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·Original Article·

Activation of extracellular signal-regulated kinase in the anterior cingulate cortex contributes to the induction of long-term potentiation in rats

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Abstract: Objective To explore the role of the extracellular signal-regulated kinase (ERK)/cAMP response element binding protein (CREB) pathway in the induction of long-term potentiation (LTP) in the anterior cingulate cortex (ACC) that may be implicated in pain-related negative emotion. **Methods** LTP of field potential was recorded in ACC slice and the expressions of phospho-ERK (pERK) and phospho-CREB (pCREB) were examined using immunohistochemistry method. **Results** LTP could be induced stably in ACC slice by high frequency stimulation (2-train, 100 Hz, 1 s), while APv (an antagonist of NMDA receptor) could block the induction of LTP in the ACC, indicating that LTP in this experiment was NMDA receptor-dependent. Bath application of PD98059 (50 μmol/L), a selective MEK inhibitor, at 30 min before tetanic stimulation could completely block the induction of LTP. Moreover, the protein level of pERK in the ACC was transiently increased after LTP induction, starting at 5 min and returning to basal at 1 h after tetanic stimulation. The protein level of pCREB was also increased after LTP induction. The up-regulation in pERK and pCREB expressions could be blocked by pretreatment of PD98059. Double immunostaining showed that after LTP induction, most pERK was co-localized with pCREB. **Conclusion** NMDA receptor and ERK-CREB pathway are necessary for the induction of LTP in rat ACC and may play important roles in pain emotion.

Keywords: long-term potentiation; extracellular signal-regulated kinase; cAMP response element binding protein; anterior cingulate cortex; rat

1 Introduction

Long-term potentiation (LTP) which refers to the activity-dependent increase in synaptic transmission, has been intensively studied in hippocampus, amygdala and spinal dorsal horn, and is believed to be a cellular model of learning, memory and pathological pain[1-5]. Accumulating evidence from morphological, electrophysiological, neuroimaging and behavioral studies, as well as clinical observations, has implicated the role of anterior cingulate cortex (ACC) in pain-related negative affect or displeasure[6-11]. However, the underlying molecular and cellular mechanisms remain unclear.

Extracellular signal-regulated kinase (ERK) is a specific subset of mitogen-activated protein kinases (MAPKs) family, and has been proven to be an important signaling molecule in many forms of synaptic plasticity[12,13]. Activation of NMDA receptor could induce a marked increase in expression level of phosphor-ERK (pERK), which is an activated

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form of ERK, through the Ca^{2+}-dependent mechanism, in hippocampal, striatal and spinal neurons[14,15]. Experimental evidence from cell lines, cultured hippocampal neurons and brain slices indicates that the activated ERK is translocated from the cytosol to the nucleus where it phosphorylates the transcription factor—cAMP response element binding protein (CREB) via the CREB kinase Rsk2, subsequently activating cAMP response element (CRE)-mediated gene expression[16-20]. Both ERK and CREB activities are implicated in neuronal plasticity including long-term memory[21-23], pain central sensitization[5,24-26], and LTP of synaptic strength in hippocampus and spinal dorsal horn[27-30]. Our recent study has indicated that the activation of NMDA receptor-dependent ERK/MAPK cascade is required for pain-related negative affect[31]. However, it is still not clear whether it is also involved in cingulate LTP. In the present study, the electrophysiological and immunohistochemical methods were employed to investigate whether the activation of ERK is necessary for the induction of cingulate LTP, which might play an important role in pain-related negative emotion.

2 Materials and methods

2.1 Subjects Experiments were carried out on coronal brain slices containing the rostral anterior cingulate cortex (rACC) obtained from young Sprague-Dawley (SD) rats (4-week old). Animals were obtained from Experimental Animal Center of Fudan University and were raised under a 12:12 light-dark cycle at the room temperature of (23±1) ºC, with food and water *ad libitum*. All the experiments were carried out in accordance with the guidelines of the International Association for the Study of Pain, and were approved by the Shanghai Animal Care and Use Committee.

