

Activation of glycine site and GluN2B subunit of NMDA receptors is necessary for ERK/CREB signaling cascade in rostral anterior cingulate cortex in rats: Implications for affective pain

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Abstract: Objective The rostral anterior cingulate cortex (rACC) is implicated in processing the emotional component of pain. N-methyl-*D*-aspartate receptors (NMDARs) are highly expressed in the rACC and mediate pain-related affect by activating a signaling pathway that involves cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and/or extracellular regulated kinase (ERK)/cAMP-response element-binding protein (CREB). The present study investigated the contributions of the NMDAR glycine site and GluN2B subunit to the activation of ERK and CREB both *in vitro* and *in vivo* in rat rACC. **Methods** Immunohistochemistry and Western blot analysis were used to separately assess the expression of phospho-ERK (pERK) and phospho-CREB (pCREB) *in vitro* and *in vivo*. Double immunostaining was also used to determine the colocalization of pERK and pCREB. **Results** Both bath application of NMDA in brain slices *in vitro* and intraplantar injection of formalin into the rat hindpaw *in vivo* induced significant up-regulation of pERK and pCREB in the rACC, which was inhibited by the NMDAR antagonist *DL*-2-amino-5-phospho-novaleric acid. Selective blockade of the NMDAR GluN2B subunit and the glycine-binding site, or degradation of endogenous *D*-serine, a co-agonist for the glycine site, significantly decreased the up-regulation of pERK and pCREB expression in the rACC. Further, the activated ERK predominantly colocalized with CREB. **Conclusion** Either the glycine site or the GluN2B subunit of NMDARs participates in the phosphorylation of ERK and CREB induced by bath application of NMDA in brain slices or hindpaw injection of 5% formalin in rats, and these might be fundamental molecular mechanisms underlying pain affect.

Keywords: N-methyl-*D*-aspartate receptor; glycine site; GluN2B; *D*-serine; extracellular regulated kinase/cAMP-response element-binding protein signaling pathway; rostral anterior cingulate cortex

1 Introduction

The encoding of the sensory and affective dimen-

sions of pain involves different neural circuits^[1]. The early medical literature reported that patients with anterior cingulate cortex (ACC) lobotomies still felt pain but found it less disturbing^[2]. Considerable evidence suggests that the ACC has substantial connections with broad brain regions including secondary visual cortex, secondary auditory cortex, thalamus, amygdala, hippocampus^[3] and primary and

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secondary somatosensory cortices^[4]. Such neuroanatomy also makes the ACC a candidate region that contributes to pain affect. Using hypnotic suggestion, Rainville and his colleagues found that activity within the ACC is correlated with the level of perceived unpleasantness when subjects are exposed to the same intensity of pain^[4], which provided the first direct evidence for a specific role of the ACC in the processing of pain affect. More recent studies further showed that the rostral ACC (rACC), but not the caudal ACC, contributes to pain affect through glutamatergic activation within this region^[5–7].

A series of experiments in our lab revealed that the acquisition of formalin-induced conditioned place avoidance (F-CPA), a pain-related avoidance learning that directly reflects the affective component of pain in rats, requires activation of the N-methyl-D-aspartate receptor (NMDAR)-dependent cAMP/PKA and/or extracellular regulated kinase (ERK)/cAMP-response element-binding protein (CREB) signaling pathway^[5]. Particularly, NMDARs play an indispensable role in the induction of pain affect^[7, 8]. Functional NMDARs are heterodimers composed of one GluN1 and GluN2 subunit. GluN2A and GluN2B, subtypes of the GluN2 subunit, together with GluN1, are highly expressed in forebrain areas^[9], especially in the rACC^[10]. Peripheral noxious stimuli elevate the expression of GluN2A and GluN2B subunits in the ACC^[10]. Selective blockade of GluN2A and GluN2B subunits or the glycine site of NMDARs abolishes the acquisition of F-CPA^[10, 11]. However, it remains unclear which NMDAR subunits or regulatory sites contribute to the cellular ERK-CREB signaling pathway in pain affect. In the present study, we investigated the effects of the NMDAR GluN2B subunit and glycine site on the activation of ERK and CREB in the rACC both *in vitro* and *in vivo*.

2 Materials and methods

2.1 Animals Young (4-week-old) and adult male Sprague-Dawley rats (220–250 g) from the Experimental Animal Center, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, were used in the *in vitro* and *in vivo* studies, respectively. The rats were housed at

23 ± 1°C under a 12:12 light-dark cycle, with food and water *ad libitum*. Before experimental manipulations, the animals were allowed a period of 3 days (for *in vitro*) or 7 days (for *in vivo*) for adaptation to the new environment. All experiments were performed 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 Reagents NMDA, DL-2-amino-5-phosphonovaleric acid (APV, an NMDAR antagonist), 7-chlorokynurenate (7-CK, a glycine site antagonist), D-amino acid oxidase (DAAO, an enzyme that degrades D-serine), ifenprodil (an NMDAR GluN2B antagonist) and the MAP kinase kinase (MEK) inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) were all from Sigma-Aldrich Co., St Louis, MO, USA. Tetrodotoxin (TTX) was from The Fisheries Research Institute, Hebei, China.

NMDA, APV and DAAO were prepared as stock solutions in ion-free water and diluted immediately before use in artificial cerebrospinal fluid (ACSF) for the *in vitro* study or in normal saline (NS) for the *in vivo* study. 7-Chlorokynurenate was dissolved in 0.1 N NaOH, diluted with ACSF or NS, respectively for the *in vitro* and *in vivo* studies, and the pH was adjusted to 7.2–7.4. Ifenprodil and PD98059 were dissolved in 10% dimethylsulfoxide (DMSO). The concentration of DMSO used in slice experiments was 0.1 %.

2.3 Brain slice preparation and drug application The young rats were anesthetized with isoflurane and decapitated. The brain was quickly removed and submerged in preoxygenated (95% O₂ and 5% CO₂) cold ACSF containing (in mmol/L): 126 NaCl, 4 KCl, 1.25 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 CaCl₂, and 10 glucose. Osmolality was adjusted to 300 mOsmol/L and pH to 7.35. Coronal slices containing the rACC (380 µm thick) were cut on a vibratome (Leica VT 1000S) and incubated in an oxygenated chamber at room temperature (22 ± 1°C) for at least 1 h before further processing.

rACC slices were treated with NMDA (50 µmol/L) for 10 min at room temperature (22 ± 1°C), with APV (50 µmol/L), 7-chlorokynurenate (50 µmol/L), ifenprodil (3 µmol/L),

DAAO (0.2 U/mL), PD98059 (50 μ mol/L) or DMSO (0.1%) being added to ACSF 40 min before NMDA stimulation. Slices serving as the control were incubated in ACSF only. The drug concentration was determined according to our previous study^[5]. The slices were then rapidly fixed in cold 4% paraformaldehyde for 60 min and rinsed in 0.01mol/L PBS for 6×10 min before immunostaining.

