Brain Function Activity Changes and Contribution of Neuroinflammatory Factors in Insular Cortex of Mice with Dry Eye-Related Chronic Corneal Pain.

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Abstract

Purpose:Chronic corneal pain is the most common symptom of dry eye disease (DED), while the central sensitization mechanisms underlying remain unclear. Methods:Excision of extra orbital lacrimal glands was used to establish dry eye (DE) model. Tear volume measurements, corneal fluorescein staining, corneal hypersensitivity and anxiety behavior were tested after surgery. The amplitude of low-frequency fluctuation (ALFF) by fMRI was used for determining brain functional activity. C-Fos, Brain-derived neurotrophic factor (BDNF), and cytokine levels in corresponding brain regions were tested. Results:Compared to the Sham group, the ALFF signals in the supplemental somatosensory area, secondary auditory cortex, agranular insular cortex, temporal association areas, and ectorhinal cortex brain areas were enhanced in DE group. ALFF signal in the insular cortex was related to corneal hypersensitivity ($p < 0.01$). C-Fos ($P < 0.001$), BDNF ($P < 0.01$), TNF-α, IL-6 and IL-1β ($P < 0.05$) increased, while IL-10 levels ($P < 0.05$) decreased in the insular cortex in the DE group. Surgery-induced corneal hypersensitivity and upregulation of inflammatory cytokines, but not anxiety, could be blocked by insular cortex injection of Tyrosine Kinase receptor B (TrkB) agonist cyclotraxin-B ($P< 0.01$). Conclusions :This research presents the map of functional brain by ALFF through rs-fMRI associated with chronic corneal pain. BDNF-TrkB signaling-related neuroinflammation in the insular cortex might contribute to dry eye-related chronic corneal pain. This measure could potentially help clinicians improve therapeutic approach to pain control and development of diagnostic approach.

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ABSTRACT :

Purpose: Chronic corneal pain is the most common symptom of dry eye disease, while the central sensitization mechanisms underlying remain unclear.

Methods: Excision of extra orbital lacrimal glands was used to establish dry eye model. Corneal hypersensitivity and anxiety behavior were tested after surgery. The amplitude of low-frequency fluctuation was used for determining brain functional activity. C-Fos, Brain-derived neurotrophic factor, and cytokine levels in corresponding brain regions were tested.

Results: Compared to the Sham group, the amplitude of low-frequency fluctuation signals in the supplemental somatosensory area, secondary auditory cortex, agranular insular cortex, temporal association areas, and ectorhinal cortex brain areas were enhanced in dry eye group. Amplitude of low-frequency fluctuation signal in the insular cortex was related to corneal hypersensitivity ($p < 0.01$). C-Fos ($P < 0.001$), brain-derived neurotrophic factor ($P < 0.01$), TNF-α, IL-6 and IL-1β ($P < 0.05$) increased, while IL-10 levels ($P < 0.05$) decreased in the insular cortex in the dry eye group. Surgery-induced corneal hypersensitivity and upregulation of inflammatory cytokines, but not anxiety, could be blocked by insular cortex injection of Tyrosine Kinase receptor B agonist cyclotraxin-B ($P< 0.01$).

Conclusions : This research presents the map of functional brain by amplitude of low-frequency fluctuation associated with chronic corneal pain. Brain-derived neurotrophic factor-Tyrosine Kinase receptor B signaling-related neuroinflammation in the insular cortex might contribute to dry eye-related chronic corneal pain. This measure could potentially help clinicians improve therapeutic approach to pain control and development of diagnostic approach.

Keywords : Dry eye disease, chronic corneal pain, central sensitization, insular cortex, amplitude of low-frequency fluctuation

Introduction

Dry eye disease (DED) affects 5%-55% of people worldwide[1]. Chronic corneal pain is the most common symptom of DED, including burning, aching, dryness, and itching[2]. Although chronic corneal pain often falls outside the scope of DED therapy, reduces work productivity, impacts the quality of life and even psychological statement[3], the underlying mechanism of chronic corneal pain related to DED remains unclear.

Interestingly, the corneal pain symptom severity does not correlate well with ocular surface findings, suggesting the pain symptoms might amplify during transferring to the higher nervous system. Recently, several studies have revealed central sensitization and neuroinflammation may contribute to the development of chronic corneal pain of DED[4]. The abnormal sensory message transmitted to the central nervous system (CNS) may affect the excitability of sensory neurons in associated brain regions and induce chronic pain symptoms. However, the representative brain areas of chronic corneal pain and cellular and molecular mechanisms have not been well screened and clarified. Rs-fMRI based on the blood oxygenation level-dependent

(BOLD) signal could approve a novel approach to detect spontaneous neuronal activity in different brain regions. The amplitude of low-frequency fluctuation (ALFF) is an index of low-frequency oscillations representing the BOLD signal fluctuations in the gray matter. It features an optimal balance between test-retest reliability and replicability of all rs-fMRI indicators[5,6]. C-Fos has been reported as a marker of neuronal activity in specific areas of brain in pain models[7]. Thus, using ALFF and c-Fos to evaluate the differences in neuronal activity of brain between chronic corneal pain model and the sham may provide novel information about central sensitization mechanisms related to chronic corneal pain.

