Despite some of the mechanisms of proton sensing in the ocular surface are becoming to be elucidated, specific studies on the different types of channels or receptors involved are needed, as well as the different types of sensory fibers involved.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This research was funded by European Union, Fondo Europeo de Desarrollo Regional (FEDER), Ministerio de Ciencia e Innovación and Instituto de Salud Carlos III of Spain, FIS P17/00296 (XG), RETICs Oftared RD16/0008/0014 (XG). Research project PID 2020-119305RB-100 (XG and NC) funded by MCIN/AEI/10.13039/501100011033. Molecule-to-man pain network (Pain-Net, 721841) MSCA-ITN-2016—Innovative Training Networks (XG). Generalitat de Catalunya 2017SGR737 (XG) and María de Maeztu MDM-2017-0729 to Institut de Neurociències, Universitat de Barcelona.

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frontiers in Pharmacology

REVIEW
published: 30 November 2021
doi: 10.3389/fphar.2021.764396

Mechanisms of Peripheral and Central Pain Sensitization: Focus on Ocular Pain

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Persistent ocular pain caused by corneal inflammation and/or nerve injury is accompanied by significant alterations along the pain axis. Both primary sensory neurons in the trigeminal nerves and secondary neurons in the spinal trigeminal nucleus are subjected to profound morphological and functional changes, leading to peripheral and central pain sensitization. Several studies using animal models of inflammatory and neuropathic ocular pain have provided insight about the mechanisms involved in these maladaptive changes. Recently, the advent of new techniques such as optogenetics or genetic neuronal labelling has allowed the investigation of identified circuits involved in nociception, both at the spinal and trigeminal level. In this review, we will describe some of the mechanisms that contribute to the perception of ocular pain at the periphery and at the spinal trigeminal nucleus. Recent advances in the discovery of molecular and cellular mechanisms contributing to peripheral and central pain sensitization of the trigeminal pathways will be also presented.

Keywords: cornea, trigeminal ganglion, peripheral and central sensitization, synaptic transmission, descending modulation, ocular pain

OPEN ACCESS

Edited by:
Dario Rusciano,
Sooft Italia SpA, Italy

Reviewed by:
Vinod Tiwari,
Indian Institute of Technology (BHU), India
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *25 August 2021*
Accepted: *09 November 2021*
Published: *30 November 2021*

Citation:
Puja G, Sonkodi B and Bardoni R (2021) Mechanisms of Peripheral and Central Pain Sensitization: Focus on Ocular Pain. Front. Pharmacol. 12:764396. doi: 10.3389/fphar.2021.764396

INTRODUCTION

Ocular pain is produced by stimulation of primary sensory neurons at the eye surface or by alterations along the ocular pain pathway. Peripheral and central sensitization at these levels is fundamental for the development of long lasting pain perception.

At the ocular surface, the cornea represents the most innervated and sensitive tissue. Its innervation is supplied exclusively by small myelinated and unmyelinated sensory fibers, which are located between the different layers of the corneal epithelium, protecting cornea integrity from potential injuries. Corneal sensory fibers are mainly associated with pain: psychophysical studies in humans have demonstrated that corneal mechanical, chemical or thermal stimulation produces aversive or nociceptive sensations (Kenshalo et al., 1960; Beuerman and Tanelian, 1979; Belmonte et al., 1999), except for the purely cold sensations provoked by low-temperature stimuli of moderate intensity (Acosta et al., 2001). Direct activation of corneal nerve terminals evokes also protective reflexes, such as eye blinks, tear formation, endocrine and cardiovascular responses (Bereiter et al., 1996; Boscan and Paton, 2002).

Primary afferent fibers innervating the cornea belong to the myelinated Aδ and unmyelinated C type and run in the trigeminal nerve (ophthalmic branch, V1), whose ganglion (trigeminal ganglion, TG) contains the somas of the primary sensory neurons. The central branches of corneal afferents reach the trigeminal spinal nucleus (Sp5) in the brainstem, where they contact the second order sensory neurons, represented by both projection and local circuit neurons (**Figure 1A**).

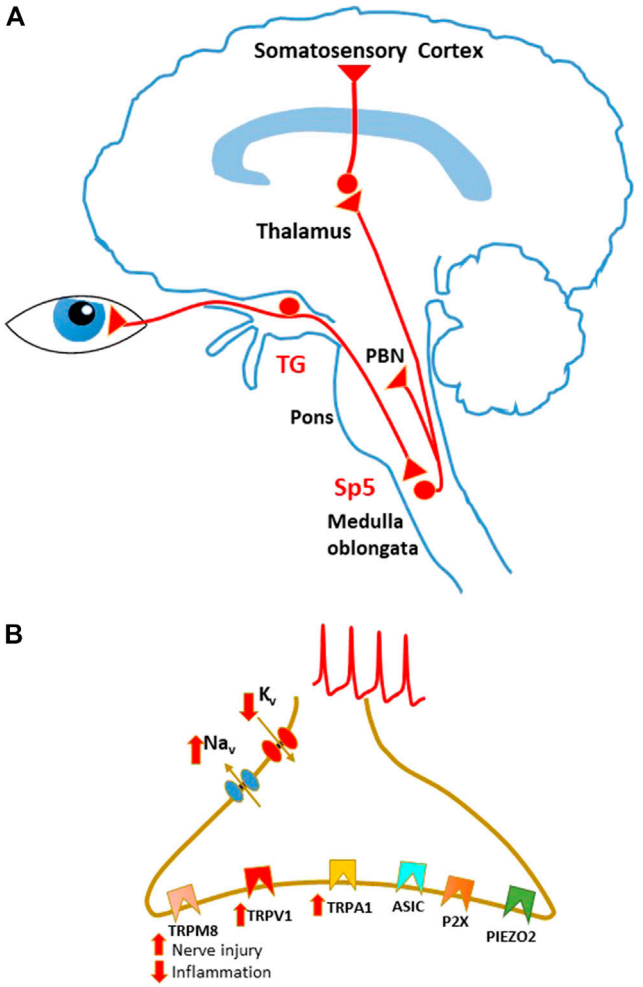

FIGURE 1 | Schematic representation of sensory pathways involved in corneal pain transmission. **(A)** Sensory pathways conveying corneal nociceptive input to the central nervous system. Corneal sensory input is transmitted by corneal nociceptors, whose cell bodies are located in the trigeminal ganglion (TG). Central terminals of nociceptors project to the spinal trigeminal nucleus (Sp5) in the brain stem. Projection neurons in these regions send ascending pathways to several areas, including the parabrachial nucleus (PBN) and the thalamus, that in turn project to higher centers. **(B)** Principal ion channels involved in corneal sensory transduction on the nociceptor peripheral terminals. During peripheral sensitization, TRPV1 and TRPA1 are usually upregulated, while TRPM8 function is enhanced in neuropathic pain and decreased during inflammation.

Corneal nerve structure and function are adversely affected by many ophthalmic and systemic disorders. Persistent ocular pain can be provoked by a long-lasting noxious stimulus or damage to the ocular surface (nociceptive pain) or can result from abnormalities in the ocular neurosensory apparatus itself (neuropathic pain). Persistent and abnormal activation of corneal nociceptors can lead to pain sensitization, occurring at peripheral and/or central sites and manifesting as spontaneous pain, hyperalgesia (increased response to a noxious stimulus), and allodynia (pain evoked by a normally innocuous stimulus) (Galor et al., 2018; Guerrero-Moreno et al., 2020).

While several mechanisms of pain sensitization occurring at the peripheral corneal afferent endings have been identified, synaptic alterations affecting second order neurons in the spinal trigeminal nucleus have not been fully investigated. On the other hand, new technical approaches developed during the last decade (such as neuronal genetic labelling and optogenetic stimulation) have provided a better comprehension of the trigeminal circuits involved in sensory transmission and pain sensitization. In this review, we will outline recent advances in understanding corneal pain processing, with particular focus on the mechanisms leading to pain sensitization.

PERIPHERAL MECHANISMS MEDIATING OCULAR PAIN

Types of Corneal Sensory Fibers

Nociceptors innervating the corneal surface can be classified as mechanonociceptors, polymodal nociceptors and cold-sensitive receptors (Gallar et al., 1993; Belmonte et al., 2004; Gonzalez-Gonzalez et al., 2017).

Mechanonociceptors (MNs) represent about 10% of corneal fibers (mostly Aδ type) and are activated exclusively by noxious mechanical forces generated by external objects, presence of foreign bodies, air pressure or distortion of epithelium layer caused by drying ocular surface. Their thresholds are low in comparison with the MNs in the skin: however, since their endings are very close to corneal surface, they are probably excited by similar actual forces (Boada, 2013). MNs respond to mechanical stimulation mainly with a short lasting, phasic discharge of action potentials (APs), thus encoding the dynamic changes of the stimulus (Belmonte and Giraldez, 1981; Belmonte et al., 1991).

A large population of corneal sensory fibers (about 40%) are polymodal nociceptors (PNs). They respond with a strong discharge in response to a broad spectrum of stimuli: mechanical energy near or above noxious level, heat (>39°C) or noxious cold, exogenous chemical liquid or gaseous irritants, bacterial toxins. PNs can be also activated by endogenous chemical mediators released by damaged corneal tissue or deriving from plasma leaking from limbal vessels. Most PNs belong to C-type fibers and generate an irregular, continuous discharge, providing information about the intensity of the stimulation (Belmonte and Giraldez, 1981; Belmonte et al., 1991; Chen et al., 1997).

About 50% of corneal afferent fibers are represented by cold-sensitive receptors (CRs), including both Aδ and C fibers. They are activated by cooling of the corneal surface (induced by corneal application of cold solution or cold air or by tear film evaporation) or by the increase of tear osmolarity (Belmonte and Giraldez, 1981; Acosta et al., 2001; Parra et al., 2014). CRs fire tonically and contribute to maintain ocular surface wetness by regulating basal tear flow and blinking rate (Hirata and Meng, 2010; Hirata et al., 2012). Based on their activation threshold, CRs can be divided in low and high threshold (LT and HT). LT receptors discharge spontaneously at rest and increase their firing rate under small decreases of the corneal temperature below the

normal value (about 34–35°C), providing a sensation of cold and dryness. HT receptors, whose activation causes a sensation of dryness and pain, do not show spontaneous activity at normal corneal temperature (they remain silent at temperatures >29°C) and are activated only by strong cooling (Hirata et al., 2012; Hirata and Rosenblatt, 2014).

Membrane Channels Involved in Corneal Sensory Transduction

Corneal nociceptors are involved in sensory transduction, that is mediated by several classes of ion channels, detecting different types of nociceptive stimuli. Opening of these channels on the nociceptor peripheral terminals generates an inflow of cations, which mediates membrane depolarization. Supra-threshold depolarizations, in turn, activate sodium and potassium voltage-dependent channels, generating APs that propagate to the neuronal soma in the TG and to the central terminals in Sp5 (**Figure 1B**).

Corneal transduction of mechanical stimuli is mainly performed by PIEZO2 channels, large membrane proteins with a homotrimeric propeller-shaped structure, comprising a central ion-conducting pore module and three peripheral mechanosensing blades with 38 transmembrane domains. Mechanical activation of this channel generates a cationic current, that depolarizes excitable cells (Coste et al., 2010; Coste et al., 2012). PIEZO2 is mainly activated by innocuous mechanical forces and is expressed in dorsal root ganglia (DRG) and TG sensory neurons, in tactile epithelial Merkel cells, and in the sensory endings of proprioceptors (Woo et al., 2014; Woo et al., 2015). Accordingly, mice carrying a conditional deletion of PIEZO2 in sensory neurons or in Merkel cells show severe deficits in tactile discrimination and movement coordination, while responses to mechanical nociceptive stimulation are unaffected or only partially diminished (Woo et al., 2014; Woo et al., 2015; Murthy et al., 2018). Recent experimental evidence suggests that PIEZO2 is also involved in different forms of mechanoceptive sensitization, such as mechanical allodynia, generated by inflammation or nerve injury (Murthy et al., 2018; Szczot et al., 2018).

In the cornea, PIEZO2 is expressed by pure MN sensory neurons and by a subpopulation of PNs (Bron et al., 2014; Fernandez-Trillo et al., 2020). In sensory-specific PIEZO2 knock out mice, electrophysiological responses to mechanical stimulation of corneal MNs and PNs were significantly reduced and the eye blink reflex was impaired (Fernandez-Trillo et al., 2020). The expression of highly sensitive mechanotransducing channels like PIEZO2 on corneal nociceptors is critical for the early detection of low-intensity mechanical stimuli, potentially harmful to the corneal epithelium.

PIEZO2 has been also identified as the principal mechanotransduction channel for proprioception (Woo et al., 2015), however strong evidence is lacking that corneal trigeminal afferents and extraocular muscle spindles contribute to proprioception (Weir et al., 2000; Rao and Prevosto, 2013). Nevertheless, it was suggested that the primary afferents of extraocular muscle spindles initiate the corneal reflex (Bratzlavsky, 1972). Under neuropathic cornea disease, somatosensory PIEZO2 channels could be microinjured mechano-energetically and could alter genetically preprogrammed reflexes with longitudinal central nervous system consequences. The repetitive reinjury of PIEZO2 channels could cause chronic pain even in the absence of secondary harsher tissue injury (Sonkodi et al., 2021a; Sonkodi et al., 2021b).

Both TRPV1 and TRPA1 channels, belonging to the transient receptor potential (TRP) family of ion channels, are involved in corneal pain transduction. All TRP channels possess a tetrameric structure, where each monomer consists of six transmembrane domains (S1-S6). A pore loop, located between S5 and S6, forms the permeation pathway to cations. In the cornea, peptidergic PNs highly express TRPV1, that is directly activated by heat, protons, and high osmolarity (Murata and Masuko, 2006; Zhang et al., 2007; Hegarty et al., 2014; reviewed in: Mergler et al., 2014; Luo et al., 2021). A subpopulation of PNs present the TRPA1 channels, responding to exogenous irritants, toxins, chemicals, strong cold, and endogenous agents (such as ROS and lipid peroxidation products) (Acosta et al., 2014; Schecterson et al., 2020). In DRG neurons heteromeric interactions between TRPV1 and TRPA1 have been reported (Akopian, 2011). Interestingly, physical association between TRPA1 and TRPV1 is regulated by the membrane adaptor protein Tmem100: when this protein is present, TRPV1 mediated inhibition on TRPA1 is reduced. This leads to the potentiation of TRPA1 activity, contributing to persistent pain (Weng et al., 2015). Although the co-expression of TRPV1 and TRPA1 in corneal sensory neurons is still debated (Gonzalez-Gonzalez et al., 2017; Schecterson et al., 2020), the presence of TRPV1-A1 complexes in corneal afferents could play an important role in pain transduction and sensitization. Beside TRPV1 and TRPA1, other channels are involved in sensory transduction in corneal PNs. These include ASICs (acid-sensing ion channels, opened by protons) and P2x (purinergic ionotropic receptors binding ATP) (Belmonte et al., 2015; Belmonte, 2019).

TRPM8 channel, another member of the TRP receptor family, is highly expressed by corneal CRs, where it is sensitive to dynamic downward shifts of temperature and to moderate osmolarity increases (Parra et al., 2010; Parra et al., 2014; Quallo et al., 2015). Additional channels contributing to cold transduction include background potassium channels, closed by cooling of the corneal surface, thereby inducing membrane depolarization and AP firing (Viana et al., 2002) and potassium K_v1 channels, whose opening sets the threshold of CR activation and counteracts the cold-induced response in PNs (Madrid et al., 2009).

Molecular Mechanisms of Peripheral Pain Sensitization

Beside sensory transduction in acute ocular pain, corneal nociceptors are also involved in several forms of peripheral sensitization, which develop during prolonged exposure to painful stimuli. Peripheral sensitization is defined as the increased responsiveness and reduced threshold of nociceptive neurons in the periphery of the sensory system, induced by local inflammation or by peripheral nerve injury.

TABLE 1 | Summary of experimental approaches used to induce and study ocular pain in animals.

Experimental procedure	Ocular pain model	References
Chemical (saline, mustard oil, capsaicin, CO_2 application), thermal, mechanical or electrical corneal stimulation	Acute corneal pain	Lasagni-Vitar et al. (2021), Bereiter and Bereiter (1996), Meng and Bereiter (1996), Martinez and Belmonte (1996), Meng et al. (1997), Meng et al. (1998), Hirata et al. (2000), Hirata et al. (2004), Khalilzadeh and Saiah (2017)
Acetic acid application to ocular surface	Corneal irritation and acute corneal pain.	Martinez and Belmonte (1996)
Topical application of benzalkonium chloride	Ocular surface inflammation	Byun et al. (2020)
Alkali burn (NaOH application on cornea)	Inflammatory and neuropathic pain	Xiang et al. (2017)
Corneal ultraviolet irradiation	Photokeratitis Corneal inflammation	Tashiro et al. (2010); Acosta et al. (2014).
Endotoxin/Lipopolysaccharide (LPS) on cornea surface	Uveitis Intraocular inflammation	Bereiter et al. (2005)
Excision of lacrimal glands	Dry eye disease (DED) Inflammatory and neuropathic pain	Rahman et al. (2015), Hatta et al. (2019), Li et al. (2019), Fakih et al. (2019), Fakih et al. (2021)
Corneal surgical lesion	Corneal refractive surgery. Inflammatory and neuropathic pain.	Luna et al. (2021)
Controlled cutting of stromal nerve fibers	Corneal nerve damage. Neuropathic pain.	Zhang et al. (2012)

In the cornea, several conditions can lead to tissue inflammation: infections caused by bacteria, viruses or fungi; eye injuries; exposure to irritant chemicals or ultraviolet radiation (UV); tear evaporation and hyperosmolarity in dry eye disease (DED). Damaged corneal tissue and immune cells release several molecules and inflammatory mediators, such as ATP, H^+, Substance P (SP), Neurokinin A, Tumor necrosis factor alpha (TNF-α), prostaglandin E2 (PGE2), and interleukins (ILs), which interact with membrane receptors/channels of nociceptor ending membrane. This may lead to the opening and/or modifications of ion channels involved in sensory transduction (directly or by activating intracellular pathways), depolarization of nerve endings, increase of nociceptor excitability, and spontaneous firing. Consistently, several electrophysiological studies have demonstrated that peripheral corneal nerves sensitize whenever exposed to inflammatory milieu or to DED conditions (Gallar et al., 2007; Kurose and Meng, 2013; Parra et al., 2014).

Peripheral sensitization is observed also in case of damage to corneal nerve fibers, leading to neuropathic pain. Corneal nerve injuries can be generated by several factors or disorders, including photorefractive surgeries, DED, cornea abrasion, chemicals, radiations, diabetes, autoimmune diseases (such as the Sjögren's syndrome), fibromyalgia, herpes zoster, and systemic medications. Injury of the corneal nerve induces initially a reduced or total loss of sensitivity of the damaged area, determining insensitivity or higher threshold to natural stimuli in the injured axons (Beuerman and Schimmelpfennig, 1980; Lee et al., 2002; Gallar et al., 2004; Cho et al., 2019). The subsequent regeneration of some damaged axons determines the formation of neuromas (i.e. axons surrounded by connective tissue and immune cells) and accumulation of ion channels in the neural stumps (Lisney and Devor, 1987; Devor et al., 1993). This can lead to an aberrant function of the peripheral nerve endings, generating spontaneous impulse bursts in absence of stimulation (ectopic activity) and/or paroxysmal firing in response to mild mechanical and chemical stimuli (Rivera et al., 2000; Luna et al., 2021).

Molecular mechanisms of sensitization involving ion channels and receptors expressed by peripheral trigeminal fibers have been thoroughly investigated by using several animal models of ocular pain (**Table 1**) (reviewed in Belmonte et al., 2015, Belmonte, 2019; Goto et al., 2016; Andersen et al., 2017; Guerrero-Moreno et al., 2020). We will present here some of the most recent studies, which have added interesting insight to this topic.

TRPV1 and TRPA1 channels undergo important changes during persistent corneal pain. *De novo* channel expression, increase of membrane trafficking and channel phosphorylation have been reported in corneal pain of both inflammatory and neuropathic origin, causing the potentiation of channel function and the increase of membrane depolarization. In an experimental model of keratitis induced by ultraviolet (UV) radiation, nocifensive responses produced by application of capsaicin and AITC (TRPV1 and TRPA1 agonists, respectively) were potentiated in irradiated eyes compared to controls (Acosta et al., 2014). In a rat model of DED (the excision of the lacrimary glands), TRPV1-mediated effects on ongoing activity and sensitivity to heat of corneal nociceptors were increased (Hatta et al., 2019). Finally, the upregulation of TRPV1, TRPA1, ASIC1, and ASIC3 mRNA was detected in the ophthalmic branch of the trigeminal nerve in a mouse model of severe DED caused by the excision of Harderian and extraorbital lacrimal glands (Fakih et al., 2021).

Voltage-dependent sodium channels (Na_v) are actively involved in corneal nociceptor sensitization: perfusion with amitriptyline (a Na_v channel blocker) was less effective in tear-deficient mice, suggesting the occurrence of changes in the expression of these channels induced by ocular dryness (Masuoka et al., 2018). In guinea pig excised eyes, previously subjected to a corneal surgical lesion, AP discharges of PNs were increased in response to chemical corneal stimulation (CO_2 application) (Luna et al., 2021). Similarly, removal of the main

lachrymal gland in guinea pigs enhanced the ongoing AP firing and the responses to cooling of corneal CRs. These effects were mediated by the increase of the sodium currents and the decrease of potassium currents in TG neurons (Kovacs et al., 2016). In a different study, acute treatment of corneal nociceptor endings with the pro-inflammatory substances TNF-α and IL-1β increased the functional availability of Na_v channels at the terminal. This caused a shift of the spike initiation zone toward the axonal end, increasing the nociceptor excitability. Interestingly, the same effect on the spike initiation zone was observed in an animal model of photokeratitis induced by UV exposure (Goldstein et al., 2019).

TRPM8 channels undergo different changes depending on the type of corneal injury (Belmonte, 2019). TRPM8 function in mouse cold sensitive corneal fibers was inhibited by perfusion of inflammatory mediators (such as bradykinin, PG, histamine), which caused a reduction of ongoing cold-evoked impulse activity, recorded *in vitro* (Zhang et al., 2012). In contrast, corneal nerve injury increased the functional expression of TRPM8 in CRs, enhancing their cold sensitivity and causing a rise in the ongoing firing activity and basal tearing (Piña et al., 2019). Removal of lacrimatory gland in mice enhanced the TRPV1 expression in corneal TRPM8+ fibers, leading to increased AP firing in response to cold and to cold allodynia (Li et al., 2019).

Corneal nociceptor terminals express neuropeptides, in particular SP and CGRP (calcitonin-gene-related peptide) (Murata and Masuko, 2006). Following corneal injury, performed through superficial epithelial abrasion, CGRP expression in peripheral nociceptor terminal was upregulated (Hegarty et al., 2018). Release of SP and CGRP exerts a proinflammatory action (defined as "neurogenic inflammation"), by promoting the release of other inflammatory mediators, cell chemotaxis, and plasma extravasation. Consistently, ocular surface inflammation in rats, induced by topical application of 0.1% benzalkonium chloride, enhanced the expression of SP in trigeminal neurons (Byun et al., 2020), while ablation of the SP gene Tac1 or blockade of SP receptor NK1 reduced ocular nociceptive responses in mice, induced by saline application (5 M NaCl) on corneal surface (Lasagni Vitar et al., 2021).

A recent study suggests that cornea epithelial cells actively participate to peripheral pain sensitization. Indeed, TRPV-4 channels expressed on these cells can act as osmotic and thermal sensors: heat or cell swelling, induced by cell hypotonicity, trigger the opening of these channels, determining calcium influx, ATP release and modulation of corneal sensory fibers (Lapajne et al., 2020).

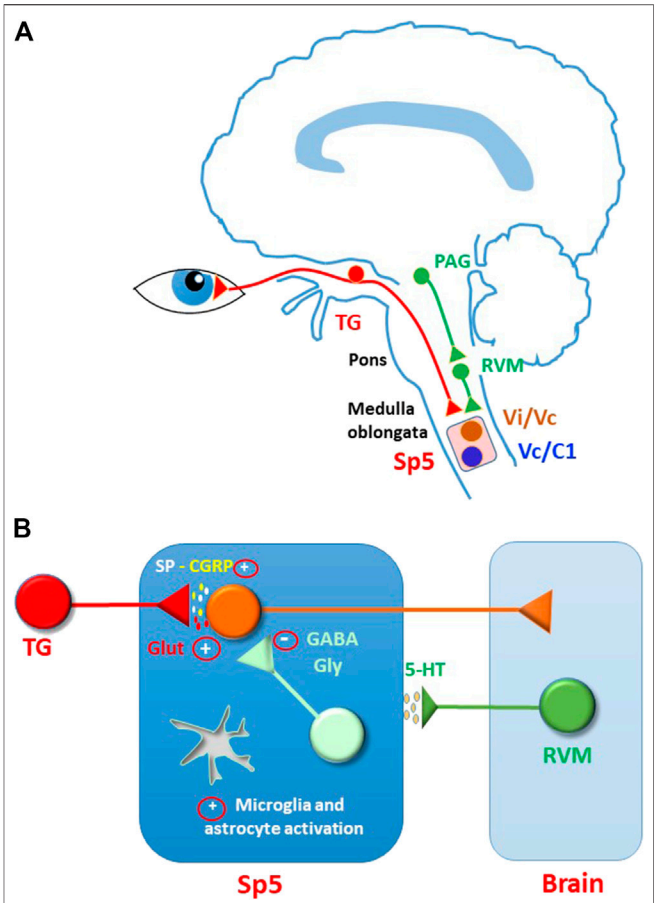

FIGURE 2 | Modulation and sensitization of sensory input in the spinal trigeminal nucleus (Sp5). **(A)** Corneal sensory input is processed in Sp5, mainly at the transition between the subnuclei interpolaris and caudalis (Vi/Vc) and at the junction between the subnucleus caudalis and the upper cervical spinal cord (Vc/C1). Sp5 activity is controlled by descending modulation, comprising serotoninergic pathways. Serotoninergic neurons are located in rostral ventral medulla (RVM) and are activated by projection neurons in periaqueductal grey area (PAG). **(B)** Hypothetical mechanisms sustaining central ocular pain sensitization in Sp5. Persistent corneal nociceptive input may induce a general increase of synaptic excitation (mostly mediated by glutamate and peptides) and a decrease of synaptic inhibition (mediated by GABA and glycine). As reported for several forms of spinal and trigeminal pain, glutamate receptors could be potentiated by increased phosphorylation and participate to plasticity phenomena, such as wind-up and LTP. Synaptic inhibition could be depressed through changes of chloride equilibrium potential, LTD, neuronal loss, decrease of transmitter release, and presynaptic facilitation. Furthermore, a switch in the function of serotoninergic modulation from anti-to pro-nociceptive could contribute to the hyperexcitability state. Further studies are needed to confirm these mechanisms in the ocular pain system.

CENTRAL MECHANISMS OF OCULAR PAIN

Eye Pain Processing in the Trigeminal Spinal Nucleus (Sp5)

Corneal sensory input is transmitted from peripheral terminals through the TG and along the central terminals to the brain stem. Initial processing of the sensory information occurs in the trigeminal spinal nucleus (Sp5), located in the medulla oblongata. This nucleus consists of three subnuclei (oralis, interpolaris, caudalis), the most caudal of which, the subnucleus caudalis, extends into the cervical spinal cord. Two regions are particularly involved in the processing of corneal pain: the transition between the subnuclei interpolaris and caudalis (Vi/Vc) and the junction between the subnucleus caudalis and the upper cervical spinal cord (Vc/C1) (Marfurt and Del Toro, 1987)

(**Figure 2A**). Beside receiving sensory inputs from several craniofacial structures, these areas are also connected to each other by intersubnuclear projections (Nasution and Shigenaga, 1987; Jacquin et al., 1990).

Ocular stimulation (mechanical, chemical or electrical) activates trigeminal nerve sensory fibers, carrying information to Vi/Vc and Vc/C1 neurons: corneal stimulation or intravitreal capsaicin induced a bimodal distribution of the cFos gene expression (a marker for intense neural activation), showing a rostral peak in Vi/Vc and a caudal peak in Vc/C1 (Lu et al., 1993; Strassman and Vos, 1993; Bereiter and Bereiter, 1996; Martinez and Belmonte, 1996; Meng and Bereiter 1996).

In vivo electrophysiological recordings from Vi/Vc and Vc/C1 in rats have identified different subpopulations of neurons, exhibiting specific properties in response to cornea stimulation. In general, neurons receiving a corneal input are for the vast majority nociceptor-specific, activated either by corneal nociceptors only or by convergent corneal and cutaneous nociceptors (Meng et al., 1997; Meng et al., 1998; Hirata et al., 1999; Hirata et al., 2004).

In the Vi/Vc, two neuronal classes have been described:

- Type I: include both corneal specific units and neurons receiving also convergent cutaneous inputs. A late excitation is evoked in these neurons in response to corneal stimulation by CO_2.
- Type II: represented only by neurons with convergent corneal and cutaneous inputs. These units are subjected to strong feedforward inhibition, since they respond to corneal stimulation with an inhibitory phase followed by late excitation. This class includes also neurons responding to acute changes of moisture status of ocular surface, importantly involved in the reflex of lacrimation.

Differently from Vi/Vc, all Vc/C1 neurons belong only to the Type I class and show convergent receptor fields.

Responses evoked by corneal electrical stimulation in Vi/Vc and Vc/C1 neurons are differently modulated by opioids: while all Vc/C1 units are inhibited by morphine, the responses of many Vi/Vc neurons are enhanced by μ receptor (MOR) agonists (Meng et al., 1998; Hirata et al., 1999). Interestingly, local administration of morphine to Vc/C1 region increased the responses to CO_2 in Vi/Vc, confirming the presence of intersubnuclear connections that could contribute to opioid analgesia in corneal pain (Meng et al., 1998; Hirata et al., 2000).

All these results demonstrate that the ophthalmic division of the trigeminal nerve provides a dual sensory representation of the cornea in the Sp5. As pointed out by Bereiter et al. (2000), this redundancy may be explained by different roles played by Vi/Vc and Vc/C1 regions in the sensory elaboration of corneal pain. The properties of Vc/C1 corneal neurons (excitation in response to cornea stimulation, inhibition by opioids) are common to other areas along the pain neuraxis and are consistent with a role of this region in the sensory-discriminative aspects of ocular pain. On the other hand, the heterogeneous responses of Vi/Vc corneal neurons to cornea stimulation and to opioids, together with the exclusive presence of neurons sensitive to the ocular moisture status, would suggest the involvement of this area in more specialized ocular functions, such as reflex control of tear formation and eye blinks, and in the recruitment of anti-nociceptive pathways. Consistently, single unit recordings from rat spinal trigeminal nucleus have identified in Vi/Vc two neuron types importantly involved in the initiation of the eye blink reflex (Henriquez and Evinger, 2007).

Second order neurons in Vi/Vc and Vc/C1 project to various brain regions including the bilateral parabrachial nuclear complex (PBN) (Cechetto et al., 1985; Panneton et al., 1994; Mitchell et al., 2004; Aicher et al., 2013; Aicher et al., 2014) and the posterior and medial contralateral thalamus (Dado and Giesler, 1990; Hirata et al., 2000; Guy et al., 2005; Saito et al., 2017). Other projections from Vi/VC and Vc/C1 neurons reach the periaqueductal gray (PAG), rostral ventral medulla, hypothalamus, and insular cortex (Bereiter et al., 2000; Sessle et al., 2000; Xiang et al., 2017). Recent anatomical and functional studies have reported that cornea stimulation activates a high number of Vc projection neurons directly targeting the PBN area, while ascending pathways to the thalamus seem to rely to more complex, polysynaptic circuits (Aicher et al., 2013; Aicher et al., 2014; Saito et al., 2017).

Neurons in PBN project to multiple brain regions, including central amygdala, hypothalamus, PAG, and ventrolateral medulla, which are considered to be involved in affective pain, autonomic and homeostatic control, and descending pain modulation (Gauriau and Bernard, 2002; Chiang et al., 2019). From the thalamus, information is sent to the somatosensory cortex (responsible for the sensory-discriminative aspects of pain) and to the limbic cortical areas (such as anterior cingulate cortex, insula and prefrontal cortex), involved in the affective/emotional components of pain. Brain imaging experiments performed on human subjects have described the "pain matrix" activated by ocular pain, which include numerous areas located in the cortices (insular, anterior cingulate, somatosensory and prefrontal cortex), in the thalamus, and in several subcortical centers (Moulton et al., 2009; Moulton et al., 2012; Tang et al., 2018).

Mechanisms of Central Sensitization in Sp5

Similarly to peripheral terminals, also corneal nociceptor central terminals and Sp5 secondary neurons undergo plastic changes during long-lasting pain stimulation (**Figure 2B**). Peripheral sensitization, due to persistent inflammation or injury to the corneal nerve, and the subsequent increased afferent input to Sp5, can lead to central pain sensitization over time (Ebrahimiadib et al., 2020; Guerrero-Moreno et al., 2020). Evidence of central sensitization to corneal pain, expressed by an increased response of Sp5 neurons to cornea stimulation and the enlargement of cutaneous receptor fields, has been reported in rats in presence of endotoxin-induced uveitis (Bereiter et al., 2005), after corneal heating (Pozo and Cervero, 1993), in a model of photokeratitis (Tashiro et al., 2010), and after removal of exorbital gland (Rahman et al., 2015). In a model of cornea alkali burn, ERK phosphorylation (a marker of neuronal activation) was detected in mouse Vc/C1 and in higher brain areas belonging to the corneal neuropathic pain matrix (Xiang et al., 2017).

Beside neurons, central sensitization produces profound modifications also in Sp5 glial cells, as observed in various

models of oro-facial pain: under trigeminal nerve injury, orofacial inflammation or migraine, several molecules are released from primary afferents, contributing to microglia and astrocyte activation (reviewed in Shinoda et al., 2019; Ye et al., 2021). Activated microglial cells and astrocytes release various pro-inflammatory cytokines (IL-1β, TNFα, and IL-6), chemokines (such as CCL-2), nerve growth factors (BDNF), and "gliotransmitters" (such as ATP, glutamate and peptides), that act on nearby glial cells and neurons, leading to an exacerbation of pain. In particular, astrocytes contribute to oro-facial pain central sensitization through several mechanisms: 1) increased phosphorylation of astrocytic Jun-N-terminal kinase (JNK) (Lin et al., 2019); 2) decrease of glutamate uptake, due to disfunction of the excitatory aminoacid transporter 2 (EAAT2) and/or of Na^+/K^+ ATPase pump (Isaksen et al., 2016; Zhou et al., 2019); 3) enhancement of synthesis and release of glutamine, a precursor of glutamate (Chiang et al., 2008); 4) increase of release of D-serine, a co-agonist of the NMDA receptor (Dieb and Hafidi, 2013); 5) potentiated function of astrocytic gap-junctions, which allow the propagation of calcium waves and the release of various gliotransmitters (Wang et al., 2014). A mechanism of microglia-astrocyte communication has been recently described: in the neuropathic pain model of infraorbital nerve injury, microglial cells release the complement component C1q, contributing to the activation of astrocytes in Sp5 and the induction of persistent orofacial pain (Asano et al., 2020).

Two recent studies indicate that glial cells play a critical role also in central sensitization to corneal pain. Ocular inflammation, induced in mice by topical instillations of benzalkonium chloride, was associated with microglia activation and enhancement of phosphorylated p38 MAPK specifically in these cells (Launay et al., 2016). Furthermore, upregulation of pro-inflammatory (IL-6, IL-1β), neuronal (ATF3, cFos) and glial (Iba1 and GFAP) markers was detected in both Vi/Vc and Vc/C1 in a mouse model of DED (Fakih et al., 2019). Interestingly, a higher immunoreactivity of the protein Piccolo (associated with the presynaptic zone and the secretion of synaptic vesicles) was also detected in the same study, suggesting a role of presynaptic plasticity in the sensitization of the nociceptive responses.

Glutamatergic Synaptic Transmission in Sp5 and Plasticity

In the spinal cord dorsal horn, pain sensitization is associated with maladaptive changes of the synaptic activity, which increase the efficacy of excitatory transmission and/or reduce the impact of synaptic inhibition (Gradwell et al., 2020). In the trigeminal nuclei similar mechanisms have been proposed, involving glutamatergic, GABA- and glycinergic transmission. Early electrophysiological studies had identified glutamatergic AMPA and NMDA receptors (AMPARs and NMDARs), together with GABA and glycine receptors, as the major synaptic receptor types involved in excitatory and inhibitory synaptic transmission in Sp5 (Hamba et al., 1998; Onodera et al., 2000; Takuma, 2001; Han et al., 2008).

The role of glutamate receptors in excitatory transmission at Sp5 and the intrinsic properties of neurons receiving these glutamatergic inputs have been extensively investigated in the superficial laminae (I- and II) of the subnucleus caudalis (Vc). Vc lamina I neurons are considered to be both modality specific and WDR (wide dynamic range) (Renehan et al., 1986; Meng et al., 1997; Hirata et al., 1999). Neurons expressing the NK1 receptor are believed to represent projection neurons sending their axons to higher brain regions, similarly to what reported in spinal cord dorsal horn (Li et al., 2000; Sedlaceck et al., 2007; Luz et al., 2019). Neurons located in the Vc lamina II of cats and rats have also been functionally classified from *in vivo* recordings as WDR or nociceptor specific. Their electrophysiological characterization has revealed the presence of four different firing patterns: tonic, phasic, delayed, and single spiking (Davies and North, 2009). The use of fluorescent reporter mice allowed to correlate the tonic firing pattern prevalently to VGAT expressing GABA- and glycinergic neurons (inhibitory interneurons), while the delayed firing was most common in somatostatin/TdTomato neurons (predominantly excitatory interneurons), consistently with findings in spinal cord dorsal horn (Pradier et al., 2019).

Unmyelinated C and thinly myelinated, small-diameter Aδ primary afferent fibers are reported to be the major input to Vc laminae I-II neurons (Jacquin et al., 1986; Ambalavanar and Morris, 1992; Crissman et al., 1996). Excitatory synapses between nociceptive primary afferents and Vc neurons are primarily mediated by the activation of AMPARs and NMDARs (Grudt and Williams, 1994; Onodera et al., 2000).

New technical approaches, such as opto- and chemogenetics, are importantly contributing to the understanding of synaptic circuit organization in the spinal cord and trigeminal nuclei. In a recent study, optogenetic stimulation of primary afferent fibers, expressing TRPV1 and channelrhodopsin 2, evoked in mouse Vc glutamatergic mono- or polysynaptic responses, exhibiting different properties depending on the neuron type (Pradier et al., 2019). A similar experimental approach could be utilized also in the study of Sp5 synaptic circuits involved in corneal pain transmission. Early studies had demonstrated that the activation of such circuits strongly relies on glutamatergic receptors, since administration of AMPAR and NMDAR antagonists to Sp5 significantly decreased the c-Fos expression induced by corneal stimulation (Bereiter and Bereiter, 1996; Bereiter et al., 1996).

Glutamatergic receptors are critically involved in central sensitization in several forms of cranio-facial pain. NMDARs contribute to neuroplastic changes induced in adult rats by neonatal capsaicin treatment or by tooth pulp nociceptive stimulation (Chiang et al., 1997; Chiang et al., 1998). Dural application of an inflammatory soup (a model of migraine) enhanced phosphorylation of the NMDAR subunits NR1 and NR2B (Maneepak et al., 2009; Wang et al., 2018), while phosphorylation of the AMPAR subunit GluR1 is involved in neuron sensitization associated with dry tongue (Nakaya et al., 2016). Interestingly, release of SP from corneal CRs was increased in a mouse model of DED and the effect was mediated by sensitized TRPV1 channels (Li et al., 2019). SP release at the CR central terminals may amplify the excitation induced by glutamatergic transmission, leading to central sensitization and cold allodynia.

Several forms of synaptic plasticity, such as wind-up, long-term potentiation (LTP) and long-term depression (LTD), have been reported to contribute to central pain sensitization, requiring the activation of glutamate receptors (Sandkűhler and Gruber-Schoffnegger, 2012; Zhuo, 2017). In the Vc subnucleus, NMDARs play a critical role in the generation of wind-up, a form of short-term plasticity consisting of the increase in the total C-fiber mediated responses after repeated electrical stimulation (Luccarini et al., 2001; Woda et al., 2004). LTP can be generated in the same region by high frequency conditioning stimulation of C fibers: this mechanism, which seems to be mainly due to the activation of metabotropic glutamate mGluR5 receptors, could contribute to the persistent increase of neuron excitability observed in orofacial pain sensitization (Hamba et al., 2000; Liang et al., 2005). Recent experimental evidence has shown that LTD can be induced in Vc neurons following optogenetic stimulation of TRPV1-expressing nociceptive afferents and the subsequent activation of postsynaptic NMDARs (Pradier et al., 2018). Analogously to what observed in the spinal cord dorsal horn (Kim et al., 2015), a prevalence of LTD at synapses between primary afferent fibers and inhibitory neurons could be involved in disinhibition of synaptic circuits and increased sensitivity to nociceptive stimulation.

In an acute brain stem slice preparation, ascending and descending excitatory and inhibitory synaptic connections between Vi e Vc have been described, mediated by glutamate and by GABA or glycine, respectively. Interestingly, synaptic plasticity occurs also at these intersubnuclear connections: at Vi excitatory synapses, theta burst stimulation of ascending pathways from Vc generated LTD, that was converted in LTP in the absence of inhibitory transmission (Song and Youn, 2014).

Inhibitory Synaptic Transmission

As already mentioned, inhibitory transmission in Sp5 is mainly mediated by GABA, acting on both $GABA_A$ and $GABA_B$ receptors, and by glycine. Numerous studies have demonstrated that GABAergic and glycinergic neurons (about 30% of the total Vc neurons) inhibit Sp5 neuronal activity by both phasic and tonic activation (Grudt and Henderson, 1988; Matthews et al., 1988; Ginestal and Matute, 1993; Kondo et al., 1995; Takeda et al., 2000; Avendano et al., 2005; Han and Youn, 2008).

As shown by Hirata et al. (2003), corneal pain signaling in Sp5 is under strong GABAergic inhibition: microinjections of the $GABA_A$ agonist muscimol into the Vi/Vc decreased nociceptive responses at Vc/C1, while local injections of muscimol at recording sites (at both Vi/Vc and Vc/C1) inhibited nociceptive transmission in all tested units.

Alterations in GABA- and glycinergic transmission play a key role in central pain sensitization. In the spinal cord dorsal horn, several mechanisms have been proposed for explaining the induction of disinhibition occurring during chronic pain. They include: 1) decrease of the number of GABA and glycinergic neurons; 2) reduction of GABA/glycine release and/or increased activity of their transporters; 3) decreased excitatory drive to inhibitory interneurons; 4) depolarization shift of chloride equilibrium potential (E_{Cl}) in both primary afferent terminals and postsynaptic neurons (reviewed in: Gradwell et al., 2020; Comitato and Bardoni, 2021). Some of these mechanisms have been identified also in the trigeminal nuclei, contributing to synaptic disinhibition in chronic cranio-facial pain. Pharmacological blockade of $GABA_A$ receptors ($GABA_A$Rs) enhanced Sp5 neuron responses to orofacial mechanical stimulation, together with an expansion of receptive fields (Takeda et al., 2000). Following the transection of the inferior alveolar nerve, the number of neurons expressing the vesicular GABA transporter (VGAT) significantly decreased after seven days (Okada-Ogawa et al., 2015). Chronic constriction injury of rat infraorbital nerve (CCI-IoN) enhanced spontaneous activity of WDR neurons in Vc and decreased the tactile thresholds in all neurons. The development of mechanical allodynia was associated with a reduction of inhibition during paired-pulse stimulation and a decreased immunoreactivity to GAD65 (a marker of GABAergic neurons) (Martin et al., 2010). Similarly, CCI-IoN caused in Vc neurons the downregulation of two additional GABA neuron markers, GAD67 and parvalbumin. Intracisternal injections of vigabatrin, a blocker of the catabolic enzyme GABA transaminase, alleviated pain behaviour and restored normal GABA cell marker expression in allodynic Vc (Dieb and Hafidi, 2014).

Chloride equilibrium potential (E_{Cl}) in primary afferent fibers and second order sensory neurons is set by the balance between the activity of two chloride transporters, NKCC1 (that accumulates Cl^- into the cell) and KCC2 (extruding Cl^-). Upregulation of NKCC1 and/or downregulation of KCC2 causes the accumulation of Cl^- inside the neuron, a shift of E_{Cl} toward more depolarized potentials, and the conversion of GABA from inhibitory to excitatory transmitter (Guo and Hu, 2014; Comitato and Bardoni, 2021). Studies about modifications of chloride transporters in Sp5 under chronic pain conditions lead to controversial results. In the CCI-IoN model changes in E_{Cl} were modest and transient and did not persist during the late phase of neuropathic pain (Castro et al., 2017). In contrast, peripheral inflammation induced by a formalin injection into the vibrissa pad produced downregulation of KCC2, causing Cl^- accumulation inside Vc neurons (Wu et al., 2009). Similar effects have been obtained after transection of the inferior alveolar nerve in rats (Okada-Ogawa et al., 2015). These data indicate that alterations in the chloride transporter expression and function in trigeminal nuclei are heterogeneous and may depend on the pain model considered.

In spinal cord dorsal horn, $GABA_A$Rs are involved in presynaptic inhibition of primary afferent terminals. The relative abundance of the NKCC1 transporter over KCC2 in dorsal root ganglion neurons sets their E_{Cl} value around -30 mV. Thus, opening of $GABA_A$Rs on primary afferent central terminals causes a membrane depolarization that inactivates voltage-dependent channels and decreases glutamate release (Guo and Hu, 2014; Betelli et al., 2015). Since KCC2 mRNA is lacking in trigeminal primary neurons, a similar mechanism of presynaptic inhibition may occur also in trigeminal nuclei (Toyoda et al., 2005). Under chronic pain conditions, an increase of terminal depolarization, mediated by $GABA_A$Rs, could turn presynaptic

inhibition into facilitation, by inducing AP firing and increase of glutamate release. In the rat CCI-IoN model the upregulation of NKCC1 in TG primary neurons and the downregulation of KCC2 in Vc neurons were reported (Wei et al., 2013). This was associated with an excitatory action of $GABA_A$Rs at both pre- and postsynaptic sites, leading to the increase of neuron excitability and possibly to presynaptic facilitation.

Descending Modulation of Sp5: Role of Serotonin

Beside the GABA- and glycinergic system, an important role in synaptic inhibition of Sp5 neurons is exerted by descending modulatory pathways (**Figures 2A,B**). Second-order neurons in the Sp5 receive descending inputs from several regions of the central nervous system, such as the rostral ventral medulla or locus coeruleus, which modulate nociceptive and sensory inputs. RVM and the locus coeruleus, in turn, receive inputs from several brain regions, including amygdala, midbrain PAG, hypothalamus, and habenula. Descending modulation to trigeminal nuclei can be either inhibitory or facilitatory: while the inhibitory action is prevalent in physiological conditions, imbalance of inhibitory and facilitatory modulation in favour of facilitation, under tissue or nerve injury, can lead to chronic pain (Chung et al., 2020; Mills et al., 2020).

Descending facilitation is prevalently driven by the serotoninergic system, originating in the RVM from the nucleus raphe magnus (NRM) and its surrounding reticular formation, and projecting onto second-order neurons in trigeminal nuclei and spinal cord dorsal horn (Kwiat and Basbaum, 1992; Sugiyo et al., 2005; Okubo et al., 2013). Both Vi/Vc and Vc/C1 regions show dense serotoninergic innervation (Steinbusch et al., 1981) and receive projections from the NRM (Beitz, 1982). The involvement of serotonin in descending facilitation has been demonstrated in different models of oro-facial pain: mechanical hyperalgesia induced by masseter inflammation was relieved by the lesioning of RVM or by depletion of serotonin in RVM neurons (Sugiyo et al., 2005; Chai et al., 2012). In a recent study, chemogenetic silencing of RVM neurons, projecting to Vc, attenuated spontaneous and bite evoked pain in the same pain model (Chung et al., 2020). In the neuropathic CCI-IoN model, activation of serotoninergic receptors caused sensitization of TRPV1 channels and hyperactivity of TRPV1 positive afferent fibers (Kim et al., 2014).