2.4 Immunohistochemistry Fixed brain slices containing the rACC were blocked with 10% donkey serum in PBS (pH 7.4) with 0.3% TritonX-100 for 1 h at room temperature. Slices were then incubated with mouse anti-pERK (1:1 000; Sigma) or rabbit anti-pCREB (1:500; Upstate Biotechnology, Lake Placid, NY, USA) antibody in PBS with 1% normal donkey serum and 0.3% Triton X-100 for 36 h at 4°C. After that, the slices were washed with PBS for 4×15 min and incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA, USA) or rhodamine-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) for 4 h at 4°C, followed by 4×15 min washes in PBS. For pERK/pCREB double immunostaining, sections were incubated for 36 h at 4°C with a mixture of mouse anti-pERK (1:1 000) and rabbit anti-pCREB (1:500) and then incubated for 4 h at 4°C with a mixture of FITC-conjugated donkey anti-mouse IgG and rhodamine-conjugated donkey anti-rabbit IgG. Other procedures were performed as above. Slices were cover-slipped with a mixture of 50% glycerin and PBS (1:1, v:v), and then observed with a Leica SP2 confocal laser-scanning microscope (TCS-NT, Leica, Germany).

2.5 Intra-rACC drug infusion Rats were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/kg), and carefully placed in a stereotaxic device with the skull exposed surgically to gain access to the rACC. APV (25 nmol), 7-chlorokynurenate (1 nmol), ifenprodil (0.2 μ g), DAAO (0.2 U), or PD98059 (1 nmol) was injected into the rACC with a 5- μ L Hamilton syringe attached to a micro-manipulator (anteroposterior AP + 2.6 mm from bregma, mediolateral $\pm$ 0.6 mm, dorsoventral - 2.5 mm) according to the atlas of Paxinos and Watson (1998)^[12] 20 min before

formalin injection. The drug doses were selected based on our previous studies^[5,10,11]. Either vehicle or drug was injected at a dose of 0.5 μ L per hemisphere over a 5-min period. The syringe was left for an additional 5 min to minimize the spread of drug along the injection track. After that, the craniotomy was filled with sponge and the skin was sutured.

2.6 Western blotting Ten minutes after intraplantar injection of 5% formalin (50 μ L), rats were sacrificed by overdose of chloral hydrate (80 mg/kg) and the brain was quickly removed. Then the rACC was dissected on ice using a Rat Brain Matrix (Stoelting, Wood Dale, IL, USA) and three coronal brain slices (1 mm thick) containing the rACC (AP 3.7–0.7 mm from bregma) were obtained. The slices were further dissected using a surgical blade to remove non-rACC parts according to the atlas and rapidly frozen in liquid nitrogen. Frozen samples were homogenized in lysis buffer (12.5 μ L/mg tissue) containing protease inhibitors (Roche) and PMSF (Sigma) and then incubated on ice for 30 min and centrifuged at 10 000 rpm for 15 min at 4°C. The supernatants were collected for Western blotting.

Equal amounts of samples (~20 μ g) were separated in 10% Tris-Tricine SDS-PAGE gel and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk for 2 h at room temperature, and incubated overnight at 4°C with mouse anti-pERK (1:3 000, Sigma), mouse anti-total ERK (1:100 000, Sigma), rabbit anti-pCREB (1:3 000, Upstate Biotechnology) or rabbit anti-total CREB (1:1 000, Sigma) primary antibody. Membranes were then washed in TBST for 3×10 min and incubated with goat anti-mouse or goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (1:1 000; Pierce, Rockford, IL, USA) secondary antibody for 2 h at 4°C. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce) and the blots were exposed to X-ray film for 1–10 min. Western blot analysis was performed at least three times, and consistent results were obtained.

2.7 Quantification and statistics For quantification of immunoreactive signals, six non-adjacent slices (380 μ m)

through the rACC were randomly selected. The pERK- and pCREB-labeled cells were counted in an area of $520 \times 480 \mu\text{m}^2$ in laminae II–III and in an area of $480 \times 520 \mu\text{m}^2$ in laminae V–VI, using a computerized image analysis system (Leica Qwin 500). For quantification of Western signals, the integrated optical density of the bands was measured using Image J software. Four to five animals were included in each group for quantification and statistics of immunohistochemistry and Western blot results. Data were analyzed by one-way ANOVA followed by *post-hoc* Dunnett's test or by Student's *t* test when only two groups were analyzed.

3 Results

3.1 Glycine site and GluN2B subunit of NMDAR and ERK activation *in vivo*

ERK phosphorylation is an indicator of ERK activation. Previous work from our lab showed that up-regulation of pERK following intraplantar formalin injection is NMDAR-dependent and necessary for the acquisition of F-CPA^[5]. To investigate the roles of the glycine site and GluN2B subunit of NMDARs in formalin nociceptive stimulation-induced ERK activation, appropriate ligands were microinjected into the rACC 20 min before intraplantar injection of 5% formalin. ERK activation was tested by Western blot. Consistent with our previous finding, the nociceptive stimulation led to a robust increase in pERK level but no significant change in total ERK level (Fig. 1A). Here, we pooled the bands for pERK1 and pERK2 and the bands for tERK1 and tERK2 for statistical analysis (Fig. 1B). Intra-rACC injection of the NMDAR antagonist APV, the glycine site antagonist 7-CK, DAAO that degrades endogenous *D*-serine degradation, the NMDAR GluN2B antagonist ifenprodil, or the ERK kinase MEK inhibitor PD98059 before formalin injection significantly suppressed formalin-induced ERK activation (Fig. 1A, B), while DMSO (10%, 0.6 $\mu\text{L}/\text{side}$, vehicle for ifenprodil and PD98059) had no such effect (Fig. 1A, B). The total ERK level remained unchanged under all treatment conditions (Fig. 1A).