In the present study, we established a model of DED by excising extra orbital glands and confirmed the changes in mechanical and chemical pain thresholds. Meanwhile, rs-fMRI was performed to investigate the alternation of brain functional activity related to chronic corneal pain. Next, c-Fos was tested in higher ALFF value brain regions. Finally, brain derived neurotrophic factor (BDNF) and the secretion of proinflammatory molecules in higher ALFF value brain regions were also explored to determine their contribution to the central sensitization mechanism. Our results provide a novel insight into the contribution of CNS to chronic corneal pain.

Materials & methods

Animals

All experiments were conducted by the animal protection adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and use policies of Fudan University. Ethical number: Animal Care and Use Committee of Fudan University A2020-017. Adult C57BL/6 male mice (average weight 20.95 ± 0.05 g) (Shanghai laboratory animal center, Shanghai, China) aged 6–8 weeks were fed in cages at 25°C± 1 °C, 50 ± 10% relative humidity, under a 12-h light-dark cycle (8 am to 8 pm), free access to food and water.

Mouse dry eye model

The mice were anesthetized and the skin under the ear was disinfected with iodine. We use ophthalmic scissors to make 5-7mm incisions under the ears. The exorbital lacrimal gland was isolated gently and removed. The skin was sutured with 5–0 non-absorbable suture silk thread (ETHICON, China). The same procedure was done to the Sham group without extraorbital lacrimal gland removal.

Corneal sensitivity test

Mice were gently immobilized by the scruff in hand. Chemical corneal sensitivity was tested by pipetting 2.5 μl of 2μM capsaicine or 200 μM menthol vertically into the right eye using a micropipette. Blinking times were counted for 30s by experimenter without prior knowledge of surgical or sham. The application of different chemical requires an interval of 15 minutes. Mechanical sensitivity was tested by von Frey filaments of 0.008 g and 0.02 g. Von Frey filaments were applied in the center of each eye five times until slightly bending every five seconds, in the morning and afternoon.

Open field test

The open field chamber was made of resin plastic (40cm × 40cm× 35cm), and a 20 cm × 20 cm center square was marked. After acclimation to test room for 60 minutes, mice were allowed 15 min to freely explore the open field. Then, mice were placed in the center of the chamber, behavior was monitored for 5 min with an overhead video-tracking system and analyzed by video tracking system (Smart 3.0, Spanish).

MRI examination

MRI experiments were performed with an 11.7T small animal system (Bruker BioSpec 117/16) with a 6-mm 4-channel surface array coil. Mice were anesthetized and positioned in the scanner with 0.5% oxygen and 1.5% isoflurane. Temperature was maintained at 36.0 ± 1.0 °C, and blood oxygen saturation values were maintained at 96–99%. Standard adjustments included the calibration of the reference frequency power and the shim gradients using MapShim (Paravision v6.1). First, T2-weighted images (T2WI) were acquired as

the anatomical reference using a spin echo sequence[8]. For rs-fMRI acquisition, standard GE-EPI was applied. The acquisition parameters and data preprocessing were as follows: field of view = 18 × 18 cm, matrix = 90 × 90, repetition time = 2000 ms, echo time = 12.31 ms, number of slices = 45, slice thickness = 0.3 mm, volumes = 240, and acquisition time = 8 min. The whole brain was covered.