Serotoninergic pain modulation in both spinal cord and Sp5 is achieved by activating heterogenous receptors (5-HTRs), ranging from 5-HT1 to 5-HT7 (Millan, 2002; Bardoni, 2019). Most of these receptors are G protein-coupled receptors, whereas only the 5-HT3 subtype is a cationic channel.

In naive animals, serotonin seems to exert a prevalent inhibitory action on Vc neurons: serotonin administration on mouse brainstem slices hyperpolarizes most neurons, by binding to 5-HT1(A) and 5-HT2 receptors (Yin, 2011). Furthermore, activation of 5-HT1R subtypes 1A and 1B/D, expressed on primary afferent terminals, inhibits glutamate release in rat brainstem slices (Jennings et al., 2004; Choi et al., 2013).

In pathological conditions, other 5-HTR subtypes seem to be involved in the facilitation of pain transmission, contributing to central sensitization in trigeminal nuclei. 5-HT2ARs, expressed on Vc $PKC\gamma^+$ neurons (a subpopulation of excitatory interneurons), contribute to the development of inflammation induced mechanical allodynia by enhancing the density of synaptic spines (Alba-Delgado et al., 2018). Activation of 5-HT3Rs sensitizes TRPV1 receptors on central primary afferent terminals (Kim et al., 2014) and contributes to the maintenance of secondary hyperalgesia in a model of rat trigeminal nerve injury (Okubo, 2013). Finally, 5-HT7Rs induce the depolarization of a subpopulation of Vc neurons in the slice preparation (Yang et al., 2014), possibly increasing the excitability of Sp5 neurons under chronic pain conditions.

Serotoninergic modulation of cornea responsive units in Sp5 has been scarcely investigated. An *in vivo* study performed on rats has shown that NRM stimulation inhibits corneal evoked responses in Vi/Vc and Vc/C1 (Meng et al., 2000), confirming that transmission of corneal nociception in Sp5 is under control of the descending pathways. A recent study has described the involvement of habenular complex in the descending control of corneal pain: nociception induced by corneal application of saline can be inhibited by administration to habenula of morphine or lidocaine. Pre-treatment of the NRM with the 5-HT3 antagonist ondansetron prevented the effect of morphine on habenula, confirming the modulatory role played by this area on serotoninergic pathways (Khalilzadeh and Saiah, 2017).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

During the last decades, numerous molecular biology, behavioural and electrophysiological studies have clarified several mechanisms occurring during corneal sensory transduction and peripheral pain sensitization. Morphological and electrophysiological analysis of trigeminal ganglion cells has also provided valuable insight about the functional properties of trigeminal primary neurons and their interactions with glial cells (Goto et al., 2016; Bista and Imlach, 2019).

Despite this progress, the characterization of the neural circuits and synaptic mechanisms involved in eye pain signaling at the spinal trigeminal nucleus is still largely incomplete. In the spinal cord, major advances have been obtained during the last decade in the understanding of the dorsal horn circuitry and plasticity. Using opto-and chemogenetic techniques, genetic labelling of neurons and advanced imaging technologies, it has been possible to selectively activate specific neuronal populations *in vitro* and *in vivo* and identify their role in somatic sensory transmission. In the brain stem, however, these high level technical approaches have been employed only very recently and many aspects of the synaptic network organization and function in trigeminal nuclei are still unknown.

As outlined in this review, persistent ocular pain produces both peripheral and central sensitization. Many questions still remain unanswered about the maladaptive changes occurring under chronic eye pain, especially those related to central sensitization in the Sp5 (**Figure 1**). First of all, the involvement of glutamate receptors in the different forms of synaptic plasticity (wind-up, LTP and LTD) during inflammatory

and/or neuropathic corneal pain has not been investigated. The induction and maintenance of LTP at synapses with excitatory Sp5 neurons and/or LTD at inhibitory interneurons could play an important role in ocular pain sensitization.

Furthermore, results obtained in other models of cranio-facial pain suggest that a reduction in the efficacy of the GABA- and glycinergic inhibitory system may be critical also in chronic eye pain. However, the mechanisms responsible for Sp5 disinhibition in the different models of ocular pain still need to be clarified.

Finally, the role of descending modulation is not well defined: although a facilitatory role of serotoninergic pathways has been proposed in models of inflammatory and neuropathic eye pain, limited information is available about the circuits and receptors involved.

Based on these considerations, the acquisition of a better understanding of central processes mediating eye pain is an urgent need. A better knowledge of the cellular and molecular mechanisms involved in ocular pain sensitization will allow the identification of new players in pain transmission and the development of more effective pharmacological approaches, devoid of central side effects, for the treatment of the different forms of chronic ocular pain.

AUTHOR CONTRIBUTIONS

RB conceptualized the review. GP, BS, and RB wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GLOSSARY

AITC allyl isothiocyanate
AP action potential
ASIC acid-sensing ion channel
ATF-3 cyclic AMP-dependent transcription factor
BDNF brain-derived neurotrophic factor
C1 upper cervical spinal cord
CCL-2 chemokine (C-C motif) ligand 2
CC-IoN constriction injury of rat infraorbital nerve
CGRP calcitonin-gene-related peptide
CR cold-sensitive receptor
DED dry eye disease
DRG dorsal root ganglia
GAD67 glutamate decarboxylase 67
GFAP glial fibrillary acidic protein
IBA1 ionized calcium binding adaptor molecule 1
IL interleukin
MAPK mitogen-activated protein kinase
MN mechanonociceptor
NRM nucleus raphe magnus
PAG periaqueductal gray
PBN parabrachial nucleus
PG prostaglandin
PKC protein kinase C
PN polymodal nociceptor
RVM rostral ventral medulla
SP substance P
Sp5 trigeminal spinal nucleus
TG trigeminal ganglion
TNF-α tumor necrosis factor–alpha
TRPA1 transient receptor potential ankyrin 1
TRPV1 transient receptor potential cation channel subfamily V member 1
UV ultraviolet radiation
Vc subnucleus caudalis
VGAT vesicular GABA transporter
Vi subnucleus interpolaris
WDR wide dynamic range

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 23 December 2021
doi: 10.3389/fphar.2021.788524

Differential Effects of Treatment Strategies in Individuals With Chronic Ocular Surface Pain With a Neuropathic Component

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Background: Dysfunction at the ocular system via nociceptive or neuropathic mechanisms can lead to chronic ocular pain. While many studies have reported on responses to treatment for nociceptive pain, fewer have focused on neuropathic ocular pain. This retrospective study assessed clinical responses to pain treatment modalities in individuals with neuropathic component ocular surface pain.

Methods: 101 individuals seen at the University of Miami Oculofacial Pain Clinic from January 2015 to August 2021 with ≥3 months of clinically diagnosed neuropathic pain were included. Patients were subcategorized (postsurgical, post-traumatic, migraine-like, and laterality) and self-reported treatment outcomes were assessed (no change, mild, moderate, or marked improvement). One-way ANOVA (analysis of variance) was used to examine relationships between follow up time and number of treatments attempted with pain improvement, and multivariable logistic regression was used to assess which modalities led to pain improvement.

Results: The mean age was 55 years, and most patients were female (64.4%) and non-Hispanic (68.3%). Migraine-like pain (40.6%) was most common, followed by postsurgical (26.7%), post-traumatic (16.8%) and unilateral pain (15.8%). The most common oral therapies were α2δ ligands (48.5%), the m common topical therapies were autologous serum tears (20.8%) and topical corticosteroids (19.8%), and the most common adjuvant was periocular nerve block (24.8%). Oral therapies reduced pain in post-traumatic (81.2%), migraine-like (73%), and unilateral (72.7%) patients, but only in a minority of postsurgical (38.5%) patients. Similarly, topicals improved pain in post-traumatic (66.7%), migraine-like (78.6%), and unilateral (70%) compared to postsurgical (43.7%) patients. Non-oral/topical adjuvants reduced pain in postsurgical (54.5%), post-traumatic (71.4%), and migraine-like patients (73.3%) only. Multivariable analyses indicated migraine-like pain improved with concomitant oral α2δ ligands and adjuvant therapies, while postsurgical pain improved with topical anti-inflammatories. Those with no improvement in pain had a

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Edited by:
Giacinto Bagetta, University of Calabria, Italy

Reviewed by:
Vinod Tiwari, Indian Institute of Technology (BHU), India
Massimo Dal Monte, University of Pisa, Italy

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *02 October 2021*
Accepted: *08 December 2021*
Published: *23 December 2021*

Citation:
Patel S, Mittal R, Felix ER, Sarantopoulos KD, Levitt RC and Galor A (2021) Differential Effects of Treatment Strategies in Individuals With Chronic Ocular Surface Pain With a Neuropathic Component. Front. Pharmacol. 12:788524. doi: 10.3389/fphar.2021.788524

shorter mean follow-up (266.25 ± 262.56 days) than those with mild (396.65 ± 283.44), moderate (652 ± 413.92), or marked improvement (837.93 ± 709.35) ($p < 0.005$). Identical patterns were noted for number of attempted medications.

Conclusion: Patients with migraine-like pain frequently experienced pain improvement, while postsurgical patients had the lowest response rates. Patients with a longer follow-up and who tried more therapies experienced more significant relief, suggesting multiple trials were necessary for pain reduction.

Keywords: ocular surface pain, cornea, dry eye disease, nociceptive pain, neuropathic pain, sensitization, central mechanisms, peripheral mechanisms

INTRODUCTION

The International Association for the Study of Pain (IASP) defines pain as a "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage." (IASP Terminology, 2020) Ocular surface pain, one form of pain that is estimated to affect 5–30% individuals ≥50 years worldwide (Mehra et al., 2020), is often characterized by patients as "dryness", "burning", "aching", or "tenderness", among other terms. While ocular surface pain was initially lumped under the heading of "dry eye disease", it is now recognized that pain can exist independently from tear dysfunction. Ocular surface pain can result from pathology at a number of sites including ongoing nociceptive issues at the level of the ocular surface and neuropathic mechanisms at the level of peripheral (e.g. cornea) or central nerves (Yu et al., 2011). In addition, nociceptive and/or neuropathic issues can occur in isolation or occur as part of a wider systemic disease (e.g. Sjögren's, fibromyalgia, migraine) (Galor et al., 2016a; Diel et al., 2020). Beyond its prevalence, ocular surface pain is often chronic and is a major cause of disability and morbidity through its negative impact on quality-of-life via impaired social, physical, and mental functioning, leading to decreased productivity (Mertzanis et al., 2005; Patel et al., 2019).

Ocular surface pain is mediated via molecular and electrical signaling across activated neural pathways at various levels. Furthermore, while physiologic and neural processes are involved in the propagation of the pain signal, complex non-neural mechanisms, such as emotional and psychological factors, also play a role in the sensation of pain. Specifically, fast tear evaporation, corneal epithelial erosions, and ocular surface inflammation are common abnormalities that may contribute to chronic ocular surface pain. In addition, insults at the level of peripheral nociceptors (e.g. cornea and conjunctivae) or central nerves (e.g. trigeminal subnucleus caudalis, thalamus, or higher centers), can contribute to pain, including nerve injury associated with infection, trauma, chemical exposure, and metabolic disorders (Mehra et al., 2020). Finally, neuro-inflammatory, behavioral, cognitive and emotional mechanisms play a significant role in the perception and maintenance of pain and its manifestations, adding to the complexity of diagnosis and treatment of this common form of chronic pain.

As such, when approaching an individual with ocular surface pain, it is important to obtain a thorough history and complete ocular and neurologic examination for all potential contributors to this form of chronic pain. The examination typically begins with an evaluation of ocular surface abnormalities as potential sources for nociceptive pain. These include testing for tear film abnormalities (e.g. decreased tear production, high or unstable tear osmolarity, presence of inflammatory mediators), abnormal anatomy (e.g. conjunctivochalasis, pterygium), trauma and toxicity (e.g. topical glaucoma medications) as well as coexisting conditions (Mehra et al., 2020). Neuropathic pain is a clinical diagnosis and several findings suggest its presence, including symptoms out of proportion to signs of disease (Ong et al., 2018), a symptoms profile of sensitivity to wind and light (the ocular equivalents of hyperalgesia and allodynia) (Kalangara et al., 2017), abnormal corneal sensitivity (Galor et al., 2020), and persistent pain despite treatment of ocular surface abnormalities (Galor et al., 2016b). Furthermore, a centralized neuropathic component is suggested if pain persists despite placement of topical anesthesia on the ocular surface (Crane et al., 2017a), or when individuals report pain to light touch around the eye (consistent with presence of tactile allodynia or secondary hyperalgesia) (Timmerman et al., 2014). Overall, this complexity highlights the need for patient-centered, comprehensive, multidisciplinary approach and multimodal therapies to best address chronic ocular surface pain. This area of study represents untapped potential in ophthalmology and pain medicine, as creating new ways of precisely diagnosing and categorizing a patient's pain could lead to novel pathways for guiding therapeutic decision-making.

Generally, nociceptive pain is targeted through use of topical therapies, while neuropathic pain can be treated with oral agents or adjunctive therapies if treatment of nociceptive pain fails and/or a neuropathic component is highly suspected. While many studies have examined treatment outcomes for nociceptive sources of ocular pain (Dermer et al., 2020; Mittal et al., 2021), fewer have examined outcomes after treatment of neuropathic ocular pain. Furthermore, available literature typically report on the effects of one therapeutic modality in a limited number of patients (Ozmen et al., 2020). To improve our fund of knowledge, this study examined clinical data from a cohort of individuals with a presumed neuropathic component to their chronic ocular surface pain, with the aim of studying

subjective clinical responses to a number of commonly utilized medications.

MATERIALS AND METHODS

Study Population

We identified 124 individuals who sought care at the University of Miami Oculofacial Pain Clinic (Bascom Palmer Eye Institute and/or the University of Miami Pain Management Clinic) between January 2015 and August 2021 and whose medical records contained a diagnosis of ocular pain (International Classification of Diseases 10 [ICD10], code H57.XX). Patients were included if they had unilateral or bilateral pain for a duration ≥3 months, with a presumed neuropathic component. The diagnosis of neuropathic ocular pain was made clinically by the treating physician based on the presence of one or more pain features that included: sensitivity to wind and light (Crane et al., 2017b; Kalangara et al., 2017), symptoms out of proportion to ocular surface signs (Ong et al., 2018), abnormal corneal sensitivity (Spierer et al., 2016), persistent pain after topical anesthetic (Crane et al., 2017a), and cutaneous allodynia around the eye. Exclusion criteria included individuals whose pain lasted <3 months, or whose pain resolved with treatment of nociceptive sources of pain (e.g. topical anti-inflammatory agents, surgical correction of anatomic abnormality, etc.). After consideration of these criteria, 101 individuals remained in the study for analysis. This retrospective review was approved by the University of Miami Institutional Review Board and followed the tenets of the Declaration of Helsinki.

Data Collection

For each subject, electronic medical record information was collected including demographics (age, gender, race, ethnicity) and clinical (past ocular, medical, and surgical history) variables. Additionally, co-morbid conditions particularly those related to chronic systemic pain (e.g. fibromyalgia, peripheral neuropathy, trigeminal neuralgia, migraine) were recorded, as was information regarding prior or current ocular pain treatments, including the use of oral neuromodulators (e.g., α2δ ligands, tricyclic and serotonin-norepinephrine reuptake inhibitors [SNRI]), topical ocular therapies (e.g., anti-inflammatory therapies, autologous serum tears), and non-oral/topical adjuvant treatments (e.g., trigeminal nerve stimulation [TNS] and interventional procedures (botulinum toxin injection, steroid-anesthetic based periocular nerve block, or sphenopalatine or superior cervical ganglion block). Time to follow up from first to last visit was also calculated in days.

TABLE 1 | Demographics, medical comorbidities, and pain characteristics, by population and by pain subgroup.

	All patients; n (%)	Postsurgical pain; n (%)	Post-traumatic pain; n (%)	Migraine-like pain; n (%)	Unilateral pain; n (%)
	101 (100%)	27 (26.7%)	17 (16.8%)	41 (40.6%)	16 (15.8%)
Demographics					
Age (mean, SD; years)	55 (17)	54 (18)	53 (18)	52 (17)	61 (15)
Gender, female	65 (64.4%)	17 (63.0%)	14 (82.4%)	25 (61.0%)	9 (56.3%)
Race, White	93 (92.1%)	24 (88.9%)	16 (88.9%)	39 (95.1%)	14 (87.5%)
Ethnicity, Hispanic	31 (30.7%)	8 (29.6%)	5 (29.1%)	11 (26.8%)	7 (43.8%)
Medical Comorbidities					
Chronic joint pain	28 (27.7%)	1 (3.7%)	7 (41.2%)	15 (36.6%)	5 (31.3%)
Fibromyalgia	8 (7.9%)	2 (7.4%)	0 (0)	4 (9.8%)	2 (12.5%)
Migraine	25 (24.8%)	6 (22.2%)	2 (11.8%)	16 (39.0%)	1 (6.3%)
Peripheral neuropathy	4 (4.0%)	3 (11.1%)	0 (0)	0 (0)	1 (6.3%)
Trigeminal neuralgia	7 (6.9%)	2 (7.4%)	0 (0)	2 (4.9%)	3 (18.8%)
Herpetic neuralgia	5 (5.0%)	0 (0)	2 (11.8%)	2 (4.9%)	1 (6.3%)
Ocular History					
Pain >1 year	93 (92.1%)	24 (88.9%)	17 (100%)	38 (92.7%)	14 (87.5%)
Post-LASIK	9 (8.9%)	9 (33.3%)	0 (0)	0 (0)	0 (0)
Post-PRK	2 (2.0%)	2 (7.4%)	0 (0)	0 (0)	0 (0)
Post-CE/iol	5 (4.9%)	5 (19.0%)	0 (0)	0 (0)	0 (0)
Pain Triggers and Descriptors					
Photophobia	50 (49.5%)	11 (40.7%)	3 (17.6%)	36 (87.8%)	0 (0)
Cutaneous Allodynia[a]	20 (19.8%)	4 (14.8%)	7 (41.2%)	7 (17.1%)	2 (12.5%)
Paresthesia (tingling)	9 (8.9%)	2 (7.4%)	0 (0)	6 (14.6%)	1 (6.3%)
Foreign Body Sensation	10 (9.9%)	3 (11.1%)	4 (23.5%)	2 (4.9%)	1 (6.3%)
Dull pain	4 (4.0%)	0 (0)	2 (11.8%)	1 (2.4%)	1 (6.3%)
Throbbing/Shooting pain	20 (19.8%)	7 (25.9%)	1 (5.8%)	9 (22%)	3 (18.8%)

[a]*Pain on light touch of the skin around the eye.*

SD = standard deviation; LASIK = laser-assisted in situ *keratomileusis; PRK = photorefractive keratectomy; CE/iol = cataract extraction and intraocular lens.*

Ocular Pain Characteristics and Pain Groups

Data on pain characteristics was collected including temporality, location (unilateral vs bilateral), descriptors (e.g., squeezing, burning, throbbing, pressure, foreign body sensation), and triggers (sensitivity to light or photophobia, cutaneous allodynia). Based on pain history and characteristics, patients were placed into one of four subcategories. The Postsurgical Pain group included those who developed ocular pain after undergoing surgery (e.g. refractive, cataract, other procedure). The Post-Traumatic Pain group included individuals whose pain began after a non-surgical trauma (chemotherapy, radiation, traumatic brain injury). The Migraine-like Pain group included individuals with bilateral pain that started spontaneously and was accompanied by photophobia, with many of these individuals having co-morbid migraine or headache syndromes. The Unilateral Pain group included individuals with spontaneous unilateral pain that did not start after surgery and was not typical for trigeminal neuralgia but none-the-less had neuropathic qualities, as outlined above.

Treatment Outcomes

Treatment outcomes were determined by examining patient subjective responses after starting a given pain modulating therapy (e.g., comparison to an established baseline pain level), graded on a scale of "no change" (no change), "mild improvement" (some alleviation of symptoms), "moderate improvement" (great improvement but persistence of minor symptoms), or "marked improvement" (resolution or near-resolution of pain).

Statistical Analyses

Analyses were performed using SPSS 22.0 (IBM SPSS Statistics for Windows, 2013). Descriptive statistics were used to summarize demographic and clinical information within the population and each pain subcategory. Information on response to treatment (improvement in pain with treatment) was collected in a binary (yes or no) and scaled (none, mild, moderate, or marked improvement) fashion, and compared between ocular pain subgroups as outlined above. One-way ANOVA (analysis of variance) was utilized to examine differences in mean clinical follow-up time as well as number of attempted oral, topical, and adjuvant medications across pain improvement groups (none, mild, moderate, or marked). Finally, individual multivariable logistic regressions models were created for each pain subgroup using the binary variable '*Clinical Improvement in Pain*' as the outcome to assess which modalities were clinically effective when utilized concomitantly.

RESULTS

Study population and Demographics

The study population consisted of 101 individuals who met inclusion and exclusion criteria. The mean age was 55 years, and most patients were female (64.4%), white (92.1%), and non-

TABLE 2 | Utilized Oral, Topical, and Adjuvant Therapies, by Population and by.

	All patients (n, % of population	Postsurgical (n, % of subgroup)	Post-traumatic (n, % of subgroup)	Migraine-like (n, % of subgroup)	Unilateral (n, % of subgroup)
Oral Agents	90 (89.1%)	26 (96.3%)	16 (94.1%)	37 (90.2%)	11 (68.8%)
Pregabalin/Gabapentin	49 (48.5%)	16 (59.3%)	11 (64.7%)	17 (41.5%)	6 (37.5%)
TCA (amitriptyline)	9 (8.9%)	3 (11.1%)	3 (17.6%)	0 (0)	3 (18.8%)
SNRI (duloxetine)	17 (16.8%)	5 (18.5%)	3 (17.6%)	6 (14.6%)	2 (12.5%)
Anticonvulsant (topiramate)	9 (8.9%)	3 (11.1%)	1 (5.9%)	3 (7.3%)	2 (12.5%)
Acetaminophen	17 (1.8%)	5 (18.5%)	3 (17.6%)	7 (17.1%)	2 (12.5%)
Any NSAID[a]	32 (31.7%)	6 (22.2%)	7 (41.2%)	12 (29.3%)	7 (43.8%)
Any muscle relaxant[b]	32 (31.7%)	1 (3.7%)	2 (11.8%)	26 (63.4%)	2 (12.5%)
Any opioid agonist/antagonist[c]	15 (14.9%)	3 (11.1%)	3 (17.6%)	5 (12.2%)	4 (25%)
Topical Agents	52 (51.5%)	16 (59.3%)	12 (70.6%)	14 (34.2%)	10 (62.5%)
AST	21 (20.8%)	8 (29.6%)	5 (29.4%)	3 (17.1%)	5 (31.3%)
Topical corticosteroid	20 (19.8%)	3 (11.1%)	3 (17.6%)	6 (14.6%)	8 (50%)
Topical cyclosporine, lifitegrast	18 (17.8%)	7 (25.9%)	2 (17.6%)	6 (14.6%)	3 (18.8%)
Topical tacrolimus	9 (8.9%)	1 (3.7%)	3 (17.6%)	3 (7.3%)	2 (12.5%)
Adjuvant Agents	39 (38.6%)	11 (40.7%)	7 (41.2%)	15 (36.6%)	6 (37.5%)
TNS	16 (15.8%)	5 (18.5%)	1 (5.9%)	9 (22%)	1 (6.3%)
Peri-ocular nerve block	25 (24.8%)	9 (33.3%)	5 (29.4%)	6 (14.6%)	5 (31.3%)
Ganglion block	6 (5.9%)	1 (3.7%)	1 (5.9%)	1 (2.4%)	3 (18.8%)
Botulinum injection	11 (10.9%)	0 (0)	0 (0)	10 (24.4%)	1 (6.3%)

[a]*Ibuprofen, Diclofenac, Meloxicam, celecoxib.*
[b]*Baclofen, Cyclobenzaprine.*
[c]*Tramadol, Naltrexone, Oxycodone.*
TCA = tricyclic antidepressant; SNRI = serotonin-norepinephrine reuptake inhibitor; NSAID = Non-Steroidal Anti-Inflammatory Drug; AST = autologous serum tears; TNS = trigeminal nerve stimulation.

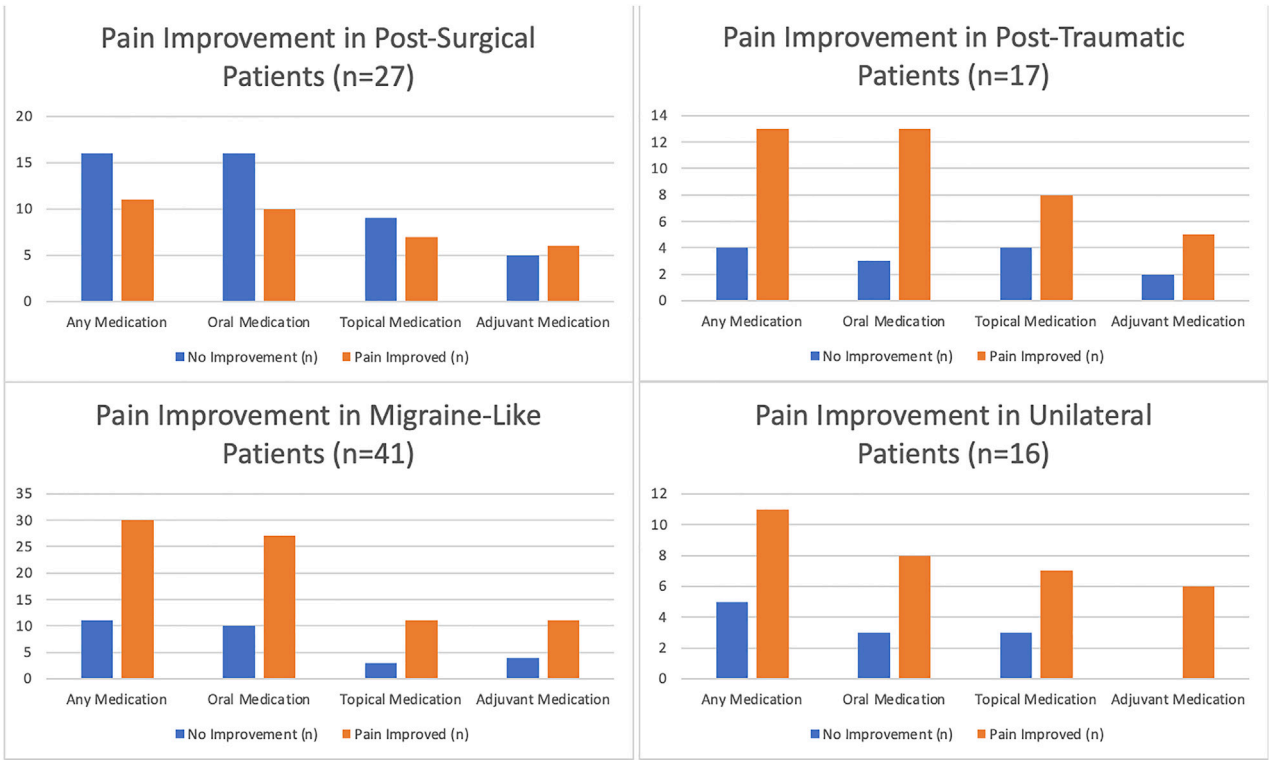

FIGURE 1 | Pain response to various treatment strategies in patients with neuropathic pain, by underlying cause.

TABLE 3 | Proportion of medications that led to improvement in pain, by pain subgroup.

	Pain improvement in response to treatment	
	None (n; % of taking)	**Any (n; % of taking)**
Postsurgical (*n* = 27)		
Any medication	16 (59.3%)	11 (40.7%)
Oral medications	16 (61.5%)	10 (38.5%)
Topical medications	9 (56.3%)	7 (43.8%)
Adjuvant therapies	5 (45.5%)	6 (54.5%)
Post-traumatic (*n* = 17)		
Any medication	4 (23.5%)	13 (76.5%)
Oral medications	3 (18.8%)	13 (81.3%)
Topical medications	4 (33.3%)	8 (66.7%)
Adjuvant therapies	2 (28.6%)	5 (71.4%)
Migraine-like (*n* = 41)		
Any medication	11 (26.8%)	30 (73.2%)
Oral medications	10 (27%)	27 (73%)
Topical medications	3 (21.4%)	11 (78.6%)
Adjuvant therapies	4 (26.7%)	11 (73.3%)
Unilateral (*n* = 16)		
Any medication	5 (31.3%)	11 (68.7%)
Oral medications	3 (27.3%)	8 (72.7%)
Topical medications	3 (30%)	7 (70%)
Adjuvant therapies	0 (0%)	6 (100%)

n = *number in the group.*

Hispanic (68.3%). Several systemic comorbidities were noted, including chronic joint pain (27.7%), migraine (24.8%), and fibromyalgia (7.9%). All individuals fit into one of the ocular pain subcategories, with migraine-like pain (40.6%) being most common, followed by postsurgical pain (26.7%), which most often occurred after refractive surgery, and finally post-traumatic pain (16.8%) and unilateral pain (15.8%). The most common pain descriptor was throbbing/shooting pain (19.8%), and many individuals reported photophobia (49.5%) as a pain trigger, as well as pain to light touch around the eye (cutaneous allodynia, 19.8%) (**Table 1**).

Subjective Response to Various Treatment Modalities Across Pain Subgroups

A variety of modalities were attempted (**Table 2**). The most common oral medications were α2δ ligands (48.5%), non-steroidal anti-inflammatory drugs (NSAIDs, 31.7%), and serotonin-norepinephrine reuptake inhibitors (SNRIs, 16.8%). Oral medications were commonly paired with topical therapy, such as autologous serum tears (AST, 20.8%) and/or a topical anti-inflammatory (e.g. topical steroid [19.8%], cyclosporine or lifitegrast [17.8%], or less commonly tacrolimus [8.9%]). Finally, a minority of patients received adjuvant therapies, like trigeminal nerve stimulation (TNS, 15.8%), steroid-anesthetic based periocular nerve block (24.8%), and/or botulinum toxin injections (10.9%).

Figure 1 and **Table 3** (and **Supplementary Tables 1–4**, Appendix) outline response to therapy, by pain subgroups. At least one oral medication reduced pain to a mild or greater degree in the majority of post-traumatic (81.2%), migraine-like (73%), and unilateral pain (72.7%) groups but in the minority of

TABLE 4 | *Population-wide Differences in Mean Follow-up Time and Medications Attempted Between Different Categories of Clinical Improvement with Treatment.*

	None	Mild	Moderate	Marked	*p*-value
FU time (days), mean ± SD	266.25 ± 262.56	396.65 ± 283.44	652 ± 413.92	837.93 ± 709.35	<0.005
Number oral meds tried, mean ± SD	1.36 ± 1.05	1.62 ± 0.85	1.67 ± 1.24	1.70 ± 0.90	0.02
Number of topical meds tried, mean ± SD	1.09 ± 1.12	1.13 ± 1.03	1.33 ± 1.05	1.30 ± 1.14	<0.005
Number of adjuvant meds tried, mean ± SD	0.44 ± 0.74	0.68 ± 0.84	0.73 ± 0.88	0.8 ± 0.67	<0.005
Number of any meds tried, mean ± SD	3.05 ± 1.50	3.32 ± 1.22	3.67 ± 1.63	3.71 ± 1.42	0.05

FU = follow-up; SD = standard deviation.

postsurgical pain patients (38.5%). Marked improvement with oral medications was most frequently noted in migraine-like patients (21.6%) compared to the other groups (postsurgical 15.4%, post-traumatic 12.5%, unilateral 0%). In a similar manner, topical medication more frequently led to a subjective improvement in pain in the post-traumatic (66.7%), migraine-like (78.6%), and unilateral (70%) groups compared to the postsurgical group (43.7%). Again, marked improvement was most common in the migraine-like group (21.4%) followed by the postsurgical group (18.8%), then the post-traumatic (8.3%) and unilateral (0%) groups. Finally, the use of one or more adjuvants reduced pain to a mild or greater degree in 54.5% of the postsurgical, 71.4% of the post-traumatic, 73.3% of the migraine-like, and 0% of the unilateral groups. Marked improvement in pain after adjuvant use was most common in the migraine-like group (20%) followed by the postsurgical group (11.1%), while in the other two groups these therapies did not lead to marked improved of pain (0%, each).

Relationship Between Subjective Pain Improvement and Follow-Up Time

Next, the relationship between follow-up time (days between initial and most recent visit) and number of medications attempted across patients with differing subjective responses to treatment were examined. Individuals who experienced no improvement had a shorter follow up time (mean = 266.25 days, SD = 262.56, range = 897) compared to those with mild (mean = 396.65, SD = 283.44, range = 1227), moderate (mean = 652, SD = 413.92, range = 1342), or marked (mean = 837.93, SD = 709.35, range = 2,222) improvement in pain. Via ANOVA, there were significant differences in mean follow-up between those with improvement and those without ($p < 0.005$). Subgroup testing also indicated that follow-up time for those with none or mild improvement in pain were non-significantly different, while those with moderate or marked improvement in pain had significantly longer follow-up periods with a clinician. Analyses further showed that patients who experienced improvement in pain tried more medications, suggesting that multiple trials were necessary to achieve increasing pain control (**Table 4**).

Multivariable Analysis of Effects of Multiple Treatments on Subjective Pain Improvement and Pain Triggers

Utilizing stepwise multivariable logistic regression analyses, we examined relationships between various treatments (independent variables) to ***any*** improvement in pain (dependent variable) in our pain subgroups. In postsurgical patients, topical cyclosporine/lifitegrast remained associated with improvement in pain (odds ratio (OR) = 1.31, 95% confidence interval (95%CI) 1.03–1.33, $p = 0.04$). Several treatments were predictive of pain improvement in the migraine-like group, including oral α2δ ligands (OR = 2.74, 95%CI 2.73–2.96, $p = 0.02$), muscle relaxants (OR = 1.36, 95%CI 1.33–1.37, $p < 0.005$), and TNS (OR = 1.20, 95%CI 1.19–1.21, $p < 0.005$). Examining these relationships with respect to pain triggers, in individuals with photophobia, oral α2δ ligands (OR = 2.18, 95%CI 1.78–2.21, $p = 0.05$) and muscle relaxants (OR = 1.32, 95%CI 1.31–1.34, $p < 0.005$) remained in the model, while in individuals with cutaneous allodynia, oral α2δ ligands (OR = 1.79, 95%CI 1.76–1.80, $p < 0.005$) and topical cyclosporine/lifitegrast (OR = 1.13, 95%CI 1.11–1.18, $p < 0.005$) remained in the model.

DISCUSSION

To summarize, we examined subjective responses to various therapies in individuals with chronic ocular surface pain with a neuropathic component. We found that despite the heterogeneity of patients, all fit into one of four pain subgroups, and that responses to treatment varied across groups, although there was significant variability within the groups. Overall, individuals with migraine-like pain reported the most frequent pain improvement (73.2%), generally with a combination of oral (α2δ ligands) and adjuvant (TNS) therapies, while the postsurgical group had the lowest overall response rate (40.7%) to the various therapies. This highlights the need for further studies to investigate other, more appropriate therapies to target the pain in the latter population. Furthermore, we found that the likelihood and degree of pain improvement increased with longer follow up time and with the number of medications utilized, indicative of inter-individual variability that necessitated multiple trials of medications to find a combination that led to clinical improvement. Given this current reality, it is essential to appropriately counsel patients on the trial-and-error approach and time frame needed to achieve clinical improvement in order to avoid early termination of care (Goyal and Hamrah, 2016).

We used various therapies in multiple compartments (oral, topical, adjuvant) due to the multiple potential locations of nerve dysfunction in our patient population (Mehra et al., 2020). Beyond nociceptive causes, peripheral (corneal) nerve abnormalities may contribute to pain in some individuals

(Galor et al., 2018a). Confocal microscopy is one tool that can detect corneal nerve abnormalities (e.g. density, length, tortuosity) in individuals with chronic ocular surface pain (Patel et al., 2020; Patel et al., 2021). In one study of 16 individuals with presumed corneal neuropathic pain (9 of 16 due to postsurgical pain after refractive surgery]), low nerve count (10.5 ± 1.4 vs 28.6 ± 2.0 nerves/frame; $p < 0.0001$) and length (10,935.5 ± 1264.3 vs 24,714.4 ± 1056.2 μm/mm^2; $p < 0.0001$) were noted compared to 12 healthy controls. Treatment with AST (20%; mean duration 3.8 ± 0.5 months, range 1–8 months) decreased pain in all individuals (mean 3.1 ± 0.3 vs baseline 9.1 ± 0.2; 0–10 scale; $p < 0.0001$) and increased nerve count (to 15.1 ± 1.6; $p < 0.0001$) and length (to 17,351.3 ± 1395.6 μm/mm^2; $p < 0.0001$) (Aggarwal et al., 2019). Overall, in our study, 62.5% (5 of 8) of postsurgical patients had mild or greater improvement with serum tears, with three of 5 (60%) reporting marked improvement.

In addition to corneal nerve abnormalities, peripheral (trigeminal non-corneal) afferents may contribute to chronic ocular surface pain (Galor et al., 2018a). Several strategies can be used to address these potential abnormalities, including TNS, nerve blocks, and botulinum toxin (Mehra et al., 2020). TNS is a non-pharmacological approach that is often used in patients with migraine; the device generates impulses at the supratrochlear and supraorbital branches of trigeminal V1 via an adhesive electrode on the head (Zayan et al., 2020; Mehra et al., 2021). Supporting the use of TNS in patients with comorbid migraine and ocular pain, an American study of 18 individuals with severe ocular pain who utilized TNS for 6 months (3.7 ± 1.9 sessions/week at month 1, 2.7 ± 2.3 sessions/week at month 6) noted lower ocular pain intensity scores at 6 months compared to baseline (3.8 ± 3.5 to 2.7 ± 3.0, $p = 0.02$, a 31.4% reduction in pain). On subgroup analyses, individuals with comorbid migraine (n = 10) had a better response than those without co-morbid migraine, but all individuals experienced pain improvement to at least a moderate level (~31.4%). Interestingly, pain improvement with TNS took time, with significant differences first noted 3 months after initiation of therapy (Mehra et al., 2021). A similar pattern emerged in a randomized placebo controlled study of TNS in migraine, highlighting that nerve modulatory therapies take time to translate into improvements in clinical manifestations (Chou et al., 2018). These findings are similar to our current study, where 66.7% (6 of 9) of individuals with migraine-like pain experienced pain improvement with TNS (33.3% mild, 33.3% moderate or greater).

Combination nerve blocks, consisting of a local anesthetic acting as a sodium channel inhibitor (for prevention of ectopic action potential generation) and long-acting corticosteroid (for potentiation of effect and additional mechanisms), have been commonly used to treat pain in an isolated anatomical area due to neuralgia (pain arising from a nerve) (Scholz et al., 1998; Galor et al., 2018a). In a case series of 11 subjects with chronic ocular pain with a presumed neuropathic component (3 migraine-like, seven postsurgical, two post-traumatic, 1 unilateral), seven experienced pain relief after nerve blockade (4 ml of 0.5% bupivacaine with 1 ml of 80 mg/ml methylprednisolone acetate), varying from hours to 7 months. This intervention was most effective in individuals with postsurgical (5 of 6) and unilateral pain (1 of 1) compared to the other pain types (0 of two migraine-like, 0 of one post-traumatic) (Small et al., 2020). Our current results reinforce these findings but in our study, all pain group types had a reasonable frequency of response to therapy, with any improvement noted most frequently in the post-traumatic (5 of 5), migraine-like (5 of 6), and unilateral (5 of 5) groups, followed by the postsurgical (4 of 9) group. Per our results, individuals in the postsurgical and migraine-like pain groups most frequently experienced moderate or greater relief (3 moderate or greater, each).

Botulinum toxin injection is another adjuvant therapy often applied to chronic ocular pain, being most frequently utilized in patients with migraine, with studies generally reporting a mild to moderate improvement in ocular symptoms after treatment (Johnson, 2007; Diel et al., 2018; Venkateswaran et al., 2020a). For example, an American study of 76 patients with chronic migraine who received BoNT-A toxin injections (100–150 U) reported a significant decrease in interictal photophobia scores (3.37 ± 2.54 from 4.89 ± 2.97, $p < 0.001$, range 0–10) after treatment (mean FU of 30.5 ± 7.65 days, range 19–56 days) (Diel et al., 2019). A similar reduction in interictal photophobia (5.27 ± 2.73 from 7.91 ± 2.05, $p < 0.001$, range 0–10) was noted in another American study of 117 patients with chronic migraine who received BoNT-A toxin injection (Diel et al., 2018). The migraine BoNT-A has been modified and used in individuals with neuropathic ocular pain but *without* a history of migraine. Four individuals treated with one session of BoNT-A (35 U given across seven forehead sites) reported a decrease in photophobia severity (3.25 ± 0.4 from 4.8 ± 0.4, range 0–5) and ocular discomfort (2.25 ± 1.0 from 4.5 ± 0.6, range 0–5) at 1 month follow-up (Venkateswaran et al., 2020b). In our current study, eight of 10 migraine-like pain patients who received botulinum toxin injections reported a subjective improvement in ocular pain (4 mild, four moderate). In addition, one patient in the unilateral pain group also experienced mild improvement in pain with the modified BoNT-A protocol.

A centralized component to pain may be suspected when chronic ocular surface pain is accompanied by photophobia, by cutaneous allodynia, and/or persistent pain after anesthesia applied onto ocular surface (Digre and Brennan, 2012). For individuals with centralized nerve pain, oral medications are a first line treatment. Commonly used oral neuromodulating agents include α2δ ligands (gabapentin or pregabalin), SNRIs (duloxetine), and TCAs (nortriptyline) (Patel et al., 2020). Such agents have a slow onset of action, with clinical effects often becoming apparent weeks to months after initiation (Mehra et al., 2020), something that further highlights the need for longer follow-up times and persistent therapies. Several case series have examined the effects of oral medications on chronic ocular surface pain–for example, in a case series of eight individuals (n = 4 postsurgical), gabapentin (starting 300 mg daily, escalation to 600–900 TID) and pregabalin (starting 75 mg daily, escalation to 150 mg BID) led to complete relief in two subjects (NRS = 0 on a 0–10 scale), marked relief in three subjects (NRS ≤2), and mild relief in one subject (NRS = 10 to 7), while two had no improvement in pain. Interestingly, the two

subjects who noted complete relief were on concomitant SNRI (duloxetine; starting 20 mg, escalation to 60 mg daily) (Small et al., 2020). These findings are similar to our analyses, which indicated that a similar proportion of individuals in the postsurgical pain group had mild or greater improvement to an α2δ ligand (n = 6 of 10; 60%), four of which had a marked improvement in pain.

A similar effect has been noted with TCAs. A British study examined 25 individuals with peripheral neuropathic pain (neuropathic symptoms and IVCM findings e.g. presence of microneuromas) who were treated with nortriptyline (10–25 mg starting dose, escalation to 100 mg daily). Pain levels 4 weeks post-treatment were ~60% lower than pre-treatment (NRS; 3.80 ± 2.39 vs 6.36 ± 2.18, $p < 0.0001$). Overall, 84% of subjects ($n = 21$) reported pain improvement [28% with >50% improvement ($n = 7$), 40% with 25–50% improvement ($n = 10$), and 32% with <25% improvement ($n = 8$)] (Ozmen et al., 2019). Because this study did not break down its population by etiology, and due to the low proportion of individuals utilizing TCAs in our population, comparisons to this study are difficult. Nonetheless, in our study, improvement in pain was rated as mild or moderate in five of nine individuals who attempted a TCA (n = 3 post-traumatic and n = 2 unilateral).

Low dose oral opioid antagonists (low dose naltrexone) have also been studied in centralized pain, with effects attributed to antihyperalgesia (Jackson et al., 2021) (transient blockade of μ- and δ opioid receptors) as well as reduced neuroinflammation (antagonistic binding to the Toll-like receptor-4) (Bostick et al., 2019). An American study of 59 patients ($n = 14$ postsurgical) with centralized neuropathic ocular pain (defined by presence of neuropathic symptoms, IVCM findings, and/or persistent pain after topical anesthetic) examined the effects of naltrexone 4.5 mg nightly (mean 14.87 ± 11.25 months) on chronic ocular surface pain. Overall, a 49.2% improvement in pain was noted from baseline (3.23 ± 2.60 from 6.13 ± 1.93, $p < 0.001$, range 0–10) (Dieckmann et al., 2021). While we grouped individuals utilizing any opioid agent into one category, naltrexone was the most common agent used; 15 individuals attempted any opioid medication in our population, and improvement in pain was seen in 10 of these patients ($n = 3$ post-traumatic, $n = 3$ migraine-like, n = 4 unilateral).

Finally, while less frequently studied, dysfunction at the autonomic nervous system may contribute to chronic ocular surface pain (Galor et al., 2018b). Along with the trigeminal nerve's sensory input, the sympathetic nervous system (SNS) projects fibers to the cornea from the superior cervical ganglion, while the parasympathetic nervous system (PSNS) sends fibers from the ciliary ganglion (Galor et al., 2018b). Autonomic dysfunction contributes to a variable degree to chronic pain conditions, like fibromyalgia (Janzen and Scudds, 1997), cluster headaches (Costa et al., 2000; Pipolo et al., 2010), and complex regional pain syndrome (Quevedo et al., 2005). In patients with parasympathetic or sympathetic contributors to pain, sphenopalatine ganglion or superior cervical ganglion blocks respectively, and/or nerve stimulation as well as intrathecal delivery of analgesic agents have been used with some success. In particular, one case report of a patient with intractable post-refractive surgery (LASIK) pain was treated initially with a trigeminal nerve stimulator and later on with intrathecal bupivacaine-fentanyl delivery. The patient has reported stable pain since 2014 with >50% (moderate) pain relief for over a year (Hayek et al., 2016). In our study, six individuals ($n = 3$ unilateral vs. $n = 1$ postsurgical, post-traumatic, migraine-like each) received a block at the aforementioned ganglia, and all patients experienced improved pain except one postsurgical patient; among those who improved, the blocks most commonly led to mild ($n = 3$) and marked ($n = 2$) improvement in pain.

As with all studies, our findings must be considered bearing in mind the study limitations, which included a retrospective evaluation of multiple therapies in a wide range of individuals with chronic ocular surface pain from varied etiologies. Yet, this weakness is also a strength considering its originality, as prior studies have only examined the effect of one therapy in a particular patient population. In reality, the majority of patients with chronic ocular surface pain will receive a number of oral, topical, and adjuvant therapies that often work concomitantly. Another limitation is sample size considerations, especially when examining pain subgroups (e.g. unilateral). As such, future studies with larger populations are needed to validate the findings of our study. Furthermore, unaccounted confounders may have affected our data, such as emotional and psychosocial contributors to pain (Lamb et al., 2010; Otis et al., 2013; Patel et al., 2019). Other studies have demonstrated that targeting these aspects with a variety of therapies, such as cognitive behavior therapy, acupuncture, and exercise, can reduce pain intensity beyond medical therapy alone (Mehra et al., 2020) and as such, these factors should be examined in future studies. This is particularly pertinent to our findings, since cognitive modification and positive counseling may enhance compliance and motivate patients to remain compliant and persistent in maintaining their continuity of care and follow ups for as long as necessary to find an efficacious treatment approach. Finally, comparison to other studies is limited considering the varying populations and pain assessments utilized.

