Alkali burn induced corneal spontaneous pain and activated neuropathic pain matrix in the central nerve system in mice

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Abstract

Purpose: To explore whether alkali burn causes corneal neuropathic pain and activates neuropathic pain matrix in the central nerve system in mice.

Methods: A corneal alkali burn mouse model (grade II) was used. Mechanical threshold in the cauterized area was tested using Von Frey hairs. Spontaneous pain behavior was investigated with conditioned place preference (CPP). Phosphor extracellular signal-regulated kinase (ERK), which is a marker for neuronal activation in chronic pain processing, was investigated in several representative areas of the neuropathic pain matrix: the two regions of the spinal trigeminal nucleus (subnucleus interpolaris/caudalis ,Vi/Vc; subnucleus caudalis/upper cervical cord , Vc/C1), insular cortex, anterior cingulated cortex (ACC), and the rostroventral medulla (RVM). Further, pharmacologically blocking pERK activation in ACC of alkali burn mice was performed in a separate study.

Results: Corneal alkali burn caused long lasting damage to the corneal subbasal nerve fibers and mice exhibited spontaneous pain behavior. By testing in several representative areas of neuropathic pain matrix in the higher nerve system, phosphor extracellular signal-regulated kinase (ERK) was significantly activated in Vc/C1, but not in Vi/Vc. Also, ERK was activated in the insular cortex, ACC, and RVM. Furthermore, pharmacologically blocking ERK activation in ACC abolished alkali burn induced corneal spontaneous pain.

Conclusion: Alkali burn could cause corneal spontaneous pain and activate neuropathic pain matrix in the central nerve system. Furthermore, activation of ERK

in ACC is required for alkali burn induced corneal spontaneous pain.

Key words: corneal alkali burn, neuropathic pain, ERK

Introduction

Chemical injuries to the eye are a significant disabling health problem and represent between 11.5%-22.1% of all ocular traumas[1]. The annual incidence of eye injury in Hong Kong is estimated to be approximately 1.25 ‰ [2] whereas in mainland China chemical burns are reported to be the second most common cause of burns [3]. Severe chemical burns usually produce extensive damage to the ocular surface, leading to complete destruction of the ocular surface, corneal opacification and permanent vision loss[4].

Studies determining the vision-related quality of life in people with ocular chemical injury revealed that ocular chemical burns have a significant and negative impact on a broad array of vision-related quality of life: visual acuity, physical function, and social function[5]. The quality of life of patients with chemical burns was also found to be significantly lower than that reported in other ocular diseases[6]. Ocular pain is very common in those patients and has significantly long-lasting effects on the patient's quality of life. As a result, these patients are usually referred to an ophthalmologist. Dry eye is thought to be a very popular complication [5], since chemical injury can destroy conjunctival goblet cells, leading to lower production or even absence of mucus in the tear film, and thus resulting in improper dispersion of the precorneal tear film. However, even in well-healed eyes or in those with only corneal injury, ocular pain represents significant morbidity. Mucus deficiency cannot be the only reason why dry eyes cause ocular pain. Corneal neuropathic pain ,which for a long time has sometimes been confused with dry eye disease, has previously been reported in

several situations, such as post LASIK surgery[7] and herpes zoster (HZ) affecting the eye[8]. However, there is no documented evidence that alkali burn eyes cause corneal chronic neuropathic pain, and furthermore, central neural mechanisms of corneal neuropathic pain need to be examined.

In the current study, utilizing the corneal alkali burn model（grade II）, we investigated whether corneal alkali burn caused corneal spontaneous pain and whether the classic pain matrix in the higher nervous system was involved in this type of cornel neuropathic pain in mice.

Material and methods:

Animals

Male C57Bl/6 mice (20–25g) were bought from Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology. Mice were maintained on a standard light/dark cycle which was scheduled by the university animal care facility. All experiments were performed during the light cycle. All procedures were carried out in accordance with the International Association for the Study of Pain, the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) after approval by the Animal Care and Use Committee of Tongji Medical College.