Fluorescent immunohistochemistry

Mice were deeply anesthetized and perfused transcardially with 20 ml of cold heparinized saline followed by 20 ml of 4% paraformaldehyde. Brains were removed and immersed in paraformaldehyde and then dehydrated with 30% sucrose for 72h. 30 μm thick sections were cut on a freezing microtome. Free-floating sections were rinsed twice in phosphate-buffered saline (PBS) and twice in PBS with 0.2% Triton X-100. Sections were blocked by incubation in PBS with 1% bovine serum albumin (V900933-100G, Sigma–Aldrich, USA) and 0.2% Triton X-100 for 45min at room temperature. Then incubated with primary rabbit anti–c-Fos (1:400; 2250S, Cell Signaling Technology, USA) for 24h. After washed three times, the sections were incubated with a secondary antibody Alexa Fluor 488 donkey anti-rabbit (1:500; Invitrogen, USA) at room temperature for 2h. The samples were then rinsed in PBS for 10min; confocal images were taken with a confocal fluorescent microscope Pannoramic MIDIII (3D Histech, Hungary) and further processed using the software CaseViewer.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extraction was conducted using Tissue Total RNA Isolation Kit V2 (RC112, Vazyme, China) according to the protocol. RNA concentration was measured using A260/A280 ratio and agarose gel electrophoresis. Total RNA (1000 ng) was individually reverse transcribed into cDNA using HiScript II Q RT Super Mix (R223-01, Vazyme, China). The reaction mix was incubated for 15 minutes at 50°C and 5 seconds at 85°C. RT-qPCR was performed by ChamQ SYBR Color qPCR Master Mix (Q411, Vazyme, China). PCR was performed using a Real-Time PCR Quantification system (ABI 7500 fast; Applied Biosystems; Thermo Fisher Scientific, Inc.). The mRNA expressions were determined by analyzing data using the 2^{-CT} method, and GAPDH served as the control of mRNA.

Correlation analysis

To assess the possible relationship between abnormal ALFF values and corneal chemical sensitivity, we conducted Pearson's correlation analyses of the ALFF values and eye blinking in the 30s after capsaicine or menthol application (significance level: $P< 0.05$).

Insular cortex administration

Cyclotraxin-B (HY-P1178, MCE, New Jersey, USA) was dissolved in physiological saline and injected into insular cortex using a dual channel microinjection pump (SP200, Shenzhen Huayang Biotechnology Co., Ltd). Infusion cannulas (RWD Life Science Co., Ltd) were placed in the target insular cortex (50 nl, bregma 0.14 mm; lateral ±3.65mm; ventral -4.0mm) and cemented to the skull using dental cement (Lang Dental Manufacturing). After surgery, dummy cannulas were inserted, and caps were screwed to keep the guide cannula from becoming occluded. Mice were allowed 7 days to recover.

Statistical analysis

The statistical analyses were performed using GraphPad Prism statistical software. We performed power analysis to calculate the number of experimental mice. Data are reported as mean +- standard error of the mean (SEM). Differences between sham and surgery groups were assessed through two-sample t-tests performed in Graphpad Prism 9.0. Values of $P< 0.05$ was considered to indicate significance. To explore the ALFF between dry eye disease mice and the sham, the standardized ALFF data were analyzed with two independent samples t-tests. The correction standard was determined by Gaussian random field (GRF), with $P<0.001$ at the voxel level and $P<0.05$ at the cluster level, indicating a significant difference.

Results

Excision of extraorbital glands induced chemical and mechanical corneal hypersensitivity and anxiety-like behavior

Dry eye model was established through extraorbital lacrimal gland removal. Palpebral closure responses to ocular application of capsaicine and menthol within 30 s were significantly increased from 14 days and lasted up to 28 days (P¡0.0001, Fig. 1a-b) compared to the Sham group. The corneal mechanical threshold measured by the proportion of blink responses to von Frey filaments 0.008 g, and 0.02 g (Fig. 1 c, d) was 50% and 60% lower in the DE group. The open field test was then applied. There was no significant difference in total traveled distance between groups, while the time in the center area was significantly reduced in DE (P< 0.001, Fig. 1f, g). In general, excision of extraorbital glands induces chemical and mechanical corneal hypersensitivity and anxiety-like behavior.

FIGURE 1

Evaluation of chemical and mechanical corneal sensitivity and anxiety-like behavior of Sham and DE animals.

Resting-state fMRI reveals a whole brain ALFF signature for chronic corneal pain.

The rs-fMRI technology was performed to examine changes in brain structure and neuronal activity in chronic corneal pain mice. Mice were scanned under resting state conditions 28 days after surgery. The final t-map of ALFF (Fig. 2) revealed significantly decreased ALFF in primary motor area (Mop), secondary motor area (Mos), primary somatosensory area (SSp)-upper limb, SSp-mouth, right SSp-nose, and right olfactory areas (OLF), right hippocampal region (HIP), right retrohippocampal region (RHP), right cortical subplate (CTX)-subplate, right anterior cingulate area (ACA)-dorsal, right prelimbic area (PL), increased ALFF in the supplemental somatosensory area (S2), gustatory system (GU), visceral area (VISC), secondary auditory cortex, dorsal (AUD), secondary auditory cortex (AuV), agranular insular cortex, dorsal (AlD), agranular insular cortex, posterior (AlP), temporal association areas (TeA), perirhinal area (PERI), Ectorhinal cortex (Ect), OLF, HIP, RHP, CTX-sp, left striatum. The details of the brain regions, voxel, and peak T value in the DE and Sham groups are shown in Table 1.