3.2 Glycine site and GluN2B subunit of NMDAR and ERK activation *in vitro*

To further confirm the roles of

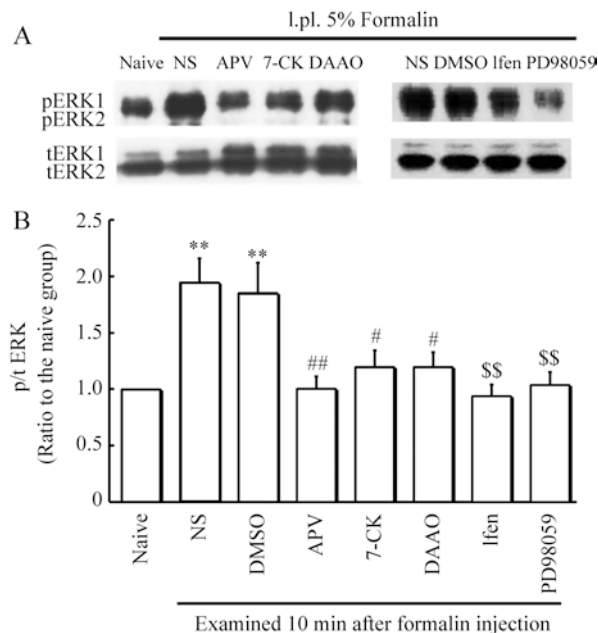


Fig. 1. Contributions of NMDAR glycine site and GluN2B subunit to ERK activation *in vivo*. A, Effects of selective blockade of the glycine site or GluN2B subunit on formalin injection-induced ERK activation. B, Densitometric analysis of phospho-ERK (pERK) and total ERK (tERK). ** $P < 0.01$ vs naive animals; # $P < 0.05$, ## $P < 0.01$ vs NS-pretreated animals; \$\$ $P < 0.01$ vs DMSO-pretreated animals. NS: normal saline. APV: *DL*-2-amino-5-phosphonovaleric acid. 7-CK: 7-chlorokynurenate. DAAO: *D*-amino acid oxidase. Ifen: ifenprodil.

the glycine site and GluN2B subunit of NMDARs in rACC ERK activation, immunohistochemical experiments were performed on brain slices. Compared to the ACSF-treated group, bath application of NMDA (50 $\mu\text{mol}/\text{L}$) for 10 min produced robust increases in ERK activation in the rACC (Fig. 2A, B, I), in the presence of 1 $\mu\text{mol}/\text{L}$ TTX to block action potentials. The up-regulated pERK was attenuated by interfering with NMDARs with APV, 7-CK, DAAO, or ifenprodil (Fig. 2D–I). Pretreatment with PD98059, the ERK kinase inhibitor, significantly decreased but did not eliminate ERK activation (Fig. 2H, I).

3.3 Glycine site and GluN2B subunit of NMDAR and CREB activation *in vitro*

CREB is downstream of ERK during cell signaling. Through binding to certain DNA regions, activated CREB facilitates the expression of genes that participate in synaptic modulation and plasticity.