CONCLUSION

Despite the study's limitations, our study presents clinical outcomes in a wide range of patients with chronic ocular surface pain, treated with a variety of oral, topical, and adjuvant therapies. Overall, there was individual variability in treatment response, although some trends were noted by pain subgroup. One likely contributor to variability is our inability to pinpoint the location(s) of nervous system dysfunction (peripheral corneal, peripheral non-ocular, central, autonomic) for each patient. Even in patients with suspected central pain, the optimal combination of oral, topical, and adjuvant therapies is not known. In our population, some patient who failed treatment with an α2δ ligand, subsequently reported subjective pain reduction with a TCA or topiramate. This points to the necessity of a trial-and-error approach, which is currently widely utilized when treating individuals with chronic ocular surface pain. Our findings point to needed areas of future research, including the development of diagnostic tests that can localize nervous system abnormalities, and then application of

personalized approaches that target these abnormalities with medications or other therapies that provide faster acting pain relief than currently available neuromodulators.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Miami Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

Supported by Department of Veterans Affairs, Veterans Health Administration, Office of Research/Development, Clinical Sciences R and D (CSRD) I01 CX002015 (Galor) and Biomedical Laboratory R and D (BLRD) Service I01 BX004893 (Galor), Department of Defense Gulf War Illness Research Program (GWIRP) W81XWH-20-1-0579 (Galor) and Vision Research Program (VRP) W81XWH-20-1-0820 (Galor), National Eye Institute R01EY026174 (Galor) and R61EY032468 (Galor), NIH Center Core Grant P30EY014801 (institutional) and Research to Prevent Blindness Unrestricted Grant (institutional); DoD W81XWH-19-1-0525 (Levitt), Wallace H. Coulter Center for Translational Research (Levitt), NIH, NINDS, UG3 NS123964 (Levitt).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.788524/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

REVIEW
published: 31 January 2022
doi: 10.3389/fphar.2021.769909

Topical Therapeutic Options in Corneal Neuropathic Pain

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Purpose of Review: Corneal neuropathic pain can be difficult to treat, particularly due to its lack of response to standard dry eye therapies. We describe a variety of topical therapeutic options that are available to treat corneal neuropathic pain with a significant or primary peripheral component. We also describe possible mechanisms of action for such topical therapies.

Recent Findings: Topical corticosteroids and blood-derived tear preparations can be helpful. Newer therapies, including topical lacosamide and low-dose naltrexone are emerging therapeutic options that may also be considered.

Summary: Corneal neuropathic pain with a significant peripheral component may be managed with a variety of topical therapeutic options.

Keywords: corneal neuropathic pain, neuropathic ocular pain, low-dose naltrexone, lacosamide, sub-basal corneal nerve plexus, serum tears

OPEN ACCESS

Edited by:
Dario Rusciano,
Sooft Italia SpA, Italy

Reviewed by:
Piera Versura,
University of Bologna, Italy
Anna Maria Roszkowska,
University of Messina, Italy

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *02 September 2021*
Accepted: *28 December 2021*
Published: *31 January 2022*

Citation:
Nortey J, Smith D, Seitzman GD and Gonzales JA (2022) Topical Therapeutic Options in Corneal Neuropathic Pain. Front. Pharmacol. 12:769909. doi: 10.3389/fphar.2021.769909

INTRODUCTION

Corneal neuropathic pain has perplexed ophthalmologists particularly because of its symptomatic masquerade as dry eye disease. Indeed, some patients with corneal neuropathic pain may have dry eye findings that are compatible with evaporative and/or aqueous deficient dry eye disease. However, these patients fail to respond to dry eye treatments (Galor et al., 2015a; Galor et al., 2016). Some patients may even be suspected of malingering or of having somatic symptom disorders. Patients often have seen a variety of ophthalmologists for second, third, or more opinions. While corneal neuropathic pain can certainly exist in the setting of aqueous sufficient and deficient dry eye, the common clinical situation encountered is a relatively unremarkable ocular surface examination in a person complaining of significant ocular discomfort (the so-called "pain without stain" patient) (Rosenthal et al., 2009). Increasing awareness of the existence of corneal neuropathic pain, a distinct clinical entity from aqueous sufficient and aqueous deficient dry eye disease, can benefit patients by advancing them on a more directed course to more specifically addresses their discomfort (Craig et al., 2017). Management of neuropathic pain, however, continues to be a challenge, in part, due to a lack of comparative clinical trials identifying the most effective treatment strategies. Often, determining whether there is a primarily peripheral or a primarily central component of neuropathic pain (or perhaps, mixed) can identify routes of therapy that may be most efficiacious (Rosenthal et al., 2009; Rosenthal and Borsook, 2012; White et al., 2014; Dieckmann et al., 2017; Ozen et al., 2017). In patients exhibiting a significant peripheral component of corneal neuropathic pain or discomfort, topical therapeutic approaches may be of benefit (Asbell, 2006; Mondy et al., 2015; Chen et al., 2019; Siedlecki et al., 2020). This paper will use both the terms "pain" and "discomfort" to describe the subjective complaints of patients suffering from corneal

TABLE 1 | Topical therapeutic options available as eye drops for peripheral corneal neuropathic pain.

Topical therapies	Mechanism of action
Corticosteroids	Anti-inflammatory properties can decrease the density of dendritic cells
Blood-derived tear preparations	Autologous or allogenic tear promote corneal epithelial cell health and are associated with fewer features of neuropathy of sub-basal nerve plexus
Lacosamide	Amino acid molecule that decreases hyperexcitability of corneal cold-sensitive nerve terminals
Low-dose naltrexone	Opioid antagonist known for its effects on bodily neuropathic pain
Enkephalin modulators	A relatively new therapy that acts as a neuropeptide inhibitor with effects on modulating pain

neuropathic pain. Some patients point out that they do not experience "pain" per se, but rather a sensation that is often difficult to define but is, nevertheless, uncomfortable.

The nerves within the cornea stem from branches of the nasociliary nerve which itself is a branch of the ophthalmic division of the trigeminal nerve also known as the first division of the fifth cranial nerve. Nasociliary nerve branches enter the peripheral cornea radially before traveling anteriorly to penetrate Bowman's layer and forming the sub-basal nerve plexus. Ocular surface pain and corneal neuropathic pain is initiated by these peripheral nerves and their free endings located within the cornea (Mehra et al., 2020). Other pain nerve fibers, called nociceptor fibers, variably respond to different stimuli. Mechanoreceptors are responsible for the sharp pain that is felt upon mechanical stimulation such as an object coming in contact with the cornea. Polymodal receptors respond to mechanical, thermal, pH, and chemical changes within the environment, and are associated with burning and stinging pains. Cold thermoreceptors respond to changes in temperature and tear osmolarity, and can be associated with ocular surface discomfort that may be generated with evaporative tear (Rosenthal and Borsook, 2012; Cho et al., 2019; Mehra et al., 2020). Because of its peripheral location in the cornea, the sub-basal nerve plexus is the peripheral-most component to efferent and afferent corneal neuropathic pain. However, there is also a significant central component to corneal neuropathic pain, which involves first- and second-order nerve input to the thalamus and, ultimately, the cortex with additional modulation from the thalamus and amygdala (Rosenthal and Borsook, 2012; Mehra et al., 2020).

Our group at the Proctor Foundation at the University of California San Francisco serves as a major tertiary center for the evaluation and management of patients with corneal neuropathic pain. Herein, we discuss the topical (eye drop) therapeutic options that are suggested for patients with a component of peripheral corneal neuropathic pain. Our experience has been that patients with peripheral corneal neuropathic pain are very motivated to understand what eye drop therapies may be of potential benefit to them. While there are a variety of eye drops that can be used in corneal neuropathic pain, no randomized controlled trials comparing one class of eye drop to another in corneal neuropathic pain currently exists. Commercially available and compounding pharmacy-only available drops are reviewed. To ensure a comprehensive review, we searched for topical (eye drop) therapeutics in PubMed using search terms, "neuropathic pain", "ocular pain", and "eye pain" and evaluated the results if the abstracts described topical therapeutics. A total of 204 records were screened and 182 records were excluded. Of the 22 reports assessed for eligibility, additional records were excluded due to focusing on post-operative treatments (4), focusing on systemic treatments (2), and a lack of relation to neuropathiic ocular pain (6). Ten studies were included in this review. Our group's experience in topical therapy was also included (**Table 1**).

ASSESSMENT OF CORNEAL NEUROPATHIC PAIN

The assessment of corneal neuropathic pain involves 1) identifying the presence of subjective ocular pain and, if possible, 2) identifying features that align with a neuropathic phenotype. Questionnaires and *in vivo* confocal microscopy can be helpful in distinguishing patients with corneal neuropathic pain from those with non-neuropathic ocular discomfort that can be associated with dry eye disease. Patients may have features of both an aqueous sufficient or aqueous deficient dry eye disease in addition to frank corneal neuropathic pain.

Questionnaires that are specific for ocular pain can be easily self-administered in the clinical setting or administered by staff and include the Ocular Pain Assessment Survey (OPAS) and the Neuropathic Pain Symptom Inventory modified for ocular pain (NPSI-Eye) (Qazi et al., 2016; Farhangi et al., 2019).

Prior to 2016, there was no standardized and validated way of assessing ocular pain intensity and aggravating factors. The OPAS was a major step forward in identifying, anatomically locating and quantifying the intensity ocular pain. Comprised of 27 questions, spanning a gold-standard visual analog system of quantifying pain intensity (Wong-Baker FACES pain rating scale), aggravating factors, and quality of life features, the OPAS is not only comprehensive but useful in monitoring response to therapy (Qazi et al., 2016). The NPSI attempts to identify neuropathic-specific ocular pain symptoms, including burning sensation (Bouhassira et al., 2004; Farhangi et al., 2019). The identification of specific neuropathic pain features is important because such patients may be less apt to respond to artificial tears, which may be helpful in patients with non-neuropathic ocular discomfort (Galor et al., 2015b).

Following questionnaires, a critical component of the clinical examination is determining a patient's subjective response to topical anesthetic. Complete improvement in discomfort or pain is in keeping with a primarily peripheral corneal neuropathic pain process. If some component of discomfort persists, this may

suggest that there is a component of the pain that is central while a complete lack of response to topical anesthetic suggests that the discomfort is primarily central. For those corneal pain processes that have at least some component of a peripheral phenotype, topical pain modulatory therapies may be of benefit. Herein, we discuss a variety of topical therapeutic options that are available to clinicians for managing peripheral corneal neuropathic pain.

Topical Corticosteroids

In both dry eye disease and neuropathic ocular pain, inflammatory cells may mediate a component of the discomfort experienced by patients. Antigen-presenting cells, including dendritic cells, can be found within the corneal epithelium and stroma, and are important players in the complex immune functions and responses exhibited by the cornea in both processes (Hamrah et al., 2003). Compared to control patients, *in vivo* corneal confocal microscopy demonstrates a higher density of dendritic cells in patients with dry eye and corneal neuropathic pain (Kheirkhah et al., 2015; Shetty et al., 2016a; Nicolle et al., 2018). Moreover, in some cases, particularly in aqueous deficient dry eye disease, dendritic cells may be larger and be composed of more individual dendrites than in aqueous sufficient dry eye disease (Kheirkhah et al., 2015). Nicolle et al. (2018) To counterbalance this, endogenous peptides, known as enkephalins, serve to mitigate pain. Moreover, notable effects on *in vivo* confocal microscopy show a decrease in density of dendritic cells after treatment (Villani et al., 2015). Dendritic cells may express inflammatory cytokines and enzymes that degrade neuropeptides, thereby augmenting corneal nociceptors which are involved in initiating the perception of pain (Shetty et al., 2016a; Shetty et al., 2016b; Khamar et al., 2019). Indeed, it is hypothesized that dendritic cell signaling may be involved in mediating and enhancing nociceptive properties in a variety of dry eye, corneal neuropathic pain, and systemic neuropathy diseases and syndromes (Klitsch et al., 2020). Animal models of dry eye disease have demonstrated that dendritic cells can secrete a variety of proteins that activate downstream chemokines and cytokines that can modulate inflammation on the ocular surface (Gandhi et al., 2013; Zhang et al., 2014). Topical corticosteroids have well known anti-inflammatory effects. Thus, decreasing the density of dendritic cells with topical corticosteroids is one approach that may help in cases of corneal neuropathic pain where confocal microscopy identification of sub-basal dendritic cells is a predominant feature (Villani et al., 2015).

Blood-Derived Tear Preparations

Blood-derived tear preparations contain various growth factors, vitamins, and cytokines and could be considered more similar to natural tears than commercially available over-the-counter artificial tear products. Such tear preparations can be derived from a patient's own serum as autologous serum tears, from platelet rich plasma, or from allogeneic sources, such as donor cord blood (Tsubota et al., 1999a; Wu et al., 2021). Serum has been shown to promote the differentiation of corneal and conjunctival epithelial cells to express mucins (Gipson et al., 2003). This allows for the migration of corneal epithelial cells in a dose-dependent fashion and may, in part promote corneal epithelial health by stimulating expression of other growth factors and receptors (Phan et al., 1987; Tsubota et al., 1999a; Tsubota et al., 1999b; Geerling et al., 2001). A healthy corneal epithelium helps protect the sub-basal nerve plexus.

There has been some debate about the efficacy of autologous serum tears for dry eye disease. An extensive review by the Cochrane Database Group to identify randomized controlled trials evaluating the efficacy of autologous serum tears compared to artificial tears did not find significant evidence of a long-term durable benefit of autologous serum tears (Pan et al., 2017; Aggarwal et al., 2019). However, some studies have found in pain associated with dry eye, cord blood is superior to placebo (Campos et al., 2020). In non-randomized, observational series, platelet rich plasma drops helped to alleviate pain in corneal surgery-related corneal trauma (Alio et al., 2018).

Neuropathic ocular pain, which is distinctly different from aqueous deficient or sufficient dry eye disease, may benefit from blood-derived tear preparations that studies of general dry eye disease have not been powered to detect. Indeed, in patients with dry eye disease, serum tears have been shown to decrease basal epithelial cell density on confocal microscopy (Mahelkova et al., 2017). While some studies using serum tears in dry eye disease did not identify significant changes in the number of Langerhans cells or activated keratocytes or in the features of the sub-basal nerve plexus, other studies found that corneal nerve morphology improved with fewer neuropathic features using blood-derived tear products (Giannaccare et al., 2017; Mahelkova et al., 2017). The average concentration of serum growth factors vary according to patients who suffer from numerous systemic disease as comparted to healthy subjects with only ocular surface disease. This may be one reason explaining some therapeutic variability of serum tears in different patient populations (Ripa et al., 2020; Siedlecki et al., 2020).

In corneal neuropathic pain, serum tears have been described to be helpful in patients experiencing discomfort to light (photoallodynia) (Aggarwal et al., 2015). Autologous serum has been shown to decrease findings of sub-basal corneal nerve beading and neuromas (Aggarwal et al., 2015). In addition, serum tears have been associated with an improvement in other nerve metrics (as assessed by semi-automated quantification by ImageJ analysis) including total nerve length, nerve number, decrease in nerve reflectivity, and decrease in nerve tortuosity (Aggarwal et al., 2019). Such improvements in nerve metrics have also been correlated with an improvement in ocular pain by patients, suggesting that the resolution of neuropathic features is associated with an improvement in corneal nerve function (Aggarwal et al., 2019). Fresh frozen plasma and platelet-enriched plasma have similar purported benefits as serum tears and are worthy of future inverstigation with regard to ocular neuropathic discomfort/pain (Wang et al., 2021).

Topical Lacosamide

Lacosamide ($C_{13}H_{18}N_2O_3$, molecular weight 250.29 g/mol, pubchem.ncibi.hlm.nih.gov accessed August 1, 2021) is an amino acid molecule that was originally developed as an

antiepileptic medication. Its main mechanism of action is to selectively enhance the slow inactivation (as opposed to the fast inactivation) of voltage-gated sodium channels (Errington et al., 2008; Niespodziany et al., 2013; Rogawski et al., 2015). Lacosamide also binds with the collapsin-response mediator protein-2 (CRMP-2), involved in modulating neurite outgrowth, which is important in establishing new neuronal projections in developing neurons as well as in regenerating nerves (Wilson and Khanna, 2015; Wang et al., 2018).

While corneal pain is sensed by mechano-nociceptors and polymodal nociceptors, cold thermoreceptors also play a role in the perception of ocular pain and discomfort (Belmonte and Gallar, 2011). Modulation of cold thermoreceptors, then, has been a potential therapeutic target. Topical lacosamide, in an *ex vivo* model, has been shown to decrease the hyperexcitability of corneal cold-sensitive nerve terminals (Kovács et al., 2016). In an aqueous deficient animal model (in which the lacrimal glands had been extirpated), the hyperexcitability as measured by the nerve terminal impulses in corneal cold-sensitive nerve terminals was decreased (Kovács et al., 2016).

Lacosamide 1% is produced from preservative-free Vimpat (UCB Inc., Smyrna, Georgia) 10 mg/ml 20 ml vial. It is important to note that Vimpat is a Schedule 5 drug and all state and federal laws and regulations regarding controlled substances must be followed when prescribing and dispensing this drug. The compounding of lacosamide 10 mg/ml is an aseptic transfer from the injection vial to the droptainers or dispensing devices. When assigning a beyond use date, pharmacies must follow corresponding state and United States Pharamcopeia (USP) General Chapter 797 regulations. According to USP General Chapter 797, a beyond use date of 14 days refrigerated may be assigned. The package insert for Vimpat states that it is not to be frozen. The product Vimpat 20 mg/20 ml is available through drug wholesalers only as packs of 10. It is important to find a pharmacy that is willing to make the initial investment in order to stock the medication for compounding use.

Topical Low-Dose Naltrexone

Naltrexone ($C_{20}H_{23}NO_4$, molecular weight 341.4 kg/mol, pubchem.ncibi.hlm.nih.gov accessed August 1, 2021) is an opioid antagonist that was originally developed as a therapeutic for opioid and alcohol addiction, typically at oral doses from 50 to 100 mg daily. Low-dose naltrexone (typically in doses from 1 to 5 mg daily) has been used for treating bodily neuropathic pain (Younger and Mackey, 2009; Metyas et al., 2018). Ultra-low-dose naltrexone (doses below 1 mg daily) may also be used (Toljan and Vrooman, 2018).

Naltrexone is noted to have effects on opioid receptors (commonly described with mu-opioid receptors as well as others) and non-opioid receptors. A non-opioid receptor that seems to be associated with the functionality of naltrexone as it pertains to pain modulation is the Toll-like receptor, which is found on macrophages and microglia. Microglia are involved in pain through the binding of a protein, high mobility group box 1, HMGB1) which binds to Toll-like receptors 2 and 4 (Watkins et al., 2007). Toll-like receptor expression has been noted to be upregulated in neuronal injury (Owens et al., 2005). Microglia may then increase the expression of tumor necrosis factor-alpha (TNF-alpha) and other inflammatory mediators *via* Toll-like receptor as well as NF-KB signaling, indicating a close relationship between inflammation and pain (Nadeau and Rivest, 2000; Thibeault et al., 2001). Ultimately, glial activation is thought to enhance neuroexcitability, which may be associated with the increased perception of pain (Watkins et al., 2007). Naltrexone has been shown to inhibit the IL-6 and TNF-alpha that is produced after Toll-like receptors have interacted with their cognate ligands, which can thereby mitigate pain in animal models (Grace et al., 2015; Cant et al., 2017).

Models of ocular surface injury (penetrating trauma or alkali injury) have been associated with retinal damage and inflammation within the brain by virtue of microglial and macrophage activation (Ferrari et al., 2014; Paschalis et al., 2018). This has suggested that modulation of ocular surface neuropathy can be beneficial in a wide array of conditions. Indeed, oral low-dose naltrexone has been described to be beneficial to patients with a central component of neuropathic ocular pain. Patients treated with oral low-dose naltrexone as monotherapy or as part of a multimodal therapeutic approach was assocated with a decrease in their mean visual analog pain score as well as decrease in their mean quality of life score as assessed by the OPAS (Dieckmann et al., 2021). Topical low-dose naltrexone has been used in a diabetic murine model demonstrating improvements in corneal nerve sensitivity to a filament (von Frey) similar to that used to test human corneal sensation (Cochet-Bonnet enesthesiometer) (Zagon et al., 2014).

Interest in naltrexone's potential to ameliorate corneal neuropathic problems, such as ulcerations and delayed re-epithelialization, have led to interest in producing contact lenses that would allow for the ability to deliver a more constant level of low-dose naltrexone to the cornea's sub-basal nerve plexus (Alvarez-Rivera et al., 2019).

Presently Naltrexone, as an eye drop, requires specialty compounding. Naltrexone eye drops are reconstituted from active pharmaceutical ingredient powder because a commercial product does not currently exist. This preparation is considered high-risk compounding because pharmacies start the process with non-sterile powder and sterilize the solution *via* membrane filtration as the final step. Naltrexone HCl USP powder is dissolved in sodium chloride 0.9% sterile injection and preserved with benzalkonium chloride solution (though a preservative-free formulation can be prepared as well). The pH may range from 4.5 to 6.2. The solution is drawn up in a luer lock syringe and sterile filtered into droptainers or dispensing devices. For this type of preparation, USP General Chapter 797 allows a Beyond Use Date of 3 days refrigerated or 45 days frozen. Strengths of naltrexone ophthalmic solution range from 0.001 to 0.2%. The compounding of high-risk preparations is performed in an International Organization for Standardization (ISO) 7 ante room and the final filtering and packaging is done in an ISO 5 laminar flow hood.

Topical Enkephalin Modulators: Future Targets

Because the neuropeptides known as enkephalins can modulate pain, there has been interest in utilizing this pathway as a

potential pain therapeutic. Enkephalins are associated with an analgesic effect, but their action is relatively short-lived due to the presence of enzymes that rapidly degrade these neuropeptides. Endogenous inhibitors of enkephalin enzymes do exist and are present in foreign body models of ocular pain (Ozdogan et al., 2020; Lasagni Vitar et al., 2021). Thus, pharmacologic inhibition of such enzymes may be a therapeutic target. In one study, the topical administration of PL265, an inhibitor of enkephalinase, in a murine model of corneal pain reduced corneal mechanical and chemical hypersensitivity (Reaux-Le Goazigo et al., 2019). Targeting corneal mu-opioid receptors with agonists may be another therapeutic target as suggested in murine models (Joubert et al., 2020).

DISCUSSION

There has been substantial progress in the development of tools that can identify corneal neuropathic pain (Qazi et al., 2016; Farhangi et al., 2019). The identification of ocular pain and neuropathic pain features with the OPAS and NPSI questionnaires is an important step forward in better distinguishing patients that are less likely to respond to lubrication like their typical dry eye counterparts. However, significant challenges remain, particularly regarding treatment of corneal neuropathic pain. *In vivo* confocal microscopy can be helpful in identifying dendritic or other inflammatory cells, which may suggest a role for a brief course of topical corticosteroids. Autologous blood-derived products are a reasonable first-line approach given their association with improvement in comfort and features of neuropathy on *in vivo* confocal. If there is a significant peripheral component, consideration can be made for other topical therapies such as lacosamide or low-dose naltrexone drops compounded through a specialty pharmacy. While longitudinal comparative studies demonstrating the most effective topical treatment strategies have yet to be performed, it is reassuring that a variety of topical therapeutic options exist for patients with a peripheral component of corneal neuropathic pain and discomfort. A concerted effort on the part of both ophthalmologists and patients offer the possibility of future randomized controlled trials that can provide high-quality evidence for managing corneal neuropathic pain.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design: JN, DS, GS, and JG; data collection: JN, DS, GS, and JG; analysis and interpretation of results: JN, DS, GS, and JG; draft manuscript preparation: JN, DS, GS, and JG. All authors reviewed the results and approved the final version of the manuscript.

FUNDING

This work was supported in part by an unrestricted grant from Research to Prevent Blindness.

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Conflict of Interest: DS was employed by A&O Compounding Pharmacy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Medicine

REVIEW
published: 08 February 2022
doi: 10.3389/fmed.2021.768649

Supraspinal Mechanisms Underlying Ocular Pain

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Supraspinal mechanisms of pain are increasingly understood to underlie neuropathic ocular conditions previously thought to be exclusively peripheral in nature. Isolating individual causes of centralized chronic conditions and differentiating them is critical to understanding the mechanisms underlying neuropathic eye pain and ultimately its treatment. Though few functional imaging studies have focused on the eye as an end-organ for the transduction of noxious stimuli, the brain networks related to pain processing have been extensively studied with functional neuroimaging over the past 20 years. This article will review the supraspinal mechanisms that underlie pain as they relate to the eye.

Keywords: pain, eye, neuroimaging, fMRI, supraspinal, brain, brainstem, ocular

OPEN ACCESS

Edited by:
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University of Miami, United States

Reviewed by:
Betul Bayraktutar,
Tufts Medical Center, United States
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Specialty section:
This article was submitted to Ophthalmology, a section of the journal Frontiers in Medicine

Received: *01 September 2021*
Accepted: *27 December 2021*
Published: *08 February 2022*

Citation:
Pondelis NJ and Moulton EA (2022) Supraspinal Mechanisms Underlying Ocular Pain. Front. Med. 8:768649. doi: 10.3389/fmed.2021.768649

INTRODUCTION

Ophthalmology as a clinical field has a preoccupation with what can be seen, particularly for patients presenting with eye pain. The patient reports eye pain as a symptom, and the clinician collects the available data to reach a diagnosis. Data come in the form of patient reports, medical history, professional acumen, and clinical findings, such as those obtained with a slit lamp exam of the anterior and posterior segment or specialized equipment that provide intensely magnified views of ocular structures. Despite the ever-expanding options for precise clinical evaluation, pain is no guarantee of a physically observable sign of tissue damage. Pain is subjective by its very nature, and similar inputs can result in bewildering and wildly inconsistent pain responses. However, modern functional neuroimaging tools have allowed scientists to investigate this symptom in the context of the inner workings of the brain.

Pain serves as a crucial system to avoid bodily injury and damage. Pain accomplishes this function by creating strong, memorable disincentives for potentially damaging activity as well as protective reflexes and convalescence-promoting behaviors to prevent or limit damage. As defined by the International Association for the Study of Pain (IASP), it is "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (1). Pain is an attention-demanding, conscious state that takes precedence over other processes (2, 3). Ocular pain can be debilitating and serves to protect a critically important sensory apparatus.

Much as visual processing involves numerous brain regions, pain perception is generated by an amalgam of signals and modifications carried by a wide network of brain regions and pathways. These regions work in delicate balance with each other and are influenced by individual neurobiological variation, resulting in an inherently subjective experience (3). The transduction of noxious information travels along multiple pathways to the brain and is processed during its transmission from the periphery as well as at the highest cortical levels. The diverse inputs

from the numerous supraspinal processing areas are eventually integrated. The result is the multidimensional perception of pain, with its intensity, unpleasantness, emotional connotation, and more.

Conceptualization of Pain

Pain has been categorized into three distinct, concurrent dimensions: affective-motivational, sensory-discriminative, and cognitive-evaluative (4). The sensory and discriminative dimensions are related to the location, characteristics, intensity, and timing of a stimulus that evokes pain. The affective and motivational aspects of pain are highly intertwined with emotion and constitute the unpleasant aspects of pain that give rise to behavioral responses. The cognitive and evaluative dimension is the means by which the brain is able to comprehend and contextualize the consequences of injury or pain, anticipate pain based on memory, and inhibit or facilitate painful sensation (5, 6). Multiple distinct brain regions and networks underlie these discrete aspects of pain and their flavoring of the pain experience (7–10).

Nociceptive vs. Neuropathic Pain

Nociception is the physiological encoding and detection of noxious stimuli by the central and peripheral nervous systems (11). Though they are often concurrent, nociception and pain can occur independently, and the terms are not directly interchangeable. Nociceptive transduction can take place during the sensation of itch, which itself is not painful; likewise, pain can exist untethered from peripheral noxious input, as with phantom limb pain (12).

Nociceptive pain "arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors" (13), a detection and warning system for the presence of intense stimuli. The transfer of nociceptive signals through supraspinal centers to the cortex generates pain, that is, triggers avoidance reflexes, unpleasant sensations, and a negative emotional state. This multifaceted experience overrides most ongoing processes and diverts attention to the detection of, and withdrawal from, a noxious stimulus (2).

Pain may persist over long periods of time and can serve a beneficial purpose by reporting the extent of injury and progression of tissue repair while promoting convalescent behavior (14). To facilitate this, after injury the central nervous system can establish long-lasting sensitivity to peripheral inputs, which may help to prevent further harm during recuperation (15, 16). These changes do not always resolve after injury and sometimes cannot be clearly linked to disease as the source. Pain recurring or persisting for longer than 3 months is defined as chronic and may be the consequence of underlying disease (chronic secondary pain) or exist without a clear cause or insult (chronic primary pain) (17, 18).

Neuropathic pain is "a result of a lesion or disease of the somatosensory nervous system" and may be peripheral or central in nature (13). As part of the repair process after peripheral nerve injury, both damaged and healthy primary nerve fibers (but not their peripheral receptors) may fire action potentials spontaneously; the resulting ectopic pain is a natural consequence of healing but is nevertheless considered peripheral neuropathic pain (16, 19). In the case of centralized neuropathic pain, the complex balance of supraspinal mechanisms underlying the CNS's signaling and modulatory capacity can become disrupted and manifest pain without significant peripheral instigation (14, 16).

Organizational Summary

The primary aim of this review will be to describe the functionality and role of brain structures related to pain processing in the context of human neuroimaging (20). We will first briefly summarize how the peripheral nervous system encodes and transmits ocular nociceptive signals to the central nervous system by major ascending pathways. We then will focus on neuroimaging of supraspinal structures related to pain. Finally, we will explore sensitization in these circuits and briefly discuss their manifestation at the network level.

SENSORY INNERVATION OF THE ANTERIOR SEGMENT

The eye contains a host of sensitive tissue, with the cornea being the most densely innervated in the body (21). Nociceptors within these areas are largely offshoots of the ophthalmic division of the trigeminal nerve, but other pathways and sensory modalities contribute to nociception and processing of the full spectrum of sensory inputs to the anterior segment and eye. These peripheral pathways have been more thoroughly described previously (22, 23). The healthy cornea is exclusively innervated by large-diameter, myelinated A-delta nerve fibers and small-diameter, unmyelinated C-fibers (22, 24). The three primary classes of corneal sensory afferents are polymodal nociceptors, mechanonociceptors, and cold thermoreceptors, each of which preferentially responds to various sensory stimuli. The polymodal C-fibers are the most abundant and can detect a wide range of stimuli, including mechanical, thermal, and chemical. Cold receptors respond to thermal changes and consist of either A-delta or C-fibers, while specific mechanoreceptors are exclusively A-delta fibers and activate upon mechanical stimulation alone. These same classes of peripheral sensory afferents have also been identified in the episclera, bulbar conjunctiva, iris, and ciliary body, while non-corneal ocular tissue, especially the eyelids, may have numerous additional types of low-threshold mechanoreceptors (21, 22, 24, 25). The trigeminal system is also responsible for the innervation of both meningeal and dural vessels, and information from peripheral receptors in these areas travels alongside other sensory information through trigeminal pathways (26, 27).

Several peripheral sensors in the anterior of the eye contain melanopsin, a photopigment that offers a light transduction mechanism that may lead to pain perception. With a peak wavelength sensitivity of 480 nm, melanopsin-based photoreception can occur in intrinsically photosensitive retinal ganglion cells (ipRGCs) and is increasingly implicated as a source for light-induced pain (28–32). These ipRGCs can generate their own signal independent of rod and cone involvement

in response to light absorption yet can additionally receive or relay input from classical RGCs and support cells (33–36). In addition to ipRGCs, melanopsin has been found in a variety of other tissue in mammals and humans, including expression and prospective inherent photosensitivity in the cornea, iris, ciliary body projections, certain vasculature, and trigeminal neurons themselves (36–40). These peripheral melanopsin-containing populations can generate a light-response without traversing the optic nerve (26, 27, 40, 41).

Nociceptive Pathways

Peripheral receptors in the anterior segment are conventionally the gateway for nociceptive transduction that leads to the experience of pain. The transfer of peripheral signals to the brain is facilitated by a number of pathways, including the trigeminothalamic pathway, the parabrachial nucleus pathway, and the melanopsin pathway.

In health, and in conjunction with their respective peripheral afferents, these pathways supply the brain with vital information regarding the health of the eye and serve as a broad and fine-tuned detection mechanism for the prevention of ocular damage. However, damage to this network can result in dysfunction of peripheral neurons, intermediaries between them and the brain, or cortical areas themselves—all resulting in pain (22). Likewise, maladaptive sensitization of these same critical nociceptive pathways can lead to unduly painful outcomes for patients. Even after direct insults to the peripheral fibers of these nerves are healed, pain can persist. Often this persistent pain involves a central component of the nociceptive pathway that can be difficult to detect, let alone resolve.

Trigeminothalamic Pathway

Ocular nociceptive and sensory information travel from peripheral sites through primary fibers of the ophthalmic trigeminal nerve to the ipsilateral trigeminal ganglion, where the neuronal bodies are somatotopically organized along with the other trigeminal branches. First-order neurons synapse to second-order neurons in the pons at the trigeminal brainstem nuclear complex (TBNC). The synapses of nociceptive and thermosensory neurons are located in the spinal trigeminal nucleus caudalis (spVc) transition zones. From the medullary dorsal horn, nociceptive information travels along groups of neurons to either the contralateral thalamus or the ipsilateral parabrachial nuclei (22).

The trigeminal connections to the thalamus are involved in the sensory-discriminative and affective-motivational components of pain (4). From the TBNC, second-order neurons destined for the thalamus leave the subnucleus caudalis, decussate, and subsequently enter the contralateral anterior trigeminothalamic tract (lemniscus). The neurons then ascend to synapse with tertiary neurons in the medial and somatosensory (lateral) thalamic nuclei (42). Nociceptive information from the thalamus is then relayed to higher brain regions where it is further processed, eventually resulting in pain perception (22, 43, 44) (**Figure 1A**).

Parabrachial Nucleus Pathway

The nociceptive inputs that pass through the parabrachial nuclei (PBN) are involved in the affective-motivational and autonomic components of pain. The parabrachial nuclei are a bilateral grouping of neurons located in the brainstem at the junction of the dorsolateral pons and midbrain, surrounding the superior cerebellar peduncle (45). The PBN receive afferent input from second-order trigeminal neurons in the spVc (46). The PBN pass the information to the central nucleus of the amygdala, the hypothalamus, periaqueductal gray and RVM, and onto parts of the spino-parabrachial pathway, which innervates the anterior cingulate (ACC) and insular (IC) cortices via the thalamus (47). These findings in non-human primates have been reproduced in humans, where noxious stimuli to the orofacial region produce increased BOLD fMRI signal intensity in the spV and subsequently several other supraspinal regions, including the PBN (48).

In addition to acting as a conduit for peripheral nociceptive information, the PBN have a wide array of functions, such as autonomic modulation (49), and are involved in pain processing, mostly as a key supraspinal region for encoding the affective component of pain (50). The PBN also play a role in pain modulation, as the region is implicated in some forms of endogenous analgesia (48), and low-frequency deep brain stimulation of the PBN provides meaningful pain relief, although these findings are intertwined with stimulation of other, more canonical analgesia-associated brain regions in certain cases (51) (**Figure 1B**).

Melanopsin Pathway

Light information from ipRGCs is largely transmitted through the optic nerve until reaching target destinations in the brain. The three primary tracts that project to the brain are the retino-thalamo-cortical pathway, the retino-midbrain pathway, and the retino-hypothalamic tract. The retino-thalamo-cortical pathway is a direct connection between ipRGC populations and the pulvinar nuclei within the posterior thalamus (26, 52, 53). The retino-midbrain-parasympathetic (or retinomesencephelatic) pathway brings photic signals from the retina directly to the olivary pretectal nucleus in the midbrain (54). The retinohypothalamic tract extends through the optic nerve before synapsing to several areas, with the major target being the suprachiasmatic nucleus (36). This tract can be subdivided into three broad types of innervation: afferents leading to hypothalamic neurons directly, referred to simply as the retinohypothalamic tract; the retino-hypothalamo-parasympathetic tract, which innervates the superior salivatory nucleus in the brainstem; and the retino-hypothalamo-sympathetic tract, which connects to the intermediolateral nucleus in the spine (54).

The melanopsin involvement in light detection in ipRGCs, anterior segment structures, and nociceptive neurons has led to the exploration of pain enhancement by light along these and the trigeminal nociceptive pathways (26, 27, 40, 55–58).

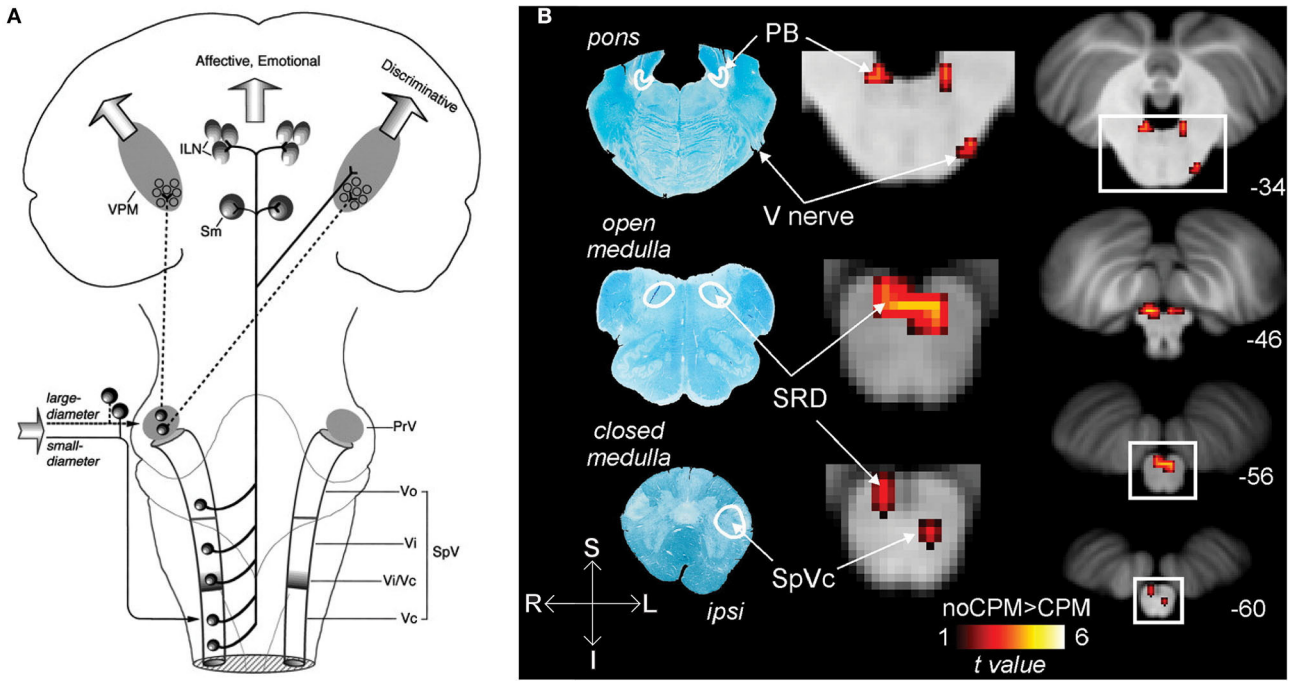

FIGURE 1 | Nociceptive pathways. **(A)** The path of afferent signal transmission from the periphery to the cortex through major projections of the trigeminothalamic pathway. Reprinted/adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature Trigeminothalamic Tract Projections. In: Schmidt R., Willis W. (eds) Encyclopedia of Pain by Ke Ren, Copyright (2007). DOI: https://doi.org/10.1007/978-3-642-28753-4_4626. **(B)** Decreased brainstem fMRI activity, including PB, during endogenous analgesia. Red/yellow indicates regions where fMRI responses to noxious stimuli demonstrated a signal decrease following conditioned pain modulation. Decreased activation was noted in the SRD, SpVc, and the trigeminal nerve along with the PB. On the left, myelin-stained sections are displayed alongside corresponding MRI images of the brainstem. Reprinted from NeuroImage, Vol 124(Part A), AM Youssef, VG Macefield, LA Henderson, Pain inhibits pain; human brainstem mechanisms, p54–62, Copyright (2020), with permission from Elsevier. DOI: https://doi.org/10.1016/j.neuroimage.2015.08.060. PrV, principal sensory nucleus; SpV, spinal trigeminal nucleus; Vo, subnuclei oralis; Vi, subnucleus interpolaris; Vc, subnucleus caudalis; PB, parabrachial nucleus; SRD, subnucleus reticularis dorsalis; SpVc, spinal trigeminal nucleus caudalis; V, trigeminal nerve; Compas: S, superior; I, inferior; R, right; L, left.

CENTRAL REPRESENTATION OF PAIN

Pain is a complex and multifaceted experience and, as such, a large number of cortical and subcortical supraspinal areas are involved in the interpretation of noxious stimuli and the resultant sensation of pain. The supraspinal areas most likely to be activated in response to a wide variety of noxious stimuli are the thalamus, secondary somatosensory cortex, anterior/mid-cingulate cortex, and the insula (7, 8, 10). In addition to these areas, studies with differing parameters and means of noxious stimulation have found additional brain regions that are involved in pain under certain conditions, including the primary somatosensory cortex, amygdala, lateral prefrontal cortex, primary and supplementary motor areas, pre-supplementary motor area, basal ganglia, cerebellum, and brainstem (10) (**Figures 2A,B**).

Distinct aspects of pain are transmitted through separate nuclei in the thalamus to higher brain structures and have been grouped into a classification scheme of medial and lateral pain systems based on the organization of innervation to and from the nuclear groupings (42). The lateral pain system is associated with the sensory-discriminative components of pain, and routes information from somatotopically arranged lateral thalamic nuclei (ventral posterior and posterior, including VPM) to the somatosensory cortices and posterior insula (42, 59–61). The medial pain system, underlying the affective-motivational pain dimension, processes and transfers pain information from non-somatotopically-organized medial dorsal, midline, and intralaminar thalamic nuclei to the cingulate cortex, prefrontal cortex, amygdala, and hypothalamus, and their subsequent projections to descending modulatory areas (42, 60, 62). As pain is an incredibly salient experience, many of these brain regions are involved not just in nociception, but also in attention and motor-response networks (9, 63) (**Figure 2C**).

Primary Somatosensory Cortex

The primary somatosensory cortex (SI, **Figure 3**) receives input from multiple thalamic nuclei, including lateral thalamic regions associated with processing sensory-discriminative aspects of noxious stimulation. SI is involved in multiple aspects of sensory encoding and integration, from non-noxious heat, proprioception, pressure, type and quality of touch to painful nociception (64–67). Beyond the thalamic connections, SI has dense cortico-cortical connections to multiple other areas, especially the secondary somatosensory cortex and insula, as well as other sensory regions, such as the visual cortex (68–70).

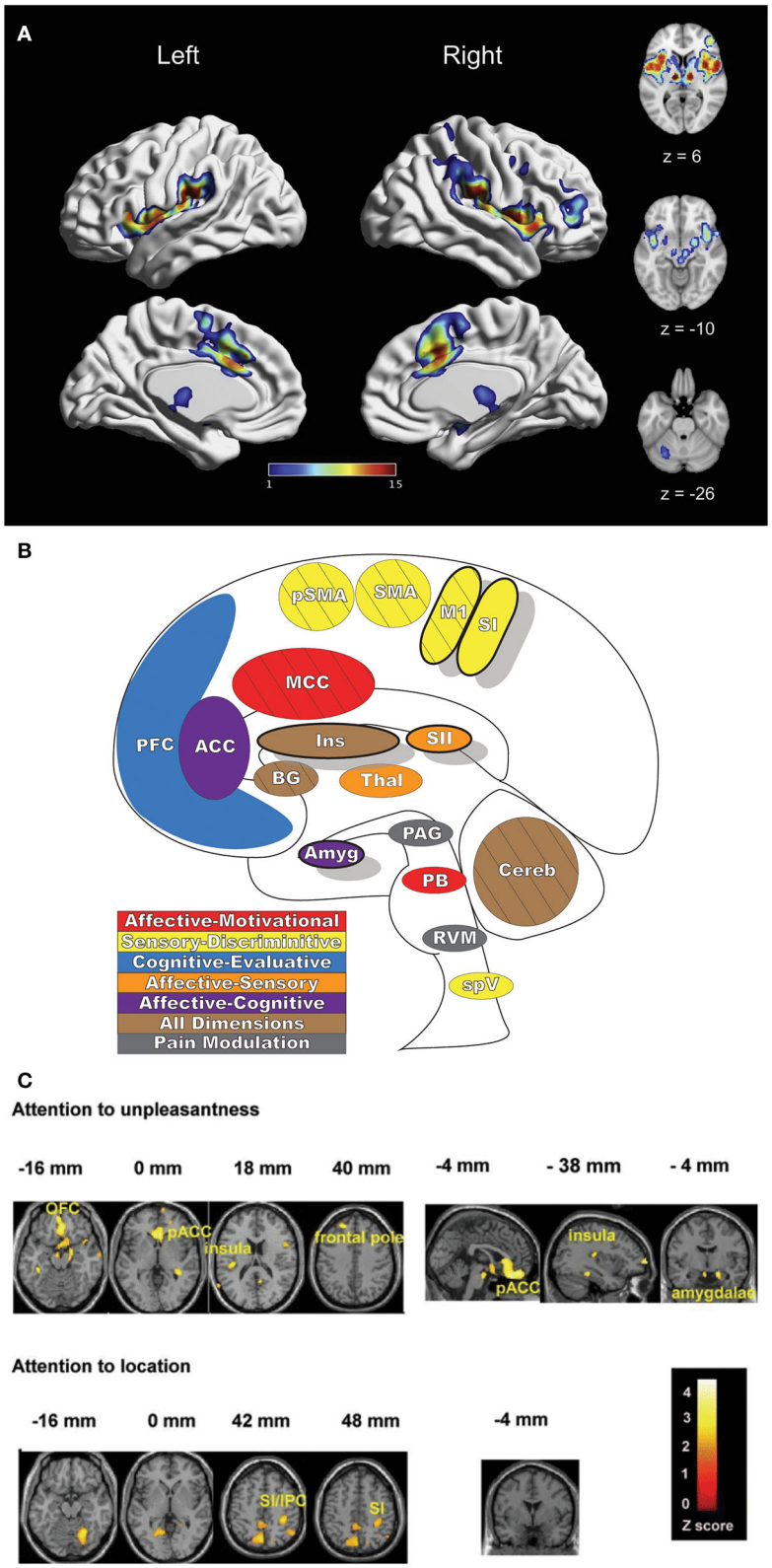

FIGURE 2 | Pain-related areas in the brain and brainstem. **(A)** A meta-analysis of pain neuroimaging studies defines a set of brain regions consistently active across 222 experiments from 200 reports, including bilateral activity in the secondary somatosensory cortex, insular cortex, midcingulate cortex, and thalamus. Voxel values

(Continued)

FIGURE 2 | increase from 1 to 15 with increasing convergence across 15 total main effects meta-analyses that each reflect pain-related activation. Reprinted from Neuroscience & Biobehavioral Reviews, Vol 112, A Xu, B Larsen, EB Baller, JC Scott, V Sharma, A Adebimpe, AI Basbaum, RH Dworkin, RR Edwards, CJ Woolf, SB Eickhoff, CR Eickhoff, TD Satterthwaite, Convergent neural representations of experimentally-induced acute pain in healthy volunteers: A large-scale fMRI meta-analysis, p300–23, Copyright (2020), with permission from Elsevier. https://doi.org/10.1016/j.neubiorev.2020.01.004. **(B)** Schematic of brain areas related to the processing of the multidimensional experience of pain. Each region is color coded to correspond to its hypothesized dimension of pain, while hatch-marks indicate processing associated with pain-related movement. Thick black borders indicate regions located more lateral to the midline. Relative size of each region is roughly proportional for structures larger than SII. **(C)** Attention to different features of a painful stimulus can shift activation patterns. Focusing on the unpleasantness of pain vs its location results in different patterns of brain activation when examined by PET, providing evidence that the unique dimensions of pain may be processed in separate brain areas. Reprinted from the European Journal of Neuroscience, Vol 21(11), B Kulkarni, DE Bentley, R Elliott, P Youell, A Watson, SW Derbyshire, RS Frackowiak, KJ Friston, AK Jones, Attention to pain localization and unpleasantness discriminates the functions of the medial and lateral pain systems, p3133-42, Copyright (2005), with permission from John Wiley and Sons. DOI: https://doi.org/10.1111/j.1460-9568.2005.04098.x. SI, primary somatosensory cortex; SII, secondary somatosensory cortex; MCC, midcingulate cortex; ACC, anterior cingulate cortex; Ins, Insular Cortex; Amyg, amygdala; PFC, prefrontal cortex; M1, primary motor cortex; SMA, supplementary motor area; pSMA, pre-supplementary motor area; BG, basal ganglia; Cereb, cerebellum; PAG, periaqueductal gray; PB, parabrachial nuclei; RVM, rostral ventromedial medulla; spV, spinal trigeminal nucleus; Thal, thalamus; OFC, orbitofrontal cortex; pACC, perigenual cingulate corex; IPC, inferior parietal cortex.

