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NMDA NR2A and NR2B Receptors in the Rostral Anterior Cingulate Cortex Contribute to Pain-related Aversion in Male Rats

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Abstract

NMDA receptors, which are implicated in pain processing, are highly expressed in forebrain areas including the anterior cingulate cortex (ACC). The ACC has been implicated in the affective response to noxious stimuli. Using a combination of immunohistochemical staining, western blot, electrophysiological recording and formalin-induced conditioned place avoidance (F-CPA) rat behavioral model that directly reflects the affective component of pain, the present study examined formalin nociceptive conditioning-induced changes in the expressions of NMDA receptor subunits NR1, NR2A, and NR2B in the rostral ACC (rACC) and its possible functional significance. We found that unilateral intraplantar (i.pl.) injection of dilute formalin with or without contextual conditioning exposure markedly increased the expressions of NMDA receptor subunits NR2A and NR2B but not NR1 in the bilateral rACC. NMDA-evoked currents in rACC neurons were significant greater in formalin-injected rats than that of naïve or normal saline-injected rats. Selectively blocking either NR2A or NR2B subunit in the rACC abolished the acquisition of F-CPA and formalin nociceptive conditioning-induced Fos expression, but did not affect formalin acute nociceptive behaviors and non-nociceptive fear stimulus-induced CPA. These results suggest that both NMDA receptor subunits NR2A and NR2B in the rACC are critically involved in pain-related aversion. Thus, a new strategy targeted at NMDA NR2A or NR2B subunit might be raised for the prevention of pain-related emotional disturbance.

Keywords

NR1; NR2A; NR2B; Anterior cingulate cortex; Formalin-induced conditioned place avoidance (F-CPA); Fos; Pain affect; Rat

1. Introduction

Accumulated evidence implicates the anterior cingulate cortex (ACC) in pain processing. Neuroimaging and electrophysiological studies in humans and animals revealed that both noxious stimuli and predictive cues of noxious stimulus activate the ACC [17,29,31]. Surgical

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ablation of the ACC attenuated the pain-related depression and unpleasantness in patients suffered from chronic pain [1,5,13]. Lesion of the ACC abolished the pain-like aversion in rats [6,15,18,19]. A number of studies have shown involvement of excitatory amino acids, especially glutamate in synaptic transmission and signal processing in the ACC [10,39,42, 45]. Our previous study indicated that blockade of NMDA but not AMPA/KA receptors in the rostral ACC (rACC) significantly inhibited formalin-induced conditioned place avoidance (F-CPA), which reflects the pain-related negative affective state and aversion learning produced by the nociceptive stimulation, and attenuated F-CPA retrieval-induced Fos expression in the ACC [20]. Furthermore, Johansen et al. [16] demonstrated that glutamatergic activation in the rACC is necessary and sufficient for pain-like aversion. It is therefore suggested that activation of glutamate NMDA receptors in the rACC is required for the induction of pain-related negative affect.

Both competitive and non-competitive NMDA receptor antagonists produce unacceptable side-effects including psychotomimesis, ataxia, sedation. Thus, the development of subtype-selective NMDA antagonists has become one of the most promising current strategies [3,23, 25]. Functional NMDA receptors contain heteromeric combinations of the NR1 subunit plus one or more NR2A-D [11,27], of which the NR1, NR2A and NR2B subunits are highly expressed in forebrain areas [26]. Our previous study has demonstrated that blockade of glycine site on NR1 subunit of NMDA receptors in the rACC abolished F-CPA [30]. Studies from Zhuo's laboratory have shown that both NMDA NR2A and NR2B subunits play critical roles in the formation of long-term potentiation (LTP) in the ACC [45]. Also, the upregulation of NMDA NR2B subunit in the ACC contributed to inflammatory behavioral sensitization, and enhanced NMDA receptor-mediated EPSCs [42]. Based on the previous works, we proposed that NMDA NR2A and/or NR2B subunits would undergo upregulation in the rACC following peripheral noxious stimulation, which may thereby contribute to the formation of pain-related negative affect.

The proto-oncogene *c-fos* has widely been used as a tool to reflect excitation of central neurons in the pain pathway. The previous results from our and other studies have also revealed that behavioral training induced increases in *c-fos* expression in several different learning paradigms, including conditioned place preference, conditioned taste aversion, passive avoidance, and pain-related aversive retrieval [7,20,21,33,44]. Moreover, we have demonstrated that NMDA receptors is coupled to activation of cAMP response element-binding protein (CREB), which in turn regulated Fos expression, in the rACC via cAMP/PKA and extracellular signal-regulated kinase (ERK) [2]. Thus, Fos expression may also be a hallmark of emotional processes. The present study, therefore, examined the levels of NMDA receptor subunits NR1, NR2A, and NR2B expression in the rACC following formalin nociceptive conditioning, and the effects of blocking NR2A or NR2B subunit on F-CPA and formalin nociceptive conditioning-induced Fos expression in the rACC.