Mouse model of grade II corneal alkali burn

According to Hughes's classification standard, a mouse model of grade II corneal

alkali burn was performed as previously described[9]. In this model, corneal injuries were not very severe and the cornea healed well with no significant ocular complications other than mild cornea stromal opacity, visible iris, low incidence of corneal ulcer and corneal neovascularization. The procedure was performed under isoflurane anesthesia (induction 4%, maintenance about 1.4%). Mice were placed under the surgical dissecting microscope in a laterally recumbent position. Topical anesthesia was applied with a single drop of 0.5%proparacaine hydrochloride (Nanjing Ruinian Best Pharmaceutical Co, Nanjing, China). A filter paper (2.0 mm diameter) which was soaked with 1.5μl 0.15 M NaOH in artificial tears was placed centrally on the mouse right cornea for 30 s. Then the eye was thoroughly irrigated with 20 mL sterilized normal saline. In control mice, the right eye only received the filter paper soaked with 1.5μl artificial tears. Right after corneal alkali burn, mice right eyes were treated topically with tobramycin-dexamethasone eye ointment (S.A. ALCONCOUVREUR N.V, Belgium). The treatment was started immediately after corneal burn (three times per day for 4 days).

Mechanical stimulation and sensitivity test:

Mechanical stimulation was performed as previously reported with minor modification[10]. The mice were gently held by hand and mechanical stimulation was performed with calibrated von Frey hairs. A set of von Frey hairs with ascending stiffness (0.005, 0.023, 0.028, 0.068, and 0.166 g) were used to assess the blink response. Each von Frey hair was applied for 1s to the burned corneal area and five times for each hair. Total response numbers were recorded.

Conditioned Place Preference (CPP)

To assess whether there was spontaneous pain on the alkali burn eye, CPP was performed as previously shown[11] with CPP apparatus. A single trial conditioning protocol is deployed in this study. Preconditioning was performed on the fourth day after corneal burn. Animals with pre-existing preference were eliminated from further testing. For conditioning, mice received corneal topical proparacaine treatment and paired with a randomly chosen chamber in the morning, and 4 hours later, treated with normal saline and paired with the other chamber in the afternoon. On the test day, time of each mouse spent in each chamber was recorded with a 15 minutes testing duration. Difference scores were calculated as difference value of test time and preconditioning time spent in the corneal topical proparacaine treatment paired chamber.

ACC cannula implantation and drug administration

Bilateral cannulation of the anterior cingulate cortex (ACC) was performed as previously described[12]. Mice were anesthetized with isoflurane (induction 4%, maintenance about 1.4%). The skull was exposed and double guide cannulas (RWD life science, Shenzhen, China) were implanted. Final coordinates for the microinjection were as following: 0.7 mm anterior to bregma, 0.3 mm lateral to the midline, and 1.75 mm ventral to the surface of the skull. Microinjection was conducted using a motorized syringe pump (RWD life science, Shenzhen, China) and a Hamilton syringe. PD98059 dissolved in 10 % DMSO was given at a dose of 10nmol to two sites of ACC (5 nmol each site) through the injection cannula. The dose was chosen by referring to Cao H's study[13]. To help prevent any solution from flowing back up the guide, the injection

cannula was left in place for 1 min after injection. Then the dummy cannula was inserted back into the guide cannula.

Whole-mount Examination of Cornea

Whole-mount staining of corneas were performed as previously described[14]. Enucleated right eyes were fixed in 4% paraformaldehyde for 40 minutes at room temperature. Corneas were dissected and washed with PBS. Permeabilization was achieved with 10 mM HCl, and 1% pepsin in a 37 ℃water bath for 10 min. Then cornea were neutralized with 0.1 M boric acid pH 8.5 for 10 min at room temperature (RT) and washed with Tris buffered saline with Tween-20 (TBST, 12.5 mM Tris pH 7.6, 0.9% NaCl, 0.1% Tween-20). For blocking, corneal samples were incubated in 1% bovine serum albumin diluted in TD buffer (phosphate buffered asline containing 1% Triton X-100 and 1% dimethyl sulfoxide) for 1 hour. Then, the tissues were incubated overnight at 4°C with a 1:100 dilution of mouse monoclonal neuron-specific anti β-tubulin III antibody (Merck Millipore, Darmstadt, German). After washing with TD buffer, corneal samples were incubated with 1:100 dilution of FITC-conjugated goat anti-mouse IgG (Jackson Immunolab, West Grove, PA, USA).