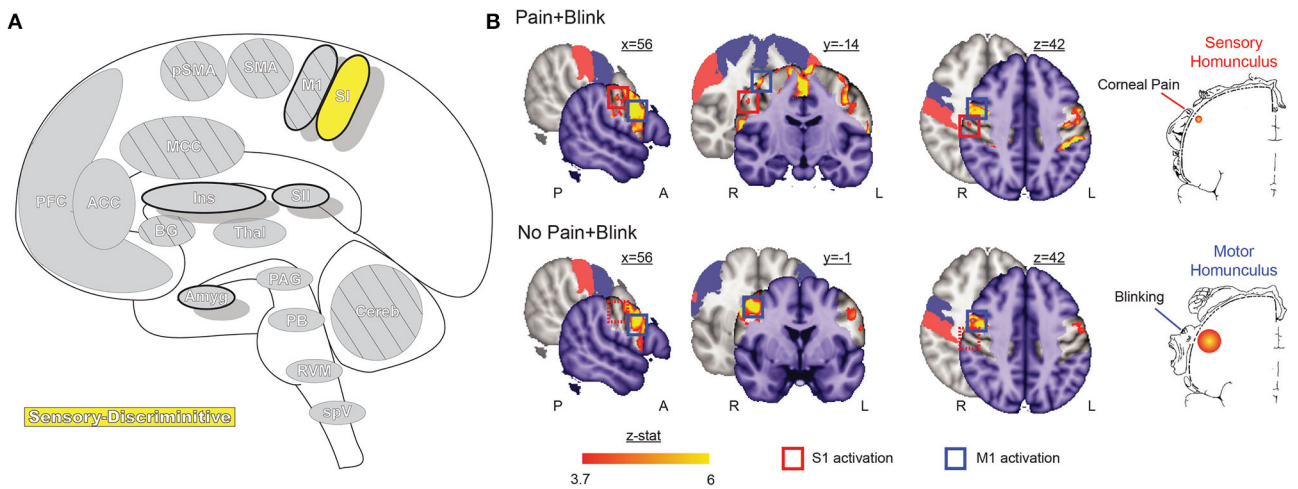

FIGURE 3 | Primary somatosensory cortex. **(A)** Brain areas active during pain: primary somatosensory cortex (SI) highlighted. **(B)** Functional imaging during exposure to bright light while in a photophobic state results in significant activation along the rostral face portion of the SI somatotopic map, contralateral to the site of corneal abrasion. SI activity is no longer present after symptoms resolve, while bilateral M1 and some bilateral SMA activation, associated with blinking, is seen in both conditions. Reprinted from PLOS ONE, Vol 7(9), EA Moulton, L Becerra, P Rosenthal, D Borsook, An approach to localizing corneal pain representation in human primary somatosensory cortex, e4463, Copyright (2012) Moulton et al., under the Creative Commons Attribution License (CC-BY). DOI: https://doi.org/10.1371/journal.pone.0044643SI, primary somatosensory cortex; M1, primary motor cortex; A, anterior; L, left; P, posterior; R, right.

The varied and often direct interconnections between these regions and SI are thought to support the role of SI in multisensory integration and actions (69–71). SI is divided into four subregions—Brodmann Areas 3a, 3b, 1, and 2—each suspected of containing a separate mirrored somatotopic map (72, 73). Areas 3a and 3b form one functional parcellation of SI, while areas 1 and 2 form the other; differences in connectivity to thalamic nuclei, other SI subregions, and multiple cortical areas, including motor and frontal cortex, suggest further divisions of function that remain to be explored (74).

Like the TBNC, the face representations in SI are somatotopically organized in an "onion-skin or dermatomal" model, wherein rostral areas are represented inferior and lateral to caudal areas (44, 68, 75). Although the representation of the eye has not been extensively mapped, an fMRI case study localized corneal pain within the rostral-most representation of the face (76).

SI nociceptive responses have been related to the sensory-discriminative aspect of pain, specifically the quality, location, and intensity of stimulus contralateral to the side in which cortical activation is observed, as described by many neuroimaging and lesion studies (64, 66, 77–79). These findings are consistent with direct electrophysiological recordings in primates (65, 67, 80–82).

Research into SI in multiple forms of pain, hyperalgesia, and allodynia describe not only functional changes but related structural changes, including somatotopic reorganization and altered gray matter (75, 83–89). These altered patterns of gray matter density and BOLD signal in the somatosensory cortex are often found during investigations of trigeminal neuropathy and chronic pain (90–93). The changes in neuron excitability, inhibition, or synaptic transmission in the primary somatosensory cortex can affect the perception of pain by its influences on other connected cortical and limbic areas

as well as subsequent altered interpretation of peripheral input (16, 94–97).

Recent investigations into the expression of pain on the face have found another relationship between SI and pain (98). The facial expression of pain can be measured for clinical and research purposes, and one method of quantification is through the Facial Action Coding System (FACS) in which non-verbal pain communications are described in Action Units (AUs). AUs are a small set of facial movements shown to consistently occur during pain that can include opening of the mouth and constriction of the muscles around the eyes, among others (99, 100). While AUs are highly variable between individuals in pain, the contraction of the orbicularis oculi muscle surrounding the eyes, a specific AU, is found consistently across subjects and in both acute and chronic pain (99). The sensory-discriminative component of pain is closely associated with orbicularis oculi contraction AU (101), while other AUs are linked to the affective component. The orbicularis oculi AU is mirrored by SI activations that correspond somatotopically to the site of painful stimulus and may serve a protective role by narrowing the eye aperture to shield the eye while preserving vision in dangerous and painful conditions (98). Pain affect-associated AUs are largely thought to be involved in communicating pain to others. Coordinated muscle contractions correspond to SI activity, and contribute toward SI responses observed with ocular pain (98).

Despite many investigations, the role of SI in pain is not fully understood, as activation is not consistently seen across many neuroimaging meta-analyses (7, 8, 10). Focal SI lesions in patients transiently decrease pain sensitivity (102, 103), and direct electrode stimulation of SI does not elicit pain (104). Increasingly, SI is viewed as an area for signal integration from multiple afferent sources, with the diverse classes of fiber inputs combining their transmissions and modifying them intracortically. The resulting signal may be greater or less than expected due to anatomical variability between subjects, non-noxious peripheral inputs, cognitive and attentional factors, and mixed excitatory and inhibitory processes (7, 10, 67, 105).

Secondary Somatosensory Cortex

The secondary somatosensory cortex (SII, **Figure 4**) receives nociceptive and innocuous somatosensory information from the thalamus simultaneously by separate but parallel neuronal connections to the pathway leading from thalamus to the SI (106–108). The region also has significant connectivity with the inferior parietal cortex and SI as well as other connections with the intraparietal sulcus, Broca's region, primary motor cortex, and pre-motor cortex (109). SII is more frequently activated than SI in response to noxious stimuli and is one of the most consistently activated brain regions to painful stimuli, along with the thalamus, medial cingulate cortex (MCC), and insula (7, 8, 110). Like SI, SII is involved in the processing of nociceptive afferent input in humans, and likewise has a role in the sensory-descriptive aspect of pain in the lateral pain system (7, 8, 104, 111–113). SII has reduced spatial resolution and receptive field size when compared with SI; unlike SI it has a role in processing other, "high-order" aspects of stimulus including attention, learning, memory, and rare or novel stimuli (113–115). SII is further activated when observing others in physical pain, and even in social-rejection related distress (116, 117). While SI activity is closely associated with the intensity of pain, SII activity is minimal for low-intensity thermal stimuli and increases quickly after exposure to high-intensity stimuli (118). However, note that both SI and SII also respond to pleasant brushing (119) and innocuous heat (105), indicating that activity in these regions is not specific to pain.

SII is frequently divided into four subregions that are loosely homologous to primate areas, termed OP1 (S2); OP2 (parietoinsular vestibular cortex); OP3 (ventral somatosensory area); and OP4 (parietal ventral area) (OP = operculum parietale) (109, 120, 121). Nomenclature of these areas can lack consistency and clarity; notably OP1 is often termed "S2" or "area SII," leading to some confusion in the literature between the subregion and the overall SII (109). Of these regions, OP1 and OP4 are the most widely studied and are considered somatosensory areas; both subregions contain a complete somatotopic map mirrored along the anatomical border separating them (122). OP1 is considered an integrative area that may facilitate higher-order complex somatosensory processing, while OP4 has greater associations with action control and sensory-motor integration (109).

Activation in SII is bilateral, and this activity increases as the stimulus intensity becomes more painful, which may include engagement of additional SII subregions (113, 118, 123–125). The bilateral activation of SII is non-symmetrical and shows greater activation contralaterally, compared to ipsilaterally (124, 126). This difference reflects the non-equal inputs to the ipsilateral and contralateral SII—contralateral SII is innervated by thalamic nuclei and SI, while ipsilateral SII receives input from contralateral SII and ipsilateral thalamic nuclei (124, 126–128). SII is implicated in identifying, discriminating between, and directing attention to stimuli, cognitively recognizing the painful nature of nociceptive activation, and integrating it with higher-level processes such as learning and memory (61, 109, 115, 118). Some evidence in experiments involving painful stimuli further suggest SII plays a role in processing pain-related emotion that may also include the detection and storage of emotion-laden information regarding potentially damaging stimuli (61). Abnormal pain processing as well as functional and anatomical changes are found in SII in a variety of painful conditions, and SII may be a target for future interventions (129–133).

Cingulate Cortex

The modern view of the cingulate cortex is a four-region model composed of the Anterior-, Mid-, and Posterior Cingulate Cortex (ACC, MCC, PCC) and the Retrosplenial Cortex (RSC), based on synaptic and functional differences in both primates and humans (134–136). Functional imaging studies in pain have helped affirm the existence of the MCC as a separate functional region rather than a transition area or subsection of the ACC or PCC. Taken together the cingulum as a whole participates in a broad array of somatosensory, emotional, and motor processes, however, fMRI recordings of painful stimuli find consistent activations in the MCC

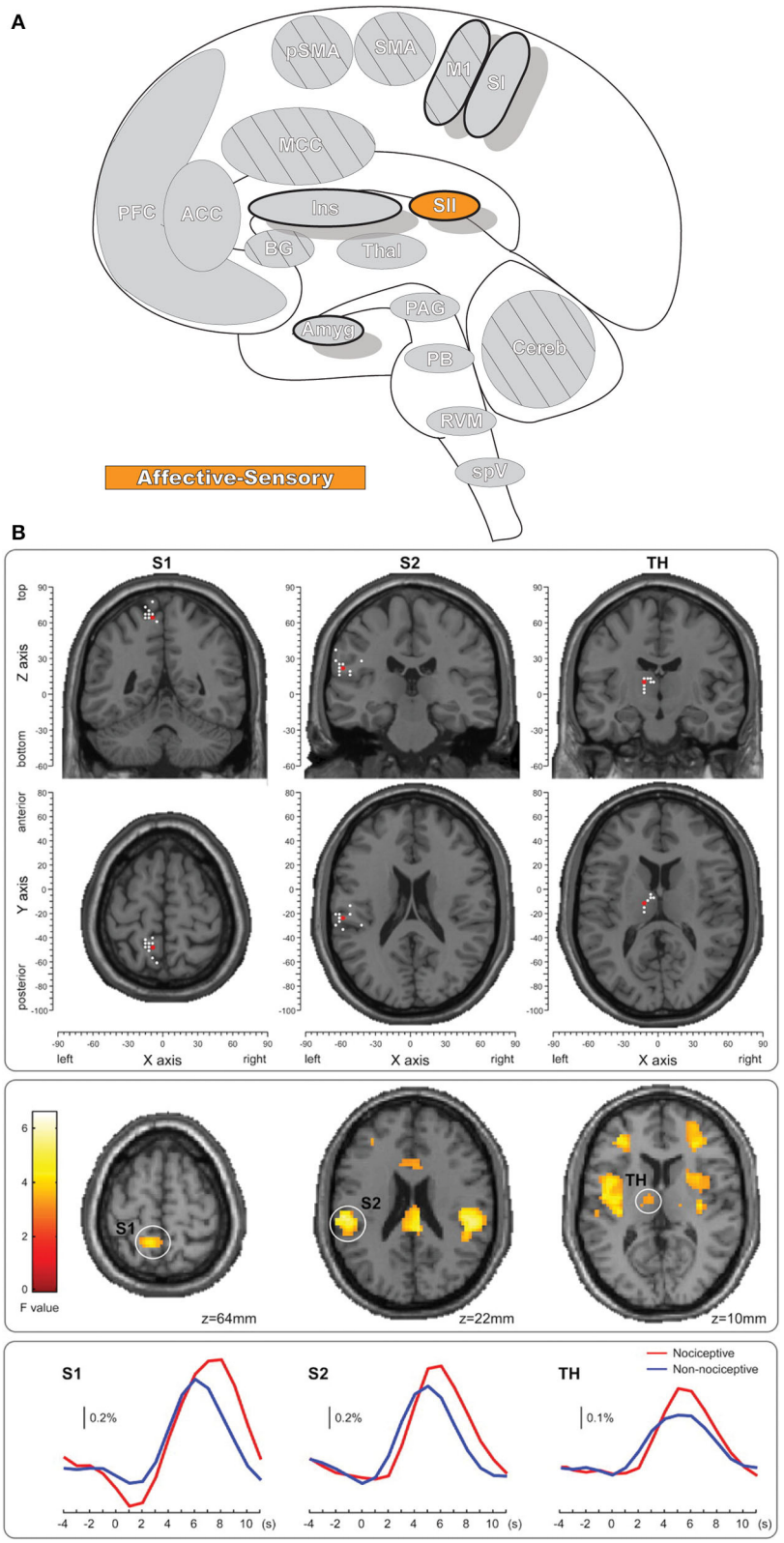

FIGURE 4 | Secondary somatosensory cortex. **(A)** Brain areas active during pain: secondary somatosensory cortex (SII) highlighted. **(B)** fMRI recordings during nociceptive and non-nociceptive stimulation in SI, SII, and Thalamus. The consistent time courses across all three regions suggest parallel information processing in the primary and secondary somatosensory cortices, along with associated activations in the thalamus. Reprinted from The Journal of Neuroscience, Vol 31(24),

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FIGURE 4 | M Liang, A Mouraux, GD Iannetti, Parallel processing of nociceptive and non-nociceptive somatosensory information in the human primary and secondary somatosensory cortices: evidence from dynamic causal modeling of functional magnetic resonance imaging data, p8976–85, Copyright (2011) Liang et al., under the Attribution-Non Commercial-Share Alike 3.0 Unported License (CC BY-NC-SA). DOI: https://doi.org/10.1523/JNEUROSCI.6207-10.2011white dots, activation maxima for each subject within a given region; red dots, activation maxima across the group within a given region.

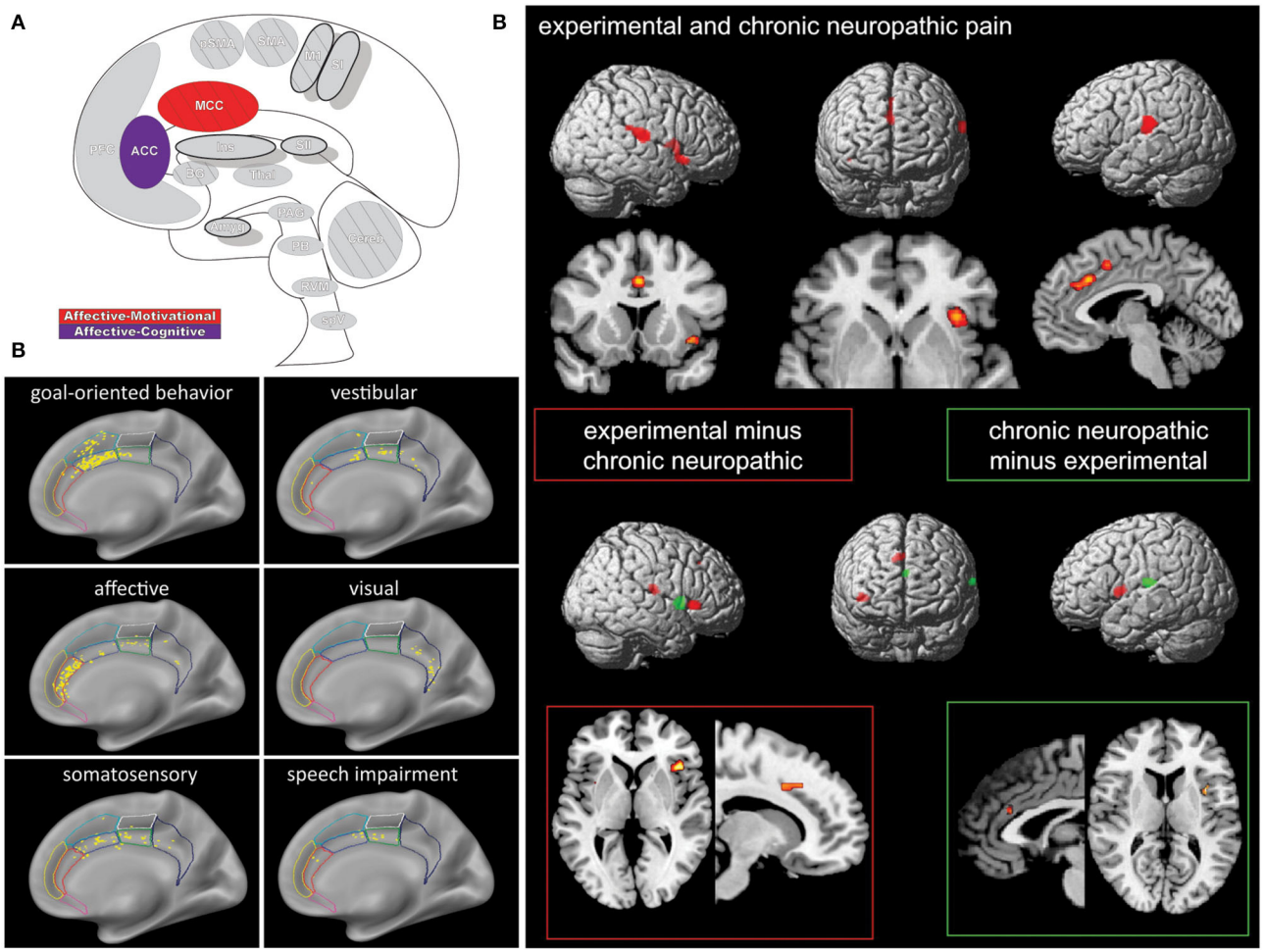

FIGURE 5 | Cingulate cortex. **(A)** Brain areas active during pain: midcingulate cortex (MCC) and anterior cingulate cortex (ACC) highlighted. **(B)** High frequency electrode stimulation across 1789 cingulate sites can elicit varying subjective and behavioral responses segregated into functional fields organized rostrocaudally along the cingulum. F Caruana, M Gerbella, P Avanzini, F Gozzo, V Pelliccia, R Mai, RO Abdollahi, F Cardinale, I Sartori, GL Russo, G Rizzolatti, Motor and emotional behaviours elicited by electrical stimulation of the human cingulate cortex, Brain, Copyright (2018), Vol 141(10), p3035–3051, by permission of Oxford University Press. DOI: https://doi.org/10.1093/brain/awy219. **(C)** Conjunction (top panel) and contrast (bottom panels) analyses of brain regions activated during chronic neuropathic and experimental pain reveal different patterns of activation, implicating several regions as potential actors in chronic pain- including the ACC. Conjunction analysis of both conditions showed activations in the ACC, MCC, SII, insula, thalamus, and supplementary motor area. Experimental - chronic neuropathic pain analysis (red box) resulted in activations in the MCC, anterior and posterior insula, and SMA. Chronic neuropathic - experimental pain (green box) revealed significant ACC, SII, and mid insular activations. Reprinted from NeuroImage, Vol 58(4), U Friebel, SB Eickhoff, M Lotze, Coordinate-based meta-analysis of experimentally induced and chronic persistent neuropathic pain, p1070–80, Copyright (2011), with permission from Elsevier. DOI: https://doi.org/10.1016/j.neuroimage.2011.07.022.

more so than other areas of the cingulum (7, 10, 136–138) (**Figure 5**).

Mid-cingulate Cortex

MCC receives projections from medial and intralaminar thalamic nuclei, including it in the medial pain system, but is also connected to other cingulate regions as well as the insula, amygdala, parietal cortex, striatum, spinal cord, motor, and pre-motor cortices, and many of these pathways are reciprocal (135, 139, 140). The MCC is further divided into anterior (aMCC) and posterior (pMCC) regions (136, 141) and partly contains two of the three cingulate motor zones (or premotor areas) (142). The anterior rostral cingulate zone (RCZa) and the posterior rostral cingulate zone (RCZp) are both somatotopically organized, containing face- and eye-related fields as well as limb motor representations

(142). These premotor areas are heavily connected to other brain motor centers, are involved in coordinated emotionally charged or context-dependent movements, such as rubbing or wincing, and are active in a variety of reward and innocuous nociceptive stimuli responses in addition to painful ones (66, 136, 139, 143, 144).

The RCZa is likely within the aMCC and displays strong functional connectivity with the prefrontal cortex, implicating the involvement of cognitive processes (145, 146). The aMCC receives relatively more medial thalamic nuclei innervation than the pMCC as well as a direct input from the amygdala and is active during fear (135, 139). Further functional and anatomical connections arise from the primary motor cortex and insula, and primate studies reveal other connections to the periaqueductal gray and spinothalamic system (145, 147). The same sites in the aMCC are activated by pain and itch and are also involved in dopaminergic reward systems (136, 139, 148). Further, activation is found in the aMCC in the expectation of pain and itch relief as well as pain empathy (136). Functional activity during pain, cognitive control, negative affect, and motor control all overlap in the aMCC, implicating it as being involved in sensorimotor integration that subsequently guides behavior (145, 147). In the context of pain, the aMCC can cognitively assess, experience, and anticipate pain and integrate negative affect into its output (136, 139, 147). The sensorimotor integration allows for a premotor signal that alters behavior and motor response selection based on context provided by numerous systems, with pain resulting in enhancement of specific avoidance and nocifensive motor actions (136, 139). The aMCC is also involved in monitoring the resulting action triggered by its pre-motor signal, sustaining it, and the reward coding of the selected behavior, participating in feedback-mediated decision making (136, 149, 150). Fear can produce many of the same movement activities as pain in the aMCC, and has a similar dynamic in autonomic areas of the ACC, which has led to the classification of fear as a premotor pain signal by some (148).

Conversely, activation in the RCZp in the pMCC is strongly associated with that in the motor cortex and more weakly with the prefrontal cortex (136, 146). The pMCC has more extensive input from the parietal lobe than the aMCC does but no connections to the amygdala and almost no activation in emotion studies (136, 139). Scratching an itch and orienting the eyes to focus on potentially noxious visual targets both show activation in pMCC, and more severe stimuli, or threat of stimuli, result in larger responses (66, 136). Multisensory information from the parietal connections is used by the pMCC to capture attention, guide quick and precise reflexive movements, and orient the body toward impending or realized external multisensory stimuli, including painful ones (136, 139, 140, 144).

Anterior Cingulate Cortex

The anterior cingulate cortex (ACC) stores emotionally-valenced memory, has a role in autonomic processes, and serves to integrate these two functionalities (139). The ACC receives medial thalamic innervation, although less so than the aMCC, the orbitofrontal cortex, amygdala, and parahippocampal gyrus (42, 139, 151). Through its OFC connections and downstream, descending pain modulatory sites, the ACC has also been implicated in pain inhibition and facilitation (151, 152). Pain relief from intervention in the ACC usually manifests as a reduction in the perceived unpleasantness or associated distress, highlighting its role in the medial pain system and affect (153). The ACC is often parcellated as two areas: the pregenual (pACC) and the subgenual (sACC) (148, 154, 155).

Activity in pACC is associated with happiness and related memories are stored there (135, 148). Activation in the region by positive memories and events represent reward values that are related to experienced pleasure and show robust functional connectivity to areas of the medial orbitofrontal cortex with similar positive associations and reward (148, 155). The pACC has projections to the facial region of the motor nucleus and is heavily involved in emotion and internal state expression through these projections and the anterior rostral cingulate zone (135, 139). Emotional awareness, common value scaling, and cost assessment are also functions carried out by the pACC, and the subregion can help make decisions involving reward/punishment tradeoffs (135, 139, 148). Opioid receptors are dense in the ACC, and the application of naloxone negates activity in the pACC during nociception, showing the key role of this area in antinociceptive processes (156).

sACC activity is maximal during negatively valenced stimuli and events, and the subregion stores memories associated with sadness (139, 148, 157). Fear also results in notable activation of the sACC (148). Like the pACC, the sACC receives OFC inputs but from the punishment-related and negatively associated lateral orbitofrontal cortex (148, 151). The sACC is strongly connected to the amygdala, lateral hypothalamus, PAG, and parabrachial nucleus (139, 148), and these outputs underscore the role this region plays as an integrative autonomic center (139, 148, 158). Enhanced sACC activity is found in numerous pain studies and is associated with reduced pain; diversion, placebo, habituation, pain adaptation, expectancy, and reward all seem to function through the activation of brainstem descending pain pathways initiated by the ACC (148, 151, 155).

ACC is activated in pain neuroimaging experiments far less frequently than the MCC, likely due to the fact that ACC activation is seen when the stimulus or pain is intense enough to engage descending pain control systems (152). Significant confusion surrounds cingulate pain neuroimaging, often due to the evolving subregional nomenclature (famously, dACC is not the same as aMCC), and the work of many meta-analyses has been devoted to reclassifying data to fit the new models (136, 148). Thus, over time, the ACC has "lost" some of its presumed functioning in pain as those nociceptive activations are correctly reassigned to other cingulate regions (148).

Insular Cortex

The insula (Ins: **Figure 6**) receives direct nociceptive input from thalamocortical pathways in primates and is a core region activated in essentially all painful experimental conditions, including a wide variety of exteroceptive and interoceptive

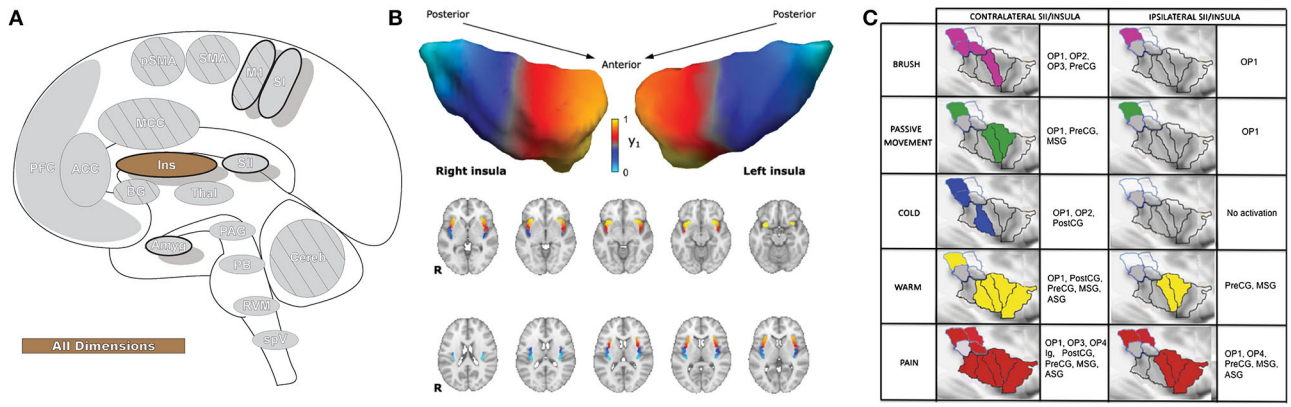

FIGURE 6 | Insular cortex. **(A)** Brain areas active during pain: insula (Ins) highlighted. **(B)** Topographic organization of connectivity (anatomical and FC) of the insula and other brain regions is arranged along a rostro-caudal gradient wherein anterior insular regions show strong connections to the anterior cingulate cortex, dorsolateral prefrontal cortex, and inferior parietal lobules (red) and the posterior insula with somatosensory regions and the parietal operculum (blue). Similarities in connectivity profiles in adjacent insular regions suggest that, rather than discrete subunits, the topographic distribution of connections is better appreciated as a spatially continuous and gradually changing gradient. Displayed as a gradient in graph form, this type of spatial connectivity analysis is referred to as a connectopy map. FC,functional connectivity- temporally synchronized low-frequency fluctuations in BOLD signal between regions that indicate they are connected in their functions. Such areas may or may not have direct anatomical connections. Reprinted from Nature: Scientific Reports, Vol 22(1), D Vereb, B Kincses, T Spisak, F Schlitt, N Szabo, P Farago, K Kocsis, B Bozsik, E Toth, A Kiraly, M Zunhammer, T Schmidt-Wilcke, U Bingel, ZT Kincses, Resting-state functional heterogeneity of the right insula contributes to pain sensitivity, p22945, Copyright (2021) Vereb et al., under the Creative Commons Attribution License (CC-BY). DOI: https://doi.org/10.1038/s41598-021-02474-x. **(C)** Operculo-insular areas (including insula and SII) respond to a wide variety of somatosensory, and painful, stimuli. Anatomically defined region of interest analyses with fMRI indicate varied functional overlap/segregation between a variety of stimuli delivered to the left hand. Reprinted from NeuroImage, Vol 60(1), L Mazzola, I Faillenot, FG Barral, F Mauguiere, R Peyron, Spatial segregation of somato-sensory and pain activations in the human operculo-insular cortex, p409–18., Copyright (2012), with permission from Elsevier. DOI: https://doi.org/10.1016/j.neuroimage.2011.12.072. PreCG,precentral insular gyrus; ASG,anterior short gyrus; MSG,middle short gyrus; posterior- PostCG,postcentral insular gyrus; Ig1,insular lobe granular area 1; Ig2,insular lobe granular layer 2; Id1,insular lobe dysgranular area 1; SII subunits: OP1, OP2, OP3, OP4 (OP, operculum parietale).

stimuli (10, 159). The insula is the only brain region that evokes pain when directly stimulated, including pain around the eye (104, 113, 160). The insula is divided into three subregions: the anterior, middle, and posterior; different regions of the insula play a role in sensory, affective, and cognitive aspects of perception (10, 161). Most resources refer to discrete insula subdivisions, and experiments are often designed around this fact. However, recent investigation has suggested the region may be better appreciated as a gradually changing topographical gradient of functional and anatomical connections along the rostrocaudal axis. While discrete subunits are described throughout this manuscript, selecting small/discrete subregions of interest for analysis may provide significant results that may not reflect the totality of activations or connectivity in a given brain region (162, 163).

Anterior insula (AI) is involved in processing emotion, including empathy, and activation of this subregion is found in affective processing (161, 164). AI has strong functional and anatomical connections with the thalamus and cognitive and emotional parts of the prefrontal cortex as well as the amygdala and some cingulate regions, particularly the ACC, and can have increased or decreased functional associations with these areas in chronic pain (163, 164). The strongest connections to the prefrontal cortex are to the dorsolateral prefrontal cortex, as well as areas associated with cognitive-evaluative processing and outcome anticipation (orbitofrontal cortex) and with the regulation of emotions and cognitive pain modulation (ventrolateral prefrontal cortex) (163, 164). Expectation and behavioral avoidance of a negative outcome activate AI, and the region is thought to impose emotional states that are informed by the evaluation of affective events (161, 164). The evaluation of the saliency of various insular inputs, attention, and the engagement of relevant brain regions and their triggered affective and emotional responses (including affective and cognitive pain modulation) are major functions of the AI (10, 161, 164–166). The AI has also been closely tied to autonomic function (167), and neural activity in this area has been correlated with the dynamic magnitude of pupillary dilation (168).

The mid insular (MI) area has connections to SI and SII (sensory-discriminative) as well as the ventrolateral prefrontal cortex (affective-emotional-cognitive), and diverse outputs to the orbitofrontal and premotor cortices, parietal and temporal brain regions, and the inferior frontal gyrus (164). Based on the diversity of input and output, the MI is viewed as a hybrid medial/lateral pain system area that integrates the diverse components of pain (164).

Posterior insular (PI) regions are thought to process interoceptive, somatosensory, visceral, and pain stimuli (7, 161, 164, 165). PI has its strongest connections to SII (structural and resting state analyses) and SI (structural) along with other somatosensory areas, and has some resting state association with the pMCC as well (163, 164). Activations in PI are found as stimuli progress from innocuous to painful in intensity with little activation in the absence of noxious input. Thalamic nuclei,

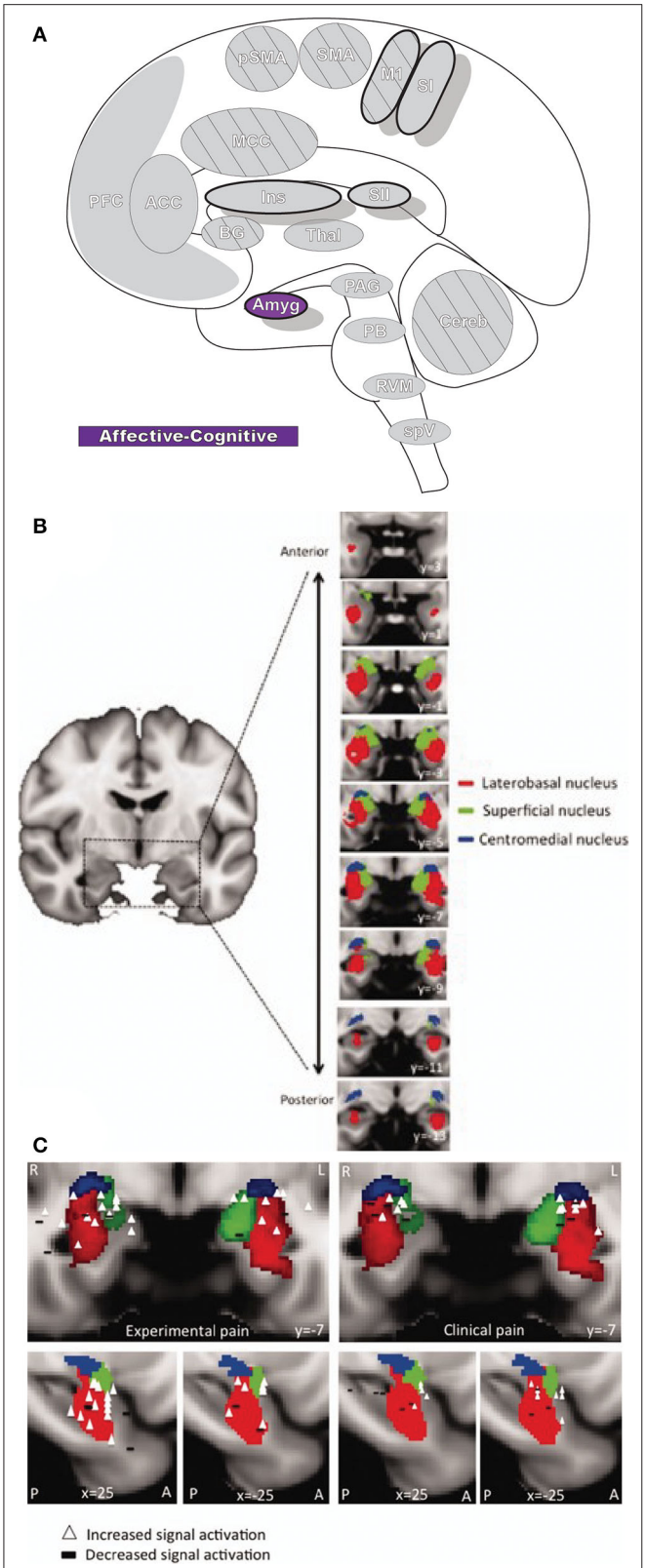

FIGURE 7 | Amygdala. **(A)** Brain areas active during pain: Amygdala (Amyg) highlighted. **(B)** MRI imaging of amygdala subunits displayed in a series of *(Continued)*

FIGURE 7 | coronal slices. Reprinted from Human Brain Mapping, Vol 35(2), LE Simons, EA Moulton, C Linnman, E Carpino, L Becerra, D Borsook. The human amygdala and pain: Evidence from neuroimaging, p527-38, Copyright (2012), with permission from John Wiley and Sons. DOI: https://doi.org/10.1002/hbm.22199. **(C)** A meta-analysis of functional neuroimaging studies reporting amygdala involvement, including 24 experimental and 17 clinical pain studies, emphasizes the participation of the amygdala in pain. The associations between laterobasal activation and clinical pain, as well as the centromedial/superficial regions and experimental pain, support previously reported anatomic and functional parcellations of the amygdala. White triangles,increased signal activation vs controls reported; black dashes,decreased signal activation vs controls reported. Reprinted from Human Brain Mapping, Vol 35(2), LE Simons, EA Moulton, C Linnman, E Carpino, L Becerra, D Borsook et al., The human amygdala and pain: Evidence from neuroimaging, p527–38, Copyright (2012), with permission from John Wiley and Sons. DOI: https://doi.org/10.1002/hbm.22199.

insula, SI, and SII together comprise the lateral pain system and sensory-discriminative pain; however, lateral thalamic nuclei have been shown to have low connection probability to PI, in contrast to many primate tracing studies.

While most other cortical areas are activated by distinct components of pain, insula appears to have sub-regions dedicated to processing and integrating a wide array of intero- and exteroceptive information as well as the focusing of cognitive and perceptive attention to the most salient of these inputs (161, 164–166, 169). Insular lesions can result in pain asymbolia, wherein patients recognize the presence of pain but are devoid of proper emotional and motor responses and may not react to visual or auditory threats (170). The inappropriate reaction to pain caused by insular damage highlights the importance of the region in serving to join the sensory and limbic systems and correctly process and integrate the affective-motivational component of pain with the other dimensions (170). While multiple studies have looked into the role of the insula in pain, as indeed it is the most consistently activated region in pain-related neuroimaging studies (7, 10, 171), the insula is also part of a prospective sensory salience network (172). As other regions in the brain have the capacity for multimodal sensory integration and can be active during painful stimulation, the question remains as to whether activations in insula truly reflect the various dimensions of pain or whether they process saliency and focus attention to particularly salient stimuli (173).

Amygdala

The amygdala (Amyg: **Figure 7**) is directly involved in emotional processing (174) and has a major role in aversive, fear-based learning and negative affect, as well as motivation and reward learning (175–178). The amygdala is a highly interconnected region of the brain, with dense afferent and efferent connections extending widely (178, 179). Nuclei of the amygdala are typically divided into superficial, laterobasal, and centromedial groupings based on cytoarchitecture and diffusion-tensor imaging studies (180–182). The superficial division is largely concerned with olfactory processes; however, some studies have found functional connectivity associations with other limbic regions that imply a potentially larger role in affect (178, 183). The laterobasal and centromedial groups are important for the transmission of

nociceptive signals and, through their diverse connections to other brain regions, are implicated in emotional and affective-motivational components of pain, as well as the cognitive-evaluative dimension (178).

The laterobasal division has extensive innervation from numerous modalities, including nociceptive input *via* the somatosensory thalamus and multiple cortical and subcortical areas, such as hippocampus, ACC, and insula (184, 185). Laterobasal amygdala is involved in associative learning, as with fear-based classical conditioning, thereby giving sensory information emotional significance, and as such is important in anxiety and fear related to pain (178, 186). Additionally, the laterobasal nuclei group has connections with parts of the striatum as well as prefrontal and frontal cortices, which contribute to pain memory and expectation, important parts of the cognitive-evaluative component of pain and the anticipation of pain (178, 187, 188).

The amygdala's centromedial nuclei are a major target of excitatory and inhibitory sensory input from other amygdala nuclei groups and they also receive nociceptive information from the medullary dorsal horn, cingulate cortex, and insula as well as the lateral parabrachial nucleus complex (47, 179). This information is projected to the nearby bed nucleus of the stria terminalis, hypothalamus, PAG, striatum, and several other brainstem regions (178, 179, 184). The connectivity between the amygdala and these areas underlies its significance in generating behavioral responses to painful stimuli as well as modulating the subsequent emotional, autonomic, behavioral, and endocrine pain responses (178, 185).

Prefrontal Cortex

Prefrontal cortex (PFC: **Figure 8**) is critical for cognitive control (the manipulation of information in pursuit of a goal) and can represent abstract information and complex rules that subsequently inform thoughts, emotions, and actions. It participates in high-order, intelligent planning and problem solving, emotion generation and regulation, and other executive functions (189–191). PFC is believed to be organized in a hierarchal rostro-caudal axis, in which posterior areas are involved in control of short-term and concrete action representations while complex, longer-term representations occur in progressively more anterior areas as information and control selection become increasingly abstract (146, 189, 192). Painful stimuli can engage many of these high-order processes, and multiple areas of PFC are involved in pain processing (190). In PFC, nociceptive signals are gathered with other contextual information (e.g., memories and emotions) into a unified, processed perception that then modulates peripheral nociception by its projections to the PAG (190).

The parcellation and nomenclature of PFC subregions is not consistent in the literature; cytoarchitectonic areas (e.g., Brodmann) can be assigned to different subregions depending on the study. Likewise, some subregion divisions can include functionally and anatomically distinct areas that may be referred to differently between studies and across disciplines (191, 193). A unified systematic nomenclature and parcellation may help ease the difficulties in investigating large-scale, integrative PFC functionality (191). The challenges and shortcomings in nomenclature are not unique to PFC—as neuroimaging techniques become more sophisticated it seems clear that establishing common representations and analysis methodologies throughout the brain can serve to advance the field (194–196). In this review, PFC is divided into orbitofrontal cortex (OFC), medial prefrontal cortex (MPFC), lateral prefrontal cortex (LPFC), and anterior prefrontal cortex (APFC), each with its own internal functional parcellations; the pACC, sACC, and aMCC are also often considered functionally to be part of the PFC (191).

LPFC activity is found frequently in neuroimaging of pain and cognitive control, and the subregion can be divided into dorsolateral and ventrolateral prefrontal cortex (DLPFC, VLPFC) (10, 189, 191, 197). DLPFC is also involved in executive function, ranging from attention, decision making, and emotional regulation to working memory and reward/value coding (197). DLPFC is a part of several brain networks, is widely involved in top-down process control and modulation, and has a similar role in the context of pain—cognitive/attentional modulation of pain, reducing emotional pain-responses, placebo analgesia, and other forms of pain suppression (197–199). Many of these phenomena engage circuits involving VLPFC and the ACC, to which DLPFC is interconnected, and are thought to be involved in the initiation of modulatory signaling to downstream effectors in the brainstem (6, 197). Pain detection and spatial discrimination are other implicated functions, as DLPFC activation has been observed in the response to, and anticipation of, painful nociceptive stimulation (190, 197). DLPFC is a site of integration between pain transmission, cognitive expectation, and evaluation of the resultant pain; the results of this processing lead to pain modulation and context-informed behavioral response to painful stimulation (197). Many investigations have found associations between DLPFC activation and enhanced pain in experimentally sensitized nociceptive circuits as well as abnormal anatomical and functional states in chronic pain (190, 197, 198).

VLPFC is innervated by AI, shows activation during pain anticipation, and is functionally associated with cognitive pain control systems (ACC, PAG, and RVM) (198, 200). VLPFC also shows increased activity in painful stimulation with activation often seen during placebo analgesia and other forms of signal manipulation, potentially contributing to pain modulation by reappraisal of the estimated threat an aversive stimulus represents (198, 201, 202). The delineation and initiation of functional process between the LPFCs by neuroimaging is complicated by close connections between the two regions (DLPFC and VLPFC), their dual involvement in expectation and emotion-regulation, and shared inverse association between pain-expectant cognitive processes and catastrophizing (201). Likewise, both DLPFC and VLPFC have been found to function abnormally in some cases of chronic pain (190, 198, 201).

MPFC, often divided into dorsomedial and ventromedial subregions, has a prominent role in aversive learning, processes the affective and cognitive components of pain, and functionally includes portions of the ACC and MCC (191, 203). Considered together, projections in primate tracings and supporting fMRI

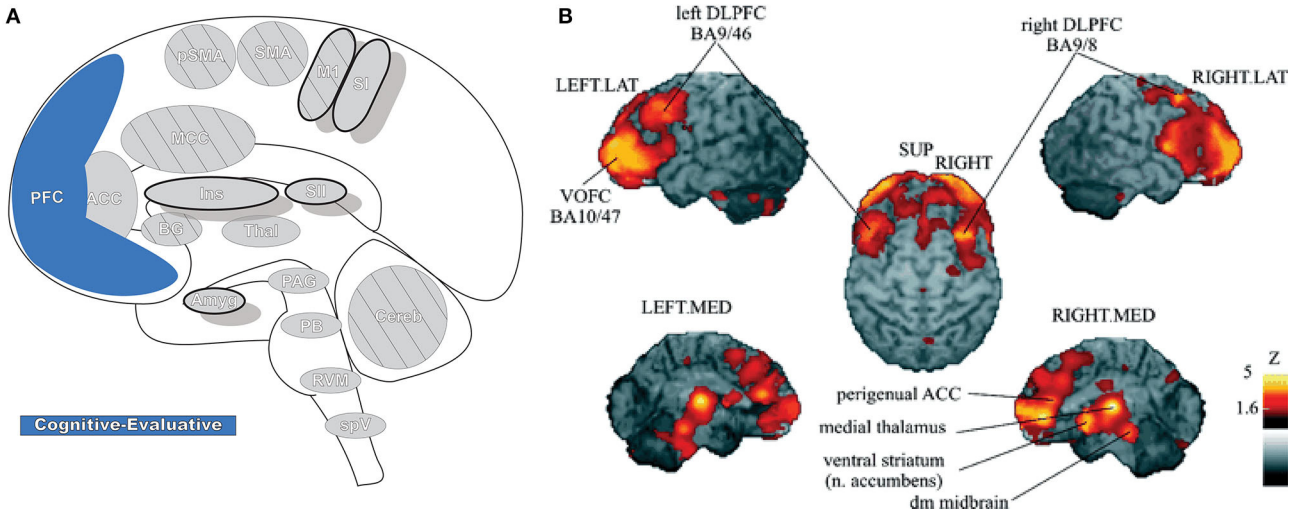

FIGURE 8 | Prefrontal cortex. **(A)** Brain areas active during pain: prefrontal cortex (PFC) highlighted. **(B)** Enhanced activation of ventral/orbitofrontal cortex (VOFC) and dorsolateral prefrontal cortex (DLPFC) during experimentally induced heat allodynia compared to equally intense heat pain stimuli. This difference demonstrates the nuanced response of the PFC in pain processing in different contexts. The basal ganglia were also found significantly more active in allodynia. Reprinted from J Lorenz, S Minoshima, KL Casey, Keeping pain out of mind: the role of the dorsolateral prefrontal cortex in pain modulation, Brain, Copyright (2003), Vol 126(5), p1079–91, by permission of Oxford University Press. DOI: https://doi.org/10.1093/brain/awg102. VOFC, ventral/orbitofrontal cortex; DLPFC, dorsolateral prefrontal cortex; LAT, lateral; MED, media; SUP, superior; dm, dorsomedial.

functional studies have found connections from MPFC to the PAG, comprising a large portion of the overall input to the critical pain modulatory region (190). Additional tracts are seen between the MPFC, amygdala, thalamus, hypothalamus, and rostral ventromedial medulla, providing a potential way that emotion (i.e., fear) can influence pain modulation as well as function in empathy toward pain or suffering (190). Cognitive inhibition of emotional and pain responses, including motor and facial expression such as orbicularis oculi contraction, is thought to be learned and can occur through coordinated activations in the MPFC, basal ganglia, and cingulate regions that can be disrupted as the perception or suggested intensity of sensory-discriminative pain increases (98, 191). MPFC connections to parietal areas underlie the processing of emotionally-valenced visual stimuli that affect associated nociceptive signaling, a form of cognitive pain modulation (190).