2. Materials and methods

2.1 Animal and reagents

Two hundreds sixty-eight adult (3–6 month old) and 27 young (3–4 week old) male Sprague-Dawley rats (The Animal Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were used in the present experiments. Rats were housed under a 12 : 12 h light–dark cycle with a room temperature of $22\pm 1^{\circ}\text{C}$, and received food and water *ad libitum*. All behavioral procedures took place during the light cycle. All experiments were carried out with the approval of the Shanghai Animal Care and Use Committee and followed the policies issued by International Association for the Study of Pain on the use of laboratory animals.

NR2A antisense (AS) and missense (MS) oligodeoxynucleotides (ODNs), NR2B subunit antagonists Ifenprodil (0.2 µg/µl, a gift from Prof. Li BM, Institute of Neurobiology, Fudan University, Shanghai, China) and Ro 25–6981 (2 µg/µl) (Tocris Cookson, St. Louis MO) were dissolved in sterile normal saline (NS).

2.2 Behavioral experiments

2.2.1 Surgery—Rats were anesthetized with intraperitoneal chloral hydrate (40 mg/kg), and securely placed into a stereotaxic device with Bregma and Lambda at a horizontal level. A 30-gauge stainless steel cannulae with a 33-gauge stainless steel stylet plug were bilaterally implanted 0.5 mm above the rACC injection site (AP = 2.6, ML = 0.6, and DV = 2.5 from Bregma) according to the atlas of Paxinos and Watson [28]. Animals were allowed to recover for 7 days, and began to gain a weight before the experiments. Rats that showed any neurological deficits resulting from the surgical procedure were excluded from the experiment.

2.2.2 Conditioned place avoidance (CPA)—CPA was conducted as previously described [30]. Place conditioning apparatus consists of three opaque acrylic compartments (a neutral compartment and two conditioning compartments with distinctive visual, olfactory and tactile cues) with removable doors to allow room isolation when necessary. The experimental process consists of three distinct sessions: a pre-conditioning session (days 1 and 2), conditioning session (day 3), and post-conditioning session (test, day 4). On the first day, rats were individually placed in the neutral compartment and allowed to explore the two conditioning compartments freely for 15 min, so that they habituated themselves to the apparatus. The time spent in each of the compartments was automatically recorded by a timer in a blind fashion. On day 2, the same trial was performed and the time spent in each compartment was measured. Rats that spent more than 80% (720 s) in one side on day 2, or that spent more than 600 s in one side on day 1 and more than 600 s on the other side on day 2 were eliminated from the experiment. On day 3, place conditioning was performed. In the morning, rats received nothing, and were randomly confined in one of the conditioning compartments for 45 min. After at least 4 h, in the afternoon, for formalin experiments, rats received a noxious stimulus (i.pl. 5% formalin 50 µl), or a control treatment (i.pl. NS) and then confined in the other conditioning compartment for 45 min. For electric foot-shock-induced CPA (S-CPA), the rat received an electric shock (0.5 mA for 2 s) every 8–10 min in the other conditioning compartment during the 45-min training session. On Day 4, the same trial was performed as day 1 or 2. The time spent in each compartment was measured.

2.2.3 Formalin test—Rats were placed in a 20×20×20 cm plexiglas chamber and allowed to habituate for about 15 min. A mirror was positioned below a chamber at a 45° angle for unobstructed observation of the rat's paws. The rat was given a unilateral hindpaw intraplantar (i.pl.) injection of 5% formalin (50 µl) and placed into the chamber. The responses to formalin injection were monitored by measuring the amount of time the animal spent with the injected paw elevated or licking and biting the paw per 5 min during the 45-min observation period. A weighted pain score for each animal was calculated using the following formula [32].

Formalin pain score = [time spent with injected paw elevated + 2(time spent licking or biting injected paw)]/300.

