

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

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22

23 **Abstract**

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

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

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

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

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

46 **Key words:** corneal alkali burn, neuropathic pain, ERK

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67 **Introduction**

68 Chemical injuries to the eye are a significant disabling health problem and represent
69 between 11.5%-22.1% of all ocular traumas¹. The annual incidence of eye injury in
70 Hong Kong is estimated to be approximately 1.25 ‰² whereas in mainland China
71 chemical burns are reported to be the second most common cause of burns³. Severe
72 chemical burns usually produce extensive damage to the ocular surface, leading to
73 complete destruction of the ocular surface, corneal opacification and permanent vision
74 loss⁴.

75 Studies determining the vision-related quality of life in people with ocular chemical
76 injury revealed that ocular chemical burns have a significant and negative impact on a
77 broad array of vision-related quality of life: visual acuity, physical function, and social
78 function⁵. The quality of life of patients with chemical burns was also found to be
79 significantly lower than that reported in other ocular diseases⁶. Ocular pain is very
80 common in those patients and has significantly long-lasting effects on the patient's
81 quality of life. As a result, these patients are usually referred to an ophthalmologist.

82 Dry eye is thought to be a very popular complication⁵, since chemical injury can
83 destroy conjunctival goblet cells, leading to lower production or even absence of
84 mucus in the tear film, and thus resulting in improper dispersion of the precorneal tear
85 film. However, even in well-healed eyes or in those with only corneal injury, ocular
86 pain represents significant morbidity. Mucus deficiency cannot be the only reason
87 why dry eyes cause ocular pain. Corneal neuropathic pain, which for a long time has
88 sometimes been confused with dry eye disease, has previously been reported in

89 several situations, such as post LASIK surgery⁷ and herpes zoster (HZ) affecting the
90 eye⁸. However, there is no documented evidence that alkali burn eyes cause corneal
91 chronic neuropathic pain, and furthermore, central neural mechanisms of corneal
92 neuropathic pain need to be examined.

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

97 **Material and methods:**

98 **Animals**

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

109 **Mouse model of grade II corneal alkali burn**

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

111 alkali burn was performed as previously described⁹. In this model, corneal injuries
112 were not very severe and the cornea healed well with no significant ocular
113 complications other than mild cornea stromal opacity, visible iris, low incidence of
114 corneal ulcer and corneal neovascularization. The procedure was performed under
115 isoflurane anesthesia (induction 4%, maintenance about 1.4%). Mice were placed
116 under the surgical dissecting microscope in a laterally recumbent position. Topical
117 anesthesia was applied with a single drop of 0.5% proparacaine hydrochloride
118 (Nanjing Ruinian Best Pharmaceutical Co, Nanjing, China). A filter paper (2.0 mm
119 diameter) which was soaked with 1.5 μ l 0.15 M NaOH in artificial tears was placed
120 centrally on the mouse right cornea for 30 s. Then the eye was thoroughly irrigated
121 with 20 mL sterilized normal saline. In control mice, the right eye only received the
122 filter paper soaked with 1.5 μ l artificial tears. Right after corneal alkali burn, mice
123 right eyes were treated topically with tobramycin-dexamethasone eye ointment
124 (S.A. ALCONCOUVREUR N.V, Belgium). The treatment was started immediately
125 after corneal burn (three times per day for 4 days).

126 **Mechanical stimulation and sensitivity test:**

127 Mechanical stimulation was performed as previously reported with minor
128 modification¹⁰. The mice were gently held by hand and mechanical stimulation was
129 performed with calibrated von Frey hairs. A set of von Frey hairs with ascending
130 stiffness (0.005, 0.023, 0.028, 0.068, and 0.166 g) were used to assess the blink
131 response. Each von Frey hair was applied for 1s to the burned corneal area and five
132 times for each hair. Total response numbers were recorded.

133 **Conditioned Place Preference (CPP)**

134 To assess whether there was spontaneous pain on the alkali burn eye, CPP was
135 performed as previously shown¹¹ with CPP apparatus. A single trial conditioning
136 protocol is deployed in this study. Preconditioning was performed on the fourth day
137 after corneal burn. Animals with pre-existing preference were eliminated from further
138 testing. For conditioning, mice received corneal topical proparacaine treatment and
139 paired with a randomly chosen chamber in the morning, and 4 hours later, treated with
140 normal saline and paired with the other chamber in the afternoon. On the test day, time
141 of each mouse spent in each chamber was recorded with a 15 minutes testing duration.
142 Difference scores were calculated as difference value of test time and preconditioning
143 time spent in the corneal topical proparacaine treatment paired chamber.