OFC, containing medial and lateral subdivisions, has connections to many pain-processing areas, including the insula, ACC, and somatosensory cortex, and shows increased activation during exposure to uncontrollable and unpredictable pain and its accompanying sensitization as well as to the fear of pain (191, 202, 204). OFC processes negative and punishment-related aspects of stimuli as well as the context-dependent value of a reward (204, 205). In the presence of both pain and reward, functional coupling between OFC and other cerebral pain centers is disrupted, resulting in higher-order signal modulation and pain inhibition (205). Like other parts of PFC, many studies have revealed alterations of this area in chronic pain, although OFC changes may not only reflect the modulation of nociceptive signaling but instead interactions between pain and reward (190, 205).

APFC, like other prefrontal regions, has been related to many functions including reward and conflict, working memory, risk and decision making, and pain (206, 207). This area has also been found to be involved in essentially all salient stimuli that may require behavioral response (206). APFC has reciprocal connections to the other PFC subregions, the parietal, insular, and anterior temporal cortices, multiple thalamic nuclei, and numerous other subcortical regions in tracer studies in primates, strongly supported by structural and functional associations in human neuroimaging (206, 207). APFC has a medial and lateral division (mAPFC and lAPFC), which have functionally distinct processes (206, 207). lAPFC in pain is thought to be involved in high-level cognitive sensory and emotional nociceptive signal integration and may modulate pain through its input to the descending antinociceptive circuit (206). On the other hand, mAPFC may have a role in memory-based aversive processing, both past and ongoing, and general stress response while its connections to the medial pain system highlight a potential role in emotional and motivational components of pain (206). Together, APFC regions perform high-level cognitive evaluation of pain, historical and present, and the results of that processing are used to guide behavior (206).

Primary Motor Area, Supplementary Motor Area, and Pre-supplementary Motor Area

Primary motor cortex (M1), supplementary motor area (SMA), and pre-supplementary motor area (Pre-SMA) are critical areas for the planning and execution of motor output in response to sensory input, and their activation is found in many pain studies (7, 8, 10) (**Figure 9**). The motor areas have dense

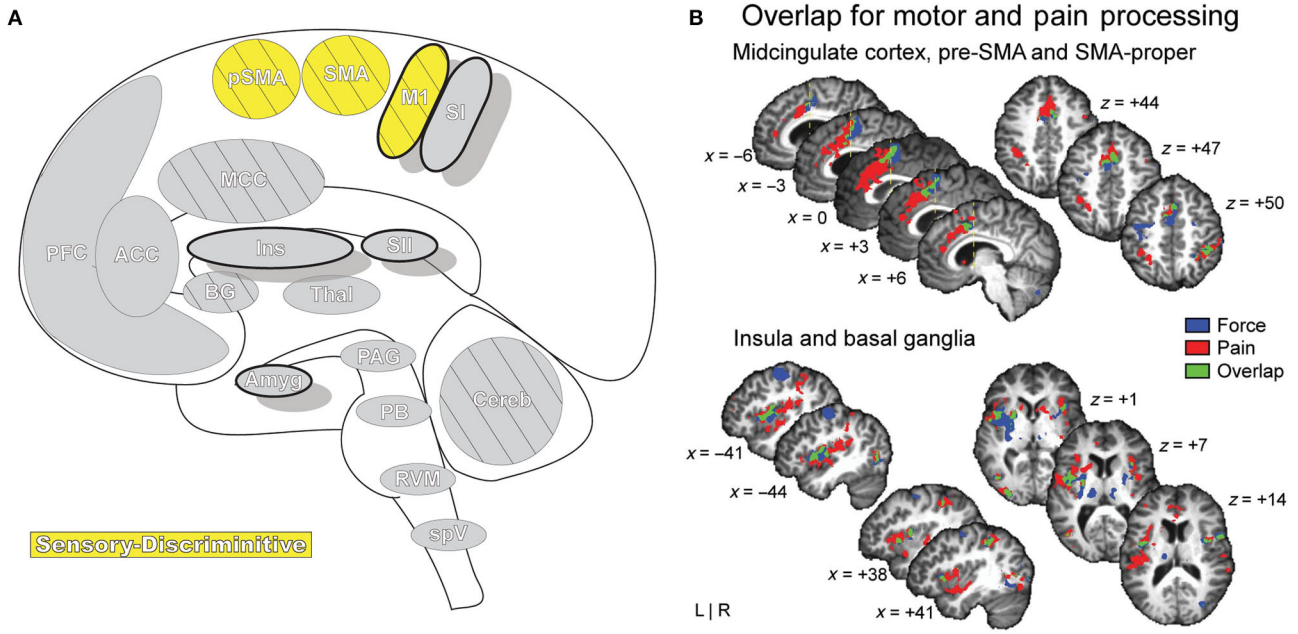

FIGURE 9 | Motor Areas. **(A)** Brain areas active during pain: primary motor area (M1), supplementary motor area (SMA/SMA-proper), pre-supplementary motor area (pSMA/pre-SMA) highlighted. **(B)** Functional activations associated with pain processing (painful heat: red) and motor control (force production: blue) overlap (green) in the SMA, pSMA, and aMCC, and display increased activation when simultaneously processing both conditions. Further results of the same group-level conjunction analysis describe overlap in pain and motor processes in the anterior insula and basal ganglia (putamen), reinforcing a dynamic established previously in the literature. Pain processes were established by painful thermal stimulation to the right hand; motor control processes were established by participants gripping a force transducer with their right hand. Reprinted from G Misra, SA Coombes, Neuroimaging Evidence of Motor Control and Pain Processing in the Human Midcingulate Cortex, Cerebral Cortex, Copyright (2014), Vol 25(7), p1906–19, by permission of Oxford University Press. DOI: https://doi.org/10.1093/cercor/bhu001.

innervation between themselves and are heavily connected to the corticospinal tract, parietal cortex, cerebellum, and thalamus (208). The collection of motor and motor planning areas are implicated in behavioral and some reflex responses to pain as well as the anticipation of pain (98, 145, 209).

Pain impacts muscle contraction and coordination and interferes with motor-skill learning (210). Many pain-related motor area activations are thought to be involved in pain-initiated movement or the suppression of pain reflexes and are involved in affective/motivational pain responses (8, 42). The pre-SMA and SMA show activity in similar regions in both pain processing and motor function as well as during the execution of visually guided movement (145). Additionally, emotion can influence motor system responses, and the system may itself code and store emotional context along with motor-processes; activation of the motor system can be seen before and during interaction with an unpleasant stimulus (209, 211). Pain and/or the expectation of pain may alter excitability in motor areas, causing inhibition of certain motor actions, such as further interaction with a negative stimulus; conversely, positively associated stimuli may facilitate motor activities leading to increased interactions (211, 212).

Motor cortex intra- and transcranial stimulation has repeatedly been shown to relieve pain in certain neuropathic conditions, but the mechanism behind this phenomenon is not fully understood (213, 214). Multiple hypotheses exist and may not be exclusionary, involving modulation and regulation of signals to PFC, cingulate cortices, thalamus, brainstem, basal nuclei, and spinal cord (214, 215). The altered excitation of nerve fibers by activation of opioid-releasing structures throughout the brain, active reappraisal of the emotional component of pain, and potential regulation of peripheral feedback imbalances may all contribute to pain relief (214–216).

Basal Ganglia

The basal ganglia (BG, **Figure 10**) are a group of subcortical forebrain nuclei that are highly connected to the cortex, brainstem, and thalamus and are best known for dopaminergic involvement in motor systems and movement control (217–219). BG are involved with planning learned motor behavior execution, directing voluntary movement, and coordinating context-dependent movement (220). BG nuclei include globus pallidus, substantia nigra, striatum, and subthalamic nucleus (219, 221). Striatum is separated into ventral and dorsal subdivisions—the ventral is closely associated with the limbic system and is partially comprised of the nucleus accumbens, while the dorsal striatum consists of caudate nucleus and putamen (219, 221). The striatum is the main input area of the BG and is innervated almost globally by the cortex; these diverse inputs are organized into sensorimotor, cognitive, and affective functional regions with overlap that may reflect integration of

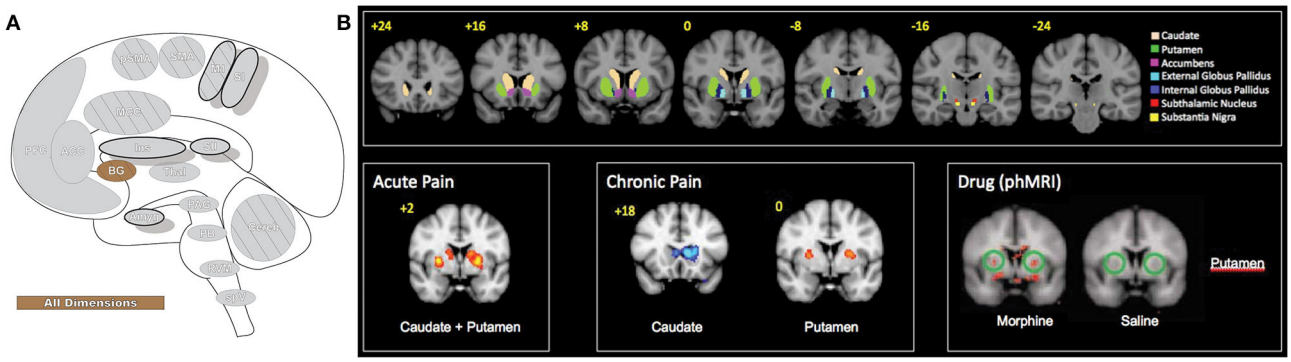

FIGURE 10 | Basal ganglia. **(A)** Brain areas active during pain: basal ganglia (BG) highlighted. **(B)** The basal ganglia participate in pain processing, from acute pain and chronic pain (cold and brush neuropathic allodynia) to morphine-induced analgesia, as revealed by pain-related patterns of fMRI BOLD activity. Upper panels: BG parcellations are color-coded and highlighted in coronal sections organized from anterior to posterior. Bottom panels: red areas indicate increased fMRI BOLD activation and blue areas indicate decreased activation. phMRI, pharmacological MRI- wherein pharmacological agents/drugs (in this case morphine) are used as stimuli to induce hemodynamic changes that are subsequently assessed by fMRI. Reprinted from Molecular Pain, Vol 6(27), D Borsook, J Upadhyay, EH Chudler, L Becerra, A key role of the basal ganglia in pain and analgesia–insights gained through human functional imaging, Copyright (2010) Borsook et al, under the Creative Commons Attribution License (CC-BY). DOI: https://doi.org/10.1186/1744-8069-6-27.

cortical information (217, 219). Globus pallidus contains major output nuclei that connect widely to other BG nuclei as well as several thalamic nuclei and midbrain structures (219). One such structure is the superior colliculus, in which BG has a non-looped connection that supports a role in regulating eye movements and behaviors resulting in orientation toward a stimulus (219).

BGs role has expanded beyond movement alone to include cognitive and emotional activity, skill and habit learning, perception, procedural memory, planning, language, and attention (218, 219, 221, 222). This diverse functionality extends to pain processing, where BG are suggested to participate in the affective-motivational, sensory-discriminative and cognitive-evaluative components of pain as well as some analgesic effects and are critical participants in the behavioral resultants of chronic pain (221). Of the BG, the regions most consistently activated in experimental pain the caudate, pallidus, and putamen subregions (10). Caudate is believed to involve pain avoidance behavior and behavioral reinforcement that may include pain (221). Pallidus encodes repertoires of behavior, and deep brain stimulation of this area causes pain inhibition (221). Putamen activates bilaterally while also contralaterally representing somatotopic nociceptive information and potentially playing a role in pain modulation (220, 221). BG have an important role in managing context-dependent movement and, through extensive thalamo-cortical-BG loops, modulate the integration of the diverse components of the pain experience and influence resultant movement behaviors (221).

Cerebellum

The cerebellum (Cereb: **Figure 11**) is best known for coordinating movement (223). This basic understanding has evolved, as interrogation of the cerebellum has revealed integrative and diverse functionality, from memory and learning to the processing of somatosensory input. The cerebellum has many supraspinal projections that are routed through the brainstem and has reciprocal connections to cortical structures involved in both sensorimotor processing and cognitive functions (224, 225). In addition to supraspinal input, direct afferent pathways pass nociceptive information from peripheral sources through midbrain nuclei to the cerebellum (225, 226). Some evidence suggests a somatotopic organization for these peripheral afferent inputs, while cerebellar regions receiving supraspinal input may be non-somatotopically organized and provide a means for emotional and cognitive information to affect cerebellar sensory-motor processes (227). The cerebellum can be divided into separate functional regions based on anatomic, neuroimaging, and resting state studies. These studies describe anterior cerebellar connections to sensorimotor-related cortical regions, which support motor functions and posterior cerebellar circuits to cognitive and associative cortical regions, which in turn may function in motor planning, nociception or memory (225, 226).

While not observed in all pain neuroimaging studies (10), the cerebellum is often active during pain (7, 8, 226). The cerebellar response to pain is most frequently seen in activation of the anterior vermis and posterior hemispheres, and similar activity in the posterior hemispheres is observed in the neuroimaging of emotion and evocative pictures, particularly those with aversive connotation (226, 228). These same regions may be activated in response to the anticipation of a painful stimulus as well as the stimulus itself (227). Activation of posterior cerebellar regions has been functionally inversely correlated to limbic areas involved in emotional processing (228), and damage to these regions has been linked to disrupted pain affect (229). A positive functional relationship is found between pain and sensorimotor areas, such as M1, SII, AI, and the PAG. The cerebellar pain response may be related to motor planning and reflexes as well as to the activation of a corticocerebellar aversive network that modulates sensitivity to negative events by connectivity to cognitive and emotional brain regions (225, 228).

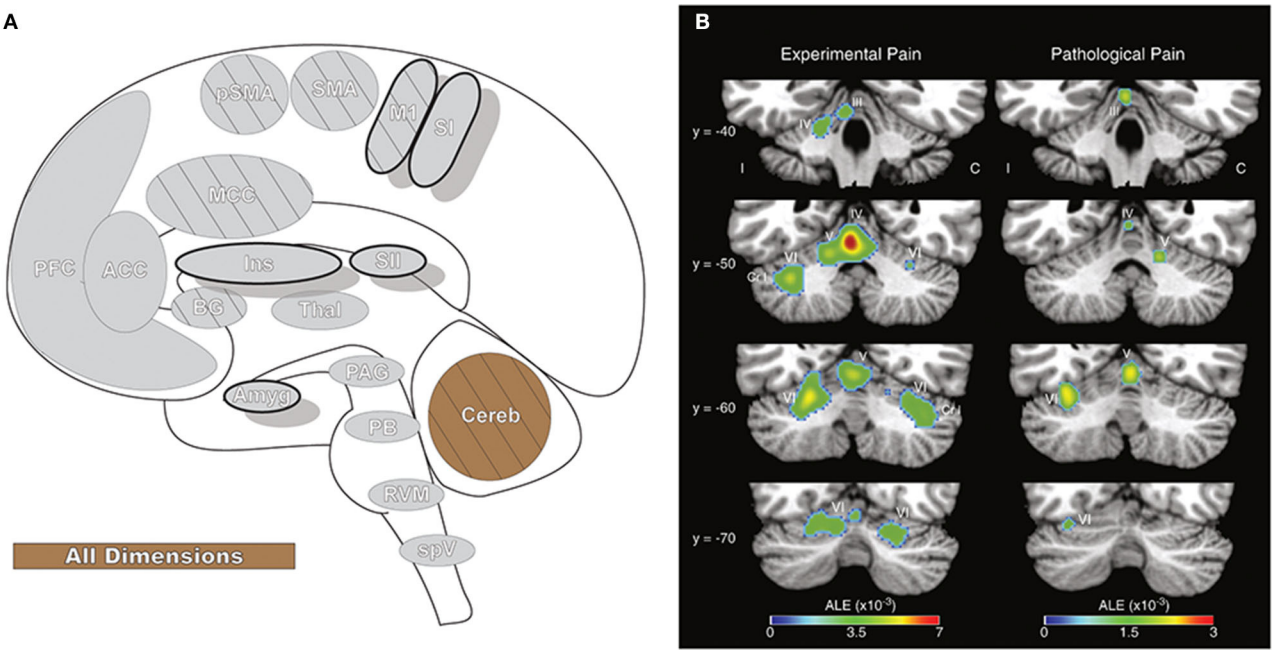

FIGURE 11 | Cerebellum. **(A)** Brain areas active during pain: cerebellum (Cereb) highlighted. **(B)** Cerebellar activation likelihood estimation (ALE), derived from meta-analysis of 56 experimental and 20 pathological pain studies, illustrates that fMRI activity is frequently present in specific cerebellar foci during pain. Reprinted from Brain Research Reviews, Vol 65(1), EA Moulton, JD Schmahmann, L Becerra, D Borsook, The cerebellum and pain: Passive integrator or active participator?, p14–27, Copyright (2010), with permission from Elsevier. DOI: https://doi.org/10.1016/j.brainresrev.2010.05.005. C, activation contralateral to painful stimuli; I, activation ipsilateral to painful stimuli; Cr I, Crus I; III-VI, cerebellar hemispheric lobules III through VI.

Brainstem

The brainstem (**Figure 12**) is a critical integrative relay between ascending inputs from primary afferents as they proceed to supraspinal areas and descending modulatory influences from supraspinal areas themselves (49). The ascending sensory system traverses the medulla, pons, and midbrain enroute to the cerebral cortex, and is modified in transit at the primary afferent synapse (i.e., spVc) as well as other brainstem regions (49, 230). Descending modulation of ascending sensory transmission is triggered by cortical and subcortical messaging to brainstem structures that can enhance or suppress the afferent signal depending on context (49, 231). That context comes in the form of situational input from amygdala, cerebellum, PFC, ACC, hypothalamus, and thalamus, which all exert "top down" pro- or antinociceptive, analgesic-mediated influence on brainstem nuclei; these circuits are referred to as the descending pain modulatory system (49, 230, 231). Bidirectional signal control is important for context-dependent pain modulation—depression of pain and antinociceptive signaling may be necessary to enact escape despite painful injury, while pronociceptive modulation promotes vigilance and protection of damaged tissue (231).

Nuclei associated with pain processing and descending modulation are situated throughout the brainstem. Within the midbrain lies the periaqueductal gray (PAG) and nucleus cuneiformis (NCF); within the pons is the parabrachial nuclei, locus coeruleus (LC), and dorsal and medial raphe nuclei; and within the medulla is the rostral ventromedial medulla (RVM), ventrolateral medulla, subnucleus reticularis dorsalis (SRD), and spV (49, 230).

PAG is a critical site in which ascending sensory and descending modulatory pathways interact and is part of the endogenous pain inhibitory system (232). In addition to pain processing and control, PAG participates in the expression of anxiety, analgesia, fear, cardiovascular function, vocalization, and reproductive behaviors (232). Diffusion tensor imaging tractography has shown PAG connections to PFC, ACC, cerebellum, hypothalamus, thalamus, nucleus accumbens, and amygdala as well as the pre- and postcentral gyri and lower brainstem nuclei, such as RVM and medullary dorsal horn (49, 232). PAG consists of four longitudinal, columnar subnuclei parallel to and surrounding the mesencephalic aqueduct: the dorsolateral (dlPAG), dorsomedial (dmPAG), lateral (lPAG), and ventrolateral (vlPAG) subdivisions (232). The subdivisions seem functionally segregated, with stimulation of lPAG and dlPAG eliciting elevated blood pressure along with active emotional coping strategies and behavioral responses (fight or flight), while vlPAG stimulation results in decreased blood pressure and passive responses (quiescence) (49, 232). Functional resting state connectivity investigations have shown additional associations and corroborated many connections identified with primate and human tract tracing, including ACC, AI, cerebellum, dorsal putamen, hippocampus, globus pallidus, and ventromedial medulla; these investigations have also revealed negative connectivity between LPFC,

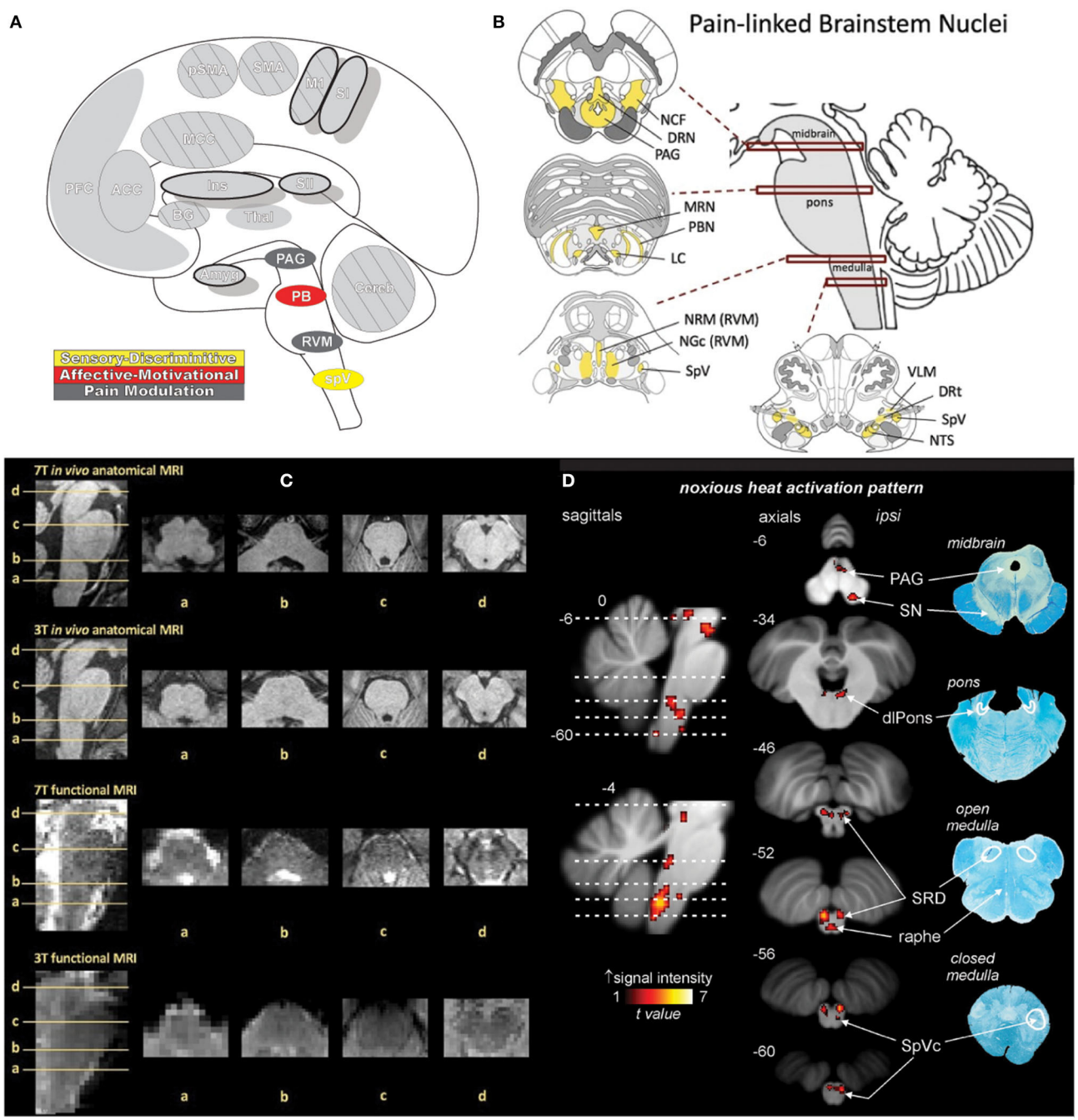

FIGURE 12 | Brainstem. **(A)** Brain areas active during pain: periaqueductal gray (PAG), parabrachial nuclei (PB), rostral ventromedial medulla (RVM), spinal trigeminal nucleus (SpV) highlighted. **(B)** Schematic of brainstem nuclei associated with pain processing. Reprinted from PAIN Reports, Vol 4(4), V Napadow, R Sclocco, LA Henderson, Brainstem neuroimaging of nociception and pain circuitries, p e745, Copyright (2019) Napadow et al., under the Creative Commons Attribution License (CC-BY). DOI: https://dx.doi.org/10.1097%2FPR9.0000000000000745. **(C)** Axial slices containing brainstem nuclei from Figure. 12(B) arranged to compare the spatial resolution and quality of anatomical and functional MRI data at different magnetic field strengths (7 Tesla and 3 Tesla). Advances in imaging techniques and technologies promise to advance neuroimaging investigation of the brainstem as subtle differences in increasingly fine and detailed structures can be appreciated by MRI. Reprinted from PAIN Reports, Vol 4(4), V Napadow, R Sclocco, LA Henderson, Brainstem neuroimaging of nociception and pain circuitries, p e745, Copyright (2019) Napadow et al., under the Creative Commons Attribution License (CC-BY). DOI: https://dx.doi.org/10.1097%2FPR9.0000000000000745. **(D)** fMRI activations in the medulla, pons, and midbrain in response to brief noxious thermal stimulation, comprising activation of ascending nociceptive pathways and descending pain modulation, highlighting the dense and complex pain circuitry present in the brainstem. Myelin-stained ex-vivo axial sections are displayed to the right of corresponding sagittal and axial MRI slices. Reprinted from NeuroImage, Vol 124(Part A), AM Youssef, VG Macefield, LA Henderson, Pain inhibits pain; human brainstem mechanisms, p54-62, Copyright (2020), with permission from Elsevier. DOI: https://doi.org/10.1016/j.neuroimage.2015.08.060. DRN, dorsal raphe nucleus; DRt, dorsal reticular nucleus; LC, locus coeruleus; MRN, median raphe nucleus; NCF, nucleus cuneiformis; NGc, nucleus gigantocellularis; NRM, nucleus raphe magnus; NTS, nucleus tractus solitarii; PAG, periaqueductal gray; PBN, parabrachial nucleus; RVM, rostral ventromedial medulla; SpV, spinal trigeminal nucleus; VLM, ventrolateral medulla; SpVc, spinal trigeminal nucleus caudalis; SRD, subnucleus reticularis dorsalis; dlPons, dorsolateral pons; PAG, periaqueductal gray; SN, substantia nigra.

PI, and post-central and occipital gyri (232). In addition to functional and anatomical differences, animal studies have found fundamentally different methods of analgesia in the subregions— vlPAG is opioid-mediated while lPAG (which receives somatotopically arranged spV nociceptive projections) and dlPAG are non-opioid mediated (230, 232). Supporting these findings in animals, electrical stimulation of PAG results in analgesia that is abolished when naloxone is administered (233).

PAG has diverse brain innervation, participates in afferent sensory transmission, and, after integrating its numerous inputs, is a principal effector of the descending pain modulation system by means of its projections to the RVM and other brainstem nuclei (49, 230). RVM carries out bidirectional pro- and anti-analgesic modulation through projections to other brainstem areas, dependent on signaling from dense connections with the PAG (49, 230). RVM receives projections from parts of LC, PBN, and thalamus in addition to those from PAG, and is the lowest "common relay" of descending pain modulation pathways, subsequently sending outputs to Vc, Vi/VC, and SpV (234).

RVM can inhibit incoming noxious signals through the activation of OFF class neurons or facilitate nociception *via* ON class neurons and, at rest, the counteracting neuronal activities are thought to be balanced (230). Most information on specific cell function comes from animal studies, as the intermingled anatomy of RVM cell populations cannot be differentiated by neuroimaging (49). However, neuroimaging and resting state studies have shown increased functional coupling between vlPAG and RVM as well as RVM and multiple subnuclei of spV in patients with trigeminal neuropathy (230, 235). Animal studies of the ventrolateral medulla and NCF have reported ON/OFF cell populations, similar to those of RVM, with presumably similar functions in afferent regulation and, like the PAG, the NCF participates in ascending signal transmission and has projections to RVM (49).

Another PAG coordinated area involved in nociceptive modulation is LC, which has reciprocal connections to both vlPAG and spV in primate tracings (49, 230). LC regulates attention and mood through noradrenergic inputs to the brain while also playing a role in pain processing, such as in cognitive-mediated distraction analgesia (49). Resting state examination in painful trigeminal neuropathy has found increased resting state connectivity strength between LC and RVM, suggesting altered noradrenergic and opioid system interactions in neuropathic pain (230). Further, LC connections with the nucleus accumbens and ACC are implicated in reward signaling from pain relief, and functional connectivity between these areas is disrupted in chronic pain. The same investigation additionally found decreased connectivity between LC and vlPAG and increased LC connection strength with SRD; these results support animal models in which LC is thought to inhibit signaling in spV through direct projections and facilitate signals indirectly through connections to SRD. SRD is also involved in analgesia produced by inhibition of one stimulus by a second (conditioned pain modulation) and achieves pain inhibition by suppressing nociceptive input in spV (48, 49).

Pain-related activation in the brainstem is less commonly found in neuroimaging studies than in many brain areas, despite the documented activity in pain and nociception throughout (7, 10). The small and complex anatomy of brainstem nuclei and susceptibility to physiological noise and other distortions may mask activation along with many other technical limitations, while in other cases the intensity of experimental stimuli may not be great enough to engage the descending pain systems (49, 236).

Thalamus

The thalamus (Thal: **Figure 13**) receives and passes along information from peripheral sources to the cortex, and every region of the cortex has reciprocal connections back to thalamus (237). Thalamus receives direct sensory input from numerous sources, including the trigeminothalamic tract and its connections to the ventroposteromedial (VPM) nucleus and medial nuclei, and is involved in multiple dimensions of the pain experience (238–240). Historically the thalamus was viewed as a relay site based on this widespread connectivity, but evidence continues to mount that it has a role in aggregating, processing, and integrating information from functional brain networks as well as mediating cortico-cortical connections (237, 241–243). In a view of the brain as a complex network of semi-independent modules, thalamus plays a key role in multimodal information processing by serving as an information-sharing nexus for cortical functional networks as well as structurally maintaining the modular organization of the brain network as a whole (237, 243–245).

Thalamus is composed of first-order nuclei and higher-order nuclei, which are discriminated based on the composition of their innervation—primarily ascending afferents and subcortical areas (first-order) or primarily cortical connections (higher-order) (237, 241, 242). Higher-order thalamic nuclei, through their extensive and reciprocal cortico-thalamo-cortical connections, allow for indirect interactions between areas of the cortex; first-order nuclei function more as a relay of modality-specific information to appropriate brain regions, but a role in information exchange between functionally disparate brain networks has been suggested as well (237).

Thalamus is widely activated in experimental pain and shows altered functionality in many forms of pain (7, 10, 246). Thalamic activation in response to nociceptive pain is often bilateral, another indication that the role of the thalamus goes beyond purely sensory signal transmission (7, 10). Attentional processes also increase bilateral thalamic activity, suggesting that the thalamus is involved in both discriminative and attentional networks (7).

Structural differences in thalamic gray and white matter are readily observable in numerous chronic pain studies, with evidence showing these changes may either be pre-existing or develop after exposure to pain over time (63, 247). Abnormal thalamic activation is a common finding in pain studies as well, as is aberrant spontaneous activity and accompanying burst discharge (246, 248, 249). Changes in thalamic perfusion are highly correlated with pain states, especially hypoperfusion, and deafferentation is one proposed explanation for reduced thalamic blood flow (250). However, deafferentation does not explain

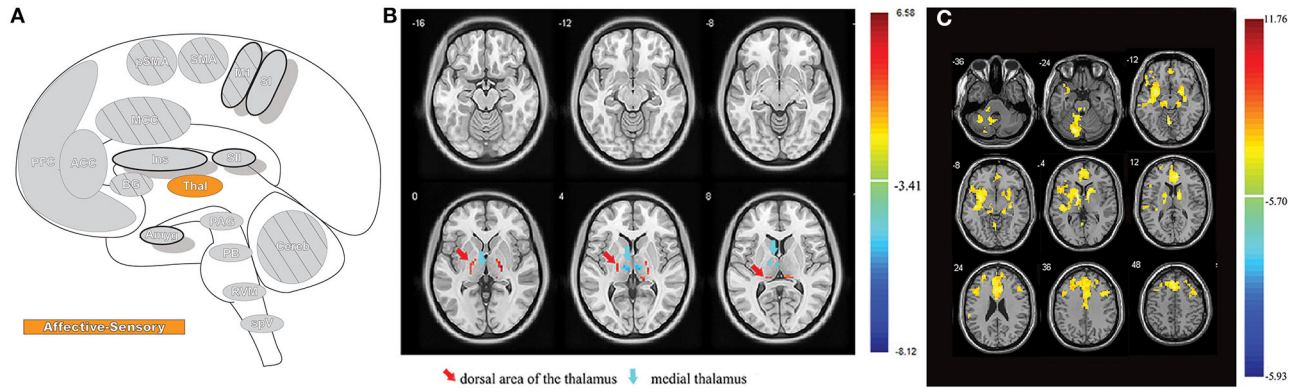

FIGURE 13 | Thalamus. **(A)** Brain areas active during pain: Thalamus (Thal) highlighted. **(B)** An examination of resting state fMRI data finds that participants with orofacial pain, experimentally induced by orthodontic separators, have significantly different patterns and intensity of spontaneous neural activity (red=increased, blue=decreased) compared to controls; differences such as these support the notion that the thalamus has a greater role in somatosensory processing than simply relaying afferent signals. Reprinted from Frontiers in Neurology, Vol 11, Y Jin, H Yang, F Zhang, J Wang, H Liu, X Yang, H Long, F Li, Q Gong, W Lai, The Medial Thalamus Plays an Important Role in the Cognitive and Emotional Modulation of Orofacial Pain: A Functional Magnetic Resonance Imaging-Based Study, p589125, Copyright (2021) Jin et al., under the Creative Commons Attribution License (CC-BY). DOI: https://doi.org/10.3389/fneur.2020.589125. **(C)** The same orofacial pain participants from **(B)** were also found to have a widespread and significant reduction (yellow areas) in resting state functional connectivity between the medial thalamus and other brain areas, emphasizing the comprehensive brain-wide interconnections of the thalamus and its engagement in pain processing. Reprinted from Frontiers in Neurology, Vol 11, Y Jin, H Yang, F Zhang, J Wang, H Liu, X Yang, H Long, F Li, Q Gong, W Lai, The Medial Thalamus Plays an Important Role in the Cognitive and Emotional Modulation of Orofacial Pain: A Functional Magnetic Resonance Imaging-Based Study, p589125, Copyright (2021) Jin et al., under the Creative Commons Attribution License (CC-BY). DOI: https://doi.org/10.3389/fneur.2020.589125.

similar findings in patients with fibromyalgia or the fact that perfusion is often found to return to normal rates after treatment in several pain conditions (250–252). Further, a recent study of multiple sclerosis found thalamic hypoperfusion to precede atrophy of the thalamic nuclei (253).

PAIN AS IT RELATES TO OCULAR PATHOLOGY AND DISRUPTED SYSTEMS

Neuroimaging has revealed a signature of pain in the brain—a network pattern of regions activated when pain is experienced (9, 248, 254). Neuroimaging techniques have also provided a way to assess tissue thickness and gray matter density of these regions as well as their response to stimulus. Identifying the nervous system's endogenous methods of change, which may subsequently result in altered structures and functional dynamics, is key to understanding how these systems can misalign and play a role in chronic pain (63).

An important feature of the nociceptive system is its capacity for plasticity, that is, for the neurons themselves to alter their structure and function (15). Repeated or intense noxious nociception can result in sensitization "increased responsiveness of nociceptive neurons to their normal input and/or recruitment of a response to normally subthreshold inputs" (13), a synaptic plasticity that leads to signal amplification resulting in pain from normally innocuous stimuli (16). When filling an adaptive role, pain amplified in this manner helps an organism stay vigilant to the threat of further damage, and neuron thresholds normalize sometime after the initiating stimulus has been resolved (16).

Peripheral Sensitization

Peripheral sensitization can occur when nociceptive neurons display increased responsiveness due to reduced activation thresholds and enhanced membrane excitability (13, 14, 16) and has been described previously in relation to the eye (23). The sensitized terminals of nociceptive neurons subsequently respond to stimulation that would normally be sub-noxious (allodynia) and have amplified and prolonged pain responses to noxious input (primary hyperalgesia) (14, 16, 19). Inflammatory pain is a common form of peripheral sensitization, initiated by the presence of inflammatory mediators, the release of which can be a consequence of nociceptive activity (2, 14, 44). Typically, once inflammation resolves the system returns to its previous balance, although the sensitized state can be maintained by ongoing inflammatory mediator release, thereby potentially causing neuropathic pain at the site of former injury (16, 19). Non-inflammatory causes of peripheral sensitization exist as well, as in the case of deafferentiation in postherpetic neuralgia, and the spontaneous and heightened activation of nociceptors, whatever the cause, is an important contributor to inducing sensitization in other portions of the nociceptive pathways (2, 14).

Peripheral sensitization alone cannot explain the severe level of pain in many cases. Clinical testing of abnormal pain, such as a proparacaine challenge in neuropathic corneal pain, can detect pain with a central origin; a component of pain that persists when the peripheral nociceptors have been silenced (255). Damage to underlying nerves transmitting the signal from the periphery is sometimes the cause, as when trigeminal neuropathic conditions cause pain amplification (22, 255). However, the changes observed in peripheral sensitization cannot account for several

phenomena, including the temporal summation of pain, tactile allodynia, and the generation of pain by innocuous input from non-injured tissue (15, 256).

Central Sensitization

In normal somatosensory sensation, low-intensity stimuli activate A-beta primary afferent nerves to produce non-painful sensations, despite close proximity to nociceptive pathways as the signals travel centrally to the cortex (2). The specific functional coupling of primary sensory neurons to their normal ascending pathways as well as the modularity of these parallel sensory and nociceptive circuits are determined by synaptic strength and the function of inhibitory neurons. Most input into neurons is subthreshold, but these connections are plastic, and departure from the normal balance of excitation and inhibition can cause exaggerated and abnormal pain.

Central sensitization is classified as "increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input. This may include increased responsiveness due to dysfunction of endogenous pain control systems" (13). Molecular, cellular, and anatomical changes can contribute to functional alterations in ocular central pain pathways, from the level of trigeminal brainstem to thalamus and up through cortical endpoints (13, 16, 22, 44, 84). Altered responsiveness in central sensitization is caused by enlarged receptive field sizes, increased membrane excitability, alteration of temporal firing dynamics, facilitated synaptic efficacy, or reduced inhibition in the neuronal circuitry of the ocular somatosensory system (16, 44). Sensitization of central neurons is often use-dependent, in which repeated activation triggers the change in synaptic functioning, and can be divided into either homosynaptic or heterosynaptic potentiation (15, 16).

Homosynaptic potentiation occurs when repeated use of a synapse facilitates subsequent activation in the same pathway, amplifying future instances of the same input (16, 257). This process is not unique to central sensitization in pain. Long Term Potentiation (LTP), the presumed hippocampal mechanism of memory, is a result of persistent homosynaptic facilitation (16). Windup is a transient form of homosynaptic potentiation in which the delivery of identical, repetitive, low-frequency noxious input results in each additional stimuli generating a larger action potential (and greater pain), but synaptic excitation returns to baseline within seconds after stimulation has ceased (16). Central sensitization remains autonomous for hours after induction and manifests after the triggering stimulus, unlike windup, which occurs during stimulation (256). Abnormal activity that triggers central sensitization by repeated nociceptive pathway use (i.e., peripheral sensitization, windup, or ectopic bursts) can cause an LTP-like homosynaptic effect (16). Future engagement of the system is facilitated, and the strengthening of synapses leads to an increase in the frequency and size of postsynaptic action potentials in TBNC (16, 44, 84). Homosynaptic functional alterations in central sensitization are contributors to primary hyperalgesia, along with peripherally sensitized areas (16).

Heterosynaptic potentiation occurs when activity in a synapse of a pathway enhances signaling in nearby, uninvolved synapses in the neuron (16). Enhancement of nearby synapses underpins the generation of pain by non-nociceptive stimuli; these heterosynaptic changes are the cause of sensitivity and pain spreading to uninjured areas (secondary hyperalgesia) as well as pain resulting from the activation of low-threshold input (allodynia) (16). In addition to the enhancement of transmission between trigeminal neuron axons and the TBNC, evidence of synaptic plasticity has been found in ACC, amygdala, PFC, and the PAG (19).

Central sensitization is a long-lasting endogenous process triggered by nociceptor input that eventually resolves when no abnormal signaling is present but can be maintained by low levels of stimulation (2). Ongoing ectopic pain (as can manifest after LASIK) or persistent peripheral sensitization (such as inflammatory dry eye disease) thus can have a role in both the generation and maintenance of central sensitization, altering the central nervous system response to stimulus for as long as the nociceptive signals persist (12, 44, 84, 257). The eventual de-escalation of the heightened pain response and reset of synaptic excitability is thought to be accomplished by inherent compensatory responses in descending pain modulatory pathways (44, 258). Damage or dysfunction in ascending pathways and descending pain modulatory processes can lead to pathological chronification of the hyper-responsive state, regardless of peripheral input. Long term maintenance of a sensitized state can be found underlying several painful craniofacial conditions and an out-of-balance descending system, especially one that promotes descending pain facilitation, can contribute to or sustain long term centralized neuropathic pain (6, 13, 14, 16, 234, 259).

Reorganization of Functional Networks

Supraspinal areas associated with pain are functionally intertwined in their activity, allowing changes in one structure to affect larger groupings of brain areas, casting doubt on some previous models of the pain-stimulus relationship (260–262). Identifying differences in structure and function in one supraspinal area may explain larger-scale patterns of change across the brain, highlighting the inherent connectivity between brain regions that work in concert.

Synchronized rhythmic fluctuations of activity in the brain measured as fMRI signal oscillations indicate the transfer of information between regions and give insight into how supraspinal areas are joined together as part of a network (245, 263, 264). While beyond the scope of this review, these network interactions are one of the largest areas of focus in modern pain research (262, 265). Network relationships can be quantified, and differences can be seen in many disease and pain states (9, 266). These altered structural, functional, and network-associative changes in neuronal processing centers can result in amplified pain beyond the afferent signal transmitted by the periphery, leading to hyperalgesia, allodynia, and even spontaneous pain (63, 262, 266–269).

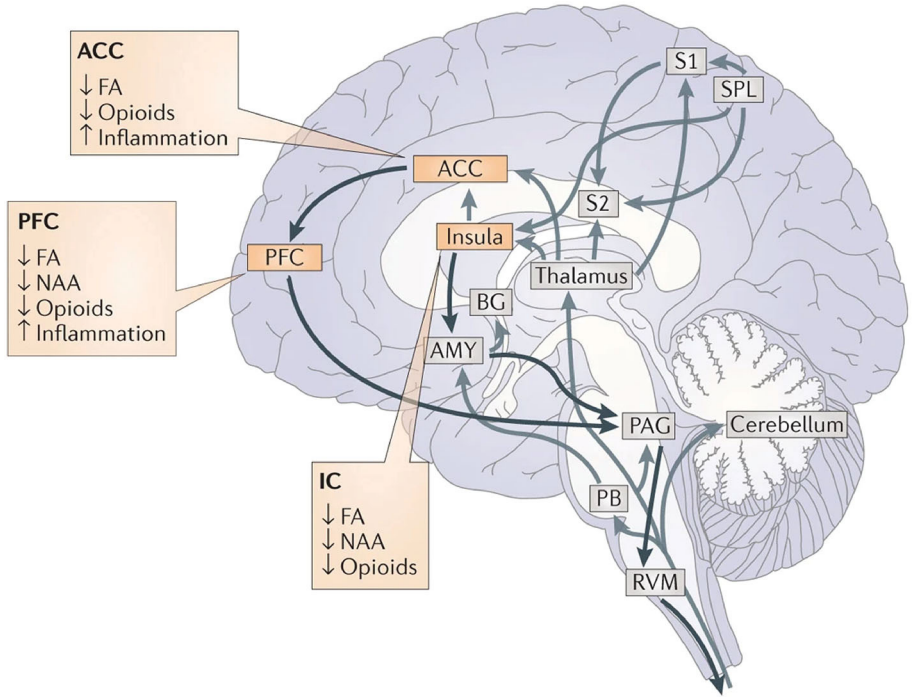

FIGURE 14 | Consistent patterns of chronic pain.The ACC, PFC, and IC consistently display decreased grey matter in chronic pain conditions, along with impaired white matter health (FA) and opioid receptor binding. Chronic pain is also associated with reductions in N-acetyl aspartate in the ACC and PFC, while studies in rodents have found increased inflammation in these regions as well. Reprinted with permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Neuroscience, Vol 14, Cognitive and emotional control of pain and its disruption in chronic pain, MC Bushnell, M Ceko, LA Low, p502–11, Copyright (2013). DOI: https://doi.org/10.1038/nrn3516. Grey arrows, pain pathways; Black arrows, descending pathways; FA,functional anisotropy: a measure of molecule diffusion that serves as an index of white matter integrity; Opioids, opioid receptor binding: a marker of the ability to bind opioids and a way to analyze the health of descending pain systems; NAA, N-acetyl aspartate: a marker of neuronal viability. ACC, anterior cingulate cortex; PFC, prefrontal cortex; IC, insular cortex; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; SPL, superior parietal lobe; BG, basal ganglia; AMY, Amygdala; PAG, periaqueductal gray; PB, parabrachial nuclei; RVM, rostral ventromedial medulla.

Neuroimaging Supraspinal Eye Pain-Qualifications and Clinical Adaptation

Neuroimaging in clinical ophthalmology is often limited to interrogating CNS causes of vision loss, nystagmus, ptosis, proptosis, diplopia, ophthalmoplegia, or optic nerve abnormalities. These may or may not be accompanied by pain (270). When pain is present it can be debilitating as the eye, and craniofacial region as whole, is subject to some of the most frequently diagnosed and intense pain conditions (234). As the eye is a critical sensory structure, the associated pain can come with intense psychological, emotional/affective components (234). The most common cause of neurological eye pain is migraine, followed by primary headaches, and trigeminal pain conditions, however, most neurological disorders can lead to referred eye pain (271). In the majority of these cases neuroimaging is not recommended unless a lesion or other underlying pathology mimicking these conditions is suspected (271). Similarly neuroimaging in patients with normal ophthalmic examinations as a pain diagnostic often does not provide a clear answer to symptoms, and applying experimental results to clinical pain realities often finds a much less direct relationship between pain and stimulus (271, 272).

The variability of pain activations between individuals can be extreme, even in an individual it is difficult to predict pain, as numerous brain regions modify sensory input along with psychological and attentional processes (6, 272). Further, no brain networks or regions associated with pain are exclusively pain related and most painful situations also engage other networks and processes, like attention, emotion and salience; the resulting overlap in brain regions active in pain and other salient experiences makes pain-specific imaging biomarkers difficult to determine (261, 272–274). Even chronic pain-associated processes and abnormalities are not pain-specific and have been observed in other conditions such as anxiety and depression (272, 273). Thus, despite evidence that certain regions are reliably activated in response to noxious stimulation, the adoption of brain imaging as a direct facsimile for pain, an inherently subjective experience, is not established (272, 273, 275).

In addition to the overlap in regional and network functions, multiple factors can influence painful stimuli-induced activations that may affect the ability to directly translate experimental work into the clinic and thus hinder the adoption of neuroimaging as a formal standard of care. Biological sex has a significant effect on pain-related brain activations, as studies have reported variance

in experienced and anticipatory pain between women and men, along with differential activations in the ACC, insula, and parietal and somatosensory cortices, among other areas (275, 276). Other factors contributing to variation in the neuroimaging of pain, although not an exhaustive list, include the duration of the painful stimulus, type of pain, mechanisms of pain, and type of disease underlying the pain; different patterns of activation can be observed in allodynia and hyperalgesia compared to normal individuals, mechanical and thermal pain have stronger or weaker activations in some brain areas when compared, and different diseases may have no functional similarities aside from increased pain thus making the results extremely specific to each condition and not widely appliable (272, 275). Despite these issues, some studies have found patterns of differential activations and even structure in some brain areas that are consistent across conditions (6) (**Figure 14**), however, other analyses find no consistent abnormalities in fMRI responses to painful stimuli in chronic pain patients- likely due to a combination of factors described above and many other uncorrected differences in experimental criteria that must be accounted for to achieve meaningful clinical translation (20, 277). While concrete and universal pain imaging biomarkers are yet to be established, neuroimaging has identified key brain regions involved in acute pain and established that CNS function is disturbed in chronic pain (272, 274).