2.2.4 Antisense oligodeoxynucleotides (ODNs) preparation and drugs delivery

—An sequence of antisense ODNs (AS, 5'-GTAGCTCTTTTAGGTGAGTCC) for NR2A (corresponding to aa 915–921) and a scrambled missense ODNs (MS, 5'-GCAGGCTAGTGGTGCTCATG) whose AT/GC ratio is similar to that of the NR2A antisense

ODNs were synthesized and purified by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). To increase stability, phosphorothioate bonds were incorporated at terminal nucleotides at the 5' and 3' ends. The ODNs were screened against the GenBank Database using the BLAST algorithm to exclude non-specificity of the antisense ODNs and to show that missense ODNs did not match any registered nucleotide sequences. The antisense sequences have been proven effective and specific in previous studies [37] (Also see Fig. 4G and 4H).

AS (2 nmol/per side) or MS (2 nmol/per side) was microinjected into the rACC 6 hours before CPA training or formalin test when the NR2A expression level in the rACC was stably decreased (data not shown). Ifenprodil (0.2 µg/µl), Ro 25-6981 (2 µg/µl) or vehicle (sterile NS), was microinjected into the rACC 20 min before CPA training or formalin test. A total volume of 0.6 µl per hemisphere of either vehicle or drugs was infused via a 1 µl Hamilton syringe with the tip protruded 0.5 mm out of the guide cannulae. The injection syringe was left in place for an additional 2 min to minimize spread of the drug along the injection track. At the end of the behavioral test, the animals received a 0.6 µl infusion of 4% methylene blue to verify the location of the injection site and the extent of infusion.

2.3 Electrophysiological experiments

2.3.1 Preparation of ACC slices—Coronal brain slices containing the rACC were obtained from young rats. After anesthetizing with ethyl ether rats were decapitated. The brain was quickly removed and submerged in pre-oxygenated (95 % O₂ - 5 % CO₂) cold ACSF at 4°C, containing 126 mM NaCl, 4.0 mM KCl, 1.25 mM MgCl₂, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM CaCl₂, and 10 mM glucose. The osmolarity was adjusted to 300 mosmol/L and the pH to 7.35. A tissue block containing the rACC was glued to the stage using cyanoacrylate glue. Slices (300 µm) were cut with a vibratome (Leica VT 1000S, Leica, Germany) and incubated in an oxygenated chamber at room temperature (24 ± 1°C) for at least 1 h before recording.

2.3.2 Whole-cell patch-clamp recordings—A single slice was transferred to a recording chamber, which was continuously perfused with oxygenated ACSF at a rate of 2–3 ml/min at room temperature. Whole cell recordings were obtained from pyramidal-shaped neurons in layer II/III of the rACC with a patch clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA) under visual control using differential interference contrast and infrared optics via a water-immersion objective (DIC-IR; Leica DMLFSA, Germany). Recording electrodes made from 1.5 mm glass capillaries were pulled on a Flaming-Brown micropipette puller (P-97, Shutter Instrument Co. Novato, CA, USA), and filled with a solution containing (in mM): 140 Gluconate, 8 NaCl, 0.5 EGTA, 2 MgATP, 0.3 Na₃GTP, 10 HEPES, pH was adjusted to 7.2 and osmolarity to 280 mosmol/l. With this solution, the recording electrodes had a resistance of 2–6 MΩ. Signals were low-pass-filtered at 2 kHz and digitized at 5 kHz with a digitizer (Digidata 1322, Axon Instruments). Pyramidal-shaped neurons were easily identified by their shape and electrophysiological properties [8]. The recordings were initially obtained in normal ACSF to identify the cell's pattern. To get the NMDA current, the membrane potential was held at -50mV, and the media were switched to an ACSF solution with low Mg²⁺ (0.1 mM) and high Ca²⁺ (3.8 mM) to partially remove the Mg²⁺ block. The concentrations of drugs applied in the perfusate were (µM): 20 NMDA, 50 AP5, 20 CNQX and 1 TTX. TTX was included in the perfusate to avoid polysynaptic phenomena [24].

2.4 Immunohistochemistry and western blots experiments

2.4.1 Immunohistochemistry—After defined survival times, rats were perfused transcardially with NS followed by 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB, pH 7.4) under the overdose Chloral Hydrate (400 mg/kg) anesthesia. The brain was