FIGURE 2

ALFF value comparison in various brain regions.

TABLE 1

Brain Regions Showing ALFF Differences in DE Group Compared to the Sham.

C-Fos was significantly upregulated in insular cortex, AuV, TeA, and Ect brain areas in chronic corneal pain mice

To further confirm involved higher activated brain areas, c-Fos staining was performed. Quantitative analysis of c-Fos expression was found much stronger in some increased ALFF brain areas. The number of c-Fos positive cells was significantly increased in the DE group in the insular cortex (Fig. 3i, P = 0.0097) and AuV, TeA, Ect (Fig. 3l, P=0.0008). The c-Fos upregulated brain areas were consistent with ALFF higher changes areas, indicating brain areas involved in central sensitization of chronic corneal pain.

FIGURE 3

C-Fos immunoreactivity in insular cortex and AuV, TeA, Ect brain section of Sham and DE mice.

The levels of BDNF and inflammatory cytokines expression increased in insular cortex, AuV, TeA, and Ect regions in chronic corneal pain mice

To clarify underlying central mechanism, we further investigated the expression of BDNF and inflammatory cytokines from the insular cortex (AID, AIP, GI, DI) and AuV, TeA, Ect brain tissue. RT-qPCR showed mRNA levels of BDNF, TNF-α, IL-6, and IL-1β (Figure 4a-4c, Figure 4e) in insular cortex of DE group statistically increased compared with the Sham group (P¡0.05), and level of IL-10 (Figure 4d) was significantly reduced (P¡0.05). Meanwhile, as shown in Figure S1, expression levels of BDNF, IL-6, and IL-1β, except

TNF-α (Figure 4 f, h, j), were significantly higher. In contrast, the expression level of IL-10 (Figure 4i) was significantly lower in AuV, TeA, Ect of DE group (P¡0.05).

FIGURE 4

Expression of trophic factor (BDNF) and inflammatory factor (TNFα, IL-6, IL-10 and IL-1β) in insular cortex and AuV, TeA, Ect brain tissue on day 28 after bilateral excision of extraorbital lacrimal glands.

Correlation analysis showed ALFF values in insular cortex is related to corneal chemical sensitivity

To further examine the contribution of ALFF changed brain areas to chronic corneal pain, we conducted a correlation analysis between ALFF values of S2, AID, AIP, AuV, TeA, Ect, and eye blinking times after chemical application. We found there was a significant correlation between the ALFF values of AID and AIP, which belong to the insular cortex, and eye blinking times after capsaicin (Figure 5b, $P< 0.001$; Figure5c, $p=0.014$) and menthol application (Figure 5h, $P< 0.001$; Figure 5i, $P< 0.001$) according to the Pearson correlation analysis. No correlation was observed in other brain regions at the $P< 0.05$ level. Therefore, we speculated insular cortex might play an essential role in chronic corneal pain.

FIGURE 5

Correlation analysis of ALFF values of S2, AID, AIP, AuV, TeA, Ect and eye blinking times after chemical application.

Local injection of cyclotraxin-B in insular cortex blocked the corneal hypersensitivity and inhibited upregulation of inflammatory cytokines in chronic corneal pain mice

The behavior paradigm and timeline are shown in Figure 6a and Figure 7a. Single-dose (Figure 6b-e) and continuous (Figure 7f-i) local infusion of cyclotraxin-B (10 μg/μL, 0.05 μL per side; MCE) into the insular cortex of DED mice had a significant effect on corneal hypersensitivity ($p < 0.0001$), suggesting a promising therapeutical effect on chronic corneal pain. The expression of pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α, was significantly reduced after consecutive injection (5 days, 10 μg/μL, 0.05 μL per side) of cyclotraxin-B in the insular cortex (P¡0.05, Figure 7b-e). However, there was no effect on DE-related anxiety-like behavior compared to saline infusion controls (Figure 7j-l).

FIGURE 6

Single-dose injection of cyclotraxin-B into the insular cortex inhibited chronic corneal pain induced chemical and mechanical corneal hypersensitivity.

FIGURE 7

Continuous injection of cyclotraxin-B into the insular cortex reduced chronic corneal pain induced corneal hypersensitivity and inhibited descending inflammatory factor upregulation.

Discussion

The primary findings in the present research are as follows: (1) ALFF signals in the supplemental somatosensory area, secondary auditory cortex, agranular insular cortex, temporal association areas, and ectorhinal cortex brain areas were enhanced in the DE group. (2) Insular cortex showed upregulated ALFF and played a dominant role in chronic corneal pain. (3) Cyclotraxin-B injection in insular cortex markedly blocked chronic corneal pain by inhibiting the upregulation of inflammatory cytokines.