144 **ACC cannula implantation and drug administration**

145 Bilateral cannulation of the anterior cingulate cortex (ACC) was performed as
146 previously described¹². Mice were anesthetized with isoflurane (induction 4%,
147 maintenance about 1.4%). The skull was exposed and double guide cannulas (RWD life
148 science, Shenzhen, China) were implanted. Final coordinates for the microinjection
149 were as following: 0.7 mm anterior to bregma, 0.3 mm lateral to the midline, and 1.75
150 mm ventral to the surface of the skull. Microinjection was conducted using a motorized
151 syringe pump (RWD life science, Shenzhen, China) and a Hamilton syringe. PD98059
152 dissolved in 10 % DMSO was given at a dose of 10nmol to two sites of ACC (5 nmol
153 each site) through the injection cannula. The dose was chosen by referring to Cao H's
154 study¹³. To help prevent any solution from flowing back up the guide, the injection

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

157 **Whole-mount Examination of Cornea**

158 Whole-mount staining of corneas were performed as previously described¹⁴.

159 Eucleated right eyes were fixed in 4% paraformaldehyde for 40 minutes at room

160 temperature. Corneas were dissected and washed with PBS. Permeabilization was

161 achieved with 10 mM HCl, and 1% pepsin in a 37 °C water bath for 10 min. Then cornea

162 were neutralized with 0.1 M boric acid pH 8.5 for 10 min at room temperature (RT)

163 and washed with Tris buffered saline with Tween-20 (TBST, 12.5 mM Tris pH 7.6, 0.9%

164 NaCl, 0.1% Tween-20). For blocking, corneal samples were incubated in 1% bovine

165 serum albumin diluted in TD buffer (phosphate buffered saline containing 1% Triton

166 X-100 and 1% dimethyl sulfoxide) for 1 hour. Then, the tissues were incubated

167 overnight at 4°C with a 1:100 dilution of mouse monoclonal neuron-specific anti β -

168 tubulin III antibody (Merck Millipore, Darmstadt, German). After washing with TD

169 buffer, corneal samples were incubated with 1:100 dilution of FITC-conjugated goat

170 anti-mouse IgG (Jackson Immunolab, West Grove, PA, USA).

171 **Immunohistochemistry**

172 After being deeply anesthetized using isoflurane (4%), mice were transcardially

173 perfused with PBS for 5 min, followed by 40ml of 4% paraformaldehyde PB solution

174 (0.1M pH 7.4). The brains were extracted and post-fixed overnight in 4%

175 paraformaldehyde at 4°C, and then cryoprotected in 30% sucrose for 24h at 4°C.

176 Coronal sections at a thickness of 20 μ m were collected. Floating sections were

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

184 **Immunoblotting**

185 Mice were sacrificed and samples were quickly dissected for Western blotting analysis.
186 Then sample tissue were homogenized in ice cold RIPA buffer which contain
187 phosphatase inhibitors (10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1
188 mM sodium orthovanadate) and protease inhibitor cocktail (sigma, American). The
189 homogenates were centrifuged and aliquots of supernatant samples were collected.
190 After protein content was tested by the Bradford method (Pierce, Rockford, IL),
191 samples (20µg of total protein) were separated by 10% SDS-PAGE and electro
192 transferred onto PVDF membrane. The membrane was probed with rabbit anti-p-
193 ERK (1:2,000, Cell Signaling Technology, Beverly, MA) at 4°C temperature overnight,
194 followed by incubation with HRP conjugated anti-rabbit secondary antibody
195 (1:10,000, Thermo Fisher Scientific Inc, Fremont ,CA). Target proteins signal were
196 detected with an enhanced chemiluminescence detection system (thermo) by using a
197 Chemi Doc system (BioRad, Hercules, CA). The membrane was then stripped and
198 used for detecting β-actin expression as internal reference protein (mouse anti-β-actin,

199 1:10,000, Thermo Fisher Scientific Inc, Fremont, CA). The protein expression levels
200 were expressed as the ratio of the optical densities of target protein to those of β -actin.