Immunohistochemistry

After being deeply anesthetized using isoflurane (4%), mice were transcardially perfused with PBS for 5 min, followed by 40ml of 4% paraformaldehyde PB solution (0.1M pH 7.4). The brains were extracted and post-fixed overnight in 4% paraformaldehyde at 4°C, and then cryoprotected in 30% sucrose for 24h at 4°C. Coronal sections at a thickness of 20μm were collected. Floating sections were

incubated overnight at 4 °C with anti-p-ERK primary antibodies diluted in blocking buffer with 10% goat serum (Cell Signaling Technology, Beverly, MA) and 0.3% Triton X-100 in PBS, followed by another incubation with FITC-conjugated goat anti-rabbit IgG (Jackson Immunolab, West Grove, PA, USA) at room temperature for 2h. Sections were imaged by an Epi-fluorescence microscope (Olympus, Melville, NY) and anatomical areas were identified using *The Mouse Brain in Stereotaxic Coordinates* [15].

Immunoblotting

Mice were sacrificed and samples were quickly dissected for Western blotting analysis. Then sample tissue were homogenized in ice cold RIPA buffer which contain phosphatase inhibitors (10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate) and protease inhibitor cocktail (sigma, American). The homogenates were centrifuged and aliquots of supernatant samples were collected. After protein content was tested by the Brandford method (Pierce, Rockford, IL), samples (20μg of total protein) were separated by 10% SDS-PAGE and electro transferred onto PVDF membrane. The membrane was probed with rabbit anti-p-ERK (1:2,000, Cell Signaling Technology, Beverly, MA) at 4℃ temperature overnight, followed by incubation with HRP conjugated anti-rabbit secondary antibody (1:10,000, Thermo Fisher Scientific Inc, Fremont ,CA). Target proteins signal were detected with an enhanced chemiluminescence detection system (thermo) by using a Chemi Doc system (BioRad, Hercules, CA). The membrane was then stripped and used for detecting β-actin expression as internal reference protein (mouse anti-β-actin,

1:10,000, Thermo Fisher Scientific Inc, Fremont, CA). The protein expression levels were expressed as the ratio of the optical densities of target protein to those of β-actin.

Statistical analysis

All data are presented as Mean±S.E.M and significance was set at $p<0.05$. Mechanical threshold were analyzed using a 2-way repeated-measures ANOVA, followed by Bonferroni post hoc test. For CPP data, two-way ANOVA (pairing versus treatment) was applied followed by Bonferroni post hoc test. Difference scores were analyzed using paired t test by comparing the difference between test time and preconditioning time in each chamber for each mouse. Western-blot data were analyzed using One-way ANOVA, followed by pair-wise comparisons using Student-Newman-Keulstest. Statistical significance was established at the 95% confidence limit.

Results

1. The mechanic threshold in cauterized area decreased after corneal alkali burn

Mechanical sensitivity of the corneal cauterized surface was examined using von Frey hairs. Baseline thresholds were tested prior to corneal alkali cauterization. In animals receiving corneal alkali burn, mechanical thresholds were significantly decreased from the second day after burning ($P<0.001$, 2-way repeated-measures ANOVA, followed by Bonferroni post-test, n=10 for baseline, day1, day3, day7, and n=7 for day14, day21, Fig1). Mechanic threshold began to increase on day 7 after alkali burn, but was still lower than baseline at the end of the observation day ($P<0.001$, 2-way repeated-measures ANOVA, followed by Bonferroni post-test). In sham-treated animals, there

was no significant change observed in corneal mechanic threshold. Taken together, these results suggest that alkali burn caused corneal mechanic sensitivity to decrease in the alkali cauterized area.