CONCLUSION

The principles for supraspinal encoding of eye pain are akin to those observed in the rest of the body. Pain is a subjective experience that engages a concert of multidimensional processes throughout the brain. Different areas encode distinct aspects of sensory and emotional processes as well as the cascade of reactive autonomic, cognitive, reflexive, and modulatory mechanisms relevant to protective behaviors and adaptation. In relation to eye pain, the brain receives afferent input from the trigeminal system, which it also modulates using descending cortico-medullary feedback and feed-forward loops. Further active areas of investigation include the transformation of acute pain to chronic pain, improved characterization and differentiation of brain networks in chronic pain conditions, sex differences in the processing of pain, interactions between the immune system and brain regions, and patient stratification for targeted therapies for specific chronic pain conditions (278).

AUTHOR CONTRIBUTIONS

EM conceived of and directed the manuscript. NP drafted the initial manuscript. NP and EM wrote the final manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Pharmacology

HYPOTHESIS AND THEORY
published: 18 March 2022
doi: 10.3389/fphar.2022.759748

Multiple Criterion and Multiple Stimulus Signal Detection Theory Analysis of Corneal Painful and Cool Pneumatic Stimuli

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Purpose: To evaluate the detectability of pneumatic corneal stimuli and response bias using multi-stimuli multi-criterion signal detection theory (MSDT).

Methods: Thirty-six participants were recruited using convenience sampling. A Waterloo Belmonte esthesiometer was used to deliver cold, mechanical, and chemical stimuli to the center of the cornea at three separate study visits. The stimulus type was assigned randomly to each visit at the start of the study. The threshold (baseline for detection theory experiment) for the assigned stimulus type was obtained using the ascending method of limits. In the cold and mechanical MSDT experiments, 100 trials (80 signal (20 each for 4 intensities) and 20 catch trials) were presented in randomized order, and participants responded with a 5-point confidence rating to each trial. In the chemical MSDT experiments, 50 trials (20 signal trials each for two intensities and 10 catch trials) were presented, and responses were provided using 4-point confidence ratings. Detection theory indices were calculated individually and as groups, which were then analyzed using mixed models and paired t-tests.

Results: Detectability (d_a) and the area under the curve (A_z) were significantly different between stimulus intensities within each stimulus type (all $p < 0.001$) but were not different between the stimulus types. Receiver operating characteristics (ROC) curves were separable between the scaled intensities for all stimulus types, and no overlaps were observed in the z-ROC space. The log-likelihood ratio ($ln\beta$) depended on stimulus intensity and psychophysical criterion for all stimulus types.

Conclusion: It is feasible to use MSDT for analyzing ocular surface sensory processing and the theory provides insight into the possible bias associated with the use of pneumatic stimuli. With noxious and non-noxious pneumatic stimulation, detectability and criteria vary systematically with stimulus intensity, a result that cannot be derived using classical psychophysics and this highlights the importance of signal detection theory and its approaches in studying ocular surface pain and thermal processing.

Keywords: cornea, pain, psychophysics, human, signal detection theory

OPEN ACCESS

Edited by:
Juana Gallar, Miguel Hernández University of Elche, Spain

Reviewed by:
Chen Li, Free University of Berlin, Germany
Juan Gonzalo Carracedo Rodríguez, Complutense University of Madrid, Spain

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *17 August 2021*
Accepted: *07 February 2022*
Published: *18 March 2022*

Citation:
Jayakumar V and Simpson T (2022) Multiple Criterion and Multiple Stimulus Signal Detection Theory Analysis of Corneal Painful and Cool Pneumatic Stimuli. Front. Pharmacol. 13:759748. doi: 10.3389/fphar.2022.759748

INTRODUCTION

The corneal neurons are classified into Aδ-fibers (thinly myelinated and fast conducting) and C-fibers (unmyelinated and slow conducting) based on the thickness of the myelin sheath surrounding them and their conduction velocities, which transmits impulses from cornea to trigeminal ganglion and farther to the brain for pain processing (Tanelian and Beuerman, 1984; Belmonte et al., 1991; MacIver and Tanelian, 1993a; Gallar et al., 1993; Chen et al., 1995; Kovács et al., 2016). Three types of corneal receptors (polymodal nociceptors, mechano-nociceptors, and cold receptors) have been identified electrophysiologically in non-primates, which detect the signal and transmit impulses either through Aδ or C-fibers (Belmonte and Giraldez, 1981; Tanelian and MacIver, 1990; MacIver and Tanelian, 1993b; Belmonte et al., 1997; Müller et al., 2003). The cold thermo-receptors and polymodal nociceptors transduce signals conducted through the C-fibers, while the mechano-nociceptors transduce information for the fast-conducting Aδ-fibers' rapid response to painful mechanical stimuli (MacIver and Tanelian, 1993a; MacIver and Tanelian, 1993b; Belmonte et al., 2004). Since there is no systematic neurophysiological examination on the effects of human corneal stimulations, the presence of receptors/channels in the human cornea has been evaluated psychophysically (Feng and Simpson, 2004; Jayakumar and Simpson, 2020). Feng and Simpson (2004) have identified multiple corneal psychophysical channels in the human cornea. Our previous report using signal detection theory (SDT) showed favorable evidence in our data toward both the nerve conduction and nociception hypotheses (Jayakumar and Simpson, 2020).

The detection of the human ocular surface stimuli is complex due to the interdependence of the components of the ocular surface sensory processing system (both within and between the cornea and conjunctiva) (Feng and Simpson, 2004; Feng and Simpson, 2005). Detection thresholds estimated using classical psychophysical methods have been used as a measure of ocular surface sensory processing, even though they have been found to vary (Murphy et al., 1996; Acosta et al., 2001; Feng and Simpson, 2003; Golebiowski et al., 2005; Situ et al., 2008; Golebiowski et al., 2011). Variable observer's decision criteria are a major influence on threshold measurements (Swets, 1961; Gescheider, 1997) and these may lead to biased decisions by observers. Examples producing these biases include time of the experiment, previous experience and training, instruction characteristics, signal probability, stimulus intensity, or presumed tolerability to pain (Swets, 1961; Chapman, 1977; Rollman, 1977; Vision, 1985; Gescheider, 1997; Macmillan and Creelman, 2005). Only 1 experimental investigation of ocular surface sensory and decision criteria derived using signal detection theory (SDT) has ever been published (Jayakumar and Simpson, 2020). In it, we showed among other things, that there was a shortcoming in understanding the criteria used by participants because the simple yes-no experiment was designed to examine only the single criterion used by each subject (Jayakumar and Simpson, 2020).

The yes-no SDT experiment involved a detection task, in which participants detected the presence of a signal (supra-threshold stimulus) against the background noise. The yes-no SDT experiment demonstrated the feasibility of using one-interval two response (yes-no) design SDT to analyze the ocular surface sensory processing (OSSP) of pneumatic stimuli. However, there were a few limitations in the experiment that needed to be addressed, such as the assumption of fixed criterion, detection indices obtained only for a single intensity, and longer experiment duration if we need to test each intensity separately in a similar protocol. Yes-no SDT assumes that participants use a single criterion throughout the experiment when responding "Yes" or "No" to a trial, similar to the assumed single (and fixed) criterion in a classical psychophysical method but with the ability to estimate bias (Green et al., 1974; Gescheider, 1997; Macmillan and Creelman, 2005). However, if the participants vary their criterion during the experiment, the variation cannot be distinguished/evaluated due to the two-response design. Pay-off matrices or changes in instructions provided before the experiment have been reported in the literature to control/alter the criterion assumed by the participants (Green et al., 1974; Gescheider, 1997; Macmillan and Creelman, 2005). However, these restrict the participants from choosing their criterion independently during the experiment. Also, in a normal/clinical/experimental environment, the cornea receives multiple stimuli of different types and intensity at the same time. For example, in a clinical environment, participants may have to detect the stimuli of different intensities while they are already experiencing discomfort from the pre-existing dry eyes or factors such as drafts and dry air conditioning (Mendell and Smith, 1990; Wolkoff et al., 2005). These limitations make the yes-no one-interval SDT design less efficient, but the flexibility of SDT is that the same experiment could be conducted with variable criteria and multiple stimuli instead of a single stimulus intensity yes-no design. SDT experiments with variable criteria are usually referred to as multi-criterion or rating SDT experiment and in rating SDT experiments, instead of reporting a yes/no detection response, participants rate their confidence with which they detected a signal compared to the background noise (Green et al., 1974; Gescheider, 1997; Stanislaw and Todorov, 1999; Falmagne, 2002; Macmillan and Creelman, 2005; Wickens, 2010). Each level is then "converted" to a yes-no design to obtain different criteria adopted by the participants during the experiment, which will be similar to conducting multiple yes-no experiments with different pay-off matrices. Either ends of the rating scale (1 and 5, if 1-5 rating scale is used) represent the most conservative or most lax criteria used by the participants during the experiment, but participants can independently choose and vary their criterion during the experiment (Green et al., 1974; Gescheider, 1988; Stanislaw and Todorov, 1999; Wickens, 2010). Also, the detection indices may be estimated for multiple intensities within a single rating SDT experiment and here we refer to this as multi-stimulus rating SDT (MSDT). (Green et al., 1974; Gescheider, 1988).

MSDT experiments with pneumatic stimuli have never been conducted to examine OSSP. In the only previously reported OSSP study using MSDT, detectability of thermal waterjet corneal stimuli was obtained from rating responses, but the results were reported as though the experiment was conducted as a yes-no SDT experiment (Beuerman and Rozsa, 1985). MSDT has been used in many other areas such as audition, memory, and pain (Clark and Mehl, 1973; Green et al., 1974; Gescheider, 1988;

Belmonte et al., 1997; Macmillan and Creelman, 2005; Weidemann and Kahana, 2016). We initiated a series of signal detection theory approaches to understanding OSSP because of its similarity to somatic pain processing instead of using the trigeminal pathway, and signal arising from similar pain receptors (Millodot, 1984; Müller et al., 1995; Belmonte and Cervero, 1997; Müller et al., 2003; Belmonte et al., 2015; Belmonte et al., 2017).

According to the International Association for the Study of Pain, pain is an "unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Bonica, 1979; Merskey, 1994), and recently Williams and Craig (2016) defined pain as "a distressing experience associated with actual or potential tissue damage with sensory, emotional, cognitive, and social components." Studies have found that psycho-social entities such as anxiety, fear, personality, confidence, decision-making, self-esteem, and stress affect the perception of painful stimuli (Leventhal and Everhart, 1979; Frenkel et al., 2009; de Visser et al., 2010). Similar issues have been suggested in the literature of corneal sensitivity (Millodot, 1984), but have never been addressed before.

According to SDT, to elicit a response for a given trial, the sensory process first detects the stimulus and this is then followed by the decision process (influenced by multiple factors) that shifts the response either in favor of signal or noise (Green et al., 1974; Wickens, 2010). Both the sensory and decision process can be measured simultaneously and independent of each other using SDT. So, the aim of this experiment was to evaluate the feasibility of using MSDT to understand the OSSP of corneal pneumatic stimuli. This paper is primarily a technical report dealing with a complex issue affecting the psychophysical measurement of ocular surface sensing.

METHODS

Forty-one participants were recruited in the study using convenience sampling from the students and staff community of the University of Waterloo. The study was conducted according to the guidelines of the Declaration of Helsinki and ethics approval was obtained from the University of Waterloo, Office of Research Ethics (Waterloo, Ontario, Canada). Informed consent was obtained from each participant and participants were allowed to discontinue at any stage of the study. The ocular surface was screened for any active signs of inflammation or infection. There were only soft contact lens wearers in this study and the lens wearers were instructed not to wear their contact lenses on the day of their study visits. The visits were scheduled to occur at the same time of the day (±30 min) to reduce diurnal variation affecting the measurement.

Sample Size

Since this MSDT experiment using ocular surface stimuli has never been performed before (Jayakumar and Simpson, 2020), we used the data from our Yes-No experiment (Jayakumar and Simpson, 2020) to calculate the sample size for this experiment using the gpower 3.1.9.6. The estimated sample size needed was 8 (two-sided pair *t*-test, alpha = 0.05, beta = 0.8) with effect size of 1.17 based on the mean ± SD of cold and mechanical stimuli.

Stimulus Characteristics

The stimulus types used in this experiment were mechanical, chemical, and cold (or cool, room temperature). A Waterloo Belmonte pneumatic esthesiometer was used to deliver each stimulus to the center of the anterior corneal surface. The mechanical stimulus was medical air, heated to 50°C (converts to 33°C at the corneal surface) at the nozzle, and the cold stimulus was a room-temperature medical air. The flow rate of the stimulus was either increased or decreased to alter the intensity of the output, depending on the response provided by the participants. In the case of the chemical stimulus, the flow rate of the stimulus was kept constant at half of the mechanical threshold to remove any mechanical effect influencing the judgment. The ratio of carbon dioxide mixing with the medical air was changed at a given flow rate to produce a chemical stimulus. The % CO_2 in the stimulus defines the intensity of the chemical sensation induced. The flow meters in the control box of the esthesiometer regulate the flow of medical air and CO_2 to the desired concentration and flow. The temperature of the chemical stimuli was the same as the mechanical stimuli. The preparation and delivery of the stimulus were automated using the custom software according to the psychophysical procedure conducted. Each stimulus type was randomly assigned to one of the three study visits at the start of the first study visit. Each visit was approximately 1 h long and was separated by at least a day to avoid fatigue effects and allow 'recovery' of the ocular surface and the pain processing system.

Ascending Method of Limits to Determine Threshold

Though it is an MSDT experiment, the detection thresholds were calculated to use as a baseline for the following MSDT experiment. At the start of the visit, detection thresholds for the assigned stimulus were measured using the ascending method of limits (AMOL). An average of three measures was considered as a threshold. The duration of the chemical stimulus was 2 s, and mechanical and cold stimuli were 3 s long. The inter-stimulus interval for cold and mechanical stimuli was 10 s; for chemical stimuli, the inter-stimulus interval was 30 s (to enable purging of the stimulus in preparation for the subsequent stimulus). The oral instructions were provided by the examiner before the start of the experiment, followed by the automated audio prompts for each trial. The training was provided. Participants were advised to blink between each trial. Participants responded yes/no to each trial using the button box and the responses were recorded in the software. If the difference in detection thresholds between 3 measures was larger than 15 ml/min or 15%, the experiment was repeated another day. If the

thresholds were still variable, the participants were excluded from the study.

Detectability Experiments

The signal intensities for the MSDT experiments were scaled based on their respective corneal detection thresholds and the signals (in the analysis and report) were referred based on relative intensity to the threshold (**Figure 1**). The scaled intensities were described later in the methods. Instructions for the detectability experiment were accompanied by a short demonstration of the trial sequence. 'Neutral' instructions were scripted and delivered to all participants at the start of the experiment, to minimize examiner induced bias and variability. The stimulus probabilities and feedbacks, indicating the correctness of the response were not provided to the participants. Instead, audio feedback confirmed each button press. Participants rated each trial using the button box and the number of button presses was stored as the rating for each trial. Participants were advised to blink between stimulus presentations.

Cold and Mechanical Detectability Experiments

The cold and mechanical MSDT experiments consisted of 100 trials with random presentations of a signal or a noise stimulus (**Figure 1**). Each experiment consisted of four signal intensities of 20 trials each and a noise stimulus of 20 trials. The signal intensities (scaled based on detection thresholds) were a sub-threshold (0.5× threshold), a threshold, and two supra-threshold (1.5× and 2× threshold) intensities. The noise stimulus was a catch trial with no stimulus. If the estimated threshold for cold or mechanical stimulus was between 15 ml/min and 20 ml/min, a flow rate of 10 ml/min was used as the intensity of the sub-threshold stimulus. If the threshold was below 15 ml/min, the trials involving sub-threshold stimulus were replaced with the blanks (catch trials) as the flow rate of 50% threshold would be well below the esthesiometer's reliable output range of 10–200 ml/min. On a given trial, either a signal (one of the four scaled stimulus intensities) or a noise (blank stimulus) trial was randomly presented, and the instructions for the noise trials were exactly the same as the signal trials.

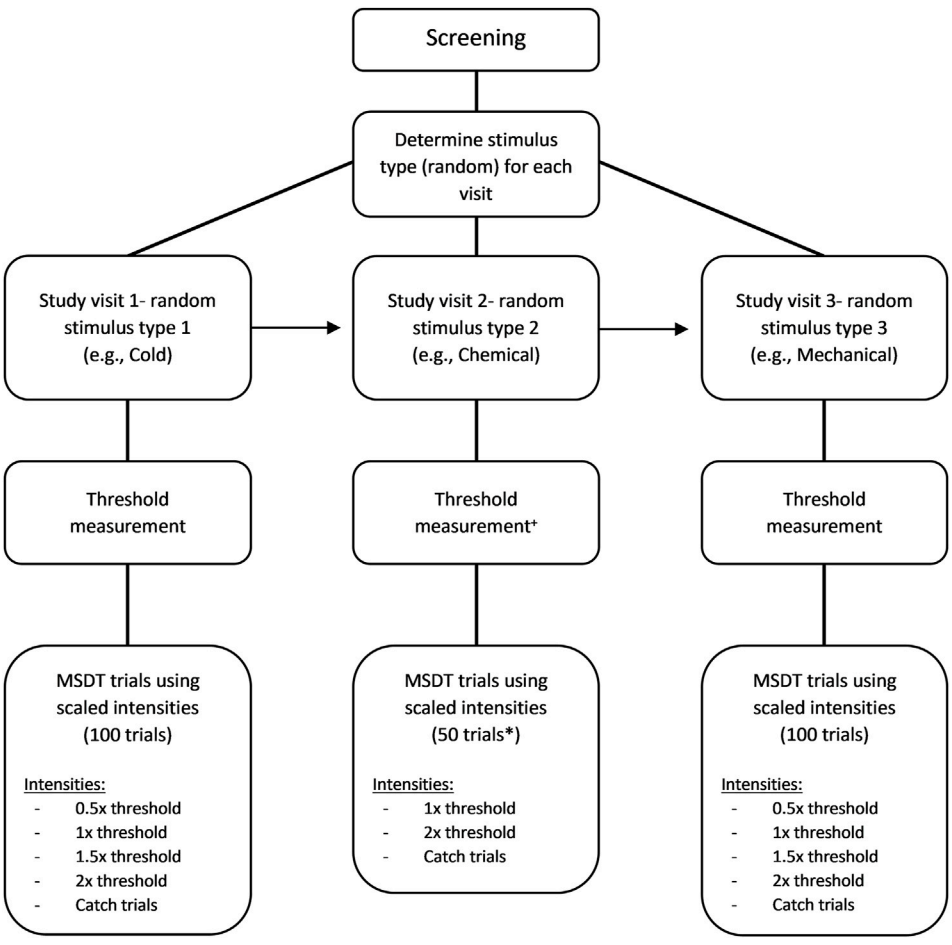

FIGURE 1 | Flow chart of the MSDT experiment. The order used in this example is cold (first visit), chemical (second visit), and mechanical (third visit) stimuli. +For chemical threshold measurement, mechanical threshold was measured first followed by the chemical threshold with half of the mechanical threshold as the flow rate of chemical stimuli. * 50 trials were used only for chemical MSDT experiments.

TABLE 1 | The confidence rating scale used by the participants to respond to a mechanical or a cold stimulus trial.

1	2	3	4	5
Definitely "No" signal was not presented	*Probably "No"* signal was not presented	Not sure/ uncertain	*Probably "Yes"* a signal was presented	*Definitely "Yes"* signal was presented

TABLE 2 | The confidence rating scale used by the participants to respond to a chemical stimulus trial.

1	2	3	4
Definitely "No" signal was not presented	*Probably "No"* signal was not presented	*Probably "Yes"* a signal was presented	*Definitely "Yes"* a signal was presented

The inter-stimulus interval and presentation time was the same as the threshold experiment. A confidence rating scale of 5 ratings was used by the participants to respond to each trial (**Table 1**). Breaks were provided after 50 trials by default or whenever participants pause the experiment using a button box.

Chemical Detectability Experiment

In order to keep the duration of this phase of experimentation approximately the same as those for mechanical and cold, we used the following protocol: The chemical MSDT experiment consisted of 50 trials with random presentations of either a signal or a noise stimulus (**Figure 1**). There were two signal intensities (the threshold and the 2x threshold) of 20 trials each and 10 noise trials. Unlike cold and mechanical MSDT experiments, the noise/ catch trials for chemical stimuli were not completely blank stimuli; instead, a medical air stimulus with 0% CO_2 was added at the same flow rate as signal trials. A confidence rating of 4 ratings was used by the participants to respond to each trial (**Table 2**). Breaks were provided after 25 trials by default or whenever participants pause the experiment using a button box.

Data Analysis

The rating data for each participant was exported to a Microsoft Excel spreadsheet. The RscorePlus software (v.5.6.1)[49] was used to calculate the detection theory parameters. These were based on assumptions of Gaussian signal and noise distributions. The RscorePlus data input file had the information on the number of rating categories, the number of signals (including catch trials), participant id, commands specific for SDT analysis along with the response frequency for each rating category. The commands included code for collapsing data in case of unsuccessful analysis, treatment of zero frequencies, and type of the SDT experiment. For this study, the SDT indices were calculated with an SINT (single-interval experiment paradigm) SDT protocol and zero frequencies were replaced with 1/number of rating categories to eliminate errors due to zero frequencies. The hit rate (HR) and false alarm rate (FAR) were calculated by cumulating the rating responses of n ratings for (n-1) decision criteria similar to the yes-no procedure. The HR and FAR were used in the calculation of detection theory parameters such as detectability (d' or d_a) and criteria. The outputs included the detection theory parameters for each signal and formatted datasheet for creating detection theory graphs using R. The equations used in calculating each detection theory parameter as provided by the software manual are listed below (Harvey, 2010):

$$d' = z(HR) - z(FAR) \ \text{(Equal variance model)}$$

$$d_a = \sqrt{\frac{2}{1+b^2}}.(z(HR) - b.z(FAR)) \ \text{(Unequal variance model)}$$

$$A_z = z^{-1}\left[\frac{d_a}{\sqrt{2}}\right]$$

$$c = -0.5\ (z(HR) + z(FAR))$$

$$\ln(\beta) = \frac{[z(FAR)^2] - [z(HR)^2]}{2}$$

The d_a provides the distance between the means of signal + noise distribution and noise distributions indicating the ability of subjects to detect signal from the background noise. The d_a and d' are numerically the same if the variance of the Gaussian distribution of noise and signal + noise are the same (Harvey, 2010; Harvey, 1992). The A_z provides the area under the curve estimate for each signal. The criteria (c and $ln\beta$) give independent bias indices for each stimulus intensity used inside the MSDT experiment. The receiver operating characteristics (ROC) curves were plotted for individual and cumulated (grouped) data. The cumulated data ROC curves were plotted using the rating data obtained by adding the response frequencies of each stimulus rating category across all the participants within the group as though a single participant received all the trials (**Figure 2**). For example, all 3600 trials (720 catch and 2880 signal trials) for mechanical stimuli were received by a single participant compared to 100 trials each by 36 participants. The R programming codes provided in the RscorePlus software package (Harvey, 2018) were used in plotting the ROCs, zROCs, and Gaussian distributions.

To analyze the bias between the types of stimuli, the multiple criterion data from the rating dataset were collapsed to a single criterion yes-no type analysis due to the difference in the rating scales between the stimulus types used by the participants to respond to the trials. The ratings were accumulated based on "liberal" and "strict" criteria. In the case of the "liberal criterion", a rating of 1 (definitely "no" there was no signal presented) was used as the frequency of "no" responses and ratings of more than

Participant id	Stimulus type	Rating 1	Rating 2	Rating 3	Rating 4	Rating 5
1	Cold sub-threshold	5	2	1	0	2
2	Cold sub-threshold	2	3	3	2	0
3	Cold sub-threshold	5	5	0	0	0
Group detection indices using the cumulated ratings data		12	10	4	2	2

FIGURE 2 | Example for the cumulated ratings to calculate group detection indices and draw group ROC curves.

1 were accumulated as the frequency of "yes" responses which would be similar to criterion 1 from the rating analysis. In the case of the 'strict criterion', a rating of 5 (definitely "yes" there was a signal) was used as the frequency of "yes" response (rating 4 for chemical stimuli) and the ratings of less than 5 were cumulated as the frequency of "no" responses which would be similar to criterion 4 (criterion 3 for chemical) from the rating analysis.

The detection theory indices were analyzed using a mixed-model analysis of variance (mixed-model ANOVA) ("lmerTest" package (Kuznetsova et al., 2017)) and paired sample *t*-test in R. The post-hoc/contrast analysis for the mixed models was performed using the "psycho" package (Makowski, 2018a). Several R packages were used in sorting, rearranging and analyzing data, and in creating and exporting graphs (Lemon, 2006; Wickham, 2007; Wickham and Winston, 2011; Xie, 2012; Hope, 2013; Bates et al., 2015; Wickham, 2016; Kuznetsova et al., 2017; Makowski, 2018b; Pinheiro et al., 2019; Wickham and Henry, 2019; Wickham and readxl, 2019; Kassambara, 2020; Wilke, 2020; Harrell, 2021; Manuilova and Andre Schuetzenmeister, 2021; Revelle, 2021; Wickman et al., 2021). An alpha value of $p \leq 0.05$ was assumed to be significant in all the analyses conducted.

RESULTS

The mean (±SD) age group of the participants was 30 ± 7.44 (range: 19–50) years. Five participants were discontinued at different stages of the study: Three discontinued due to variable detection thresholds obtained while repeating the AMOL and 2 participants discontinued due to high threshold. As mentioned earlier, the detection theory indices for all participants were calculated in two formats: 1) calculated using the cumulated rating data (for each rating) and 2) calculated from each participant's rating data. The average detection thresholds for cold, mechanical, and chemical stimuli were 26 ± 2.10 (ml/min at room temperature), 29 ± 2.25 (ml/min at corneal temperature), and 25 ± 2.30 (%).

Comparisons of detection theory indices between stimulus types follow.

Detectability

The average (±SE) d_a of each stimulus type and intensity are listed in **Table 3**. As mentioned earlier in the methods, the stimuli for detection theory experiments were scaled based on the threshold and the term "threshold" in detection theory experiments is used to indicate the intensity of the stimulus and not the outcome of the experiment. Since the detection theory parameters for the chemical sub-threshold and 1.5x threshold intensity stimuli were not evaluated, the statistical analyses were conducted independently for each intensity level between stimulus types. A paired sample *t*-test was conducted to compare the d_a between cold and mechanical stimuli of sub-threshold and 1.5x threshold intensity. The d_a's of both sub-threshold and 1.5x threshold intensity were not significantly different between the stimulus types ($p > 0.05$). On the other hand, a mixed-model analysis was conducted to compare the d_a's between the stimulus types of thresholds and 2× threshold intensity. The d_a's of the threshold intensity stimuli were not significantly different between the stimulus types [$F(2, 70) = 2.988$, $p = 0.057$], though the box plot showed a higher d_a for chemical stimuli in comparison to cold and mechanical stimuli (**Figure 3**). The d_a's of the 2× threshold intensity were not significantly different between stimulus types. A similar analysis for the A_z also showed similar comparisons as the d_a.

Within Stimulus Comparisons

Cold Stimulus

The ROC curves plotted using the cumulated ratings showed a good separation in the d_a between the scaled stimulus intensities (**Figure 4**). The ROC curve of cold sub-threshold intensity stimuli was inverted, indicating a negative d_a. The z-ROC curves for all stimuli were almost parallel to the chance line and only the z-ROC of the sub-threshold intensity stimuli was below the chance line similar to the ROC curve. The slopes of the supra-threshold z-ROC were less than 1, but the curves did not cross each other or other curves within the stimulus

TABLE 3 | Average (±SE) d_a for all three stimulus types and stimulus intensities.

SDT Parameters	Stimulus intensity	Cold (non-noxious)	Mechanical (noxious)	Chemical (noxious)
Detectability (d_a) (mean ± SE)	Sub-threshold	−0.15 ± 0.13	0.10 ± 0.14	NA
	Threshold	0.66 ± 0.12	0.68 ± 0.11	0.97 ± 0.12
	1.5× threshold	1.33 ± 0.17	1.57 ± 0.17	NA
	2× threshold	1.90 ± 0.17	2.08 ± 0.19	1.88 ± 0.16

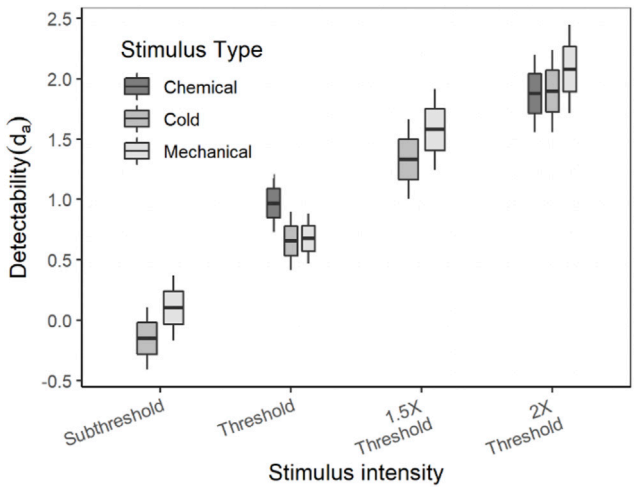

FIGURE 3 | Comparison of d_a between stimulus intensities and stimulus types.

type. A mixed-model analysis was conducted to compare the d_a of the cold stimuli between the intensities. A significant main effect of stimulus intensity [F (3,130) = 29.91, $p < 0.001$] was observed for d_a between the cold stimulus intensities (**Figure 5**). The contrast analysis showed that the d_a of each intensity was significantly different from the other. Similarly, the analysis of the area under the curve was also found to be significantly different between the intensities [F (3, 94.96) = 129.91, $p < 0.001$].

Chemical Stimulus

The ROC for the cumulated ratings of all participants showed good separation between the d_as of the threshold and 2x threshold intensity chemical stimuli (**Figure 6**). The slope of the z-ROC of the 2× threshold intensity stimuli was parallel to the chance line, whereas the slope was slightly less than 1 for threshold intensity stimuli. A paired sample *t*-test was conducted, and a significant difference was observed between the d_a's of the threshold (0.97 ± 0.12) and 2× threshold (1.88 ± 0.16) intensity stimuli; t (35) = −5.93, $p < 0.001$ (**Figure 7**). Similarly, the A_z was also significantly different between the two stimulus intensities [t (35) = −5.41, $p < 0.001$] (**Figure 7**).

Mechanical Stimulus

Similar to the cold and chemical stimuli, there was good separation between the ROC curves of different stimulus intensities (**Figures 8**, **9**). The slopes of z-ROC were less than one and the z-ROC of sub-threshold intensity crossed the chance line. The mixed-model analysis showed that the d_a's of the mechanical stimuli were significantly different between the intensities used in the experiment [F (3,100.92) = 66.46, $p < 0.001$] (**Figure 9**). A_z showed a similar significant main effect of the intensities [F (3,100.63) = 60.96, $p < 0.001$] (**Figure 9**).

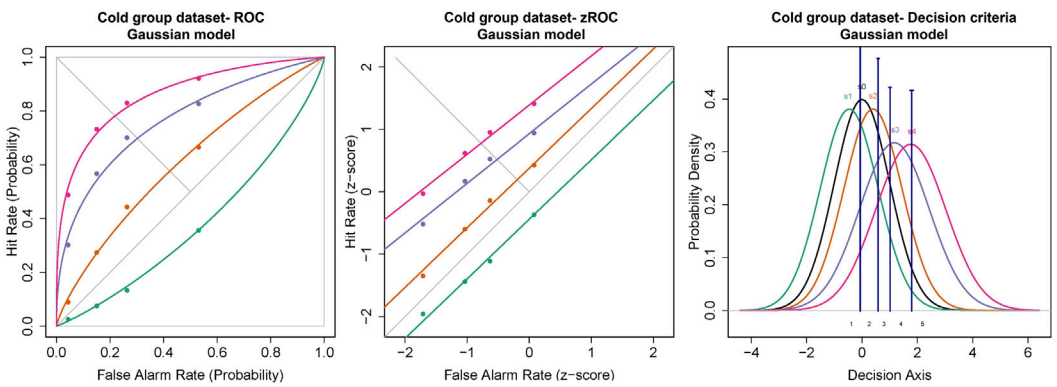

FIGURE 4 | ROC and Gaussian distribution for the cold stimuli. The green line represents the sub-threshold stimuli followed by orange (threshold), purple (1.5× threshold), and pink (2× threshold). The black line (S_0) in density functions represent the noise distribution.

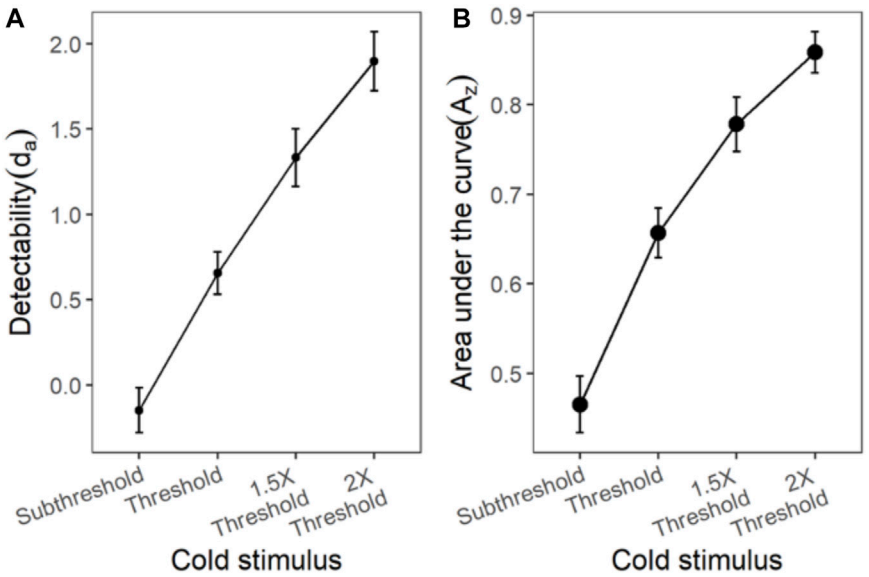

FIGURE 5 | The d_a and A_z transducer functions for cold stimuli. Each horizontal axis is stimulus intensity and in the left-hand panel, the *y*-axis is detectability (d_a) and in the right-hand panel, the *y*-axis is area under the curve (A_z). The points are the means and error bars are the SE of the estimates.

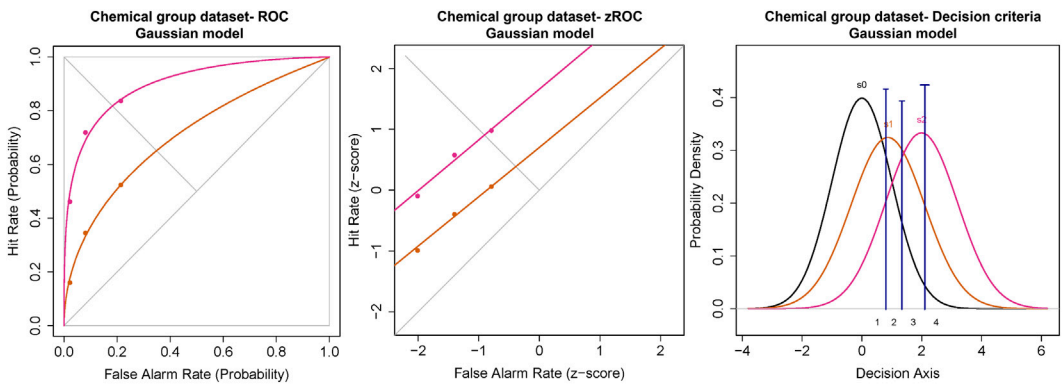

FIGURE 6 | ROC and Gaussian distribution for the chemical stimuli. The orange line represents the threshold stimuli followed by pink (2x threshold). The density functions in black (s_0) represent the noise distribution.

Criterion

Both c and *lnβ* were analyzed in this experiment but only the results for *lnβ* are discussed due to the length of the manuscript.

Cold Stimulus Criterion lnβ

Mixed-model analysis of *lnβ* also showed a significant main effect of psychophysical criterion [F (3,95.94) = 15.34, $p < 0.001$] and stimulus intensity [F (3,104.83) = 32.50, $p < 0.001$]. A significant interaction was also observed between the psychophysical criterion and intensity [F (9,285.85) = 51.59, $p < 0.001$] (**Figure 10**).

Mechanical Stimulus Criterion lnβ

There were significant main effects of psychophysical criterion [F (3,105) = 49.44, $p < 0.001$] and stimulus intensity [F (3,101.64) = 7.56, $p < 0.001$] as well as a significant interaction between the stimulus intensity and psychophysical criterion [F (9,304.08) = 38.38, $p < 0.001$] (**Figure 10**).

Chemical Stimulus Criterion *lnβ*

A significant main effect of psychophysical criterion was observed [F (2,70) = 52.10, $p < 0.001$] along with a significant interaction between the stimulus intensities and psychophysical criterion [F (2,70) = 19.68, $p < 0.001$]. However, *lnβ* was not significantly different between stimulus intensities (**Figure 10**).

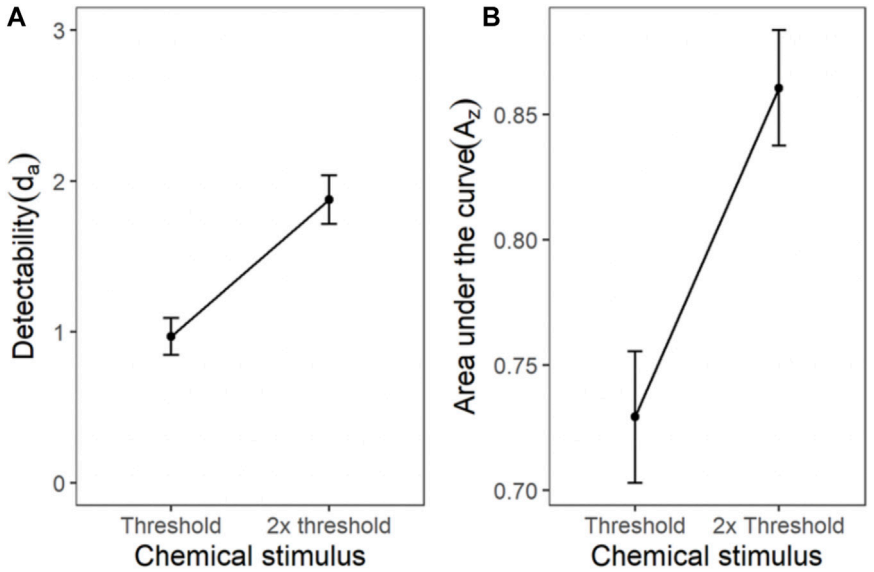

FIGURE 7 | The transducer function for d_a and A_z of chemical threshold and 2x threshold stimuli. Each horizontal axis is stimulus intensity and in the left-hand panel, the *y*-axis is detectability (d_a) and in the right-hand panel, the *y*-axis is area under the curve (A_z). The points are the means and error bars are the SE of the estimates.

DISCUSSION

The primary purpose of this experiment was to determine the feasibility of conducting an MSDT experiment using painful and cooling pneumatic ocular surface stimuli. We have shown that SDT may be used in a yes/no experiment, but there were drawbacks, some of which might be overcome if multiple stimulus intensities and participants using multiple criteria were possible (Jayakumar and Simpson, 2020). We showed that this more complex experimental design was feasible: Participants were able to concentrate during the experiments and were very well-behaved sensory (A_z and d_a) and criteria (here, $ln\beta$) metrics were reliably derivable (Jayakumar and Simpson, 2020). Because of the results reported here, additional predictor variables related to patient anxiety and decision making could be studied and their effects on d' and $ln\beta$ evaluated[1].

There were several results indicating the internal validity of the data we found. Although this paper is primarily about the feasibility of signal detectability (and in signal detection theory, "thresholds" do not exist), the detection thresholds (used in deriving stimulus intensities for the MSDT experiments) obtained in this study were consistent with previous studies that measured corneal detection thresholds as a primary outcome measure (Feng and Simpson, 2003; Situ et al., 2008; BasuthkarSundarRao and Simpson, 2014; Jayakumar et al., 2015; Alabi and Simpson, 2020). In addition, the MSDT data for pneumatic stimuli used in this study followed the assumptions of SDT, which were evident in the ROC curves and Gaussian distributions reported in the results (**Figures 4**, **6**, **8**). The ROC curves obtained were well behaved (with low residuals for each ROC line) for all stimulus types and the curves (both in ROC and z-ROC space) for intensities within each stimulus type did not overlap, indicating independent detectabilities for the scaled intensities. The z-ROC curves were almost parallel to the chance (45°) line, indicating the adherence of the obtained data to 1) Gaussian assumptions and 2) approximately equal variance in basic signal detection theory. The d_as calculated were similar using both cumulated rating data method and the average of the individual detectabilities (**Table 4**). This similarity in the d_a between the two methods indicates that the group detectability can be computed either from individual d_a's or from group averaged d_a's for ocular surface stimuli scaled based on detection thresholds. These results collectively point to the feasibility and internal (and face) validity of using MSDT (with intensities scaled based on detection thresholds) in analyzing the OSSP of the pneumatic stimuli.

Another metric of experimental feasibility is the number of participants who could not complete the experimental protocol. It is not useful if a substantial proportion of participants cannot do the experiments, even if the data from (a smaller number of) participants are well behaved. Two participants could not complete all the experiments due to their high baseline detection thresholds, and three participants could not complete due to variable detection thresholds. Neither of these groups of participants could not be used because of the signal detection theory aspects of the experiments: They were excluded because of the preliminary results, so considering the complex and noisy nature of the OSSP system, the results were very promising and clearly indicate the feasibility of these study methods.

[1]Ocular Surface Sensory Processing and Signal Detection Theory, September 2021, A PhD thesis presented by Varadharajan Jayakumar to the University of Waterloo.

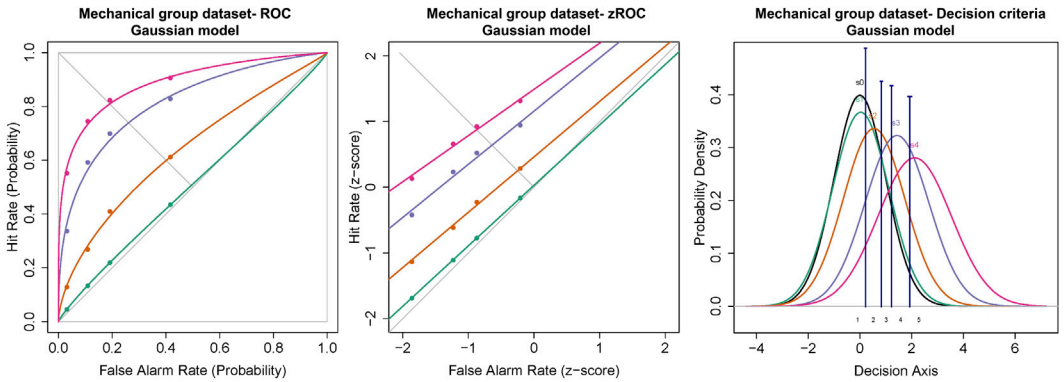

FIGURE 8 | ROC, zROC, and Gaussian distribution for the mechanical stimuli. The green line represents the sub-threshold stimuli followed by orange (threshold), purple (1.5x threshold), and pink (2x threshold). The black line (S_0) in density functions represent the noise distribution.

Detection Theory Indices

The d_a (obtained from ROC using cumulated data) of all three stimulus types (the intensities of which were scaled based on their respective detection thresholds), showed a systematic increase with increase in the intensity of the stimuli. Such behavior of these transducer functions was, of course, expected: Similar increases in the average d_a have been observed in the transducer functions in other senses (e.g., vision) (Nachmias, 1972). In addition, in this experiment, although 2 of the 3 stimuli were nociceptive (mechanical and chemical), as is apparent in **Figures 5**, **7**, **9**, there were no differences between nociceptive and non-nociceptive transducer functions.

$ln\beta$ showed a relatively complex dependency on the psychophysical criterion and stimulus intensity, especially as participant criterion increased. This complexity is somewhat scientifically problematic, because, ideally, one might prefer bias metrics should be approximately independent of stimulus intensity and the interaction is a further complication. What this does, however, is highlight the problem with psychophysical methods that do not derive any criterion metric, such as traditional Fechnerian methods. Detection thresholds combine sensory and decision components, and they cannot be disambiguated. If, as we show in our experiment, there are complex relationships between intensity and criteria, then methods that cannot disentangle these 2 are more difficult to interpret.

Detectability

Stimulus detectability may be derived in several ways: Using each hit rate (saying a stimulus was present when it was) and false alarm rate (saying a stimulus was present when it was not) the equations from individual/group data in the introduction may be used. Using collections of hit rate and false alarm rate for each criterion used, one might derive ROC curves from which detectability may also be derived. In this work there were consistent results that made it clear that it did not matter what approach was used. The transducer functions and the ROC curves all strongly pointed to the same conclusion that there was a clear separation of threshold scaled stimuli for both painful and cold corneal stimulation, again, pointing to the utility of a reliable SDT detection metrics being obtainable using the experimental design selected, as well as providing compelling evidence of the external validity of our data. The results are perfectly in line with several aspects of signal detection theory that predict how detectability scales with intensity and how criteria shift along ROC (iso-detection) curves.

We hypothesised that d_a derived using corneal pneumatic stimuli would be different between the intensities and also, on the basis of our earlier work (Tanelian and Beuerman, 1984), between the stimulus types (nocimetric and non-nocimetric). SDT proposes the sensory process as a continuous output, detectability, that is a function of the separation of a noise distribution and a signal-plus-noise distribution, unlike the threshold theory that defines the stimulus as always detectable once it crosses a threshold (and not detectable below threshold) (Wickens, 2010). The change in the detectability with stimulus intensity was evident in our experiment for each of the three types of stimuli, something reported previously in other senses, e.g., (Tanner and Swets, 1954; Stromeyer et al., 1982; Stromeyer et al., 1984). Since other ocular MSDT studies are not available for comparison within the ocular somatosensory system, the human response to similarly scaled stimulation might need to be examined indirectly. Alabi and Simpson (Alabi, 2018; Alabi and Simpson, 2019; Alabi and Simpson, 2020) observed a dose-effect increase in the autonomic responses such as redness, pupillary response, and accommodation for pneumatic stimuli. Situ et al. (Situ and Simpson, 2010) also reported an increase in the tearing response (using tear meniscus height measurement) and these taken together point to similar monotonic scaling of the human psychophysiological response to painful and cooling corneal stimulation. We and others have contributed reports of increases in ratings of attributes of ocular surface stimulation with increasing stimulus intensity in humans (Chen et al., 1995; Belmonte et al., 1999; Acosta et al., 2001; Feng and Simpson, 2004; Situ, 2010; Wu et al., 2015; Situ et al., 2019). Our detectability results, although with stimuli that are 'circum-threshold' or slightly suprathreshold are, therefore, in line with other work in

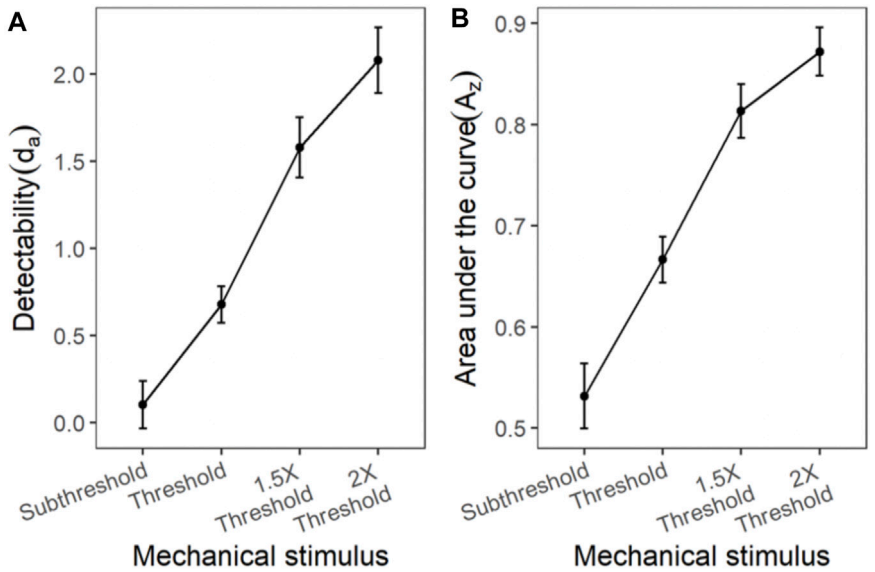

FIGURE 9 | The transducer function for d_a and A_z of mechanical stimuli. Each horizontal axis is stimulus intensity and in the left-hand panel, the *y*-axis is detectability (d_a) and in the right-hand panel, the *y*-axis is area under the curve (A_z). The points are the means and error bars are the SE of the estimates.

humans that show physiological and perceptual responses to the stimuli we used without the complication of the effect of participants' criteria.