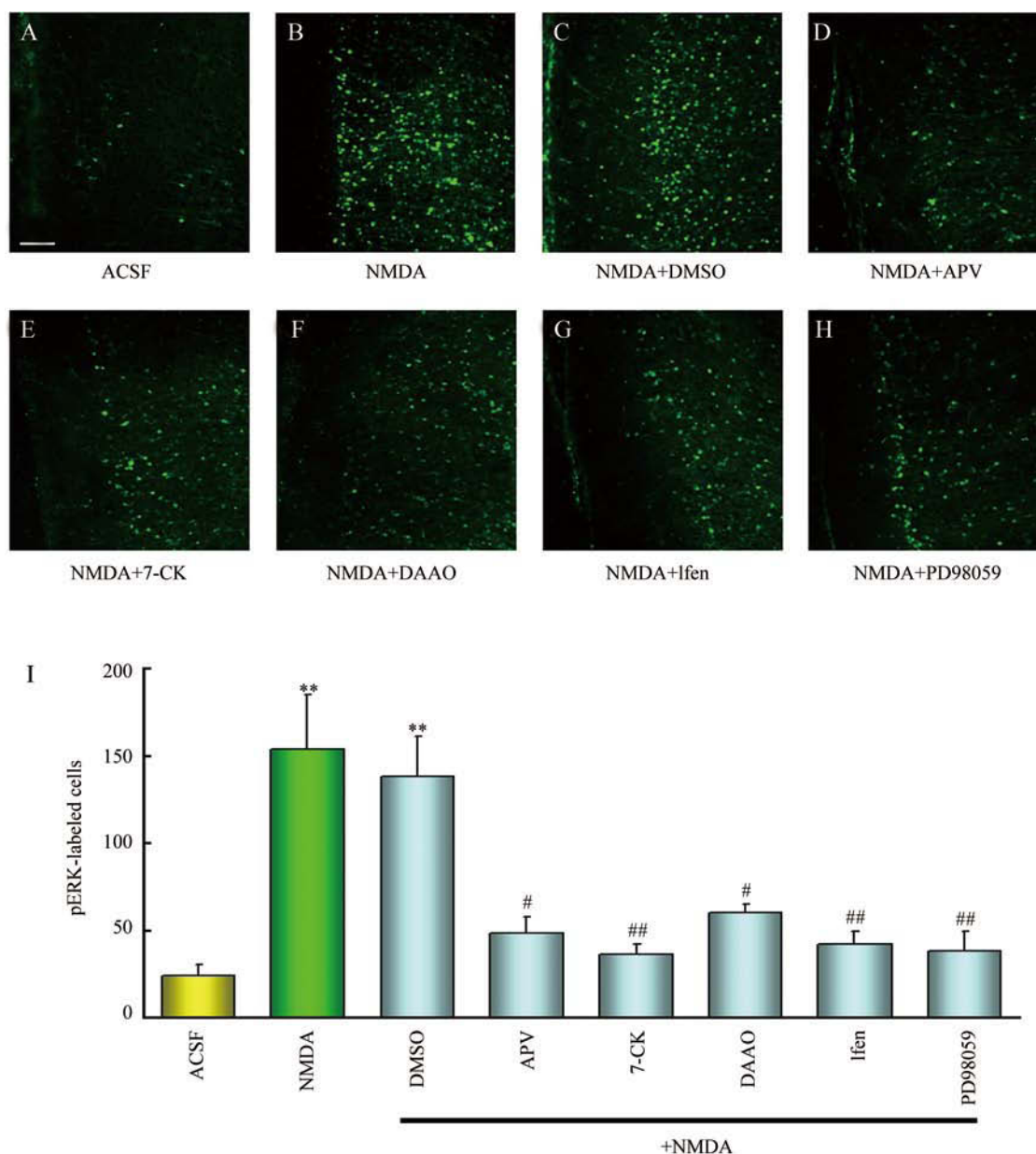


Fig. 2. Contributions of NMDAR glycine site and GluN2B subunit to ERK activation *in vitro*. A–H, Effects of selective blockade of glycine site or GluN2B subunit on NMDA-induced ERK activation. Brain slices containing rACC were treated with ACSF (A), NMDA (B), DMSO + NMDA (C), APV + NMDA (D), 7-CK + NMDA (E), DAAO + NMDA (F), ifenprodil + NMDA (G) and PD98059 + NMDA (H). Scale bar, 100 μ m. I: Quantification of pERK-positive cells. ** $P < 0.01$ vs ACSF; # $P < 0.05$, ## $P < 0.01$ vs DMSO + NMDA. ACSF: artificial cerebrospinal fluid. NMDA: N-methyl-D-aspartate. APV: DL-2-amino-5-phosphonovaleric acid. 7-CK: 7-chlorokynurenate. DAAO: D-amino acid oxidase. Ifen: ifenprodil.

Substantial evidence shows that CREB plays a crucial role in the formation of long-term potentiation (LTP) which underlies the basis of long-term memory and associative learning. Since LTP can be induced in ACC slices^[13,14] and the essence of the F-CPA model is associative learning, we

considered that CREB might be a downstream target of activated ERK induced by NMDA application or formalin injection. To test this, we first determined whether activated ERK and CREB are expressed in the same neurons in the rACC. Double immunostaining showed that bath ap-

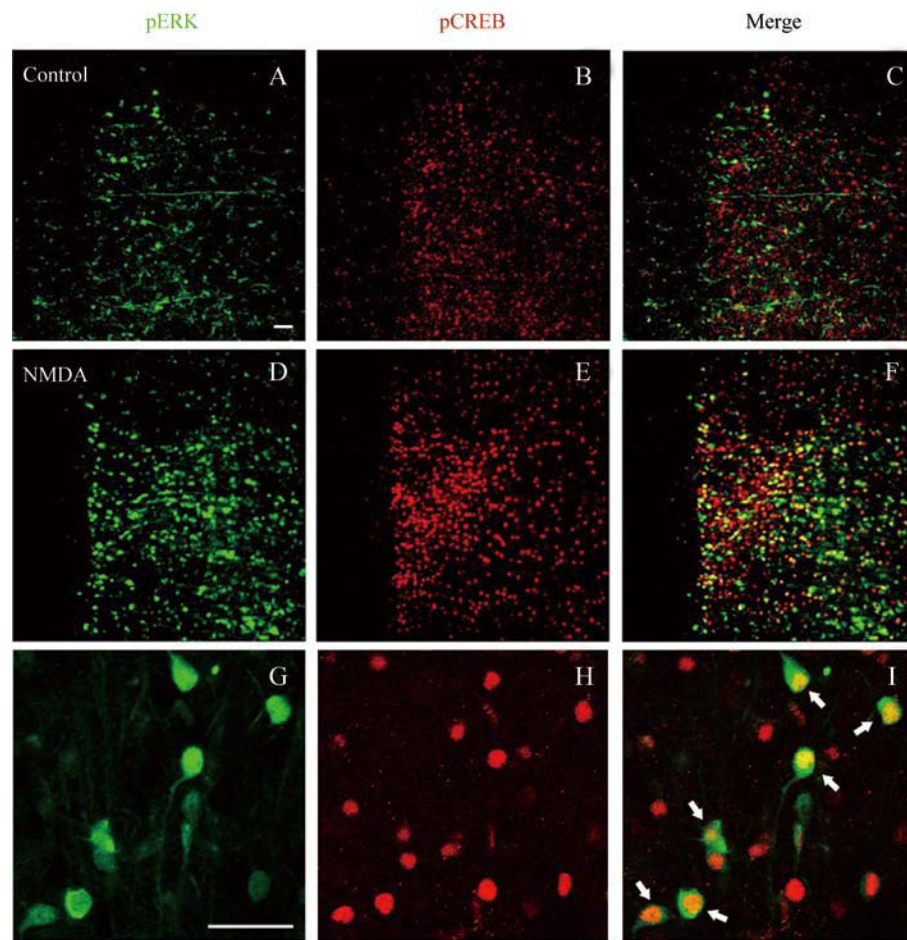


Fig. 3. Double immunofluorescence revealed colocalization of ERK activation (phospho-ERK, green) with CREB activation (phospho-CREB, red) in rACC slices. Slices containing the rACC were treated with artificial cerebrospinal fluid alone (Control, A–C) or with N-methyl-D-aspartate (NMDA) (D–F). G–I, Higher magnifications of the rACC slice treated with NMDA. pERK: phospho-ERK. pCREB: phospho-CREB. arrows indicate double-labeled cells. Scale bars: 40 μ m.

plication of NMDA induced robust activation of ERK and CREB, and activated ERK was predominantly colocalized with pCREB in the rACC slice (Fig. 3).

In addition, NMDA markedly increased the number of pCREB-positive cells in rACC slices (Fig. 4A, I). Meanwhile, PD98059, a MEK inhibitor that blocks ERK activation, prevented the phosphorylation of CREB induced by NMDA in rACC slices (Fig. 4H, I). These results are consistent with previous evidence that activated ERK translocates to the nucleus, and phosphorylates and switches on the transcriptional activity of CREB^[15]. Moreover, APV, 7-CK, DAAO and ifenprodil all inhibited the CREB activation induced by NMDA application in rACC slices (Fig. 4D–G, I).

3.4 Glycine site and GluN2B subunit of NMDAR and CREB activation *in vivo* The expression of pCREB and the contributions of the NMDAR glycine site and GluN2B subunit to CREB activation were also confirmed *in vivo*. Intraplantar formalin injection induced a significant increase of pCREB expression in the rACC (Fig. 5A), and this was blocked by pre-administration of the NMDAR antagonist APV or the ERK kinase inhibitor PD98059 (Fig. 5A), suggesting that formalin injection-induced CREB phosphorylation is NMDAR- and/or ERK cascade-dependent. Also, interference with NMDARs by 7-CK, DAAO or ifenprodil suppressed the nociception-induced up-regulation of pCREB (Fig. 5A, B).