Recent research suggested that some dry eye patients described different responses to the same treatment, implying different pathophysiological mechanisms between patients with and without chronic corneal pain[9, 10]. It is well known that persistent peripheral ocular nerve damage and inflammation might cause structures and functional changes in the CNS[11]. However, the evidence of the central sensitization mechanism underlying chronic corneal pain is still scarce.

In this study, we applied ALFF by rs-fMRI to map the functional brain associated with chronic corneal pain and distinguish spontaneous cerebral neuro-activities. ALFF has been used to evaluate functional abnormalities of brain in some pain-related diseases such as discogenic low-back and leg pain, migraine, phantom limb pain, and trigeminal neuralgia[12-14]. However, there's only few rs-fMRI alterations research about acute corneal pain in DED. Yan. H. et al. revealed ReHo values alterations in the limbic-cortical circuits in patients with DED[15]. Zhi-Ming Pan et al. showed higher ALFF values in parahippocampal gyri and caudate in acute eye pain patients [16]. In our present study, the results demonstrated increase ALFF in S2, GU, VISC, AUDp, AuV, AlD, AlP, TeA, PERI, Ect, OLF, HIP, RHP, CTXsp, STR brain areas. We detected increased ALFF in S2. This is different from the previous case reported which uncovered noxious acute stimulation of the cornea could produce somatotopic activation in primary somatosensory cortex[17]. This may be due to the difference in acute and chronic noxious stimulation to peripheral corneal nerves.

ALFF analysis also revealed some brain areas involved in anxiety. Many studies demonstrated that DE patients experienced increased anxiety, chronic pain can aggravate emotional disorders such as anxiety and depression[18]. In current study, ALFF showed significant changes in temporal areas in DE mice. The temporal cortex has been reported involved in anxiety and could record the physical impact of negative emotions [19,20]. Moreover, we also noticed an increase ALFF in the primary auditory area, which contributes to anxiety disorders[21].

C-Fos expression was also used to verify the ALFF changed brain areas and find abnormal neuronal activity brain areas. C-Fos expression has previously been found to have significant upregulation in frontal cortex, the third ventricle, fewer in hippocampal area and the spinal nucleus of the trigeminal nerve in the medulla after menthol-stimulated rabbits' cornea[22,23]. In line with the rs-fMRI results, the upregulated mount of c-Fos verified the abnormal neuronal activity mapping especially in S2, AID, AIP, AuV, TeA, Ect in chronic corneal pain mice.

Then, we found a significant correlation between ALFF values of the insular cortex and eye blinking times after chemical application. This result is in line with the documented role of the insular cortex in trigeminal neuropathic pain, which showed insular cortex representation of dynamic mechanical allodynia[24]. The insular cortex receives afferents from sensory thalamic nuclei involved in widely different functions, such as pain perception and speech production, to the processing of social emotions[25].

Experimental results in the present study showed mRNA expression of pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α, in addition to BDNF, increased, reflecting an underlying over-active state related to altered neuron function. The present result is also consistent with previous research that pro-inflammatory cytokines, neuronal and microglial markers were upregulated in the trigeminal brainstem sensory complex of DED animals[4]. BDNF, crucial in synaptic transmission and neuronal plasticity, has been defined as a neurotransmitter and neuromodulator[26], and the BDNF-TrkB signaling pathway could mediate inflammatory responses[27]. BDNF could affect central sensitization by activation of NMDA receptor[28]. Remarkably, it has been reported that BDNF enhances the excitability of the small-diameter TRG neurons projecting onto the Vi/Vc after trigeminal inflammatory hyperalgesia[29]. So, we speculated an essential role of BDNF-derived neuroinflammation changes in the central mechanism of chronic corneal pain. The local inhibition of TrkB in the insular cortex of DE mice suppressed chronic pain and increased inflammation factors significantly.

The present research is the first attempt to apply ALFF in chronic corneal pain, but a few issues need to be declared. Our study mainly concentrated on one major brain area, other brain regions associated with chronic corneal pain have not been further confirmed. Besides Trk-B, the different type of BDNF receptors, such as the p75 neurotrophin receptor, and the signaling mechanism involved may also be established in the future.

Conclusion

In summary, the fMRI mapping showed potential brain areas of the CNS involved in chronic corneal pain. The insular cortex is one of the brain areas involved in dry eye-related chronic pain. And increased expressions of BDNF and neuroinflammation in insular cortex may contribute to the activation of central pain pathways

in dry eye disease.

Conflicts of Interest:

The authors declare no conflicts of interest.