2.2 Electrophysiological recording After being anesthetized with isoflurane, rats were decapitated. The brain was quickly removed and submerged in pre-oxygenated (95% O_2 and 5% CO_2) cold artificial cerebrospinal fluid (ACSF) containing 126 mmol/L NaCl, 4.0 mmol/L KCl, 1.25 mmol/L $MgCl_2$, 26 mmol/L $NaHCO_3$, 1.25 mmol/L NaH_2PO_4, 2.5 mmol/L $CaCl_2$ and 10 mmol/L glucose. The osmolarity and pH were adjusted to 300 mOsm and 7.35, respectively. Slices (380 μm thick) were cut with a vibratome (Leica VT1000S, Germany) and transferred to an oxygenated chamber at the room temperature of (22±1) ºC for at least 1 h before recording. A single rACC slice was transferred to a recording chamber, which was continuously perfused with oxygenated ACSF at a rate of 2-3 mL/min at 30 ºC. A bipolar tungsten stimulating electrode was placed in the layer V of the slice and extracellular field excitatory synaptic potentials (fEPSPs) were recorded using a glass microelectrode placed in layer II/III. Test stimulation (0.033 Hz) was delivered at a stimulus intensity 50 % of the maximum response, and responses were monitored for at least 15 min to ensure a stable baseline. Tetanic stimulation consisted of 2 trains of 1s, 100 Hz stimulation at the double test stimulus intensity, with an interval of 30 s. PD98059 (50 μmol/L; Sigma) or vehicle (0.2% DMSO) was applied 40 min prior to the tetanic stimulation and maintained for an additional 20 min after the stimulation.

2.3 Immunohistochemistry The brain slice immunostaining was performed as described previously[32]. After being stimulated for different times, the slices were quickly transferred into 4% parafomaldehyde and fixed for 1 h. After being washed in 0.01 mol/L PBS for 20 min for 3 times, the slices were immersed in 0.3% Triton X-100 in PBS for another 1h. Then they were washed for 3 times for the total time of 60 min. The ACC slices were blocked in PBS containing 10% goat and donkey serums for 1 h at room temperature, and incubated with mouse anti-pERK (1:2 000; Sigma) or rabbit anti-pCREB (1:500; Upstate Biotechnology) primary antibody prepared in PBS containing 1% normal donkey serum and 0.3% Triton X-100 for 1 h at room temperature and 48 h at 4 ºC. All the slices were washed fully 6 times with each time of 20 min. The sections were incubated in fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA) or rhodamine-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) for 1 h at room temperature and 3 h at 4 ºC, then they were extensively washed again in PBS for 2 h for 6 times. For pERK/pCREB double immunofluorescence, the sections were incubated with rabbitanti-pCREB at 4 ºC for 16 h and then transferred to mixed primary antibodies containing mouseanti-pERK and rabbitanti-pCREB antibodies, for another 24 h at 4 ºC. All the slices were mounted on slides and coverslipped

with 50% glycerol in 0.1 mol/L PBS. Positive signals for pERK, pCREB and double-labeling of pERK/pCREB were assessed using Leica laser confocal microscope. Digitized images were captured using Leica TCS SP2 software.

2.4 Statistical analysis For the quantification of immunoreactive signals, 4-5 ACC slices were selected. The numbers of pERK- and pCREB-positive cells were counted in a region that was captured inside the optic field (a square box, 750 μm × 750 μm) under the 20 × magnification, using a computerized image analysis system (Leica Qwin 500, Germany). Differences between groups were analyzed using one-way ANOVA followed by post hoc Dunnett's test or using student's *t*-test when only 2 groups were applied. For electrophysiological experiments, data were collected and analyzed using CLAMP-fit (Axon Instruments) and SigmaPlot software. Data were expressed as mean±SEM. $P < 0.05$ was considered as significantly different.