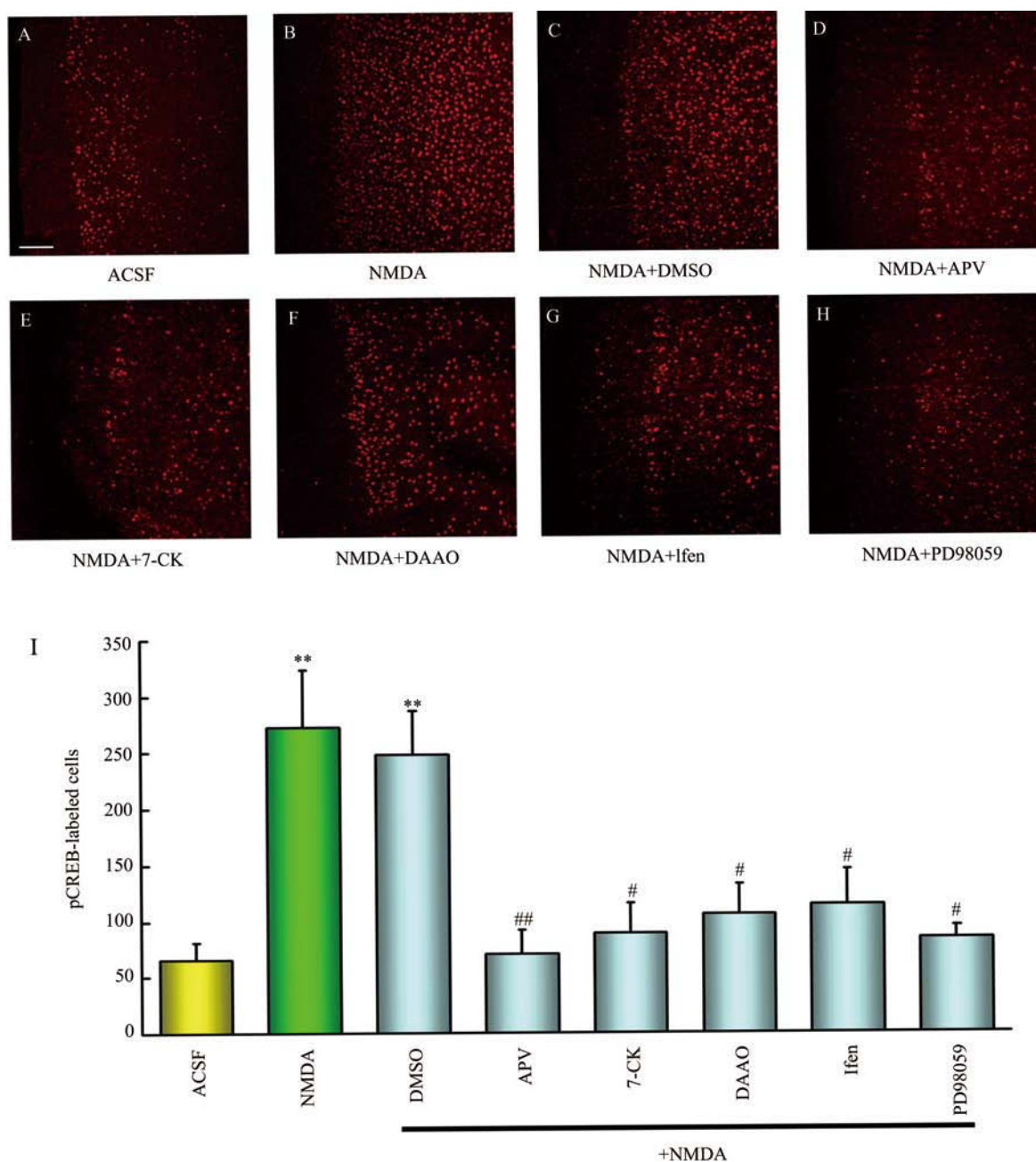


Fig. 4. Contributions of NMDAR glycine site and GluN2B subunit to CREB activation *in vitro*. A-H, Effects of selective blockade of the glycine site or GluN2B subunit on NMDA-induced CREB activation. Slices containing the rACC were treated with ACSF (A), NMDA (B), DMSO + NMDA (C), APV + NMDA (D), 7-CK + NMDA (E), DAAO + NMDA (F), ifenprodil + NMDA (G) or PD98059 + NMDA (H). Scale bar, 100 μ m. I: Quantification of pCREB-positive cells. ** $P < 0.01$ vs ACSF alone; * $P < 0.05$, ## $P < 0.01$ vs DMSO + NMDA. ACSF: artificial cerebrospinal fluid. NMDA: N-methyl-D-aspartate. APV: DL-2-amino-5-phosphonovaleric acid. 7-CK: 7-chlorokynorene. DAAO: D-amino acid oxidase. Ifen: ifenprodil.

4 Discussion

In the present study, we showed that (1) bath applica-

tion of NMDA or intraplantar injection of formalin induced significant activation of ERK and CREB within the rACC in rats; (2) this up-regulation of pERK and pCREB expres-

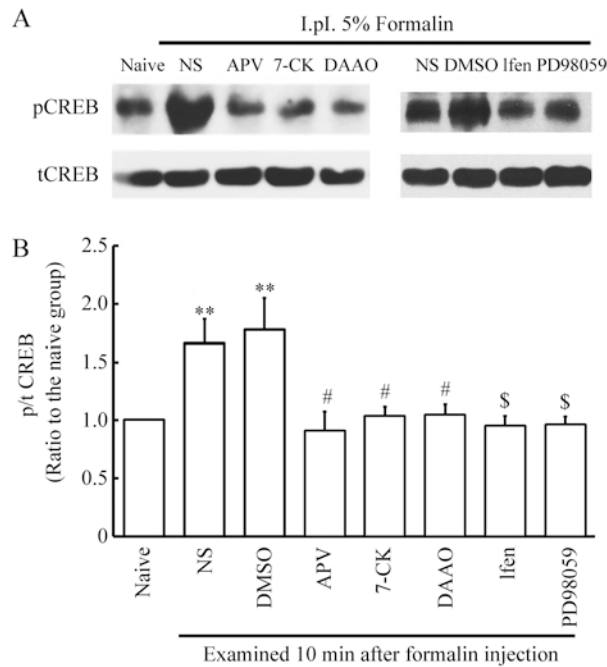


Fig. 5. Contributions of NMDAR glycine site and GluN2B subunit to CREB activation *in vivo*. A, Effects of selective blockade of the glycine site or GluN2B subunits on formalin injection-induced CREB activation. B, Densitometric analysis of phospho-CREB (pCREB) and total CREB (tCREB). ** $P < 0.01$ vs naive animals; # $P < 0.05$ vs formalin + NS; \$ $P < 0.05$ vs formalin + DMSO. NS: normal saline. APV: DL-2-amino-5-phosphonovaleric acid. 7-CK: 7-chlorokynurenate. DAAO: D-amino acid oxidase. Ifen: ifenprodil.