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References

1. Stapleton F, Alves M, Bunya V.Y, et al. (2017) TFOS DEWS II Epidemiology Report. *Ocul Surf* 15, 334-365.
2. Yan H, Shan X, Wei S, et al. (2020) Abnormal Spontaneous Brain Activities of Limbic-Cortical Circuits in Patients With Dry Eye Disease. *Front Hum Neurosci* 14, 574758.
3. Kalangara J.P, Galor A, Levitt R.C, et al. Characteristics of Ocular Pain Complaints in Patients With Idiopathic Dry Eye Symptoms.*Eye Contact Lens.* 2017; 43:192-198.
4. Fakih D, Zhao Z, Nicolle P, et al. (2019) Chronic dry eye induced corneal hypersensitivity, neuroinflammatory responses, and synaptic plasticity in the mouse trigeminal brainstem. *J Neuroinflammation* 16, 268.
5. Zhang B, Jung M, Tu Y, et al. (2019) Identifying brain regions associated with the neuropathology of chronic low back pain: a resting-state amplitude of low-frequency fluctuation study. *Br J Anaesth* 123, e303-e311.
6. Chen X, Lu B, Yan C.G. (2018) Reproducibility of R-fMRI metrics on the impact of different strategies for multiple comparison corrEction and sample sizes. *Hum Brain Mapp* 39, 300e18.
7. Touj S, Tokunaga R, Al Aïn S, Bronchti G, Piché M. (2019) Pain Hypersensitivity is Associated with Increased Amygdala Volume and c-Fos Immunoreactivity in Anophthalmic Mice. *Neuroscience* 418, 37-49.
8. Qu T, Qi Y, Yu S, et al. (2019) Dynamic Changes of Functional Neuronal Activities Between the Auditory Pathway and Limbic Systems Contribute to Noise-Induced Tinnitus with a Normal Audiogram. *Neuroscience*408, 31-45.
9. Belmonte C, Nichols J.J, Cox S.M, et al. (2017) TFOS DEWS II pain and sensation report. *Ocul Surf* 15, 404e37.

1. Siedlecki A.N, Smith S.D, Siedlecki A.R, et al. (2020) Ocular pain response to treatment in dry eye patients. *Ocul Surf* 18, 305-311.
2. Melik Parsadaniantz S, Rostène W, Baudouin C, et al. (2018) Understanding chronic ocular pain. *Biol Aujourdhui* 212, 1-11.
3. Du J.G, Xiao H, Zuo Y.X. (2018) Amplitude of Low Frequency Fluctuation (ALFF) study of the spontaneous brain activities of patients with phantom limb pain. *Eur Rev Med Pharmacol Sci* 22, 7164-7171.
4. Zhou F, Gu L, Hong S, et al. (2018) Altered low-frequency oscillation amplitude of resting state-fMRI in patients with discogenic low-back and leg pain. *J Pain Res* 11, 165-176.
5. Chen Y, Xiang C.Q, Liu W.F, et al. (2019) Application of amplitude of low-frequency fluctuation to altered spontaneous neuronal activity in classical trigeminal neuralgia patients: A resting-state functional MRI study. *Mol Med Rep.* 20,1707-1715.
6. Yan H, Shan X, Wei S, et al. (2020) Abnormal Spontaneous Brain Activities of Limbic-Cortical Circuits in Patients With Dry Eye Disease. *Front Hum Neurosci* 14, 574758.
7. Pan Z.M, Li H.J, Bao J, et al. (2018) Altered intrinsic brain activities in patients with acute eye pain using amplitude of low-frequency fluctuation: a resting-state fMRI study. *Neuropsychiatr Dis Treat* 14, 251-257.