3 Results

Firstly, the role of ERK in LTP induction was examined in the rACC. In control rACC slices, 2-train tetanic stimulation produced a significant long-lasting potentiation of fEPSPs, which could last for over 2 h in some slices (Fig. 1A, B). Consistent with the previous report[33], rACC LTP could be completely blocked by a selective NMDA receptor antagonist APv (50 μmol/L), indicating that rACC LTP is dependent on the activation of NMDA receptor (Fig. 2A). Application of PD98059 (50 μmol/L) resulted in complete inhibition of LTP induction, with no effects on basal synaptic transmission, and the fEPSP slope at 30 min was (103.2±7.2)% of basal response. The control group exerted no detectable change on the induction of LTP, and the fEPSP slope at 30 min was (174.9±13.7)% of basal response (Fig. 2B). Two-way ANOVA analysis showed a significant effect for group ($F_{1,9} = 20.49$; $P < 0.01$), a significant effect of time ($F_{119, 1071} = 2.59$, $P < 0.01$), and a nonsignificant effect of group × time interaction ($F_{119, 1071} = 0.52$, $P > 0.05$).

To further confirm whether LTP-inducing stimulation causes ERK activation, pERK expression level was examined in rACC slices that had received the tetanic stimulation for different time. The slices were fixed at 5, 10, 30, and 60 min after the treatment, respectively. Results revealed a significant increase in the number of pERK-positive cells at all the time points examined, with a peak at 10 min after the tetanic stimulation (One-way ANOVA, $F_{4, 21} = 3.611$, $P < 0.01$). Moreover, PD98059 almost completely blocked tetanization-induced ERK activation at 10 min (Fig. 3A, B).

Since activated ERK can translocate to the neucleus, leading to the phosphorylation of transcription factor, the activation of CREB following LTP induction and ERK activation was also investigated. Similar to ERK activation, CREB was activated following the high frequency stimulation.

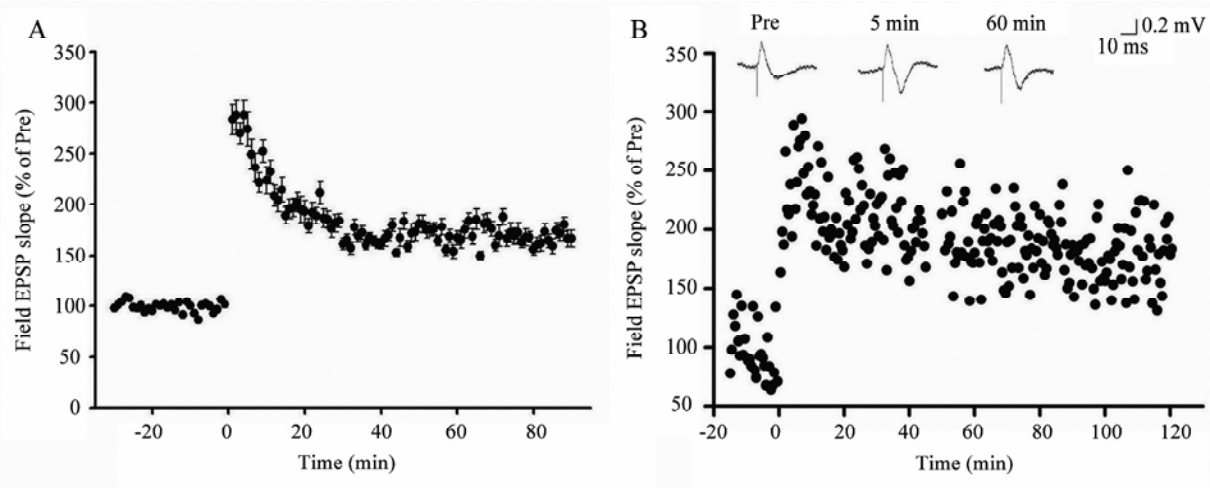

Fig. 1 LTP was induced by tetanic stimulation in rat ACC. A: 2-train tetanic stimulation induced LTP of fEPSPs in the rACC slices, $n = 8$. B: one example showing that LTP could last for 2 h. Insert were the representative traces of the EPSPs at 5 min and 60 min after tetanic stimulation.