2. Corneal alkali burn induced ocular spontaneous pain behaviors

To further explore whether corneal alkali burn caused ocular spontaneous pain, we introduced CPP testing. The CPP test training began on the fourth day after corneal alkali cauterization. When compared to those mice tested with CPP, corneal alkali burn mice spent significantly more time in the proparacaine paired chamber (391±76 seconds) than in the saline paired chamber (288±42 seconds, $P<0.01$, two-way ANOVA followed by Bonferroni post hoc test; Fig 2A), indicating that corneal alkali burn mice showed proparacaine (corneal topical administration) paired preference. Control group mice spent similar amounts of time in two chambers. These data are supported by the analysis of difference scores, which revealed that only in alkali burn mice was there proparacaine-induced pain relief (Fig2B). Taken together, there is evidence that pain continues in mice after corneal alkali burn.

3. Corneal alkali burn cause nerve fibers injury in cauterized area

The densities of nerve fibers in subbasal level of the cornea were detected by anti-β tubulin III stain. On day 7, the densities of nerve fibers in the subbasal decreased significantly in alkali burn eyes (Fig3B), than that in control eyes (Fig3A), respectively. On day 21 after alkali burn, there was some irregularly distributed nerve fibers (cluster or neuroma looking, Fig3C). However, the density of nerve fiber is obviously lower than in control mice. Taken together, these results suggest that alkali burn causes

corneal subbasal nerve fiber injury and in the cauterized area, re-epithelialization and reinnervation were not synchronized.

4. Corneal alkali burn induce ERK phosphorylation in Vc/C1 neuron, but not Vi/Vc area

The trigeminal sensory complex in the brainstem is the first relay in the central nervous system for the corneal sensory nerve. Corneal sensory nerve projections are mainly in two regions: the trigeminal subnucleus interpolaris/caudalis (Vi/Vc) transition and the subnucleus caudalis/upper cervical cord (Vc/C1) junction regions[16] . Next we detected the pERK expression in Vi/Vc and Vc/C1 7 days after the injury. A large number of pERK positive stain cells were observed in the superficial laminae of the Vc/C1 on the ipsilateral cauterization side (Fig4B). No obvious pERK positive stain cells were observed in the Vi/Vc transition area (Fig4D). In control mice, no obvious pERK positive stain cells were found neither in the region of Vc/C1, nor in Vi/Vc transition area (Fig4A, C). These results indicate that the region of Vc/C1 involved in alkali burn induced corneal chronic pain processing.

5. Corneal alkali burn induce ERK phosphorylation in multiple neuropathic pain related regions in higher nervous system

Multiple brain regions composed of several interacting networks contribute to the development of chronic neuropathic pain such as the insular cortex, the anterior cingulate cortex (ACC), and the rostroventromedial medulla (RVM). In this study we explored whether corneal alkali burn also causes neural network activation in the higher nervous system, by testing ERK phosphorylation in several representative areas of the

insular cortex, ACC and RVM. A large number of pERK positive stain cells were observed in ACC, insular cortex and RVM 7 days after alkali injury (Fig5B, D, and F). However, no pERK stain positive cells were observed in those regions of control mice (Fig5A, C, and E). These results indicate that chronic neuropathic pain related regions in the high nerve system are also involved in alkali burn induced corneal nerve injury.

6. Inhibition of ERK activation in ACC block corneal alkali burn induced spontaneous pain

To determine the role of ACC ERK activation in corneal alkali burn induced spontaneous pain, separate groups of mice received ACC pERK inhibitor (5 nmol per side) or normal saline pre-cauterization 6 days following cauterization. Mice underwent the single trial conditioning to ocular surface proparacaineon the seventh day after cauterization. Preconditioning time spent in the CPP chambers were equivalent in all groups, therefore the data were pooled for graphical representation. For sham-treated mice, there was no difference in time spent between the proparacaine paired chamber (335±46 seconds) and the normal saline paired chamber (343±39 seconds, $P>0.05$, two-way ANOVA followed by Bonferroni post hoc test; Fig6A). Corneal alkali burn mice with ACC normal saline treatment spent significantly more time in the proparacaine paired chamber (389±51 seconds) than in the saline paired chamber (256 ±37 seconds, $P<0.01$, two-way ANOVA followed by Bonferroni post hoc test). This indicates that ocular surface proparacaine induced CPP in the corneal alkali burn mice that received ACC injection of normal saline. However, for alkali burn mice with pERK inhibitor administration, there was no difference in time spent between the proparacaine