8. Moulton EA, Becerra L, Rosenthal P, et al. (2012) An approach to localizing corneal pain representation in human primary somatosensory cortex. *PLoS One* 7, e44643.
9. Wan K.H, Chen L.J, Young A.L. (2016) Depression and anxiety in dry eye disease: a systematic review and meta-analysis. *Eye (Lond)* 30, 1558-1567.
10. Drevets W.C. (1998) Functional neuroimaging studies of depression: the anatomy of melancholia. *Annu Rev Med* 49, 341–361.
11. Giotakos O. Neurobiology of emotional trauma.(2020) *Psychiatriki* 31, 162-171.
12. Wigestrand M.B, Schiff H.C, Fyhn M, et al. (2016) Primary auditory cortex regulates threat memory specificity. *Learn Mem* 24, 55-58.
13. Lee G.J, Kim YJ, Lee K, et al. (2021) Patterns of brain c-Fos expression in response to feeding behavior in acute and chronic inflammatory pain condition. *Neuroreport* 32, 1269-1277.
14. Wang J, Zhou R, Gao W. (2017) The neural pathway for lacrimal gland tear secretion in New Zealand White rabbits. *Neurosci Lett* 649, 14-19.
15. Alvarez P, Dieb W, Hafidi A, et al. (2009) Insular cortex representation of dynamic mechanical allodynia in trigeminal neuropathic rats. *Neurobiol Dis* 33,89-95.
16. Nieuwenhuys R. (2012) The insular cortex: a review. *Prog Brain Res* 195, 123-163.
17. Pezet S, Malcangio M, McMahon S.B. (2002) BDNF: a neuromodulator in nociceptive pathways? *Brain Res Brain Res Rev* 40, 240-249.
18. Jin M, Sheng W, Han L, et al. (2018) Activation of BDNF-TrkB signaling pathway-regulated brain inflammation in pentylenetetrazole-induced seizures in zebrafish. *Fish Shellfish Immunol* 83, 26-36.
19. Groth R, Aanonsen L. (2002) Spinal brain-derived neurotrophic factor (BDNF) produces hyperalgesia in normal mice while antisense directed against either BDNF or trkB, prevent inflammation-induced hyperalgesia. *Pain* 100, 171-181.
20. Takeda M, Takahashi M, Kitagawa J, et al. (2013) Brain-derived neurotrophic factor enhances the excitability of small-diameter trigeminal ganglion neurons projecting to the trigeminal nucleus interpolaris/caudalis transition zone following masseter muscle inflammation. *Mol Pain* 9, 49.

Figure Legends

FIGURE 1

Evaluation of chemical and mechanical corneal sensitivity and anxiety-like behavior of Sham and DE animals. a, b Chemical corneal sensitivity was evaluated using a drop of capsaicine (100 μM) (a) and methol (100 μM) recording the palpebral closure frequency for 30s. Sham, n =8 mice; DE, n = 8 mice. c, d Mechanical corneal sensitivity tested by recording proportion of blink responses to application of von Frey filaments 0.008 g (c) and 0.02 g (d) to the cornea in Sham and DE mice. e Representative activity tracking OFT in Sham and DE mice. f,g Time in center region (f) and total distance traveled (g) during OFT. Sham, n = 8 mice; DE, n = 8 mice. ***P< 0.001, ****P < 0.0001, ns, not significant. DE, dry eye; OFT, open field test.

FIGUE 2

ALFF value comparison in various brain regions. In comparison with the sham group, DE group had significantly increased ALFF in S2, GU, VISC, AUDp, AuV, AlD, AlP, TeA, PERI, Ect, OLF, HIP, RHP, CTXsp, STR (left), while significantly decreased ALFF in the Mop, Mos, SSp-ul, SSp-m and OLF (right), HIP (right), RHP (right), CTXsp (right), SSp-n (right), ACAd (right), PL (right). The Numbers at the top and bottom of the color bar represent the t value of the statistical result. Besides, the red presents the enhancement ALFF signal and the blue shows the decrease ALFF signal. ALFF, amplitude of low-frequency fluctuation; S2, supplemental somatosensory area; GU, gustatory system; VISC, visceral area; AuD, secondary auditory cx, dorsal; AlD, agranular insular cortex, dorsal; AlP, agranular insular cortex, posterior; TeA, temporal association areas; PERI, perirhinal area; Ect, Ectorhinal cortex; OLF, olfactory areas; HIP, hippocampal region; RHP, retrohippocampal region; CTX, cortical subplate; STR, striatum; Mop, primary motor area; Mos, secondary motor area; SSp,primary somatosensory area; ACAd, anterior cingulate area, dorsal part; PL,prelimbic area.

FIGURE 3

C-Fos immunoreactivity in insular cortex and AuV, TeA, Ect brain section of Sham and DE mice. a,d Anatomical mouse brain atlas. Structural elements of the pain matrix are displayed in red frame. b-c, e-f, g-h, j-k C-Fos immunoreactivity of Sham and DE mice at d28. b, e Scale bar = 200 μm. c, f Scale bar = 50 μm. g-h, j-k Scale bar = 100 μm i The number of c-Fos positive neurons per section was significantly greater in DE mice compared with sham mice in insular cortex (i, P=0.0097, n = 3 per group). l, The number of c-Fos positive neurons per section was also significantly greater in DE mice compared with sham mice in AuV, TeA and Ect (l, P=0.0008, n = 4 per group). ***P < 0.001 relative to the sham group. Results are expressed as the mean ±SEM at d28 post-surgery. AuV, secondary auditory cortex, ventral area; Tea, temporal association areas; Ect, Ectorhinal cortex; DE, dry eye.