removed, postfixed in the fixative solution for 4 h at 4 °C, and immersed from 10–30 % (w/v) gradient sucrose in 0.1 M PB for 24–48 hrs at 4 °C for cryoprotection. Thirty-µm coronal sections were cut with a freezing microtome (Leica 1900, German). All the sections were blocked with 10% normal goat serum (sigma) in 0.01 M PBS with 0.3 % triton-X-100 overnight at 4 °C, then incubated with rabbit anti-NR1 (1:200, Chemicon), rabbit anti-NR2A (1:2000, Chemicon), rabbit anti-NR2B (1:2000, Chemicon), or rabbit anti-Fos (1:1000, Santa Cruz Biotechnology) primary antibodies in PBS with 1 % normal goat serum and 0.3 % triton-X-100 for 48 hours at 4°C. The sections were then incubated for 2 hrs at 4 °C with fluorescein isothiocyanate (FITC) –conjugated goat anti-rabbit IgG (1:200, Jackson Immunolab). For NR1-NeuN/GFAP/OX-42, NR2A-NeuN/GFAP/OX-42, or NR2B-NeuN/GFAP/OX-42 double immunofluorescence, sections were incubated with a mixture of rabbit anti-NR1, NR2A, NR2B and mouse anti-NeuN (neuronal marker, 1: 500, Chemicon), GFAP (astrocytic marker, 1:1000, Sigma), or OX-42 (microglial marker, 1:500, Serotec) separately for 48 hours at 4°C, followed by a mixture of FITC-and rhodamine-conjugated (1:200, Jackson immunolab) secondary antibodies for 2 hrs at 4 °C. The sections were observed with a Leica SP2 confocal laser scanning microscope (Mannheim, Germany) using laser beams of 488 and 543 nm with appropriate emission filters for FITC (510–525 nm) and rhodamine (590–610 nm).

2.4.2 Western Blots—For Western Blot analysis, rats were killed by overdose of Chloral hydrate (80 mg/kg) after defined survival times, and the rACC tissues were quickly removed. The tissue extracts were prepared following the procedure described in detail previously [42], with minor modifications. The extract samples (5.0 mg/ml, 10 µl) were loaded, subjected to 12 % SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes (Millipore Immobilon-p Transfer Membrane) using Mini-Protean 3 electrophoresis system and Mini Trans-Blot electrophoretic transfer system (Bio-Tanon, Shanghai, China). The membranes were blocked with 5 % milk in PBS with 0.1 % Tween -20 for 1h at room temperature and then incubated with the antibodies against NR1, NR2A, NR2B, Fos, or tubulin at working dilutions of 1: 400, 1: 1500, 1:1500, 1:3000, 1:1000, 1:2000, and 1: 10000 respectively, overnight at 4°C. The blots were washed, incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1500, Pierce, Rockford, IL) for 2 h at 4°C, and finally visualized with enhanced chemiluminescence (SuperSignal Wester Femto Maximum Sensitivity substrate, Pierce).

2.5 Quantification and statistics

For the quantification of immunoreactive signals, six non-adjacent sections (30 µm) through the rACC were randomly selected. The number of NR1-, NR2A-, and NR2B immuno-positive (IP) cells were counted in a region that was captured inside the optic field (a square box, 750 × 750 µm) under 20 magnification, using a computerized image analysis system (Leica Qwin 500, Germany). This size of the box as shown in figure 1 was kept the same in all conditions. For the quantification of western signals, X-ray films with blotting bands were scanned. Image-pro analysis system was then used to measure the integrated optic density of the bands. Four to eight rats were included in each group for quantification of western blot and immunohistochemistry results. Differences between groups were compared using student's t-test. In the case of CPA experiments, the CPA scores represent the time spent in the paired compartment on day 2 minus the time spent in the same compartment on day 4. The differences in CPA scores among drug-treated groups were compared using one-way ANOVA followed by *post hoc* Dunnett's test. In addition, the absolute time spent in the treatment-paired compartment on the pre-conditioning day versus the post-conditioning day was compared in vehicle or drug-treated animals using paired *t-test*. For formalin-induced nociceptive behaviors, formalin pain scores per 5 min were analyzed using a two-way (drug treatment × time) ANOVA with a post hoc comparison for analysis of the differences at each 5-min period. In the case of electrophysiological experiments, data were collected and analyzed using

pCLAMP 8.1 software (Axon Instruments). Statistical significance of the results was determined with one-way ANOVA followed by *post hoc* Dunnett's test. All data were expressed as mean $\pm$ SEM, and the accepted level of statistical significance for all experiments was $p < 0.05$.

2.6 Experimental design

2.6.1 Experiment 1: Effects of i.pl. formalin on the expression of NMDA receptor subunits NR1, NR2A, and NR2B in the rACC—For i.pl. formalin injection without contextual conditioning exposure experiments, adult rats received an i.pl. injection of 5 % formalin (50 μ l) or aequales NS (as control) and stayed at home cage for 6 hrs, then were perfused with fixative or decapitated directly and the brain was removed for immunohistochemistry or western blot analysis.