paired chamber (324±61 seconds) and the normal saline paired chamber (349±38 seconds, $P>0.05$, two-way ANOVA followed by Bonferroni post hoc test).This demonstrates that the administration of ACC pERK inhibitor blocked the ocular surface proparacaine-induced CPP. Difference scores also confirm that only corneal alkali burn mice with ACC given normal saline showed increased time spent in the ocular surface proparacaine paired chamber than in the saline paired chamber (Fig6B). Western-blot confirmed that pERK inhibitor effectively inhibited the ERK activation in ACC. However, the expression of total ERK remained unchanged (FigC, D). These results suggest that pharmacological inhibition of ERK activation in ACC could effectively block corneal alkali burn induced ocular spontaneous pain.

Discussion

Chemical burns, especially alkali injuries, represent potentially serious ocular injuries. Post corneal alkali burn pain is a very common complication in patients, with healing taking prolonged time after injury. An ophthalmologist is usually the first to examine the patient and make a diagnosis of dry eye following pain complaint. However, ocular pain rarely responds to traditional dry eye therapy. A possible explanation is that the pain is due to chronic neuropathic pain disorder which results from nerve injury, since corneal chronic pain has already been reported following LASIK surgery, chronic inflammation and herpes zoster (HZ) eye infection. Here in this study, by employing a corneal alkali burn model (Grade II), we found that corneal alkali burn did induce corneal long lasting nerve injury and corneal spontaneous pain in mice. Phosphor extracellular signal-regulated kinase (ERK), a marker for neuronal activation in chronic

pain processing was significantly activated in several representative areas of neuropathic pain matrix in the central nerve system: the subnucleus caudalis/upper cervical cord (Vc/C1), insular cortex, anterior cingulated cortex (ACC), and rostroventral medulla (RVM). More importantly, we found that pharmacologically blocked pERK activation in ACC could abolish alkali burn induced corneal spontaneous pain.

Ophthalmologists have traditionally paid little attention to the mechanism involved in eye pain and only relied on sporadic experimental studies devoted to clarifying the properties and neural basis of ocular pain. Mechanisms based on the central nerve system also need to be explored. For corneal alkali burn, most existing studies focus on mechanisms of neovascularization, tissue adhesions and so on, but underestimate post injury pain. However, ocular pain affects extensively a patient's quality of life. Alkaline agents possess both hydrophilic and lipophilic properties. They saponify the fatty acids of cell membranes, even penetrate the corneal stroma[17], and directly cause nerve fiber injury in any level of cornea where it reaches. More than this, subsequent inflammation responses in damaged tissues lead to further nerve damage or irritation. In this study, by using a corneal grade II alkali burn model, alkali burn caused loss of the subbasal nerves plexus and during corneal re-innervation, nerve fibers may form neuromas. This is the most plausible reason for spontaneous pain. By testing with CPP, mice preferred to stay in the corneal topical proparacaine paired chamber. Analgesia induced place preference showed ongoing pain in corneal alkali burn mice. This is in line with the available evidence on clinical situations. Clinically,

ocular pain is the most intensive pain, because the density of corneal pain receptors has been estimated to be 40 times that of dental pulp and 300-600 times that of the skin[18]. Another interesting phenomenon is that the cauterized area mechanical threshold decreased significantly, even of the corneal already re-epithelialization. That could be because alkali burn caused nerve injury which resulted in nociceptor loss. Although the cornea already gets re-epithelialized, new nociceptors generation may take much longer. These results are also in line with clinical findings that report that burns with alkaline decrease corneal sensitivity[19].