Figure 2 shows that there is a systematic increase in d' with increase in stimulus intensity. A cursory understanding might suggest that this is nothing more than suprathreshold scaling [say a manifestation of Stevens Power Law (Wickham and Henry, 2019)]. This is not that simplistic: In a suprathreshold scaling experiment, an observer reports some value (derived using magnitude estimation or another form of scaling) that matches the subjective (perceived) intensity of the stimulus. This has 2 components, an intensity component and a criterion component. SDT methods enable a separation of this scalar value into a vector with 2 pieces, the sensory and the decision component. Detectabilty is one of those components and is not "simply" related to a suprathreshold score, because it acknowledges (and is mathematically derived from) the experimental fact that it (d') includes scores related to the ***absence*** as well as the ***presence*** of the stimulus. The interpretation that this then somehow is just the same as the suprathreshold scaling ignores another primary observation we made: The decision component ***also*** is a function of stimulus intensity (in a more complicated way as is shown by the interaction with stimulus intensity in **Figure 9**). Finally, we used a subthreshold stimulus for mechanical and cooling stimuli, that when using conventional suprathreshold methods could simply not be feasible since it would ***not*** be perceived by the observer for the majority of the stimulus presentations. Because of the multiple criterion method used, this extremely low stimulus intensity did not detract from what was feasible experimentally and so detectability and criteria metrics were derived as expected from SDT. Finally, it should be pointed out how badly behaved some suprathreshold scaling functions actually are, with many saturating and inverted perceived intensity vs. stimulus intensity functions, illustrating that suprathreshold scaling methods used do not always result in outcomes that are as might be predicted physiologically.

Physiological Interpretations

These results were almost perfectly in accordance with signal detection theory. The basic physiological implications are therefore fairly direct. The distribution of firing frequency of quiescent sensory neurons is Gaussian and against that distribution, decisions about sensory stimulation are made–is the Gaussian distribution of firing frequency of the stimulated (ocular surface) neuron (or system of neurons) different from that when there is no noise. In the context of the effect of a drug on the eye that alters this process, there are a number of ways to affect the outcome. In the first place, the distribution of the noise could be altered, either by reducing spontaneous firing or changing (reducing) the variance of the noise distribution. In these instances, a criterion stimulus would be more detectible (something not necessarily desirable if the eye is already uncomfortable). If it were desirable to reduce the effect of a painful/unpleasant stimulus, the drug could affect the stimulated distribution by reducing the firing frequency or altering the firing frequency variability so that detectability was lowered. Another possibility is to alter the decision so that the detectability is unchanged, but the firing frequencies are interpreted in a more conservative way, say, so that the observer patient is either less likely to call a criterion stimulus a stimulus (i.e., report that it is absent) or be less certain about the presence of a painful/uncomfortable stimulus. This is not to say that different from the interpretation of work on placebos using signal detection theory (Clark, 1969; Rollman, 1977; Allan and Siegel, 2002). This, of course, would imply more central acting and not peripheral acting pharmacological activity. This dichotomy of action based on detection theory is in

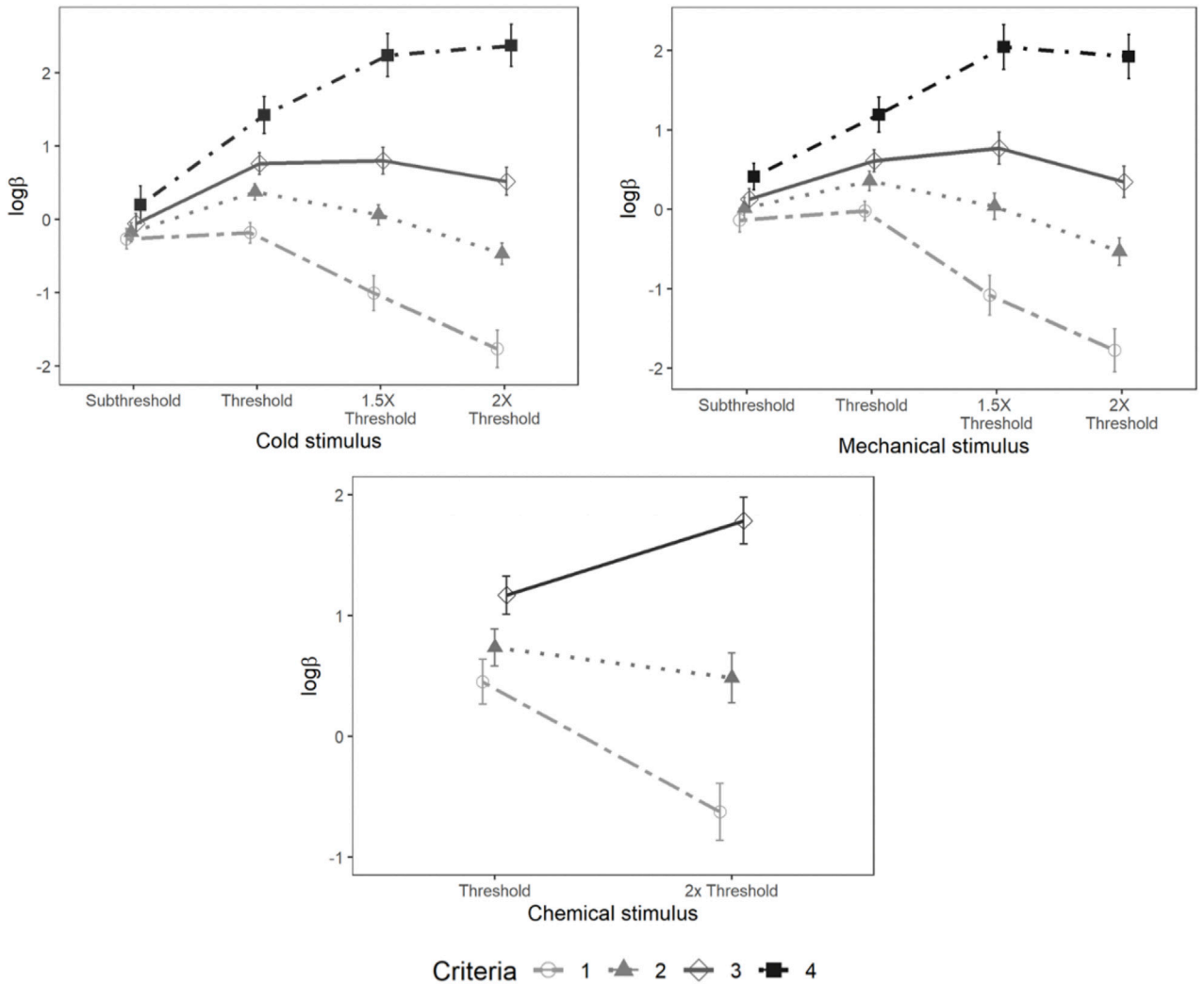

FIGURE 10 | Loglikelihood ratio for cold, mechanical, and chemical stimulus. Each horizontal axis is stimulus intensity and vertical axis is *ln*β. The points are the means and error bars are the SE of the estimates. The lines indicate different criteria.

TABLE 4 | Comparison of d_a obtained using cumulated and individual rating data.

Stimulus type	Stimulus intensity	d_a using cumulated rating data	Average d_a calculated from the d_a of each participant
Cold	Sub-threshold	–0.46	–0.15 ± 0.13
	Threshold	0.40	0.66 ± 0.12
	1.5× threshold	1.17	1.33 ± 0.17
	2× threshold	1.77	1.90 ± 0.17
Mechanical	Sub-threshold	0.03	0.10 ± 0.14
	Threshold	0.55	0.68 ± 0.11
	1.5× threshold	1.43	1.57 ± 0.17
	2× threshold	2.11	2.08 ± 0.19
Chemical	Threshold	0.87	0.97 ± 0.12
	2× threshold	1.99	1.88 ± 0.16

line with the model of pain processing being a combination of a sensory/intensity dimension with an affective motivational modulation aspect (Williams and Craig, 2016). The simple clinical relevance of both the sensory and criteria metrics is however yet to be explored, as this work was a test of basic detection theory concepts. In particular, the measurement of bias is important, but more testing is also needed to evaluate ways to control/manipulate bias before it can be routinely applied in clinical measurements.

Limitations

There were a few instruments and psychophysical method related limitations in this experiment. The instrument related limitations were the Belmonte esthesiometer's stimulus range and the time taken to prepare the chemical stimuli. The Waterloo Belmonte esthesiometer has a reliable stimulus flow rate range of 10–200 ml/min. In addition, the maximum concentration of added CO_2 in chemical stimuli can be only 100%. Since the MSDT experiment has stimuli of intensities at the detection threshold, as well as sub-threshold (0.5x detection threshold) and supra-threshold (1.5x and 2x detection threshold) levels, limitations arose when the scaled intensities fell outside the stimulus range available. For example, if the participant had a high chemical detection threshold of 70%, both supra-threshold intensities (105 and 140%) are outside the physical range of concentrations possible. Similarly, if the participant had a high mechanical detection threshold of 115 ml/min, the 2× supra-threshold (230 ml/min) stimuli would be outside the stimulus range available from the Waterloo Belmonte instrument. The 2 of 41 participants with these high detection thresholds were excluded from the experiment.

Another limitation of our esthesiometer was the time taken between chemical stimuli to purge the esthesiometer delivery tubes for each subsequent stimulus. To keep each stimulus-type experiment approximately the same duration, we used fewer chemical intensities and fewer chemical trials and were then able to keep an approximately constant stimulus probability across nocimetric and non-nocimetric stimulus types. The number of ratings were also reduced to minimize rating categories with no responses. A therefore unavoidable (obvious) consequence of these changes was observed in the analysis when detection indices were compared between stimulus types due to the difference in the number of ratings and number of intensities between stimulus types. Although complicating the inferences that could be made because of the unbalanced design, this did not influence our ability to compare stimulus types, however.

The training was provided to participants to familiarize them with the experimental set-up, the audio prompts during the experiment, and how to use the response button box. Participants were also instructed about different intensities before the MSDT trials. Because of the inclusion of (separate) anxiety measurement (Wickham, 2016) between experiments, feedback was not provided after each response; part of the experiment was to monitor anxiety change during the experiment. Future work may be needed to evaluate the exact effect of more extensive training and the effects of perceptual learning on the sensory and decision metrics used in this experiment as well as whether feedback would affect the results reported here.

In conclusion, we showed 1) MSDT is feasible for analyzing ocular surface sensory processing and 2) detectability and bias may be reliably extracted when using pneumatic stimuli. Specifically, detectability (d_a) of scaled threshold intensities systematically increases and the bias psychophysical criterion ($ln\beta$) systematically varies with stimulus intensity. In humans, during ocular surface processing of noxious and non-noxious pneumatic stimulation, detectability and criteria vary systematically with stimulus intensity, a result that cannot be derived using classical psychophysics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Univesity of Waterloo, Office of Research. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TS conceived the experimental design, JV collected the data and performed most of the statistical analysis. JV and TS wrote and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by an infrastructure grant from CFI and an operating grant from NSERC to TS.

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frontiers | Frontiers in Pharmacology

ORIGINAL RESEARCH
published: 05 April 2022
doi: 10.3389/fphar.2022.827244

Title: P2x7 Receptor Activation and Estrogen Status Drive Neuroinflammatory Mechanisms in a Rat Model for Dry Eye

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Dry eye disease (DED) is recognized as a chronic inflammatory condition with an increase in tear osmolarity and loss of tear film integrity. DED is often accompanied by adverse ocular symptoms which are more prevalent in females than males. The basis for ocular hyperalgesia in DED remains uncertain; however, both peripheral and central neural mechanisms are implicated. A model for aqueous deficient DED, exorbital gland excision, was used to determine if activation of the purinergic receptor subtype 7, P2X7R, expressed by non-neural cells in peripheral and central trigeminal nerve pathways, contributed to persistent ocular hyperalgesia. Densitometry of trigeminal brainstem sections revealed increases in P2X7R, the myeloid cell marker Iba1, and the inflammasome, NLRP3, of estradiol-treated DED females compared to estradiol-treated sham females, while expression in DED males and DED females not given estradiol displayed minor changes. No evidence of immune cell infiltration into the trigeminal brainstem was seen in DED rats; however, markers for microglia activation (Iba1) were increased in all groups. Isolated microglia expressed increased levels of P2X7R and P2X4R, IL-1β (Interleukin-1β), NLRP3, and iNOS (nitric oxide synthase). Further, estradiol-treated DED females displayed greater increases in P2X7R, IL-1β and NLRP3 expression compared to untreated DED females. Orbicularis oculi muscle activity (OOemg) evoked by ocular instillation of hypertonic saline (HS) was recorded as a surrogate measure of ocular hyperalgesia and was markedly enhanced in all DED groups compared to sham rats. Systemic minocycline reduced HS-evoked OOemg in all DED groups compared to sham rats. Local microinjection in the caudal trigeminal brainstem of an antagonist for P2X7R (A804598) greatly reduced HS-evoked OOemg activity in all DE groups, while responses in sham groups were not affected. Intra-trigeminal ganglion injection of siRNA for P2X7R significantly reduced HS-evoked OOemg activity in all DED groups, while evoked responses in sham animals were not affected. These results indicated that activation of P2X7R at central and peripheral sites in trigeminal pain pathways contributed to an increase in ocular hyperalgesia and microglia activation in DED males and females. Estrogen treatment in females further amplified ocular hyperalgesia and neuroimmune responses in this model for aqueous deficient DED.

OPEN ACCESS

Edited by:
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Reviewed by:
Alexander V. Zholos, Taras Shevchenko National University of Kyiv, Ukraine
Anna Matynia, University of California, Los Angeles, United States

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *01 December 2021*
Accepted: *16 March 2022*
Published: *05 April 2022*

Citation:
Bereiter DA, Rahman M, Ahmed F, Thompson R, Luong N and Olson JK (2022) Title: P2x7 Receptor Activation and Estrogen Status Drive Neuroinflammatory Mechanisms in a Rat Model for Dry Eye. Front. Pharmacol. 13:827244. doi: 10.3389/fphar.2022.827244

Keywords: dry eye, microglia, estrogen (17b-estradiol), trigeminal afferent pathway, purinergic (P2X) receptors

INTRODUCTION

Dry eye disease (DED) is a chronic inflammatory condition that is influenced by multiple intrinsic and external factors (Craig et al., 2017; Pflugfelder and Paiva 2017). Persistent adverse symptoms are the main reasons patients seek medical attention for DED (Rosenthal et al., 2009; Galor et al., 2015) which can range from a sense of ocular dryness to severe pain (Begley et al., 2001; Kalangara et al., 2017). Management of ocular symptoms in moderate to severe cases of DED is often inadequate (Asbell and Spiegel 2010; Williamson et al., 2014; Siedlecki et al., 2020). Although considerable progress has been made in the diagnosis of DED (Wolffsohn et al., 2017), the neural mechanisms that mediate ocular hyperalgesia are not well defined. It is widely accepted that most chronic pain conditions involve both peripheral and central neural mechanisms (Baron et al., 2013; Grace et al., 2021); however, studies concerned with the mechanisms of adverse symptoms in DED have emphasized peripheral factors (Belmonte et al., 2017). Peripheral biomarkers alone are not sufficient to predict the intensity of adverse ocular symptoms (Bron et al., 2014; Sullivan 2014) suggesting that CNS as well as peripheral neural mechanisms are involved.

Neuroimmune interactions are critical for the maintenance of inflammatory and neuropathic pain (Ji et al., 2016; Hore and Denk 2019). Tear hyperosmolarity is a prominent diagnostic feature of DED which is thought to trigger ocular infiltration of immune cells in a "vicious cycle" resulting in chronic inflammation (Baudouin et al., 2013). Tears of DED patients contain elevated levels of pro-inflammatory cytokines and adenosine triphosphate (ATP) (Guzman-Aranguez et al., 2017; Willcox et al., 2017). ATP released from injured cells enhances inflammation and immune responses through activation of the purinergic receptors such as P2X7R (Burnstock 2016; Di Virgilio et al., 2017). Disruption of P2X7R markedly reduces behavioral correlates of inflammatory and neuropathic pain in animals (Chessell et al., 2005), while mutations of the P2X7R gene significantly influence pain intensity in humans (Sorge et al., 2012; Kambur et al., 2018). P2X7R is highly expressed by several immune cell types, while expression by neurons remains controversial (Kaczmarek-Hajek et al., 2018). In the trigeminal sensory system, P2X7R is expressed by satellite glia which surround trigeminal ganglion (TG) neurons (Nowodworska et al., 2017; Inoue and Tsuda, 2021) and by microglia in the trigeminal brainstem (Ito et al., 2013). The threshold concentration of ATP necessary for P2X7R activation is higher than for other ionotropic purinergic receptors consistent with a role during moderate to severe inflammatory conditions. P2X7R activation is critical for assembly of the inflammasome, NLRP3, which through caspase-1 activation is necessary for IL-1β production and release by glia and immune cells (Burnstock 2016; Di Virgilio et al., 2017).

Women are diagnosed with DED more often and display more severe ocular symptoms than men (Sullivan et al., 2017; Vehof et al., 2018); however, the basis for sex differences in symptomatic DED remains unresolved. In an animal model for aqueous deficient DED, female mice displayed greater nociceptive and anxiety-like behaviors than males (Mecum et al., 2020). Estrogen status has long been recognized as a contributing factor for sex differences in pain behavior (Amandusson and Blomqvist 2013). While sex differences in microglia activation have been reported (Guneykaya et al., 2018), the relative contributions of activated microglia in male and female animals to pain processing are not well defined (Sorge et al., 2015; Taves et al., 2015; Lopes et al., 2017; Fernandez-Zafra et al., 2019). The present study tested the hypothesis that elevated estrogen status in ovariectomized (OvXE) female rats is a significant factor in mediating microglia activation and evoked orbicularis oculi muscle activity (OOemg) in a model for aqueous deficient DED. These results demonstrated that activation of P2X7R at peripheral and central sites in trigeminal pain pathways played a significant role in mediating neuroimmune responses and ocular hyperalgesia in DED.

MATERIALS AND METHODS

Animals. A total of 288 adult male, ovariectomized female (OvX) and estradiol-treated OvX female (OvXE) rats (250–350 g, Sprague-Dawley, Harlan, Indianapolis, IN) were used in these experiments. OvXE rats were given a single bolus injection of estradiol (E2, 30 µg/kg, sc) the day before tissue collection or muscle recording to simulate the proestrus surge in estrogens in normal cycling rats (Naftolin et al., 1972). The estrogen status of female rats was determined on the day of the experiment by vaginal cytology. Vaginal lavage samples from OvX rats contained small nucleated leukocytes, while samples from OvXE rats displayed mainly large nucleated epithelial cells consistent with low and high estrogen conditions, respectively. Animals were housed in pairs and given access to food and water ad libitum. Climate and lighting were controlled (25 ± 2°C, 12:12-h light/dark cycle, light on at 7: 00 a.m.). The animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (United States) and according to guidelines set by The National Institutes of Health Guide for the Care and the Use of Laboratory Animals (PHS Law 99-158, revised 2015).

Exorbital gland excision. Rats were anesthetized with isoflurane (5%) and the overlying masseter muscle was exposed. A small skin incision was made over the masseter muscle to remove the left exorbital gland. The wound margin was treated with 2% xylocaine gel and the incision was closed with absorbable sutures. The gland was exposed in sham rats but was not removed. Carprofen (25 mg/kg, i.p) was given as a

single dose after surgery. Rats survived for 2 or 14 days after gland removal for experiments that involved tissue collection and cellular and molecular analyses. Orbicularis oculi muscle recording was preformed 14 days after surgery.

Tear Volume and Orbicularis Oculi Muscle Electromyography (OOemg) Recording.

Rats were anesthetized with urethane (1.2–1.5 g/kg, ip) and spontaneous tear volume was measured by the increase in wet length of phenol red thread (ZONE-QUICK, Menicon INC., San Mateo, CA) at 14 days after surgery. The thread was gently placed in contact with the cornea/conjunctiva at its inferior-lateral edge and tear volume was measured over 2 min. Following tear volume measurement, a cannula was positioned in the left femoral artery to monitor mean arterial blood pressure and was maintained at 90–110 mmHg. Wound margins were infiltrated with 2% lidocaine and body temperature was kept at 38°C with a heating blanket. Rats were allowed to breathe spontaneously. The rat was placed in a stereotaxic frame and Teflon-coated copper wires (0.12 mm diameter) were implanted by a 26-gauge needle near the center of the upper and lower OO muscles, proximal to the lid margins, and grounded by a wire inserted in the neck muscle and at least 1 h elapsed before recording began (Rahman et al., 2017).

OOemg activity was sampled at 1,000 Hz, amplified (x10 k), filtered (bandwidth 10–300 Hz), displayed and stored offline (ADInstruments, Colorado spring, CO, United States). OOemg activity was recorded continuously for 6 min from 3 min before (baseline activity) until 3 min after stimulus onset. Recorded activity was rectified and stored as 1 s bins for off-line analyses. Total OOemg activity was calculated initially from the raw signal and the integrated area under the curve (AUC) for the 3 min epoch (μV-s per 3 min) sampled after each stimulus minus the 3 min epoch recorded immediately prior to stimulation. Ocular surface stimulation consisted of instillation of normal saline (0.15M NaCl) and hypertonic saline (HS) in concentrations of 1 and 2.5 M NaCl. Previously we determined that HS-evoked total OOemg activity consisted mainly (>90% of total) of a period of long duration activity (>200 ms) and a minor contribution from short duration (<200 ms) and that total OOemg activity was a valid measure of ocular hyperalgesia in a rat model for DED (Rahman et al., 2017; Bereiter et al., 2018).

Effects of Drug Treatments on OOemg Activity

OOemg and systemic minocycline. The non-specific anti-inflammatory agent and glial cell inhibitor, minocycline, was given systemically (40 mg/kg, ip) for 4 days prior to recording (four to five rats per group). At 14d after gland removal (4 days after the onset of minocycline treatment) rats were prepared for OOemg recording as detailed above. OOemg activity was evoked by ocular instillation of normal saline (0.15M) followed by increasing concentrations of HS (1.0, 2.5 M) applied at 30 min intervals. Each solution remained on the eye for 3–4 min before rinsing with artificial tears. Although the highest osmolar concentrations of HS used in this study was greater than that reported to evoke pain sensation in humans (Liu et al., 2009) or squint-like behavior in conscious rats (Yorek et al., 2016) (600–1,000 mOsm (by sucrose) versus 900-2200mOsM (by NaCl), respectively), we found that brief repeated application of these higher HS concentrations did not induce desensitization or tachyphylaxis of evoked OOemg activity.

OOemg and local inhibition of purinergic P2X7R in trigeminal brainstem. Rats were prepared for OOemg recording (10 rats per group) as noted above. Next the dorsal surface of the caudal brainstem was exposed surgically to allow microinjection of the selective P2X7 receptor antagonist, A804598 (10 μM, 0.2μL, Tocris) into trigeminal subnucleus caudalis (Vc) ipsilateral to the ocular stimulus. OOemg activity was evoked by ocular instillation of 2.5 M NaCl before drug injection. After 20 min A804598 or vehicle (PBS) was injected into Vc and 2.5M NaCl was applied to the ocular surface at 10, 30 and 50 min after drug injection. Drugs were prepared fresh each day. In separate animals, A804598 (10 mg/kg, sc) was given daily for 4 days and then the trigeminal brainstem was removed to determine the effects of drug treatment on markers for microglia activation in sham and 14 days DED rats.

OOemg after intra-trigeminal ganglion (TG) injection of siRNA for P2X7R. Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p) and maintained with isoflurane (1–2%). The animals were placed in a stereotaxic apparatus, the scalp was exposed, and a hole was drilled into the left parietal bone (3.5–4 mm anterior to the auricle and 3–4 mm lateral to the midline, and 8 mm below the cortical surface). The siRNA solution (600 μg, 200 nL, Stealth RNAi for P2X7R, RSS-310828 #83546611, validated by Invitrogen, Carlsbad, CA) or the Stealth RNAi negative control (#12935112, lot 857,979, Invitrogen), was injected into the left TG ~10 days after exorbital gland removal via a 33-gauge needle inserted through a 26-gauge guided cannula positioned stereotaxically and was kept in position at least 10 min after the injection to minimize leakage. The wound margin was closed with sutures and povidone-iodine solution was applied to the wound area. A single dose of carprofen (25 mg/kg, i.p) was injected in each animal to minimize post-surgical pain. Animals survived for 3 days after the intra-trigeminal ganglion injection (i.e., 10 days after gland removal). OOemg activity was evoked by ocular instillation of normal saline (0.15M) followed by increasing concentrations of HS (1.0, 2.5 M) applied at 30 min intervals. Each solution remained on the eye for 3–4 min before rinsing with artificial tears. At end of the recording session the TG and spinal trigeminal brainstem (Vsp) were removed and prepared for protein analyses by western blot. P2X7R protein levels were measured in TG samples to confirm transcription knockdown, while Iba1 was measured in Vsp samples to assess the effects on microglia actvation.

Immunohistochemistry. Male, OvX and OvXE rats (sham, 2 days, 14 days-post surgery, four rats per group) were anesthetized with pentobarbital (70 mg/kg) and then perfused with phosphate buffered saline (PBS) followed by

4% paraformaldehyde (PFA). The caudal brainstem was removed and placed in PFA overnight at 4°C. Transverse tissue sections were cut at 30μm on a vibratome and free-floating sections were blocked for 1 h (PBS, 0.1% Triton X-100, 1% donkey serum) and then incubated overnight at 4°C with primary antibodies (Ab) at 1:1,000 dilution for microglia (Iba-1, MABN92, Millipore), P2X7R (APR004, Alomone), NLRP3 (orb101128, Biorbyt) and GFAP (ABnova MAB10760, Walnut, CA, 1:500). Sections were washed in PBS (3 × 5 min) and incubated in secondary Ab (donkey antirabbit IgG biotin, AP182B, Millipore) for 90 min. Staining was visualized by Vector ABC compound (ABC kit, PK-4000, peroxidase standard) for 1 h at room temperature and color developed with diaminobenzidine tetrahydrochloride (peroxidase substrate, SK-4100) for 90 s. Sections were washed, air dried and mounted. Stained sections from sham and 14d DED rats were analyzed by light microscopy (Olympus BX51) at 10X magnification and quantified by densitometry (4 sections/rat) using ImageJ software. Controls for immunohistochemistry were processed by incubating sections without primary antibodies. Sections were analyzed ipsilateral to exorbital gland removal without prior knowledge of treatment. ***Immunofluorescence.*** Briefly, representative examples of trigeminal brainstem tissues were paraffin embedded and cut at 20 μm on a microtome. Sections were dewaxed, hydrated and stained with primary antibodies at a 1: 300 dilution: anti- P2X7 Cell Signaling # 13,809, Danvers, MA; anti-Iba-1, Millipore #MABN92, Temecula, CA; anti- NLRP3/ Cryopyrin, Biorbyt #101128, St. Louis, MO). Sections were rinsed and incubated with appropriate secondary antibodies at 1:500 dilution: anti rabbit CY5 or anti-mouse CY2, Jackson Immunoresearch #s 711-175-152 and 715-226-151, West Grove, PA. Images were captured using a Zeiss LSM700 confocal microscope with 40 × objective.

Western Blot. Aliquots of protein (20 μg) were run on 4–20% polyacrylamide gels, transferred to 0.45 μm membranes, and incubated with anti- P2X7R antibody (1-1,000 dilution, Cell Signaling, #13809, Danvers, MA. Anti- GAPDH was used as normalizing antibody (1, 1:000 dilution, Sigma Chemical, # WH0002597M1, St. Louis MO). Secondary antibodies were IRDye 800CW anti-mouse and IRDye 680RD anti-rabbit, 1-15,000 dilution, LICOR, Lincoln NE. Membranes were scanned on LICOR Odyssey infrared scanner.

Flow cytometry. Rats were anesthetized and perfused through the heart with PBS, and the spinal trigeminal brainstem (Vsp), which included subnucleus oralis (Vo), interpolaris (Vi) and caudalis (Vc) regions, was dissected (3 rats per group). The tissue samples were minced and digested with collagenase type IV (Invitrogen) and DNAse (Invitrogen) for 30 min at 37°C. The tissue was then dissociated through nylon mesh before the mononuclear cells were separated on a 70/30 percoll gradient. The mononuclear cells were washed with FACS buffer (PBS with 5% normal goat serum) and blocked with antibody to CD16/32 (BD Bioscience) at 4°C for 30 min and then incubated for 45 min at 4°C with fluorescently labeled antibodies specific for CD45, CD11b, CD8, and CD4. The cells were analyzed on a FACScalibur (BD Bioscience) based on live cells. The CD45 intermediate $CD11b^+$ cells were resident microglia, and the CD45 high cells were analyzed to determine the number of macrophage ($CD11b^+$), $CD4^+$ T cells, and $CD8^+$ T cells.

Cell sorting. Rats were perfused with PBS, and the Vsp, which included Vo, Vi and Vc subnuclei, was dissected. The brainstems were used in Neural Tissue Dissociation Kit (P) following the protocol. The resulting cells were separated on a 70/30 percoll gradient. The mononuclear cells were incubated with $CD11b^+$ microglia MicroBeads (Miltenyi) and separated on a column to obtain a specific population of microglia. The isolated microglia were >99% pure based on flow cytometric analysis.

RNA isolation and quantitative real time PCR (qRT-PCR). RNA was isolated from microglia using SV Total RNA Isolation kit which contains a DNAse reaction (Promega). First strand cDNA was generated from 1 μg of total RNA using oligo $(dT)_{12\text{-}18}$ primers and Advantage for RT-PCR kit in a final volume of 100 μL (Clontech). qRT-PCR was conducted in triplicate with Rotor-Gene SYBR green RT-PCR kit (Qiagen). Briefly, 0.5μM primers, 1X SYBR Green reagent, and 2μL of cDNA were combined in 10μL reactions. The primers were specific for β-actin, IL-1β, TNFα, NLRP3, iNOS, BDNF, and TLR4. qRT-PCR was conducted on a Rotor-Gene Qiagen Q instrument using hot start with cycle combinations, 40 cycles: 95°C for 15s; 60°C for 20s; 72°C for 15s, followed by a melt from 75 to 95°C. Quantitation of the mRNA was based on standard curves derived from cDNA standards for each primer pair. Specific mRNA expression was normalized to β-actin expression.

Experimental Design and Statistical Analysis

Figure 1 Flow cytometry data were collected from three rats per group. The percentages in the quadrants were based on total mononuclear cells isolated from the trigeminal brainstem using flow cytometry software by FCS Express. **Figures 2**, **3**, **5**. Densitometry data was calculated from four sections per rat (4 rats per group) and expressed as percent positive area. Sections were analyzed without prior knowledge of treatment. Values were compared by one-way analysis of variance (ANOVA) corrected for repeated measures (GraphPad Prism v. 9) on one factor and individual group differences assessed by Tukeys or by Neuman-Keuls. The data were presented as mean ± SEM. **Figures 4**, **6**, **9**. Microglia were isolated from the Vsp (8 rats per group). Significant differences were determined by one-way ANOVA and Bonferroni's multiple comparison test based on values from sham rats of the corresponding treatment group. The data were presented as mean ± SEM. **Figures 7**, **8**, **10**. Total OOemg activity was assessed by two-way ANOVA (GraphPad Prism v. 9) and corrected for repeated measures on one factor. Significant treatment effects were assessed by Tukey's or Newman-Keuls after ANOVA. The data were presented as mean ± SEM and the significant level set at $p < 0.05$, n = 5-6 rats per group. Sample size was based on results from previous studies (Rahman et al., 2017; Bereiter et al., 2018), which we calculated would provide 80% power at

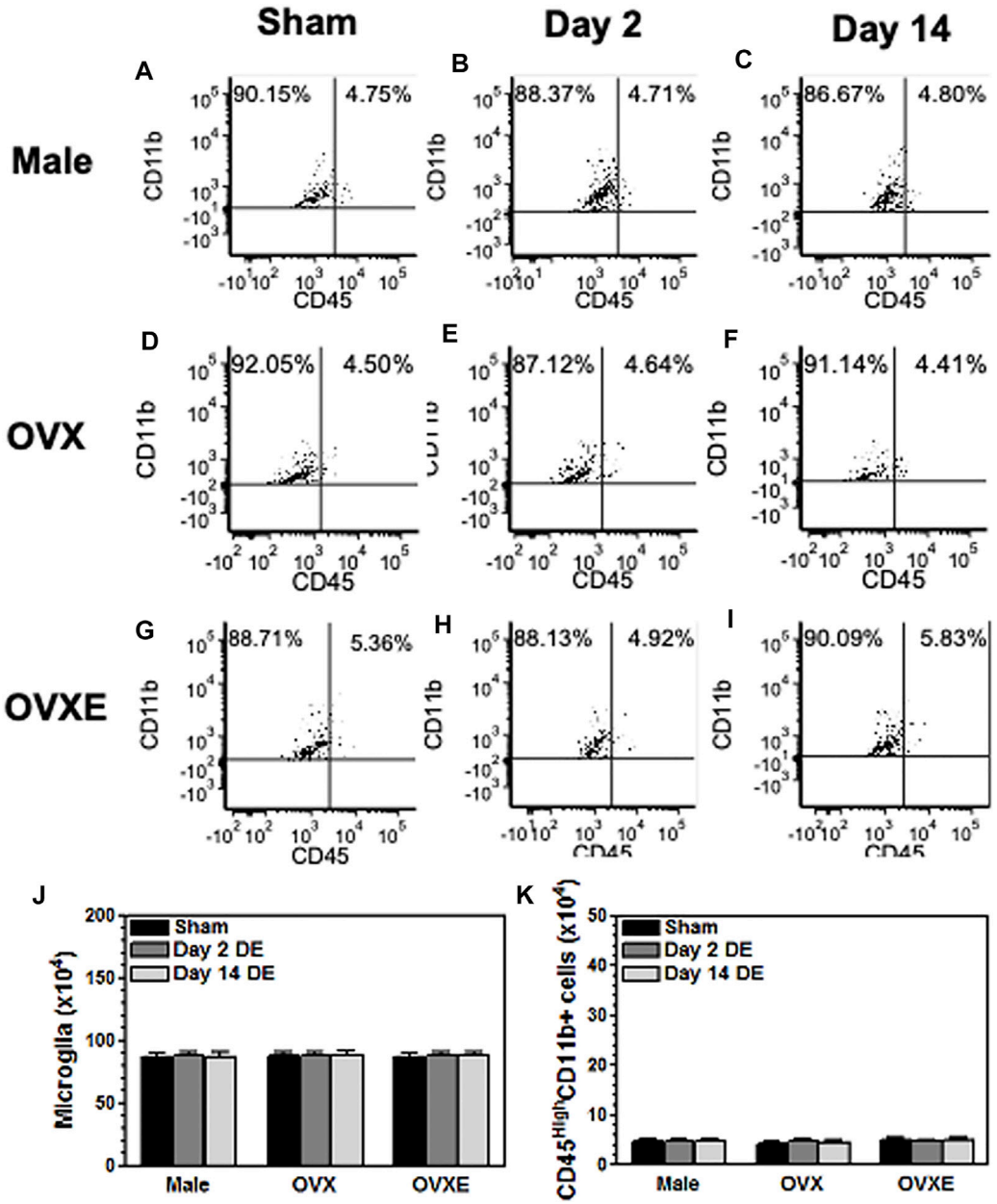

FIGURE 1 | Peripheral immune cells do not infiltrate the brainstem during DED. Male, OVX, and OVXE rats had the left exorbital gland removed or were sham treated **(A,D,G)** (3 rats per group). At 2 days **(B,E,H)** and 14 days **(C,F,I)** post-surgery, rats were perfused with PBS and brainstem tissue was dissociated with trypsin and collagenase prior to separation on a 70/30 percoll gradient. The mononuclear cells were incubated with fluorescently labeled antibody for CD45 and CD11b. The cells were analyzed by flow cytometry gating on live cells. The infiltrating monocytes/macrophage identified as CD45highCD11b^{+} and microglia identified as CD45intermediateCD11b^{+}. The percentage in each quadrant is based on total mononuclear cells isolated from the brainstem. These dot plots represent data from one of three independent repeated experiments. The total number of microglia (CD45intermediateCD11b+) **(J)** and monocytes/macrophage (CD45HighCD11b^{+}) **(K)** were calculated for each of the three experiments and combined in the graphs **(J,K)**. *N* = 3 rats per group.

$p < 0.05$. Three female rats were excluded from further analysis due to low blood pressure at the time of recording. The experiments used sham and DED rats and selected in random order. **Figure 11**. Protein levels for Iba1 were quantified in Vsp samples from OvX rats by densitometry (4 rats per treatment group) and analyzed by ANOVA.

RESULTS

Tear Volume After Exorbital Gland Removal

Male, OvX and OvXE rats displayed no signs of ocular hyperemia or inflammation and gained weight normally over the 14 days after exorbital gland excision. Although fluorescein staining was

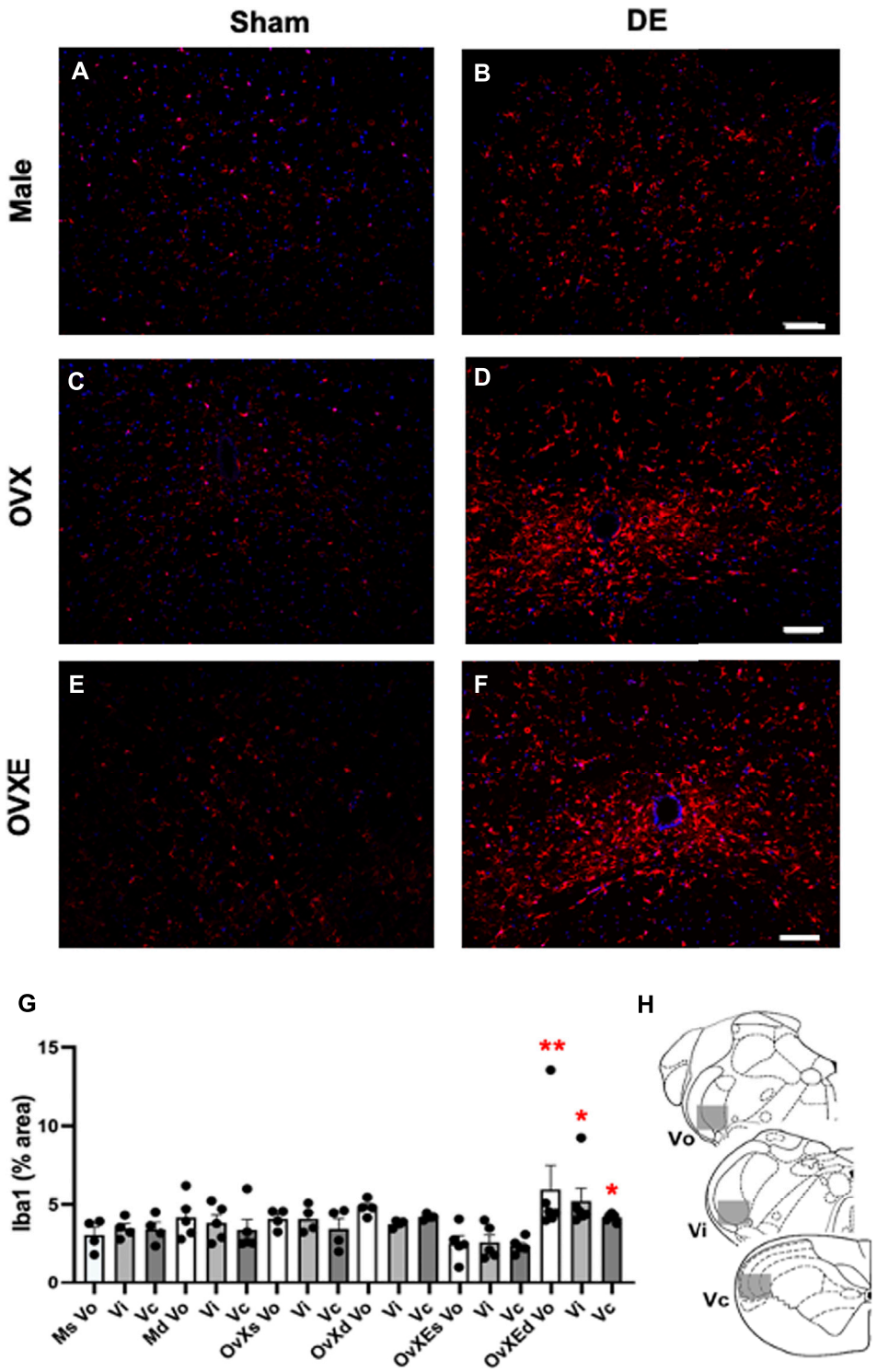

FIGURE 2 | Microglia are activated in the Vsp during DED and express Iba-1. Male, OVX, and OVXE 14 days DED rats **(B,D,F)** and sham rats **(A,C,E)** were perfused with 4% PFA, fixed and brainstem sections stained with anti-Iba1 (red) and dapi (blue). The micrographic examples are of trigeminal subnucleus caudalis (Vc). Scale bar = 80 μm. **(G)** Densitometry was conducted on light microscopy-stained sections from Vo, Vi, Vc regions using ImageJ on four sections per rat with four rats/group (mean ± SEM). Abbreviations: Ms, male sham; Md, male DED; OvXs, OvX sham; OvXd, OvX DED; OvXEs, OvXE sham; OvXEd, OvXE DED. $^{*}p < 0.05$, $^{**}p < 0.01$ versus sham group. **(H)** Shaded areas represent regions of trigeminal brainstem that were sampled. $N = 4$ rats per group.

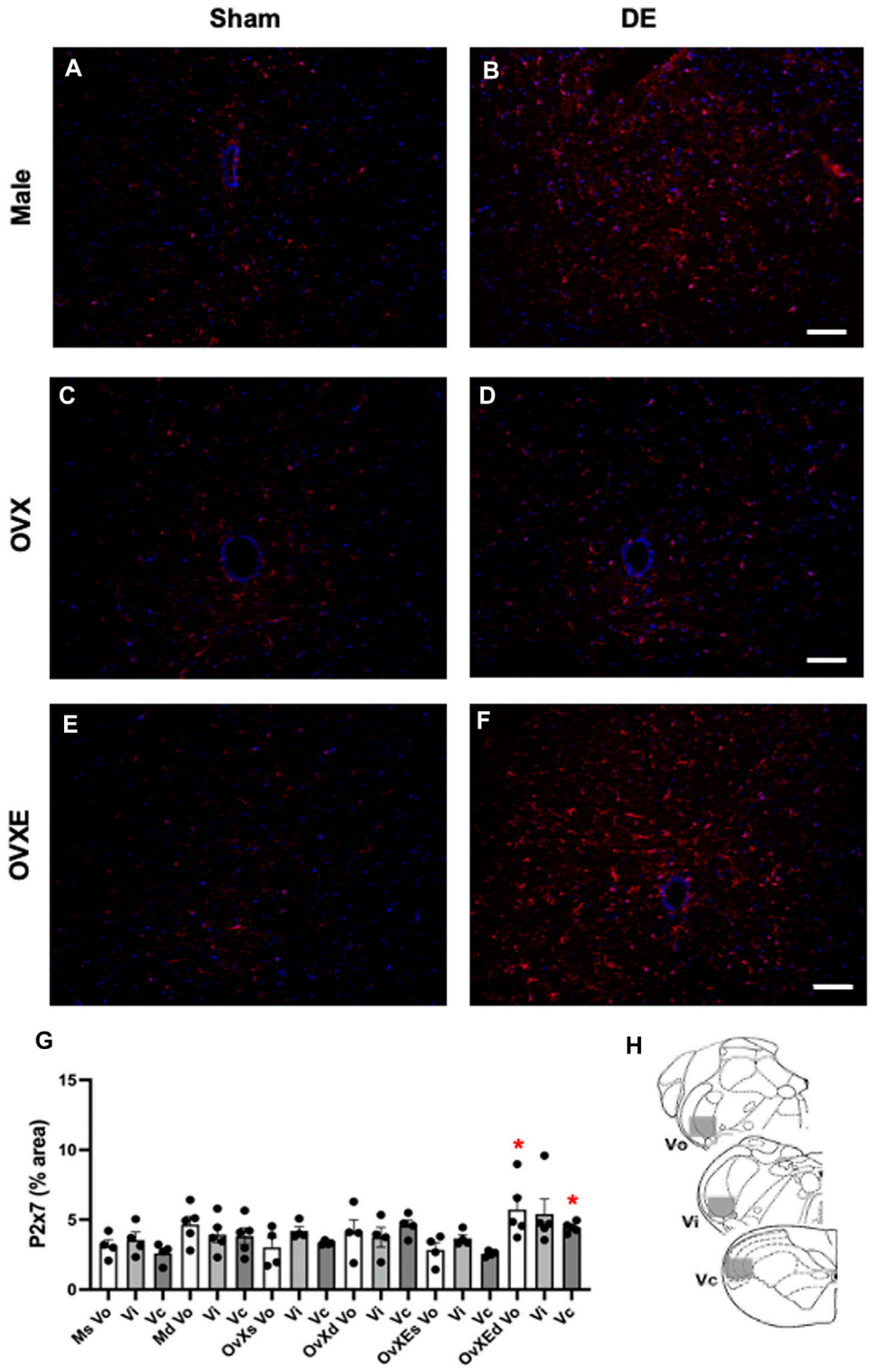

FIGURE 3 | Microglia express P2X7R in the Vsp during DED. Male, OVX, and OVXE 14 days DED rats **(B,D,F)** and sham rats **(A,C,E)** were perfused with 4% PFA, fixed and brainstem sections stained with anti-Iba1 (red) and dapi (blue). The micrographic examples are of trigeminal subnucleus caudalis (Vc). Scale bar = 80 μm. **(G)** Densitometry was conducted on Vo, Vi, Vc regions using ImageJ (mean ± SEM). Abbreviations: Ms, male sham; Md, male DED; OvXs, OvX sham; OvXd, OvX DED; OvXEs, OvXE sham; OvXEd, OvXE DED. *$p < 0.05$ versus sham group. **(H)** Shaded areas represent regions of trigeminal brainstem that were sampled. $N = 4$ sections per rat and four rats/group.

not performed here, others have used very similar methods for exorbital gland excision and reported no significant change in staining for at least 4 weeks (Meng et al., 2015). Resting tear volume was measured in 90 rats (sham, $n = 45$; DED, $n = 45$). At 14 days after surgery, the tear volume across all DED groups averaged 8.6 ± 0.2 mm/2 min (mean ± SEM) ipsilateral to gland removal and 19.3 ± 0.2 mm/2 min (mean ± SEM) in the contralateral eye ($F_{1,88} = 423$, $p < 0.001$). In sham rats, tear volume averaged 18.9 ± 0.2 mm/2 min from the left and right eyes. There were no significant sex differences in tear volume for sham or DED groups ($p > 0.1$).