sion was diminished by pre-administration of the NMDAR antagonist APV or the ERK kinase inhibitor PD98059; (3) inducing dysfunction of NMDARs by either targeting the glycine site with 7-CK or DAAO, or targeting the GluN2B subunit with ifenprodil also significantly attenuated the up-regulation of pERK and pCREB expression; and (4) phosphorylated ERK was well colocalized with pCREB in the rACC. These results indicate that both the NMDAR glycine site and the GluN2B subunit are required for ERK and CREB activation within the rACC. Our previous studies demonstrated the necessity for NMDAR and ERK activation in the induction of affective pain^[5,8,11]. Thus, we conclude that the glycine site and GluN2B subunit activation coupled to intracellular ERK/CREB signaling is a key factor in the encoding of the affective dimension of pain.

The NMDAR plays a crucial role in a variety of phy-

siological and pathological processes, including different forms of LTP, learning/memory, pain hyperalgesia, neurodegeneration and psychosis^[16-19]. Morphological studies have shown that NMDARs including the GluN1, GluN2A and GluN2B subtypes are highly expressed in the ACC. Accumulating evidence shows that activation of NMDARs increases postsynaptic Ca^{2+} concentration^[20] and consequently activates multiple signaling cascades such as the ERK pathway^[21]. Elevated Ca^{2+} increases intracellular cAMP levels, leading to PKA activation and therefore phosphorylates ERK through Rap1, then B-Raf^[15]. Our studies *in vitro* and *in vivo* showed that bath application of NMDA or intraplantar injection of formalin increased pERK expression in the rat rACC, and this was blocked by APV, implicating NMDARs in ERK activation, which is consistent with our and other groups' findings^[5,22-27]. In particular, our present data demonstrated that 7-CK or DAAO suppressed the up-regulation of pERK induced by NMDA or nociceptive stimulation, suggesting involvement of the NMDAR glycine site in ERK activation. Ifenprodil, a GluN2B antagonist, also reduced the up-regulation of pERK induced by NMDA, indicating that GluN2B subunit couples to intracellular signaling and then activates ERK, which is consistent with the finding of Krapivinsky *et al.*^[28] that NMDARs mediate ERK activity via interaction of the GluN2B subunit with RasGRF1^[28]. However, Kim *et al.*^[29] showed that the NMDAR GluN2B subunit is coupled to inhibition rather than activation of Ras-ERK. It is difficult to completely explain the discrepancy at present, except to note the differences in brain regions (hippocampus vs rACC) and neuronal models (cultured neurons vs slices).

Ca^{2+} influx through NMDARs can also stimulate neuronal gene expression via transcription factors^[30]. Our previous study showed that CREB is one of the downstream targets of the ERK signaling pathway in rACC neurons, and that it triggers CREB-mediated transcription of genes such as *c-fos* during the induction of pain affect^[5,31]. Here, we showed that NMDA application and formalin injection which induced ERK activation were also coupled to CREB phosphorylation. Importantly, pERK was largely colocalized with pCREB in the rACC slice. Moreover,

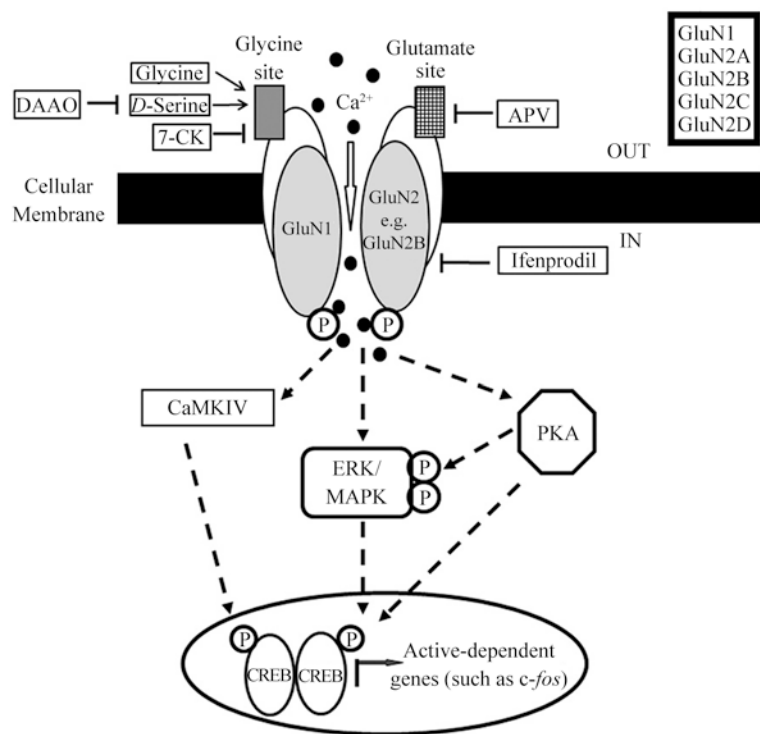


Fig. 6. Possible cellular and molecular mechanisms underlying affective pain. Noxious stimuli such as formalin injection activate the NMDAR, including its GluN1 and GluN2 subunits in the anterior cingulate cortex. Activation of the glycine site and GluN2B subunit of NMDARs induces phosphorylation of ERK/MAPK, and then activates the translocation of ERK to the nucleus and phosphorylates CREB. Dysfunction of NMDARs induced by APV, ifenprodil, 7-CK or DAAO inhibits the intracellular ERK/CREB signaling pathway. DAAO: D-amino acid oxidase. 7-CK: 7-chlorokynurenate. PKA: protein kinase A. CaMKIV: Ca²⁺ calmodulin-dependent protein kinase IV.