Injury on the periphery never develops into chronic pain but usually involve anatomical and neuro-chemical changes in both the peripheral and central nerve systems. According to various study results, activation of ERK is a biomarker for neuronal activation and central sensitization following noxious stimulation and tissue injury[20]. Corneal sensory nerves originate in the trigeminal ganglion (TG) and the second order neuron located in two regions of the sensory trigeminal complex (V): the trigeminal subnucleus interpolaris/caudalis (Vi/Vc) transition and the subnucleus caudalis/upper cervical cord (Vc/C1) junction region[16]. Noxious stimulation of the cornea has shown a c-fos positive neuron distribution in these two regions[21]. However, it is not clear that these two regions are involved in chronic corneal neuropathic pain. Here in this study, we found that ERK significantly activated in Vc/C1 regions, but not in Vi/Vc 7 days after corneal alkali burn. This indicates that these two regions contribute differently in alkali burn induced corneal chronic neuropathic pain. Several lines of evidence also suggest that these regions have different functions: the Vc/C1 region integrates noxious

353 stimuli from the cornea, whereas the Vi/Vc region seems to be more related to the
354 control of lacrimation and the blinking reflex[22, 23].
355 Multiple nuclei in the higher nervous system are composed of several interacting
356 networks that contribute to the development and maintenance of neuropathic pain.
357 These functional areas form several pain matrices which provide the sensory specificity
358 of the pain experience, the attentional modulation, the pain consciousness, the
359 subjective afferent and efferent modification and so on[24]. Nerve injury triggers long-
360 term plastic changes in these areas that eventually contribute to the development and
361 maintenance of chronic pain. In this study, corneal alkali burn induced ERK activation
362 in the insular cortex, ACC, and RVM. The insular cortex is a representative area for the
363 nociceptive matrix while the ACC is the second matrix for attentional-perceptive and
364 the RVM a representative area for descending pain modulation[24]. Corneal alkali burn
365 induced activation in these areas indicating that the mechanisms based on neuropathic
366 pain matrix in the central nerve system involved the development and maintenance of
367 chronic corneal neuropathic pain.
368 Spontaneous pain is the most important hallmark of nerve injury induced neuropathic
369 pain[25, 26]. The ACC has been implicated in multiple components of pain[27, 28] and it has
370 been reported that it plays a key role in spontaneous pain[29, 30]. Our results demonstrated
371 that ERK was activated in post alkali burn pain. This is in line with research that
372 reported that ERK is activated in the ACC during the induction and expression of
373 chronic pain after never injury or stimulation[13, 31]. In this study we found that ACC
374 pERK inhibitor administration fully blocked the CPP which was induced by corneal

topical proparacaine treatment in alkali burn mice. This result suggests that corneal spontaneous pain requires ERK activation in ACC. Multiple lines of evidence link ACC synaptic plasticity to chronic pain states[29, 32, 33]. Long-term potentiation (LTP) is one form of synaptic plasticity in ACC[34]. ERK has also been shown to be necessary for the induction of LTP in the ACC[35]. Based on previous understanding of the role of ERK in sustaining LTP and its role in synapse plasticity, it may be reasonable to speculate that this mechanism may also apply to corneal alkali burn induced spontaneous pain. However, the precise mechanisms by which the pathway contributes to causing ERK activation in the ACC, and downstream mechanisms underlying ERK's action in the ACC need further investigation.

In conclusion, Alkali burn could cause corneal spontaneous pain and activate neuropathic pain matrix in the central nerve system. Furthermore, activation of ERK in ACC is required for alkali burn induced corneal spontaneous pain. Thus mechanisms based on neuropathic pain matrix in central nerve system may be involved in the development and maintenance of chronic corneal neuropathic pain. In future, therapeutic options which aim to treat neuropathic pain need keep in mind the needs of these patients.

Conflict of interest statement

The work is original, and there is no conflict of interest to disclose.

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Figure legend

Fig1: Corneal alkali burn induced mechanic threshold decreased in cauterized area. The mechanic threshold in cauterized area to von Frey filament probing was measured and mechanical threshold decreased significantly from the second day (day1) after corneal alkali burn. Data are expressed in Mean ± SEM. **P*<0.01, compared with the control mice; ♯*P*<0.01, compared with the second day (day1) after corneal alkali burn, n = 10 for baseline, day1, day3, day5, day7 and n=7 for

day14, day 21 of each group.