Microglia Activation in the Trigeminal Brainstem During DED

To determine whether DED was associated with an infiltration of immune cells into the trigeminal brainstem, the spinal trigeminal nucleus (Vsp), which included subnucleus oralis (Vo), interpolaris (Vi) and caudalis (Vc), was dissected from male, OvX, and OvXE rats at 2 days or 14 days post-surgery or following sham surgery. After dissociation the mononuclear cells were isolated and then incubated with fluorescently labeled antibodies to CD11b and CD45 prior to analysis by flow cytometry. Peripheral immune cells express high levels of CD45 while microglia express intermediate levels of CD45. CD11b is present on peripheral monocytes/macrophage and microglia. No infiltrating immune cells (CD45 high cells) were seen in samples from sham groups based on $CD11b^+$ and CD45 intermediate expression. Similarly, samples collected at 2 days or 14 days from DED male, OvX, and OvXE rats also had only resident microglia in the brainstem and no evidence of infiltrating immune cells (**Figure 1**).

Immunohistochemistry (IHC) and densitometry was used to determine if resident microglia in the Vsp were activated in DED rats. The Vsp was removed from sham and 14 days DED male, OvX and OvXE rats. The tissue was fixed, sectioned, stained with anti-Iba-1 and analyzed by confocal microscopy and densitometry for activation of microglia (**Figure 2**). Overall treatment effects indicated differences between sham and DED rats ($F_{5,22} = 3.11$, $p < 0.05$). Individual comparisons revealed that microglia at Vo, Vi and Vc levels of Vsp from OvXE rats had a higher expression of Iba1 at 14 days post-surgery compared to OvXE sham rats ($p < 0.05$), while only marginal changes were seen in OvX and males at 14 days post-surgery (**Figure 2G**). These results suggested that high estrogen status and loss of tear volume interact to enhance microglia activation in the Vsp.

Purinergic P2X7 receptors (P2X7R) are primarily expressed by microglia, the predominant immune competent cell in the CNS, and bind ATP released by injured or stressed neurons to promote expression of cytokines and effector molecules. The effect of DED on P2X7R expression in the Vsp was determined by IHC and densitometry of light microscopy-stained sections (**Figure 3G**). Note that the fluorescent-stained sections in **Figure 3** represent examples only. Overall treatment effects indicated a significant difference for P2X7R between sham and DED rats ($F_{2,22} = 3.25$, $p < 0.025$). Individual comparisons revealed that P2X7R at Vo and Vc from OvXE rats had a higher expression at 14 days post-surgery compared to OvXE sham rats ($p < 0.05$), while P2X7R expression was not significantly different between OvXE and either OvX or males at 14 days post-surgery (**Figure 3G**). The expression levels of P2X7R in Vsp subnuclei of OvX and male DED rats were not significantly different from the corresponding sham groups. To determine whether gland removal specifically affected microglia expression of purinergic receptor subtypes, microglia were isolated from whole Vsp from male, OvX, and OvXE rats at 2 and 14 days post-surgery and from sham controls by cell sorting and then analyzed for the expression of P2X7R and P2X4R by real-time PCR (**Figure 4**). The expression of P2X4R was increased in 2 days and 14 days DED rats in all the groups

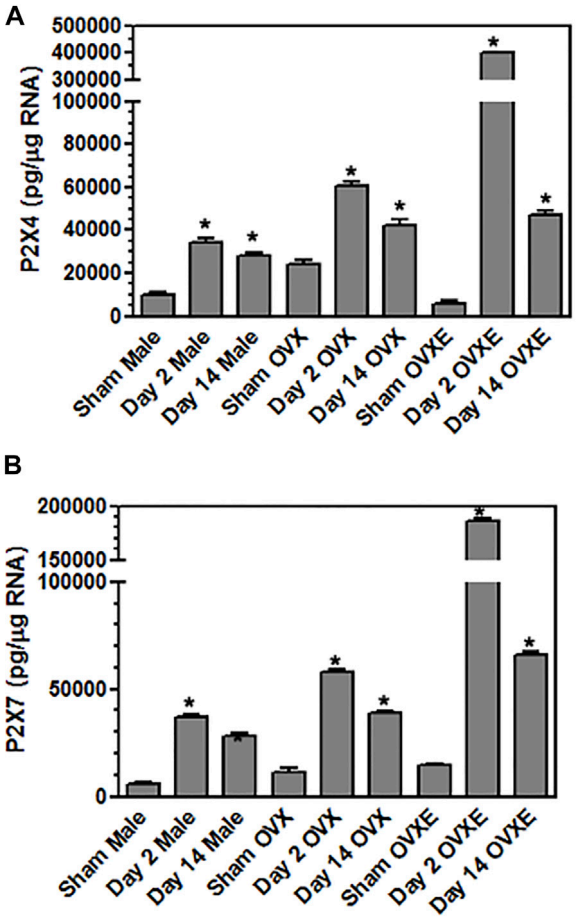

FIGURE 4 | Microglia express purinergic receptors in an estrogen dependent manner during DED. Male, OVX, and OVXE rats had the left exorbital gland removed or were sham treated and at 2 and 14 days post-surgery, rats were perfused with PBS, trigeminal brainstem was removed and tissue dissociated prior to separation on a 70/30 percoll gradient. The mononuclear cells were incubated with fluorescently labeled antibody for CD45 and CD11b. The microglia were sorted on a cell sorter based on $CD45^{intermediate}CD11b^+$ cells. The isolated microglia were lysed, RNA isolated, converted to cDNA, and analyzed by real time PCR for expression of **(A)** P2X4, **(B)** P2X7. Significant differences were determined by one-way ANOVA and Bonferroni's multiple comparison test (*$p < 0.001$) based on sham rat microglia for the corresponding group (mean ± SEM). $N = 8$ rats per group. Data presented as mean ± SEM.

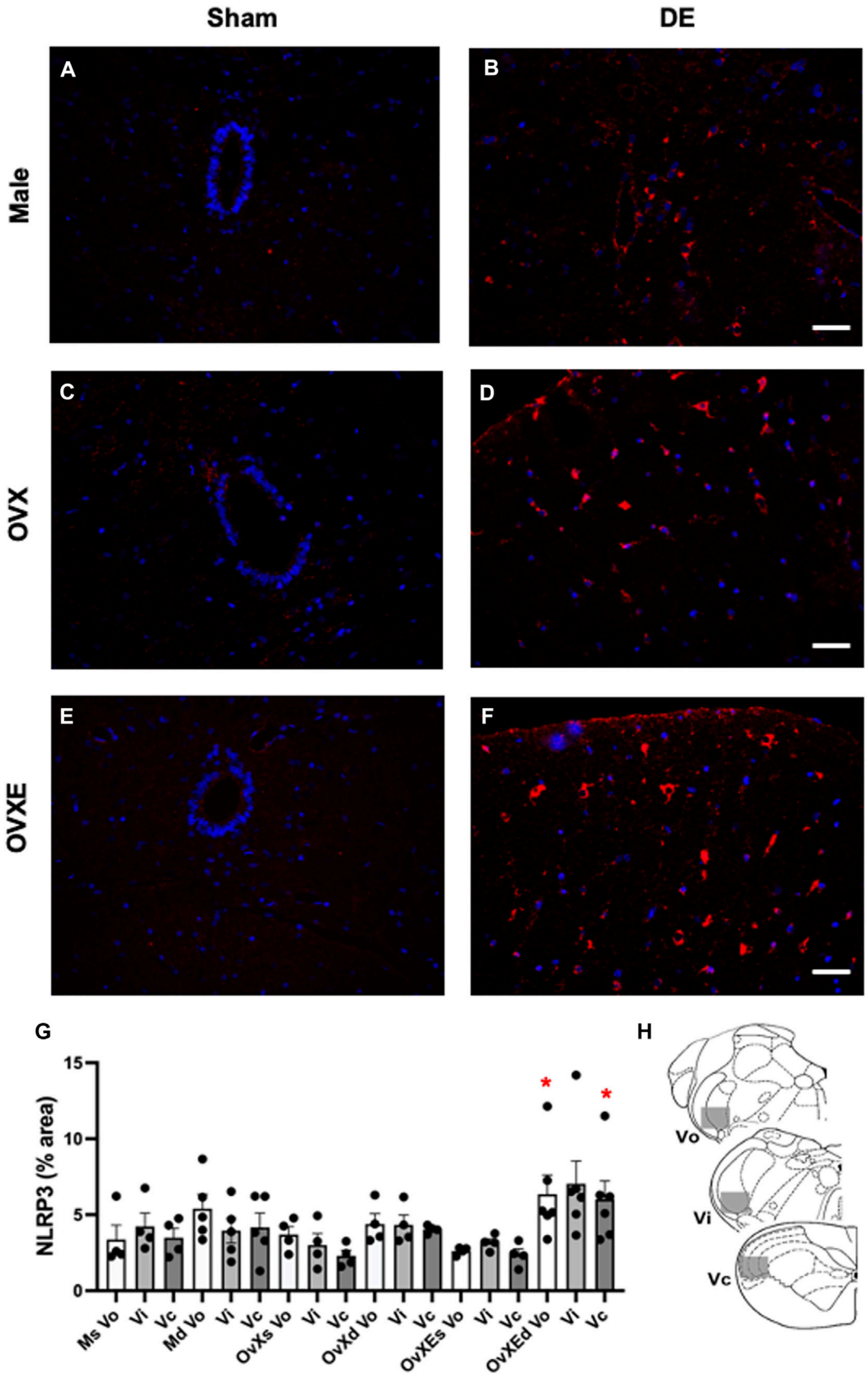

FIGURE 5 | Microglia have increased NLRP3 expression in the brainstem during DED. Male, OVX, and OVXE 14 days DED rats **(B,D,F)** and sham rats **(A,C,E)** were

(*Continued*)

FIGURE 5 | perfused with 4% PFA, fixed and brainstem sections stained with anti-Iba1 (red) and dapi (blue). The micrographic examples are of trigeminal subnucleus caudalis (Vc). Scale bar = 80 μm. **(G)** Densitometry was conducted on light microscopy-stained sections at Vo, Vi, Vc regions using ImageJ on four sections per rat with four rats/group (mean ± SEM) Abbreviations: Ms, male sham; Md, male DED; OvXs, OvX sham; OvXd, OvX DED; OvXEs, OvXE sham; OvXEd, OvXE DED. *$p < 0.05$ versus sham group. **(H)** Shaded areas represent regions of trigeminal brainstem that were sampled. $N = 4$ rats per group.

with the highest level seen under high estrogen conditions (OvXE) (**Figure 4A**). Similarly, P2X7R expression was increased at 2 days in all DED groups and at 14 days post-surgery in OvX and OvXE, with OvXE rats displaying the highest level of expression (**Figure 4B**). Overall, microglia displayed increased expression of the purinergic receptors P2X4 and P2X7 at 2 days post-surgery and remained elevated in 14 days DED OvX and OvXE rats. Notably, P2X7R expression was highest in the OvXE group at both 2 and 14 days after gland removal.

Microglia become activated to express cytokines and effector molecules in response to stimuli from their environment. P2X7R is critical for nucleotide-binding domain-like receptor 3 (NLRP3) inflammasome assembly and subsequently for mature IL-1β release. To determine the effect of reduced tear volume on NLPR3 expression Vsp tissue sections from the Vo, Vi, and Vc regions were examined by immunohistochemistry (**Figure 5**). Overall treatment effects indicated differences between sham and DED rats ($F_{5,21} = 3.72$, $p < 0.025$). Individual comparisons revealed that microglia at Vo and Vc levels of Vsp from OvXE rats had a higher expression of NLRP3 at 14 days post-surgery compared to sham rats ($p < 0.05$), while NLRP3 was only marginally increased in OvX and males at 14 days post-surgery (**Figure 5G**). To determine whether microglia activated during DED express pro-inflammatory cytokines and effector molecules, which may promote neuroinflammation associated with pain, microglia were isolated from the trigeminal brainstem at 2 and 14 days post-surgery. Microglia were analyzed by real-time PCR for the expression of the pro-inflammatory cytokines, IL-1β and TNFα; inducible nitric oxide (iNOS) and brain-derived neurotrophic factor (BDNF); NLRP3 inflammasome; and Toll-like receptor 4 (TLR4) (**Figure 6**). An increase in NLRP3 expression by isolated microglia was seen at 2- and 14-day post-surgery in all groups, while the OvXE group had the highest level of expression (**Figure 6A**). Microglia displayed increased expression of IL-1β at 2 and 14 days DED in male, OVX, and OVXE groups, with greatest increase in expression in OvXE rats (**Figure 6B**). Microglia displayed a marked increase in the expression of effector molecule, iNOS, at 2 and 14 days DED in all the groups, with the highest increase observed in microglia from OvXE rats (**Figure 6C**). Toll-like receptor 4 (TLR4) expression on microglia was increased at 2 days DED and at 14 days DED with the highest expression in OvXE rats (**Figure 6D**). Although microglia also displayed significant increases the expression of tumor necrosis factor alpha (TNFα) and BDNF during DED, the overall expression levels were low (**Figures 6E,F**). The increase in NLRP3 expression was associated with the increased expression of IL-1β during DED. Densitometry analyses for GFAP, a marker for astrocytes, revealed no change in expression in DED rats (range = 3.95–5.33% area) compared to sham animals (range = 2.55–4.71% area) ($F_{5,22} = 1.49$, $p > 0.1$). Overall, these results revealed that microglia became activated in the trigeminal brainstem during DED to express cytokines and effector molecules which promote neuroinflammation.

Inhibition of Microglia or P2X7R Activation Reduces OOemg Activity

Three approaches were used to determine if inhibiting the activation of microglia or P2X7R affected ocular hyperalgesia as seen by changes in orbicularis oculi muscle electromyography (OOemg) evoked by HS. *Systemic minocycline.* In the first approach, the non-selective glial cell inhibitor, minocycline, was administered systemically daily for 4 days (40 mg/kg) prior to recording and OOemg activity was evoked by instillation of hypertonic saline (HS) in sham and 14 days DED rats. As seen in **Figure 7A**, males displayed significant increases in HS-evoked OOemg under sham ($F_{2,38} = 14.7$, $p < 0.001$) and DED conditions ($F_{2,38} = 94.7$, $p < 0.001$). Minocycline did not affect the OOemg responses in sham males ($F_{1,9} = 0.15$, $p > 0.1$), whereas evoked responses in DED males were greatly reduced ($F_{1,10} = 14.44$, $p < 0.001$). As seen in **Figure 7B**, OvX females also displayed significant increases in HS-evoked OOemg under sham ($F_{2,36} = 14.8$, $p < 0.001$) and DED conditions $F_{2,36} = 121$, $p < 0.001$). Minocycline did not affect the OOemg responses in sham OvX females ($F_{1,9} = 3.2$, $p < 0.1$), whereas the responses in DED OvX females were greatly reduced ($F_{1,9} = 14.75$, $p < 0.001$). Similarly, OvXE females displayed significant increases in HS-evoked OOemg under sham ($F_{2,36} = 12.1$, $p < 0.001$) and DED conditions ($F_{2,36} = 205$, $p < 0.001$, **Figure 7C**). Unexpectedly, minocycline treatment caused a small increase in HS-evoked OOemg responses in sham OvXE females compared to untreated OvXE females ($F_{1,9} = 12.6$, $p < 0.01$); however, OOemg responses in DED OvXE females were greatly reduced ($F_{1,9} = 42.18$, $p < 0.001$), although responses remained elevated compared to untreated OvXE females. These data indicated that minocycline significantly reduced HS-evoked OOemg activity in males as well as in females under low and high estrogen conditions in DED rats.

Local microinjection of a specific antagonist for P2X7R. The specific antagonist for P2X7R, A804598 (10 μM, 0.2 μL), was injected into the Vc ipsilateral to exorbital gland excision. As seen in **Figure 8A**, A804598 markedly reduced HS-evoked OOemg in males ($F_{7,72} = 16.97$, $p < 0.001$), whereas responses in sham animals were not affected. As seen in **Figure 8B**, HS-evoked OOemg was markedly reduced in OvX females ($F_{7,72} = 27.38$, $p < 0.001$) and the reduction in evoked OOemg after Vc injection of A804598 was restricted to DED animals, while responses in sham animals were not affected. Similarly, HS-evoked OOemg was markedly reduced in OvXE DED rats ($F_{7,72} = 50.73$, $p < 0.001$), whereas the evoked responses in sham animals was not affected

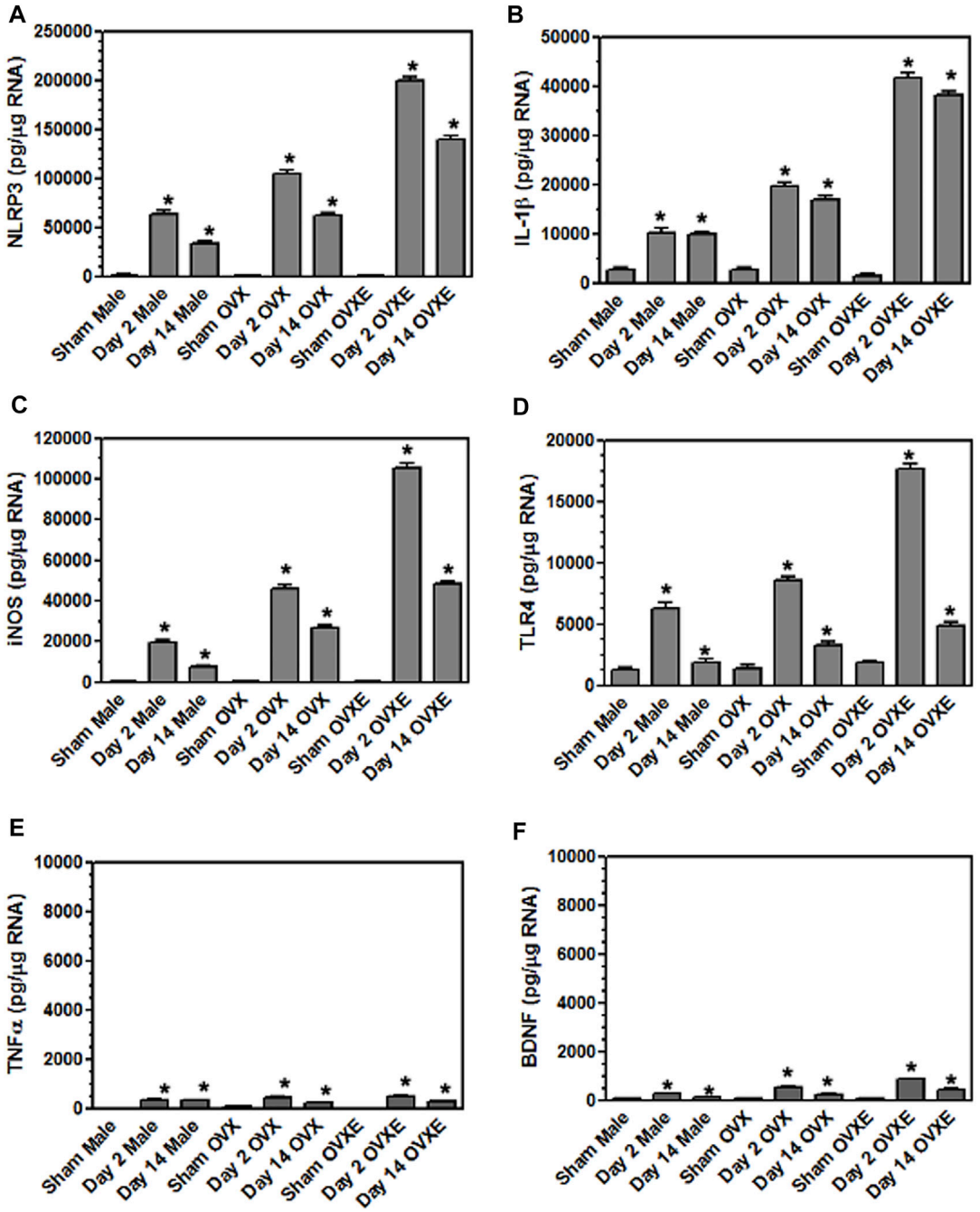

FIGURE 6 | Microglia express inflammatory cytokines and effector molecules in an estrogen dependent manner during DED. Male, OVX, and OVXE rats had their exorbital glands removed or sham treated. At 2 days and 14 days post-surgery, brainstem tissue was prepared and analyzed by real time PCR as in **Figure 4** for expression of: **(A)** IL-1β, **(B)** NLRP3, **(C)** iNOS, **(D)** TLR4, **(E)** TNFα, and **(F)** BDNF. $p < 0.001$ versus sham group. $N = 8$ rats per group.

(**Figure 8C**). The magnitude of the drug-induced inhibition of evoked OOemg was significantly greater for OvXE DED animals compared to that seen for male or OvX females ($F_{5,180} = 34.4$, $p < 0.001$).

To determine whether A804598 would reduce microglia activation in DED or in sham male, OvX, and OvXE rats were administered A804598 (10 mg/kg/day × 4 days, sc) and at 14 days post-surgery, microglia were isolated from the trigeminal brainstem. Isolated microglia were analyzed for the expression of purinergic receptors, P2X4R and P2X7R, IL-1β, NLRP3, and iNOS, by real-time PCR (**Figure 9**). A804598 significantly reduced the expression of P2X4R and P2X7R by isolated

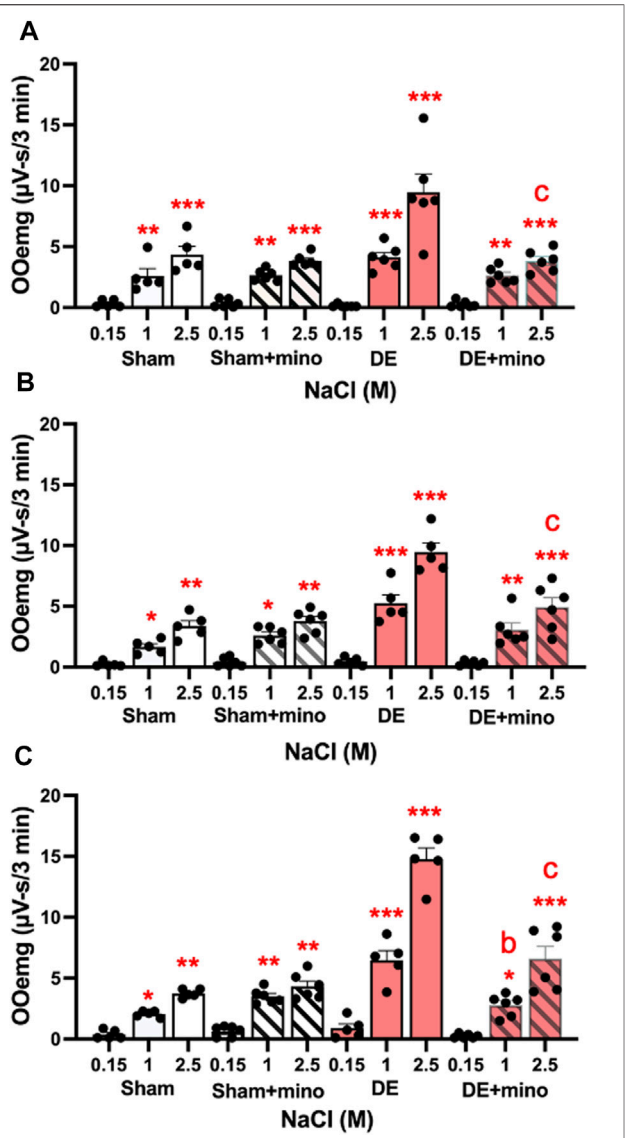

FIGURE 7 | Minocycline reduces HS-evoked OOemg activity in DED males and females. Minocycline (40 mg/kg/day, sc, X 4 days) significantly reduced the enhanced HS-evoked OOemg activity recorded 14 days after exorbital gland removal. Test stimuli = ocular instillation of NaCl (0.15, 1.0, 2.5 M). Test stimuli applied at 30 min intervals. **(A)** Males; **(B)** OvX; **(C)** OvXE. Open bars = sham; open shaded bars = sham + minocycline; open red bars = 14 days DED; red shaded bars = 14 days DED + minocycline. *p < 0.05, **p < 0.01, ***p < 0.001 versus 0.15 M NaCl; b = p< 0.01; c = p< 0.001 versus pre-drug. Sham, n = 5-6 rats per group; DED, n = 6 per group. Data presented as mean ± SEM.

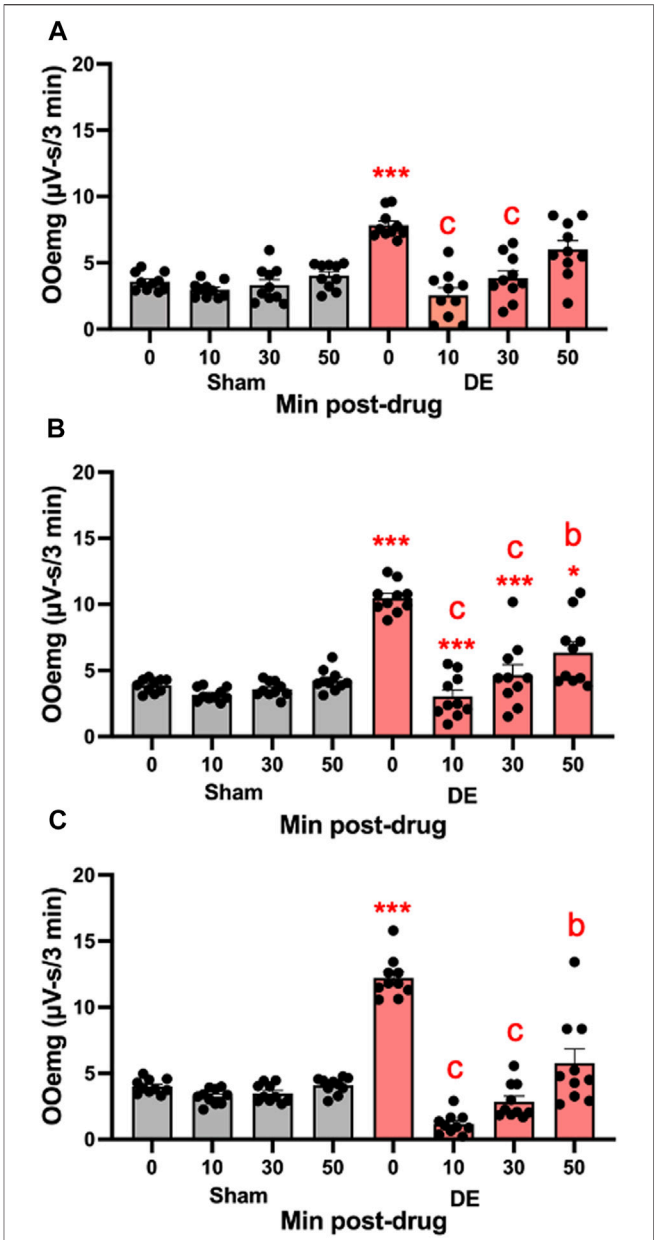

FIGURE 8 | Local inhibition of P2X7R in Vc reduces HS-evoked OOemg activity during DED. The selective P2X7R antagonist (A804598) was microinjected (10 μM, 0.2 μL) into the Vc of sham and 14 days DED rats 30 min prior to the test stimulus (ocular instillation of 2.5 M NaCl). **(A)** Males, **(B)** OvX females, **(C)** OvXE. Gray bars = sham; red bars = 14-day DED. *p < 0.01, p < 0.001 versus 0.15 M NaCl; b = p< 0.01, c = p< 0.001 versus pre-drug. N = 10 rats per group.

microglia in all DED groups. The expression of P2X4R and P2X7R in OvX and OvXE DED groups was reduced to sham levels, whereas male DED rats displayed smaller reductions compared to sham males. The P2X7R antagonist also decreased the expression of IL-1β, NLRP3, and iNOS by microglia in male, OvX, and OvXE DED rats. These results demonstrated that inhibition of the P2X7R reduced the activation of microglia in male, OvX, and OvXE DED rats.

Transcriptional inhibition of P2X7R in TG reduces HS-evoked OOemg activity. The third approach addressed the role of peripheral P2X7R in ocular hyperalgesia. In the TG P2X7R is expressed mainly by satellite glia that surround neurons. To determine the effect of local peripheral inhibition of P2X7R, siRNA for P2X7R was microinjected into the left TG and 3 days later (i.e., 11 days after surgery) HS-evoked OOemg activity was

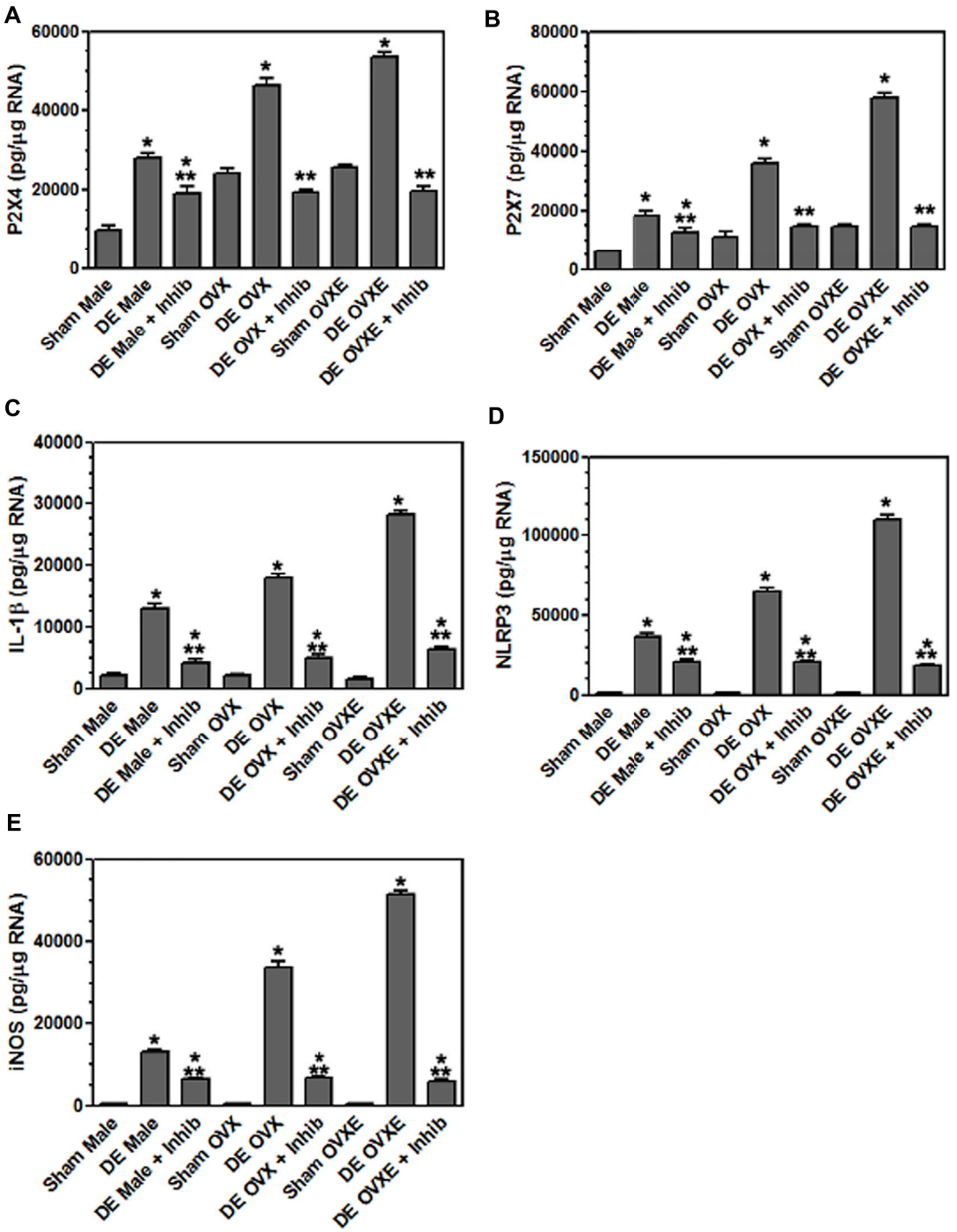

FIGURE 9 | Inhibition of P2X7 reduces inflammatory cytokine expression in microglia. Male, OVX, and OVXE rats had the left exorbital gland removed and were administered P2X7 inhibitor (A804598, 10 mg/kg, sc X 4 days) or untreated. At 14 days post-surgery, rats were perfused with PBS and microglia were isolated and analyzed as in **Figure 4**. **(A)** P2X4, **(B)** P2X7, **(C)** IL-1β, **(D)** NLRP3 and **(E)** iNOS. $^{**}p < 0.01$ versus sham group. $N = 8$ rats per group. Data presented as mean ± SEM.

recorded. As seen in **Figure 10A**, siRNA for P2X7R caused a significant reduction in HS-evoked OOemg in male DED rats, while responses in sham males were not affected ($F_{3,16} = 20.39$, $p < 0.001$). Similarly, siRNA injection in OvX females caused a significant reduction in HS-evoked OOemg in DED rats, whereas responses in sham OvX females were not different from non-injected rats ($F_{3,16} = 20.38$, $p < 0.001$, **Figure 10B**). As seen in **Figure 10C**, there was a marked reduction in HS-evoked OOemg responses in OvXE DED rats after siRNA injection, whereas responses in sham OvXE rats were similar to non-injected sham

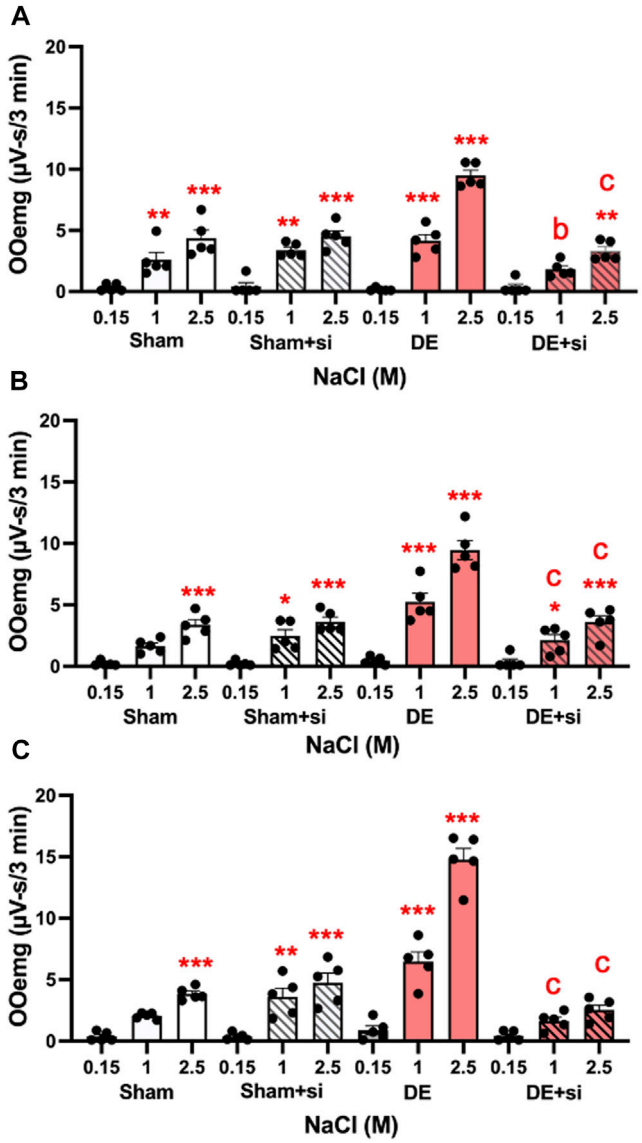

FIGURE 10 | Knockdown of peripheral P2X7R by siRNA injection into TG reduces HS-evoked OOemg in DED rats. siRNA for P2X7R inhibits TMJ-evoked OOemg activity in: **(A)** male, **(B)** OvX and **(C)** OvXE females 3 days prior to recording. Note that responses to HS-evoked OOemg responses in sham rats were not affected. Open bars = sham; open shaded bars = sham + siRNA: Open red bars = 14 days DED; open red shaded bars = 14-day DED + siRNA. *$p < 0.05$, **$p < 0.01$ versus 0.15 M NaCl stimulation; $a = p < 0.05$, $b = p < 0.01$ siRNA treated versus untreated rats. $N = 5$ rats per group. Data presented as mean ± SEM.

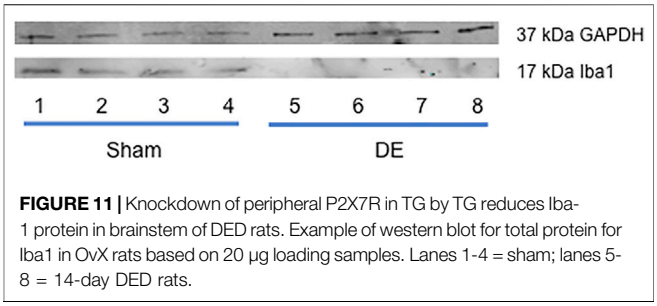

FIGURE 11 | Knockdown of peripheral P2X7R in TG by TG reduces Iba-1 protein in brainstem of DED rats. Example of western blot for total protein for Iba1 in OvX rats based on 20 μg loading samples. Lanes 1-4 = sham; lanes 5-8 = 14-day DED rats.

OvXE rats ($F_{3,16} = 44.38$, $p < 0.001$). Note also that siRNA injection in all DED groups significantly reduced the OOemg responses to 1 M NaCl, an osmolar concentration similar to that found at the center of the ocular surface in severe DED patients (Liu et al., 2009). Knockdown of P2X7R was confirmed by western blot analyses. Intra-TG injection of siRNA markedly reduced P2X7R protein levels in the TG of DED males compared to vehicle injected DED males (0.137 ± 0.003 versus 1.589 ± 0.245 relative intensity, $F_{1,6} = 43.2$, $p < 0.001$). Similarly, TG injection of siRNA in DED OvXE females was greatly reduced compared to vehicle injected OvXE females (0.009 ± 0.001 versus 0.213 ± 0.027 relative intensity) ($F_{1,6} = 53.43$, $p < 0.0001$). To determine the effect of intra-TG injection of siRNA for P2X7R on microglia activation in trigeminal brainstem, Vsp tissues were collected from OvX females for western blot analyses of Iba-1 (**Figure 11**). These results demonstrated that intra-TG injection of siRNA for P2X7R in DED rats caused a marked reduction in protein levels of Iba-1 compared to vehicle injected DED OvX females (0.098 ± 0.003 versus 0.605 ± 0.053, mean ± SEM, relative intensity) ($F_{1,6} = 89.14$, $p < 0.0001$).

DISCUSSION

The present study used a rat model for aqueous deficient DED to address two unresolved issues concerning ocular hyperalgesia and the contribution of neuroimmune responses. First, does activation of P2X7R in trigeminal pain pathways play a role in ocular hyperalgesia and immune cell activation? Secondly, does estrogen status play a significant role in ocular hyperalgesia and immune cell activation? These results revealed that activation of P2X7R in the TG and Vsp contributed to enhanced ocular surface-evoked nociceptive behavior and neuroimmune function in DED rats. Estrogen treatment further enhanced ocular hyperalgesia and microglia activation in a P2X7R-dependent manner in female DED rats.

Tear hyperosmolarity and loss of tear film integrity are diagnostic features of DED (Baudouin et al., 2013; Craig et al., 2017; Willcox et al., 2017). Exorbital gland excision is a valid model for aqueous deficient DED which results in a ~40% reduction in tear volume (Fujihara et al., 2001; Meng et al., 2015; Rahman et al., 2015; Mecum et al., 2019) and elevates levels of pro-inflammatory molecules in the anterior eye segment (Joossen et al., 2016), consistent with clinical signs seen in DED patients (Na et al., 2012; Nicolle et al., 2018). A challenge for preclinical studies of ocular pain is the ability to measure behaviors that can be reasonably interpreted as eye pain in humans. Several methods have been used to estimate chronic ocular hyperalgesia in animals: spontaneous and evoked eyeblink rates, palpebral opening and forelimb eye wiping to ocular surface stimulation (de Castro et al., 1998; Bates et al., 2010; Yorek et al., 2016; Fakih et al., 2019; Mecum et al., 2020). Recently, we reported that HS-evoked OOemg activity was a reliable surrogate for ocular hyperalgesia in anesthetized male rats

(Rahman et al., 2017). HS-evoked long duration, squint-like OOemg activity that was enhanced in DED rats, corresponded well to cornea-evoked eye wiping behavior and involved TRPV1 activation, results that were consistent with the notion of HS-evoked OOemg as a nociceptive behavior (Bereiter et al., 2018).

The initiation and maintenance of nociceptive behavior in preclinical studies of DED likely involves peripheral and central neural mechanisms. In the periphery hyperosmolar stress induces an increase in ATP levels in the tears of DED patients which is the preferred nucleotide for activation of P2X7R (Guzman-Aranguez et al., 2017). P2X7R is closely linked to inflammation and is necessary for NLRP3 inflammasome assembly and the production and release of proinflammatory cytokines such as IL-1β (Di Virgilio et al., 2017). P2X7R is expressed by satellite glia that surround TG neurons and mediates crosstalk between neurons and non-neural cells in the TG (Belzer et al., 2011; Nowodworska et al., 2017). The present study found that microinjection of siRNA for P2X7R into the TG markedly reduced HS-evoked OOemg activity and Iba-1 expression by microglia in trigeminal brainstem of DED rats. P2X7R also is expressed by corneal and conjunctival epithelial cells (Minns et al., 2016) and NLRP3 suggesting that increased levels of proinflammatory cytokines derive from multiple sources in DED (Zheng et al., 2014). P2X7R also is expressed by goblet cells and is critical for mucin secretion suggesting a role in mechanical transduction by corneal nociceptors (Puro 2021). Loss of lubrication at the ocular surface leads to increased friction during lid wiping and subsequent enhanced corneal nociceptor activation during periods of increased blinking (van Setten 2020). These data suggest that loss of tear film integrity induces P2X7R activation in the eye and TG which, through increased secretion of proinflammatory molecules, contributes to ocular symptoms and neuroimmune responses in DED. It noteworthy that P2X7R requires a higher concentration of ATP for activation and displays limited desensitization compared to other purinergic receptor subtypes suggesting the contribution to DED symptoms and ocular surface homeostasis may be greatest under conditions of moderate to severe inflammation.

Microglia are resident immune cells in the CNS and play a critical role in maintenance of chronic pain (Chen et al., 2018; Inoue and Tsuda 2018). Although microglia display rapid and persistent activation following even brief periods of nociceptor activity (Hathway et al., 2009; Gruber-Schoffnegger et al., 2013), the mechanisms for microglia involvement in chronic pain are not completely known. We confirmed that the expression of P2X7R and Iba-1 derived from resident microglia and not from infiltrating monocytes by flow cytometry and cell sorting followed by qRT-PCR analyses. These results agreed with previous preclinical studies of neuropathic and inflammatory pain in which spinal cord samples were analyzed flow cytometry and found no evidence of monocyte infiltration (Denk et al., 2016; Lopes et al., 2017; Fernandez-Zafra et al., 2019). In the present study, isolated microglia also expressed elevated levels of P2X4R in Vsp tissue samples at 2 and 14 days after exorbital gland removal. Although P2X7 and P2X4 do not normally form heterotrimers, immunoprecipitation studies indicate interaction of purinergic subtypes may act to influence immune cell function (Boumechache et al., 2009; Sakaki et al., 2013; Perez-Flores et al., 2015). Thus, it cannot be excluded that P2X4 as well as P2X7 may have contributed to the increase ocular hyperalgesia and expression of proinflammatory cytokines in this model of DED. Given the differences in threshold concentrations of nucleotides necessary to activate P2X4 and P2X7, microglia that express both subtypes could be expected to respond to a greater range of concentrations and patterns of molecular signals following nociceptor stimulation (Kato et al., 2016). P2X7R activation in microglia induces multiple downstream signaling pathways and is sufficient to cause long-term potentiation of dorsal horn neurons (Chu et al., 2010; Kronschlager et al., 2016). iNOS expression also was markedly increased in isolated microglia from Vsp tissue samples which was consistent with the involvement of glutamatergic pathways (Freire et al., 2009; Gruber-Schoffnegger et al., 2013).

The second goal of this study was to determine whether estrogen status played a significant role in ocular hyperalgesia and neuroinflammation in DED. These results strongly indicated that acute estrogen treatment in ovariectomized female rats enhanced HS-evoked OOemg activity and the expression of P2X7R in the Vsp. Densitometry of Vsp tissue sections revealed that Iba-1, NLRP3 and P2X7R positive areas were greater in Vc of OvXE DED groups than in sham OvXE rats. The Vc receives a significant direct input from TG neurons that supply the ocular surface (Marfurt and del Toro 1987; Marfurt and Echtenkamp 1988; Panneton et al., 2010). The Vc also displayed a high number of estrogen receptor positive neurons compared to other portions of the Vsp (Bereiter et al., 2005; VanderHorst et al., 2005). Estrogen treatment in DED rats induced significantly higher levels of expression by isolated microglia from whole Vsp samples for P2X7R, P2x4R, NLRP3, IL-1β, TLR4 and iNOS compared to sham OvXE rats or to OvX or male animals. This suggested an interaction between estrogen treatment and loss of tear volume which increased the level of inflammatory molecules compared to either treatment alone. P2X7, together with P2X4 and Toll-like receptors (TLRs), mediates assembly of the NLRP3 inflammasome which leads to activation of caspase-1 and cleavage of pro-IL-1β into mature IL-1β. Although TNFα and BDNF expression also were increased in isolated microglia of DED rats, these expression levels were much lower than for other pro-inflammatory molecules. To determine if P2X7R activation in the Vsp altered ocular hyperalgesia in an estrogen-dependent manner, the selective P2X7R antagonist, A804598, was microinjected into the Vc in DED and sham rats. These results revealed a significant reduction in HS-evoked OOemg in all DED groups and only minor changes in sham animals. To determine whether the A804598-induced reduction in HS-evoked OOemg activity in DED rats depended on P2X7R, the drug was administered systemically for 4 days prior to tissue collection. Isolated microglia displayed significant reductions in P2X7R, P2x4R, NLRP3, IL-1β and iNOS in all DED groups. These data suggested that P2X7R activation was necessary for enhanced ocular hyperalgesia in DED.

Several studies have reported that intrathecal injection of minocycline reduced mechanical hyperalgesia in males but not female rodents in nerve injury and arthritic pain models (Sorge et al., 2015; Mapplebeck et al., 2018; Fernandez-Zafra et al., 2019). By contrast, we found that systemic minocycline reduced HS-evoked OOemg activity of DED rats of both sexes. Several methodological differences may have contributed to this discrepancy. First, our DED model did not involve direct nerve injury, but rather resulted in changes in tear film integrity and corneal sensitivity that developed over several days (Meng et al., 2015). Second, previous studies applied minocycline as a single intrathecal injection, while we administered minocycline systemically in four daily doses. Interestingly, in a model for bone cancer which also develops slowly, intrathecal administration of minocycline effectively reduced mechanical hyperalgesia in female rats (Yang et al., 2015). Lastly, we cannot exclude that estrogen treatment given as a single injection to OvX female rats the day before OOemg recording or tissue collection may have led to results that may have differed from those seen in normal cycling female rats.

These data indicated that P2X7R activation in peripheral and central sites in the trigeminal pain pathway was a significant factor in mediating enhanced ocular hyperalgesia and neuroinflammation in this model for aqueous deficient DED. Estrogen status likely played a significant role in mediating the magnitude of evoked OOemg activity and markers for neuroinflammation in DED.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Minnesota IACUC #1802-35557A.

AUTHOR CONTRIBUTIONS

MR, RT, JO, and DB designed research; JO, MR, FA, NL, and RT conducted experiments; JO, MR, RT, and DB analyzed results; DB, JO, and MR wrote the paper.

FUNDING

Publications fees paid by internal funds from the School of Dentistry, University of Minnesota. This project was supported by grant from the National Eye Institute (EY 028143) to DAB from the Department of Defense (MS 160141) to JO.

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