our data also showed that blockade of the glycine site or GluN2B subunit of NMDARs significantly reduced the up-regulation of phospho-CREB induced by NMDA perfusion of brain slices or by formalin injection in rat hindpaw, suggesting that both the glycine site and GluN2B subunits in the rACC are required for CREB activation. This finding is in agreement with numerous studies describing NMDAR-dependent processes^[28,32,33]. Phosphorylation of the GluN1 subunit at Ser897 or tyrosine phosphorylation of GluN2B might play important roles when coupled to ERK-CREB signaling^[34,35]. However, another study reported that blockade of the GluN2B subunit does not inhibit ischemia-induced phosphorylation of CREB and the subsequent up-regulation of CREB target genes such as *cpg15* and *bdnf*^[36]. This discrepancy may be due to the differences in brain regions (rACC vs hippocampus) and animal models (inflammatory pain vs ischemia by vessel occlusion). Besides, target genes that are regulated by CREB-mediated transcription in the rACC and the regulation of these genes by different NMDAR subunits remain to be investigated. It should be noted that the expression of pCREB was more

intensive than that of pERK, and was widely distributed in the rACC, indicating the pERK-independent activation of pCREB. Following activation of NMDARs, increased Ca²⁺ can elevate intracellular cAMP levels, leading to the activation of PKA, which might translocate to the nucleus and activate CREB. Another candidate for elevated Ca²⁺ might be Ca²⁺/calmodulin-dependent protein kinases (CaM kinases). Mounting evidence shows that CaMKIV mediates the Ca²⁺-dependent activation of CREB^[37,38].

Importantly, a new finding of the present study was that pretreatment with DAAO either *in vivo* or *in vitro* strikingly inhibited the up-regulation of pERK and pCREB expression, suggesting that endogenous D-serine may be a critical factor in ERK/CREB signaling. Furthermore, Matsuda *et al.* showed that D-serine enhances the extinction of auditory-cued fear conditioning through NMDAR-induced ERK signaling in mice^[39]. It is clear that high levels of D-serine exist in the mammalian brain^[40]. Immunohistochemical studies have revealed an overlapping distribution of D-serine and NMDAR immunoreactivity in the forebrain^[41,42]. Extracellular D-serine levels are similar or even

higher than glycine levels in some areas, as measured by *in vivo* microdialysis^[43]. Furthermore, *D*-serine is functionally 100-fold more effective than glycine at potentiating NMDAR-mediated synaptic action in hypoglossal motoneurons^[44]. A series of studies has suggested that *D*-serine is the endogenous ligand for glycine sites of the NMDAR in some regions, such as the forebrain, telencephalon and developing cerebellum, whereas glycine predominates in other regions such as adult cerebellum, olfactory bulb, and hindbrain^[41,42,45,46]. Given that both competitive and non-competitive NMDAR antagonists have severe side-effects, the glycine co-agonist site of NMDARs has become the preferred target for pharmacological intervention. The present study added evidence to support the proposal that *D*-serine is a potential target for the prevention of chronic pain-induced emotional disturbance.

The molecular mechanisms underlying the emotional aspect of pain have not yet been elucidated. The different roles of NMDAR subunits might be largely dependent on the activation of their intracellular signaling pathways. We demonstrated here that activation of either the glycine site or the GluN2B subunit of NMDARs induced ERK, then CREB phosphorylation, in response to NMDA treatment or formalin injection (Fig. 6). Given that the glycine site, GluN2B, and ERK within the rACC are all indispensable for the induction of pain affect^[5,10,11], the coupling of activation of the glycine site or the GluN2B subunit to the ERK-CREB signaling pathway might be the molecular and cellular mechanism underlying affective pain.

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References:

- [1] Sowards TV, Sowards MA. Separate, parallel sensory and hedonic pathways in the mammalian somatosensory system. *Brain Res Bull* 2002, 58: 243–260.
- [2] Foltz EL, White LE. Pain ‘relief’ by frontal cingulotomy. *J Neurosurg* 1962, 19: 89–100.
- [3] Gao YJ, Zhao ZQ, Zhang YQ. The afferent projection of the rostral anterior cingulate cortex in the rat: a fluorogold retrograde tracing study. *Chin J Neuroanatomy* 2005, 21(4): 355–359 (in Chinese).
- [4] Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC. Pain affect encoded in human anterior cingulate but not somatosensory cortex. *Science* 1997, 277: 968–971.
- [5] Cao H, Gao YJ, Ren WH, Duan KZ, Li TT, Cui YH, *et al.* Activation of extracellular signal-regulated kinase in the anterior cingulate cortex contributes to the induction and expression of affective pain. *J Neurosci* 2009, 29(10): 3307–3321.
- [6] Johansen JP, Fields HL, Manning BH. The affective component of pain in rodents: Direct evidence for a contribution of the anterior cingulate cortex. *Proc Natl Acad Sci U S A* 2001, 98(14): 8077–8082.
- [7] Johansen JP and Fields HL. Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. *Nat Neurosci* 2004, 7(4): 398–403.
- [8] Lei LG, Sun S, Gao YJ, Zhao ZQ, Zhang YQ. NMDA receptors in the anterior cingulate cortex mediate pain-related aversion. *Exp Neurol* 2004, 189: 413–421.
- [9] Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 1994, 12: 529–540.
- [10] Li TT, Ren WH, Xiao X, Nan J, Cheng LZ, Zhang XH, *et al.* NMDA NR2A and NR2B receptors in the rostral anterior cingulate cortex contribute to pain-related aversion in male rats. *Pain* 2009, 146: 183–193.
- [11] Ren WH, Guo JD, Cao H, Wang H, Wang PF, Sha H, *et al.* Is endogenous *D*-serine in the rostral anterior cingulate cortex necessary for pain-related negative affect? *J Neurochem* 2006, 98(4): 1344–1344.
- [12] Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. New York: Academic, 1998.
- [13] Zhao MG, Toyoda H, Lee YS, Wu LJ, Ko SW, Zhang XH, *et al.* Roles of NMDA NR2B subtype receptor in prefrontal long-term potentiation and contextual fear memory. *Neuron* 2005, 47: 859–872.
- [14] Cao H, Cui YH, Zhao ZQ, Cao XH, Zhang YQ. Activation of extracellular signal-regulated kinase in the anterior cingulate cortex contributes to the induction of long-term potentiation in rats. *Neurosci Bull* 2009, 25 (5): 301–308.
- [15] Impey S, Obrietan K, Storm DR. Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron* 1999, 23: 11–14.
- [16] Andersen JD, Pouzet B. Spatial memory deficits induced by perinatal treatment of rats with PCP and reversal effect of *D*-serine. *Neuropsychopharmacology* 2004, 29: 1080–1090.
- [17] Malenka RC, Nicoll RA. Long term potential – a decade of progress? *Science* 1999, 285: 1870–1874.
- [18] Newcomer JW, Farber NB, Jevtovic-Todorovic V, Selke G, Melson AK, Hershey T, *et al.* Ketamine-induced NMDA receptor hypofunction as a model of memory impairment and psychosis. *Neurop-*