201 **Statistical analysis**

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

210 **Results**

211 **1. The mechanic threshold in cauterized area decreased after corneal alkali** 212 **burn**

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

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

224 **2. Corneal alkali burn induced ocular spontaneous pain behaviors**

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

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

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

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

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

247 The trigeminal sensory complex in the brainstem is the first relay in the central nervous
248 system for the corneal sensory nerve. Corneal sensory nerve projections are mainly in
249 two regions: the trigeminal subnucleus interpolaris/caudalis (Vi/Vc) transition and the
250 subnucleus caudalis/upper cervical cord (Vc/C1) junction regions¹⁶. Next we detected
251 the pERK expression in Vi/Vc and Vc/C1 7 days after the injury. A large number of
252 pERK positive stain cells were observed in the superficial laminae of the Vc/C1 on the
253 ipsilateral cauterization side (Fig4B). No obvious pERK positive stain cells were
254 observed in the Vi/Vc transition area (Fig4D). In control mice, no obvious pERK
255 positive stain cells were found neither in the region of Vc/C1, nor in Vi/Vc transition
256 area (Fig4A, C). These results indicate that the region of Vc/C1 involved in alkali burn
257 induced corneal chronic pain processing.

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

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

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

270 **6. Inhibition of ERK activation in ACC block corneal alkali burn induced spontaneous** 271 **pain**

272 To determine the role of ACC ERK activation in corneal alkali burn induced
273 spontaneous pain, separate groups of mice received ACC pERK inhibitor (5 nmol per
274 side) or normal saline pre-cauterization 6 days following cauterization. Mice underwent
275 the single trial conditioning to ocular surface proparacaine on the seventh day after
276 cauterization. Preconditioning time spent in the CPP chambers were equivalent in all
277 groups, therefore the data were pooled for graphical representation. For sham-treated
278 mice, there was no difference in time spent between the proparacaine paired chamber
279 (335 ± 46 seconds) and the normal saline paired chamber (343 ± 39 seconds, $P > 0.05$,
280 two-way ANOVA followed by Bonferroni post hoc test; Fig6A). Corneal alkali burn
281 mice with ACC normal saline treatment spent significantly more time in the
282 proparacaine paired chamber (389 ± 51 seconds) than in the saline paired chamber (256
283 ± 37 seconds, $P < 0.01$, two-way ANOVA followed by Bonferroni post hoc test). This
284 indicates that ocular surface proparacaine induced CPP in the corneal alkali burn mice
285 that received ACC injection of normal saline. However, for alkali burn mice with pERK
286 inhibitor administration, there was no difference in time spent between the proparacaine

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

297 **Discussion**

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

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

315 Ophthalmologists have traditionally paid little attention to the mechanism involved in
316 eye pain and only relied on sporadic experimental studies devoted to clarifying the
317 properties and neural basis of ocular pain. Mechanisms based on the central nerve
318 system also need to be explored. For corneal alkali burn, most existing studies focus
319 on mechanisms of neovascularization, tissue adhesions and so on, but underestimate
320 post injury pain. However, ocular pain affects extensively a patient's quality of life.
321 Alkaline agents possess both hydrophilic and lipophilic properties.
322 They saponify the fatty acids of cell membranes, even penetrate the corneal stroma¹⁷,
323 and directly cause nerve fiber injury in any level of cornea where it reaches. More than
324 this, subsequent inflammation responses in damaged tissues lead to further nerve
325 damage or irritation. In this study, by using a corneal grade II alkali burn model, alkali
326 burn caused loss of the subbasal nerves plexus and during corneal re-innervation, nerve
327 fibers may form neuromas. This is the most plausible reason for spontaneous pain. By
328 testing with CPP, mice preferred to stay in the corneal topical proparacaine paired
329 chamber. Analgesia induced place preference showed ongoing pain in corneal alkali
330 burn mice. This is in line with the available evidence on clinical situations. Clinically,

331 ocular pain is the most intensive pain, because the density of corneal pain receptors has
332 been estimated to be 40 times that of dental pulp and 300-600 times that of the skin¹⁸.
333 Another interesting phenomenon is that the cauterized area mechanical threshold
334 decreased significantly, even of the corneal already re-epithelialization. That could be
335 because alkali burn caused nerve injury which resulted in nociceptor loss. Although the
336 cornea already gets re-epithelialized, new nociceptors generation may take much longer.
337 These results are also in line with clinical findings that report that burns with alkaline
338 decrease corneal sensitivity¹⁹.