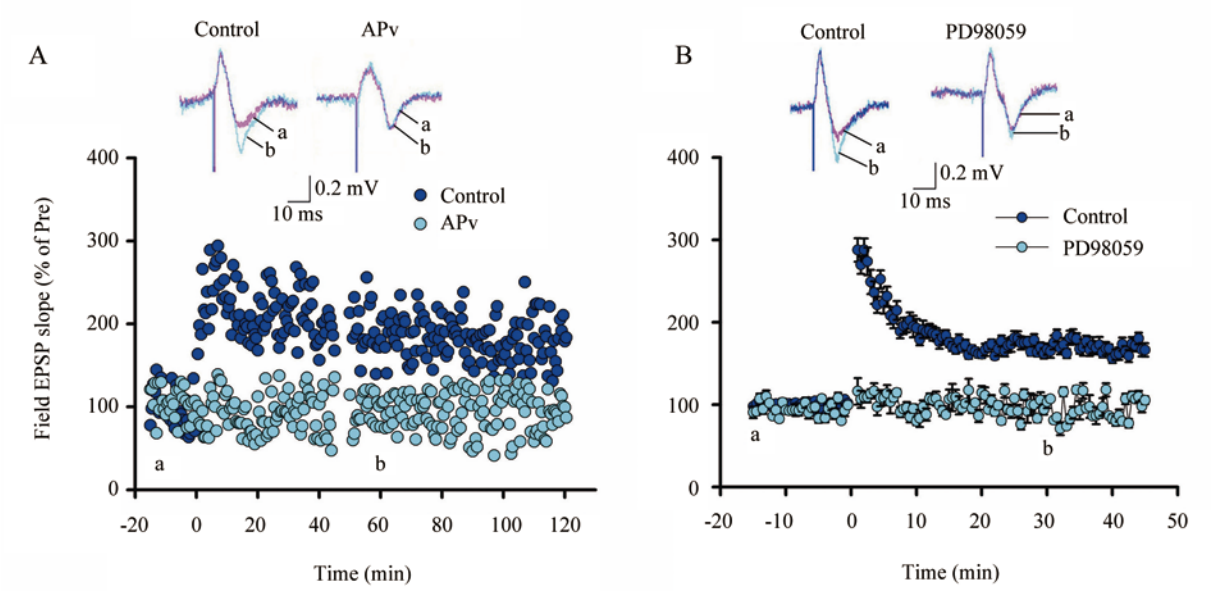

Fig. 2 ERK activation was required for the induction of LTP. A: LTP was dependent on activation of NMDA receptor. B: bath application of PD98059 (50 μmol/L) could completely block the induction of LTP in the rACC. Inserts were the representative traces of the EPSPs from control and PD98059-treated slices (a) before and (b) 30 min after tetanic stimulation. The slices were incubated with PD98059 or vehicle (0.2 % DMSO) for 40 min prior to tetanic stimulation and additional 20 min after tetanization.