FIGURE 4

Expression of trophic factor (BDNF) and inflammatory factor (TNFα, IL-6, IL-10 and IL-1β) in insular cortex and AuV, TeA, Ect brain tissue on day 28 after bilateral excision of extraorbital lacrimal glands. *P<0.05, **P<0.01, n=5. Levels of BDNF, TNFα, IL-6, IL-10 and IL-1β (a, b, c, d, e, mice insular cortex brain tissue; f, g, h, i, j, mice AuV, TeA, Ect brain tissue) mRNA were determined by qPCR. Results are expressed as the mean ±SEM at d28 post-surgery. BDNF, brain derived neurotrophic factor; TNFα, tumor necrosis factor α· IL-6, Interleukin-6; IL-10, Interleukin-10; IL-1β, Interleukin-1β.

FIGURE 5

Correlation analysis of ALFF values of S2, AID, AIP, AuV, TeA, Ect and eye blinking times after chemical application. Correlation analysis of ALFF values of S2, AID, AIP, AuV, TeA, Ect and eye blinking times after capsicin application (a-f). Correlation analysis of ALFF values of S2, AID, AIP, AuV, TeA, Ect and eye blinking times after menthol application (g-l). Significant correlation between the ALFF values of AID and eye blinking times after capsaicine (r=0.957,P¡0.001) and menthol application (r=0.948, P¡0.001) according to the pearson correlation analysis. Meanwhile, significant correlation between the ALFF values of AIP and eye blinking times after capsaicine (r=0.744,P=0.014) and menthol application (r=0.942, P¡0.001) according to the pearson correlation analysis.

FIGURE 6

Single-dose injection of cyclotraxin-B into the insular cortex inhibited chronic corneal pain induced chemical and mechanical corneal hypersensitivity. (a) Diagram and timeline schema of the experimental design. Grey arrows mark local infusion of cyclotraxin-B or saline into the insular cortex. Bilateral extraorbital glands were excised on day 0. Insular cortex cannula was inserted on day 21 after surgery. Dark grey circles mark behavior test including chemical and mechanical corneal sensitivity test. (b-e) Effect of treatment with single dose injection of BDNF antagonist cyclotraxin-B induces a statistically significant antinociceptive effect in chemical and mechanical corneal sensitivity. The DE+Cyclotraxin-B group represents that of dry eye mice receiving an intra-insular cortical microinjection of cyclotraxin-B (10 μg/μL, 0.05 μL per side; MCE). (n=8, ***P < 0.001and ****P < 0.0001 versus DE+Saline group). ELG, extraorbital gland; CTB, cyclotraxin-B; DE, dry eye.

FIGURE 7

Continuous injection of cyclotraxin-B into the insular cortex reduced chronic corneal pain induced corneal hypersensitivity and inhibited descending inflammatory factor upregulation. (a) Diagram and timeline schema of the experimental design. Grey arrows mark local infusion of cyclotraxin-B or saline into the insular cortex. Bilateral extraorbital glands were excised on day 0. Insular cortex cannula was inserted on day 21 after surgery. Dark grey circles mark behavior test including chemical and mechanical corneal sensitivity test. Black circles mark open field test. Light grey circles mark tissue sampling with qRT-PCR. (b-e) The protein expression of TNFα, IL-6, IL-10 and IL-1β in DE mice after consecutive administration of cyclotraxin-B (10 μg/μL, 0.05 μL per side for five days) (n=4, *P < 0.05 and **P < 0.01 versus DE group). (f-l) Effect of treatment with BDNF antagonist cyclotraxin-B induces a statistically significant antinociceptive effect in chemical

and mechanical corneal sensitivity, whereas during anxiolytic effect is unremarkable. The DE+Cyclotraxin-B group represents that of dry eye mice receiving an intra-insular cortical microinjection of cyclotraxin-B (10 μg/μL, 0.05 μL per side; MCE). (f-i) The chemical and mechanical thresholds after administration of cyclotraxin-B in DE mice (n=8, **P < 0.01, ***P < 0.001 and ****p < 0.0001 versus DE+Saline group). (j-l) j Representative activity tracking OFT in Sham, DE, DE+ Cyclotraxin-B, DE+Saline mice. k,l Time in center region (k) and total distance traveled (l) during OFT. (n = 6, ****p < 0.0001 versus Sham group). DE, dry eye; CTB, cyclotraxin-B; QPCR, quantitative polymerase chain reaction; NS, normal saline; ns, not significant; TNFα, tumor necrosis factor α· IL-6, Interleukin-6; IL-10, Interleukin-10; IL-1β, Interleukin-1β. OFT, open field test.

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