339 Injury on the periphery never develops into chronic pain but usually involve anatomical
340 and neuro-chemical changes in both the peripheral and central nerve systems.
341 According to various study results, activation of ERK is a biomarker for neuronal
342 activation and central sensitization following noxious stimulation and tissue injury²⁰.
343 Corneal sensory nerves originate in the trigeminal ganglion (TG) and the second order
344 neuron located in two regions of the sensory trigeminal complex (V): the trigeminal
345 subnucleus interpolaris/caudalis (Vi/Vc) transition and the subnucleus caudalis/upper
346 cervical cord (Vc/C1) junction region¹⁶. Noxious stimulation of the cornea has shown
347 a c-fos positive neuron distribution in these two regions²¹. However, it is not clear that
348 these two regions are involved in chronic corneal neuropathic pain. Here in this study,
349 we found that ERK significantly activated in Vc/C1 regions, but not in Vi/Vc 7 days
350 after corneal alkali burn. This indicates that these two regions contribute differently in
351 alkali burn induced corneal chronic neuropathic pain. Several lines of evidence also
352 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 on²⁴. 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 modulation²⁴. 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 nerve 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

375 topical proparacaine treatment in alkali burn mice. This result suggests that corneal
376 spontaneous pain requires ERK activation in ACC. Multiple lines of evidence link ACC
377 synaptic plasticity to chronic pain states^{29, 32, 33}. Long-term potentiation (LTP) is one
378 form of synaptic plasticity in ACC³⁴. ERK has also been shown to be necessary for the
379 induction of LTP in the ACC³⁵. Based on previous understanding of the role of ERK in
380 sustaining LTP and its role in synapse plasticity, it may be reasonable to speculate that
381 this mechanism may also apply to corneal alkali burn induced spontaneous pain.
382 However, the precise mechanisms by which the pathway contributes to causing ERK
383 activation in the ACC, and downstream mechanisms underlying ERK's action in the
384 ACC need further investigation.

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

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393 **Conflict of interest statement**

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

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398 **Reference**

- 399 1. Clare G, Suleman H, Bunce C, et al. Amniotic membrane transplantation for acute ocular burns.
400 *Cochrane Database Syst Rev* 2012;CD009379.
- 401 2. Yu TS, Liu H, Hui K. A case-control study of eye injuries in the workplace in Hong Kong.
402 *Ophthalmology* 2004;111:70-74.
- 403 3. Xie Y, Tan Y, Tang S. Epidemiology of 377 patients with chemical burns in Guangdong province.
404 *Burns* 2004;30:569-572.
- 405 4. Pfister RR. Chemical injuries of the eye. *Ophthalmology* 1983;90:1246-1253.
- 406 5. Le Q, Chen Y, Wang X, et al. Vision-related quality of life in patients with ocular chemical burns.
407 *Invest Ophthalmol Vis Sci* 2011;52:8951-8956.
- 408 6. Smretschnig E, Falkner-Radler CI, Binder S, et al. Vision-Related Quality of Life and Visual
409 Function after Retinal Detachment Surgery. *Retina* 2016;36:967-973.
- 410 7. Theophanous C, Jacobs DS, Hamrah P. Corneal Neuralgia after LASIK. *Optom Vis Sci*
411 2015;92:e233-240.
- 412 8. Kaufman SC. Anterior segment complications of herpes zoster ophthalmicus. *Ophthalmology*
413 2008;115:S24-32.
- 414 9. Bai JQ, Qin HF, Zhao SH. Research on mouse model of grade II corneal alkali burn. *Int J*
415 *Ophthalmol* 2016;9:487-490.
- 416 10. Bates BD, Mitchell K, Keller JM, et al. Prolonged analgesic response of cornea to topical
417 resiniferatoxin, a potent TRPV1 agonist. *Pain* 2010;149:522-528.
- 418 11. He Y, Tian X, Hu X, et al. Negative reinforcement reveals non-evoked ongoing pain in mice with
419 tissue or nerve injury. *J Pain* 2012;13:598-607.
- 420 12. Xu H, Wu LJ, Wang H, et al. Presynaptic and postsynaptic amplifications of neuropathic pain in
421 the anterior cingulate cortex. *J Neurosci* 2008;28:7445-7453.
- 422 13. Cao H, Gao YJ, Ren WH, et al. Activation of extracellular signal-regulated kinase in the anterior
423 cingulate cortex contributes to the induction and expression of affective pain. *J Neurosci* 2009;29:3307-
424 3321.
- 425 14. Carmona FD, Ou J, Jimenez R, et al. Development of the cornea of true moles (Talpidae):
426 morphogenesis and expression of PAX6 and cytokeratins. *J Anat* 2010;217:488-500.
- 427 15. Paxinos KBJFG (ed) *The Mouse Brain in Stereotaxic Coordinates Third Edition*. New York:
428 Academic Press is an imprint of Elsevier; 2007.
- 429 16. Marfurt CF, Del Toro DR. Corneal sensory pathway in the rat: a horseradish peroxidase tracing
430 study. *J Comp Neurol* 1987;261:450-459.
- 431 17. Grant WM, Kern HL. Action of alkalis on the corneal stroma. *AMA Arch Ophthalmol*
432 1955;54:931-939.
- 433 18. Muller LJ, Marfurt CF, Kruse F, et al. Corneal nerves: structure, contents and function. *Exp Eye*
434 *Res* 2003;76:521-542.
- 435 19. Boberg-Ans J. Experience in clinical examination of corneal sensitivity; corneal sensitivity and the
436 naso-lacrimal reflex after retrobulbar anaesthesia. *Br J Ophthalmol* 1955;39:705-726.
- 437 20. Gao YJ, Ji RR. c-Fos and pERK, which is a better marker for neuronal activation and central
438 sensitization after noxious stimulation and tissue injury? *Open Pain J* 2009;2:11-17.