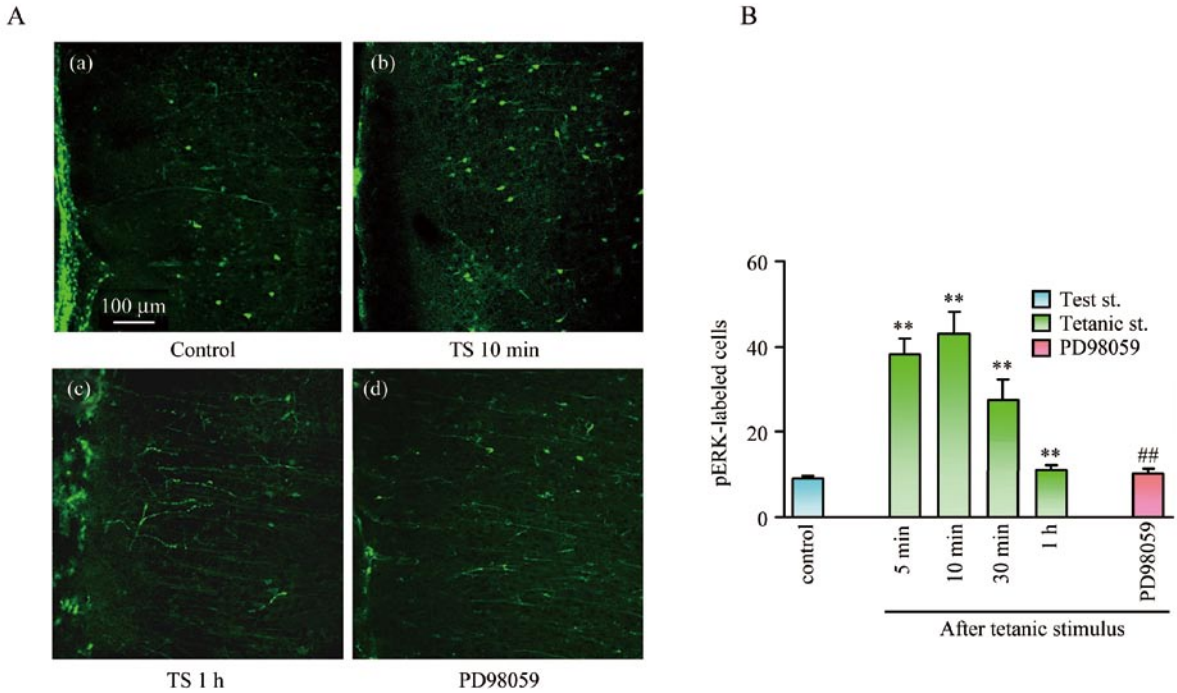

Fig. 3 ERK was activated following LTP-inducing stimulation. A: immunohistochemistry detection of pERK expression in the rACC slices (a) under control conditions, (b) 10 min after tetanic stimulation, (c) 1 h after tetanic stimulation, (d) 10 min after tetanic stimulation with PD98059 treatment. B: histograms showing the number of pERK-positive neurons in the rACC. **$P < 0.01$ *vs* control; $^{\#\#}P < 0.01$ *vs* 10 min after tetanic stimulus (n = 4-6). Scale bar=100 μm.