- synchopharmacology 1999, 20: 106–118.
- [19] Zhang HT, Crissman AM, Dorairaj NR, Chandler LJ, O'Donnell JM. Inhibition of cyclic AMP phosphodiesterase (PDE4) reverses memory deficits associated with NMDA receptor antagonism. *Neuropsychopharmacology* 2000, 23: 198–204.
- [20] Ghosh A, Greenberg ME. Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science* 1995, 268: 239–247.
- [21] Sweatt JD. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J Neurochem* 2001, 76: 1–10.
- [22] Wei F, Vadakkan KI, Toyoda H, Wu LJ, Zhao MG, Xu H, *et al.* Calcium calmodulin-stimulated adenylyl cyclases contribute to activation of extracellular signal-regulated kinase in spinal dorsal horn neurons in adult rats and mice. *J Neurosci* 2006, 26: 851–861.
- [23] English JD, Sweatt JD. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *J Biol Chem* 1997, 272: 19103–19106.
- [24] Finkbeiner S, Greenberg ME. Ca^{2+} -dependent routes to Ras: mechanisms for neuronal survival, differentiation, and plasticity? *Neuron* 1996, 16: 233–236.
- [25] Ji RR, Zhang YQ. The role of MAPK kinase cascades in cell signaling, neural plasticity and pain facilitation. *Neurosci Bull* 2005, 21(1): 1–9.
- [26] Wu HW, Li HF, Guo J. N-methyl-D-aspartate receptors mediate diphosphorylation of extracellular signal-regulated kinases through Src family tyrosine kinases and Ca^{2+} /calmodulin-dependent protein kinase II in rat hippocampus after cerebral ischemia. *Neurosci Bull* 2007, 23(2): 107–112.
- [27] Xia Z, Dudek H, Miranti CK, Greenberg ME. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J Neurosci* 1996, 16: 5425–5536.
- [28] Krapivinsky G, Krapivinsky L, Manasian Y, Ivanov A, Tyzio R, Pellegrino C, *et al.* The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1. *Neuron* 2003, 40: 775–784.
- [29] Kim MJ, Dunah AW, Wang YT, Sheng M. Differential roles of NR2A and NR2B-containing NMDA receptors in RAS-ERK signaling and AMPA receptor trafficking. *Neuron* 2005, 46: 745–760.
- [30] Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, *et al.* Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science* 1993, 260: 238–241.
- [31] Lei LG, Zhang YQ, Zhao ZQ. Pain-related aversion and Fos expression in the central nervous system in rats. *Neuroreport* 2004, 15: 67–71.
- [32] Bement MKH, Sluka KA. Co-localization of p-CREB and p-NR1 in spinothalamic neurons in a chronic muscle pain model. *Neurosci Lett* 2007, 418: 22–27.
- [33] Lee DK, Bian S, Rahman MA, Shim YB, Shim I, Choe ES. Repeated cocaine administration increases N-methyl-D-aspartate NR1 subunit, extracellular signal-regulated kinase and cyclic AMP response element-binding protein phosphorylation and glutamate release in the rat dorsal striatum. *Eur J Pharmacol* 2008, 590: 157–162.
- [34] Chen L, Miyamoto Y, Furuya K, Mori N, Sokabe M. PREGS induces LTP in the hippocampal dentate gyrus of adult rats via the tyrosine phosphorylation international cooperative research CREB signaling. *J Neurophysiol* 2007, 98: 1538–1548.
- [35] Dudman JT, Eaton ME, Rajadhyaksha A, Macias W, Taher M, Barczak M, *et al.* Dopamine D1 receptors mediate CREB phosphorylation via phosphorylation of the NMDA receptor at Ser897–NR1. *J Neurochem* 2003, 87: 922–934.
- [36] Chen M, Lu TJ, Chen XJ, Zhou Y, Chen Q, Feng XY, *et al.* Differential roles of NMDA receptor subtypes in ischemic neuronal cell death and ischemic tolerance. *Stoke* 2008, 39: 3042–3048.
- [37] Matthews RP, Guthrie CR, Wailes LM, Zhao X, Means AR, McKnight GS. Calcium/Calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol Cell Biol* 1994, 14 (9): 6107–6116.
- [38] Zhang YQ, Zhao ZQ, Ji RR. Emotional distress and related memory of pain: a neurobiological review. *Neurosci. Bull.* 2005, 21(1): 10–18.
- [39] Matsuda S, Matsuzawa D, Nakazawa K, Sutoh C, Ohtsuka H, Ishii D, *et al.* D-serine enhances extinction of auditory cued fear conditioning via ERK1/2 phosphorylation in mice. *Prog Neuro-Psychopharmacol Biol Psychiatry* 2010, 34: 895–902.
- [40] Hashimoto A, Oka T. Free D-aspartate and D-serine in the mammalian brain and periphery. *Prog Neurobiol* 1997, 52: 325–353.
- [41] Schell MJ, Brady RO Jr, Molliver ME, Snyder SH. D-serine as a neuromodulator: regional and developmental localizations in rat brain glia resemble NMDA receptors. *J Neurosci* 1997, 17: 1604–1615.
- [42] Schell MJ, Molliver ME, Snyder SH. D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. *Proc Natl Acad Sci U S A* 1995, 92: 3948–3952.
- [43] Hashimoto A, Oka T, Nishikawa T. Extracellular concentration of endogenous free D-serine in the rat brain as revealed by *in vivo* microdialysis. *Neuroscience* 1995, 66: 635–643.
- [44] Berger AJ, Dieudonne S, Ascher P. Glycine uptake governs glycine site occupancy at NMDA receptors of excitatory synapses. *J Neurophysiol* 1998, 80: 3336–3340.
- [45] Mothet JP, Parent AT, Wolosker H, Brady RO Jr, Linden DJ, Ferris CD, *et al.* D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci U S A* 2000, 97: 4926–4931.
- [46] Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR, *et al.* D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. *Proc Natl Acad Sci U S A* 2003, 100: 6789–6794.