- 439 21. Meng ID, Bereiter DA. Differential distribution of Fos-like immunoreactivity in the spinal
440 trigeminal nucleus after noxious and innocuous thermal and chemical stimulation of rat cornea.
441 *Neuroscience* 1996;72:243-254.
- 442 22. Kurose M, Meng ID. Corneal dry-responsive neurons in the spinal trigeminal nucleus respond to
443 innocuous cooling in the rat. *J Neurophysiol* 2013;109:2517-2522.
- 444 23. Stapleton F, Marfurt C, Golebiowski B, et al. The TFOS International Workshop on Contact Lens
445 Discomfort: report of the subcommittee on neurobiology. *Invest Ophthalmol Vis Sci* 2013;54:TFOS71-
446 97.
- 447 24. Garcia-Larrea L, Peyron R. Pain matrices and neuropathic pain matrices: a review. *Pain* 2013;154
448 Suppl 1:S29-43.
- 449 25. Backonja MM, Stacey B. Neuropathic pain symptoms relative to overall pain rating. *J Pain*
450 2004;5:491-497.
- 451 26. Baron R, Binder A, Wasner G. Neuropathic pain: diagnosis, pathophysiological mechanisms, and
452 treatment. *Lancet Neurol* 2010;9:807-819.
- 453 27. Johansen JP, Fields HL. Glutamatergic activation of anterior cingulate cortex produces an aversive
454 teaching signal. *Nat Neurosci* 2004;7:398-403.
- 455 28. Qu C, King T, Okun A, et al. Lesion of the rostral anterior cingulate cortex eliminates the
456 aversiveness of spontaneous neuropathic pain following partial or complete axotomy. *Pain*
457 2011;152:1641-1648.
- 458 29. Li XY, Ko HG, Chen T, et al. Alleviating neuropathic pain hypersensitivity by inhibiting PKMzeta
459 in the anterior cingulate cortex. *Science* 2010;330:1400-1404.
- 460 30. King T, Qu C, Okun A, et al. Contribution of PKMzeta-dependent and independent amplification
461 to components of experimental neuropathic pain. *Pain* 2012;153:1263-1273.
- 462 31. Wei F, Zhuo M. Activation of Erk in the anterior cingulate cortex during the induction and
463 expression of chronic pain. *Mol Pain* 2008;4:28.
- 464 32. Descalzi G, Kim S, Zhuo M. Presynaptic and postsynaptic cortical mechanisms of chronic pain.
465 *Mol Neurobiol* 2009;40:253-259.
- 466 33. Ruscheweyh R, Wilder-Smith O, Drdla R, et al. Long-term potentiation in spinal nociceptive
467 pathways as a novel target for pain therapy. *Mol Pain* 2011;7:20.
- 468 34. Sandkuhler J. Understanding LTP in pain pathways. *Mol Pain* 2007;3:9.
- 469 35. Toyoda H, Zhao MG, Xu H, et al. Requirement of extracellular signal-regulated kinase/mitogen-
470 activated protein kinase for long-term potentiation in adult mouse anterior cingulate cortex. *Mol Pain*
471 2007;3:36.