Fig2: Corneal topically proparacaine administration produce CPP in corneal alkali burn mice. Mice were tested to ensure the absence of chamber bias (‘‘pre’’) after 3 days preconditioning. A: Corneal alkali burn mice showed significant chamber preference, spending more time in proparacaine paired chambers than saline paired chamber (P<0 .01, two-way ANOVA; n = 8 for each group). B: Difference scores (test time-preconditioning time spent in the proparacaine paired chamber) confirmed that only corneal alkali burn mice showed proparacaine paired chamber preference. P<0.01, paired t-test; n = 8 for each group.

Fig3.Corneal alkali burn cause nerve fibers injury in subbasal level of cauterized area. Whole-mount examinations of cornea were stained with anti- β tubulin III. A: Cornea from control group (sham treated) B: Cornea from mice 7 days after alkali burn. C: Cornea from mice 21 days after alkali burn. White triangle indicate irregular distributed nerve fiber (cluster or neuroma looking), scale bar=200 μ m.

Fig4: Corneal alkali burn induce ERK phosphorylation in Vc/C1 neuron, but not Vi/Vc area. Frozen sections of brain tissue from each group were stained with pERK. Nervous nuclei were marked by white line referring *The Mouse Brain in Stereotaxic Coordinates* [15]. A: Slice represents Vc/C1 area from the control group B: Slice represents Vc/C1 area from mice 7 days after alkali burn. White triangles indicate positive stain of pERK cells. C: Slice represents Vi/Vc area from control group. D: Slice represents Vi/Vc area from mice 7 days after alkali burn. Scale bar=200 μ m. Sp5C= spinal trigeminal nucleus, caudal part, Sp5I= spinal trigeminal nucleus, interpolar part.

Fig5: Corneal alkali burn induce ERK phosphorylation in multiple neuropathic pain related regions in higher nervous system. Frozen sections of brain tissue from each group were stained with pERK. Nervous nuclei were marked by white line using *The Mouse Brain in Stereotaxic Coordinates*[15]. A: Slice represents insular cortex area from control group, S2=secondary somatosensory cortex,CPu= caudate putamen (striatum), Cl=claustrum, En=endopiriform claustrum, Pir=piriform cortex. B: Slice represents insular cortex area from mice 7 days after alkali burn. C: Slice represents ACC area from control group. M2=secondary motor cortex, Cg1=cingulate cortex, area 1, Cg2= cingulate cortex, area 2. D: Slice represents ACC area from mice 7 days after alkali burn. E: Slice represents RVM area from control group, Gi= gigantocellular reticular nucleus, GiA=gigantocellular reticular nucleus, alpha part, LPGi=lateral paragigantocellular nucleus, PPy=parapyramidal nucleus, RMg=raphe magnus nucleus, ml=medial lemniscus, RPa=raphe pallidus nucleus, py=pyramidal tract. F: Slice represents RVM area from mice 7 days after alkali burn. Scale bar=200 μ m.

Fig6: Inhibition of ERK activation in ACC block corneal alkali burn induced spontaneous pain. Mice were tested to ensure the absence of chamber bias (‘‘pre’’) after 3 days preconditioning. A: Corneal alkali burn mice with ACC normal saline administration showed significant chamber preference, spending more time in proparacaine paired chambers than saline paired chamber (**P<0.01, two-way ANOVA; n=8 for each group. Control mice and corneal alkali burn mice with

ACC PD98059 administration did not show significant bias to proparacaine paired chamber, nor saline paired chamber. B: Difference scores (test time-preconditioning time spent in the proparacaine paired chamber) confirmed that only corneal alkali burn mice with ACC saline treatment showed proparacaine paired chamber preference. $^{**}P<0.01$, paired t-test; n=8for each group. C and D: Western-blot and quantitative analysis confirmed that PD98059effectively inhibited the ERK activation in ACC, ** $P<0.01$, when compared with control group.