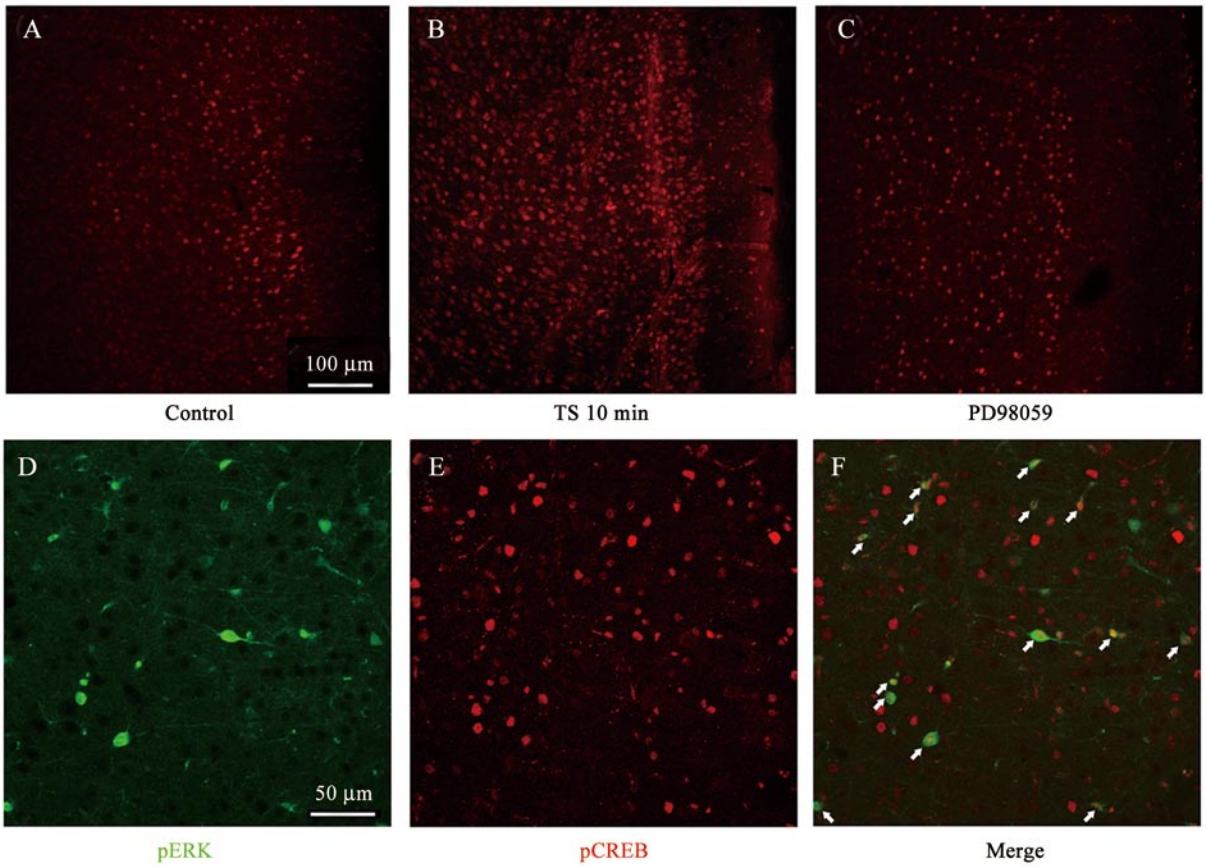

Fig. 4 CREB was activated following LTP-inducing stimulation. A-C: pCREB immunostaining in the rACC slices (A) under control conditions, (B) 10 min after tetanic stimulation, and (C) 10 min after tetanic stimulation with PD98059 treatment. Scale bar=100 μm. D-F: double immunofluorescence (F, merge) revealed that tetanic stimulation-induced pERK activation (D, green) was largely co-localized with pCREB (E, red). Scale bar=50 μm.

Moreover, PD98059 could partially block the activation of CREB at 10 min (Fig. 4A-C). Importantly, almost all the pERK was co-localized with pCREB (Fig. 4D-F).

4 Discussion

The functional significance of the ERK/MAPK signaling in synaptic plasticity has been well investigated in mammalian central nervous system (CNS). In the hippocampus, ERK is activated by strong tetanic stimulation that induces LTP[22]. Inhibition of ERK activation could block associative learning and delay the LTP induced by multiple-train tetanic stimulation[18,21]. In the visual cortex, it has been reported that blockade of ERK activation prevents LTP in the developing visual cortex and blocks the ocular dominance shift induced by monocular deprivation[34]. In the spinal cord, postsynaptic inhibition of ERK pathway blocks LTP in superficial dorsal horn neurons[30]. Studies of Zhuo M *et al.* have demonstrated that at higher concentrations (50 μmol/L), MEK inhibitor PD98059 or U0126 could completely block the induction of ACC LTP that was induced by 3 different protocols in adult mice using whole-cell patch-clamp recordings[35]. Our recent study has indicated that ERK is activated in the rACC neurons following peripheral inflammation and retrieval of nociceptive experience. Furthermore, inhibition of ERK activation could block the induction and expression of pain-related negative emotion[31]. In the present study, we further confirm that ERK activation is required for the induction of LTP in rat rACC using extracellular field potentials recording. Moreover, we observed for the first time that ERK in the rACC slice was activated within 5 min of LTP induction, reach-