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477 **Figure legend**

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

483 day14, day 21 of each group.

484

485 **Fig2: Corneal topically proparacaine administration produce CPP in corneal alkali burn**

486 **mice.** Mice were tested to ensure the absence of chamber bias (“pre”) after 3 days

487 preconditioning. A: Corneal alkali burn mice showed significant chamber preference, spending

488 more time in proparacaine paired chambers than saline paired chamber ($P < 0.01$, two-way

489 ANOVA; $n = 8$ for each group). B: Difference scores (test time-preconditioning time spent in the

490 proparacaine paired chamber) confirmed that only corneal alkali burn mice showed proparacaine

491 paired chamber preference. $P < 0.01$, paired t-test; $n = 8$ for each group.

492

493 **Fig3. Corneal alkali burn cause nerve fibers injury in subbasal level of cauterized area.**

494 Whole-mount examinations of cornea were stained with anti- β tubulin III. A: Cornea from control

495 group (sham treated) B: Cornea from mice 7 days after alkali burn. C: Cornea from mice 21 days

496 after alkali burn. White triangle indicate irregular distributed nerve fiber (cluster or neuroma

497 looking), scale bar=200 μ m.

498

499 **Fig4: Corneal alkali burn induce ERK phosphorylation in Vc/C1 neuron, but not Vi/Vc area.**

500 Frozen sections of brain tissue from each group were stained with pERK. Nervous nuclei were

501 marked by white line referring *The Mouse Brain in Stereotaxic Coordinates*¹⁵. A: Slice represents

502 Vc/C1 area from the control group B: Slice represents Vc/C1 area from mice 7 days after alkali

503 burn. White triangles indicate positive stain of pERK cells. C: Slice represents Vi/Vc area from

504 control group. D: Slice represents Vi/Vc area from mice 7 days after alkali burn. Scale bar=200 μ

505 m. Sp5C= spinal trigeminal nucleus, caudal part, Sp5I= spinal trigeminal nucleus, interpolar part.

506

507 **Fig5: Corneal alkali burn induce ERK phosphorylation in multiple neuropathic pain related**

508 **regions in higher nervous system.** Frozen sections of brain tissue from each group were stained

509 with pERK. Nervous nuclei were marked by white line using *The Mouse Brain in Stereotaxic*

510 *Coordinates*¹⁵. A: Slice represents insular cortex area from control group, S2=secondary

511 somatosensory cortex, CPu= caudate putamen (striatum), Cl=claustrum, En=endopiriform

512 claustrum, Pir=piriform cortex. B: Slice represents insular cortex area from mice 7 days after alkali

513 burn. C: Slice represents ACC area from control group. M2=secondary motor cortex, Cg1=cingulate

514 cortex, area 1, Cg2= cingulate cortex, area 2. D: Slice represents ACC area from mice 7 days after

515 alkali burn. E: Slice represents RVM area from control group, Gi= gigantocellular reticular nucleus,

516 GiA=gigantocellular reticular nucleus, alpha part, LPGi=lateral paragigantocellular nucleus,

517 PPy=parapyramidal nucleus, RMg=raphe magnus nucleus, ml=medial lemniscus, RPa=raphe

518 pallidus nucleus, py=pyramidal tract. F: Slice represents RVM area from mice 7 days after alkali

519 burn. Scale bar=200 μ m.

520

521 **Fig6: Inhibition of ERK activation in ACC block corneal alkali burn induced spontaneous**

522 **pain.** Mice were tested to ensure the absence of chamber bias (“pre”) after 3 days preconditioning.

523 A: Corneal alkali burn mice with ACC normal saline administration showed significant chamber

524 preference, spending more time in proparacaine paired chambers than saline paired chamber

525 (** $P < 0.01$, two-way ANOVA; $n = 8$ for each group). Control mice and corneal alkali burn mice with

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