ing the peak at 10 min and returning to baseline levels at 1 h, indicating that pERK is not persistently activated during LTP in the rACC. This phenomenon is consistent with previous studies in the hippocampus, in which p42 MAPK (pERK2) was activated within 2 min and returned to baseline at 45 min after LTP induction. Also, a recent report that PD98059 had no effect on the maintenance of LTP in the ACC supports the notion that ongoing activation of the ERK/MAPK pathway is not necessary for the maintenance of rACC LTP. However, our recent study has shown a different time course of ERK activation following inflammation and pain retrieval. We do not know how the 100 Hz, 1 s tetanic stimulation of rACC slices relates to an associative formalin-pairing stimuli in a behaving animal. The differences in the time course of the change in ERK activation between pain-related aversion and LTP may suggest that the *in vivo* kinetics during persistent pain is distinct from *in vitro* LTP.

The downstream effectors modulated by ERK during synaptic plasticity are diverse. ERK activation has been implicated in CREB-mediated gene transcription in cortical, hippocampal and spinal cord neurons[14, 18, 36, 37]. Here we demonstrate that tetanic stimulation-induced ERK activation in the rACC is also coupled to CREB phosphorylation. pERK was largely co-localized with pCREB in the same rACC neurons after high frequency stimulation (Fig. 4F). Co-activation of ERK and CREB in the same neurons provides a basis for ERK-mediated CREB phosphorylation in the rACC. CREB phosphorylation has been thought to initiate transcriptions of several target proteins to ultimately enhance synaptic efficacy, and be critical for induction of NMDA receptor-dependent LTP[38]. Mutant mice that lack the α and δ isoforms of CREB exhibit significant defects in a late phase of LTP[27].

In conclusion, our data indicate that LTP could be induced stably and is required for ERK activation in rat rACC. ERK and CREB are activated following LTP induction, which may provide a potential target for the treatment of pain-related negative emotion.

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大鼠前扣带皮层细胞外信号激酶参与长时程增强的诱导

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摘要 目的 探讨细胞外信号激酶(ERK)/cAMP 反应原件蛋白 (CREB) 信号通路对大鼠前扣带皮层神经元长时程增强 (LTP) 诱导的影响。**方法** 采用离体脑片场电位记录方法观察ERK激酶抑制剂对大鼠前扣带皮层 (ACC) LTP诱导的影响，采用免疫组织化学方法观察ACC脑片在强直刺激后不同时间点磷酸化ERK (pERK)和磷酸化CREB (pCREB)的表达情况。**结果** 在大鼠ACC脑薄片上，高频刺激 (2-train, 100 Hz, 1 s) 能诱导出稳定的场电位 (fEPSP) 长时程增强。预先给予 NMDA 受体竞争性拮抗剂 APv (50 μmol/L)可完全阻断 LTP 的产生，提示本实验中 ACC 神经元 LTP 是 NMDA 受体依赖性的。灌流液中预先给予 MEK 抑制剂 PD98059 (50 μmol/L)能完全阻断 LTP 的产生。取高频刺激后不同时间点的脑片进行免疫组化检测，结果显示，高频刺激后 5 min 时，pERK 表达显著升高，在 10 min 达到最高峰，1 h 后回复到基础表达水平。同样，高频刺激后 ACC 脑片中 pCREB 的表达也显著增加。预先灌流液中给予MEK抑制剂PD98059能够阻断高频刺激引起的pERK和pCREB表达上调。免疫双标结果显示几乎所有的pERK都能与pCREB共定位于同一个神经细胞。**结论** 大鼠前扣带皮层中NMDA受体和ERK/CREB信号通路是长时程增强诱导所必需的。

关键词：长时程增强；细胞外信号调节激酶；cAMP 反应元件结合蛋白；前扣带皮层；大鼠