For i.pl. formalin injection with conditioning exposure experiments, adult rats received the same training trial in day 1–3 of conditioning procedure as F-CPA but remaining in formalin-paired compartment for 45 min after i.pl. formalin on day 3, then stayed at home cage for 5 hours and 15 min before scarification (also see Fig. 2A). Control rats received the same conditioning exposure but formalin was replaced with NS.

2.6.2 Experiment 2: Effects of i.pl. formalin on NMDA-evoked currents in rACC neurons—Young rats received an i.pl. injection of 5 % formalin (50 μ l) or aequales NS (as control) and stayed at home cage for 6 hrs, then were decapitated and the brain was removed for preparation of rACC slices and electrophysiological recording.

2.6.3 Experiment 3: Effects of NR2A antisense ODNs or NR2B antagonists on CPA or formalin acute pain—Adult rats received the same training trial as CPA, but on the conditioning day (day 3). Microinjection of the rACC was performed 6 hrs (for NR2A AS or MS ODNs) or 20 min (for NR2B antagonists or NS) before i.pl. formalin or foot-shock (also see Fig. 4D and 5D). Formalin acute pain tests were performed 6 hrs or 20 min after intra-rACC of ODNs or antagonists respectively.

2.6.4 Experiment 4: Effects of NR2A antisense ODNs or NR2B antagonists on formalin-induced Fos expression in the rACC—Adult rats received the same training trial in day 1–3 of conditioning procedure as F-CPA but remaining in formalin-paired compartment for 45 min after i.pl. formalin on day 3, then scarification after 1 hour and 15 min for immunohistochemistry and western blot analysis. Intra-rACC was performed 6 hrs (for NR2A AS or MS ODNs) or 20 min (for NR2B antagonists or NS) before i.pl. formalin (also see Fig. 6A and 6C).

All the behavioral test, electrophysiological recording and quantification of immunohistochemical and western blot experiments were performed blind with respect to treatments.

3. Results

3.1 Basal expression of NMDA receptor subunits NR1, NR2A, and NR2B in the rACC

The basal expression of NMDA receptor NR1 subunit was higher on the rACC in non-stimulated or normal saline (NS) treated control rats (Fig 1A and 1B). The NR1-IP cells were widely distributed on both sides of the rACC. These NR1-IP cells were found in neurons, because they co-expressed the neuronal marker NeuN (Fig. 1A). Double immunofluorescence showed that NR1 did not co-localize with GFAP, an astrocytic marker, or OX-42, a microglial

marker, although GFAP-IP or OX-42-IP cells were positioned in close contact with NR1-positive cells with the overlapping processes (Fig. 1A).

Relative to NR1, NR2A subunit in the bilateral rACC exhibited a moderate basal expression in non-stimulated or NS injected control rats (Fig. 1C and 1D). The distributions of NR2A-IP cells in the rACC were similar to NR1 subunit. NR2B subunit in the bilateral rACC exhibited a similar basal expression and distribution to NR2A (Fig. 1E and 1F). Double immunofluorescence showed that almost all the NR2A- and NR2B-IP cells on the rACC were neurons (Fig. 1C and 1E).

3.2 NR2A and NR2B but not NR1 in the rACC up-regulated following formalin nociceptive conditioning

After i.pl. injection of formalin, the expression of NR1 in the ipsilateral and contralateral rACC did not obviously change at 6 hrs. The number of NR1-IP cells in the bilateral rACC was not significantly different between formalin- and NS-injected groups (*t-test*, $p > 0.05$, $n = 6-8$) (Fig. 1A and 1B).

Contrast to NR1, formalin injection induced a significant increase in NR2A expression in both sides of the rACC at 6 hrs (*t-test*, $p < 0.05$, $n = 6-8$) (Fig. 1C and 1D). No significant difference was found in the number of NR2A-IP cells between the ipsilateral and contralateral rACC.

Similar to NR2A, a significant upregulation of NR2B expression in both sides of the rACC was observed at 6 hrs after formalin injection (*t-test*, $p < 0.05$, $n = 6-8$) (Fig. 1E and 1F). No significant difference was found in the number of NR2B-IP cells between both sides of the rACC.

To determine if contextual conditioning exposure affects NR1, NR2A, and NR2B expression, we performed additional experiments with western blot analysis to compare the levels of these subunits in the rACC after i.pl. formalin injection with or without contextual conditioning exposure. Rats received the same training trial in day 1–3 of conditioning procedure as F-CPA but remained in formalin-paired compartment for 45 min, then euthanasia after 5 hours and 15 min on day 3 (Fig. 2A). Control rats received the same conditioning exposure but formalin was replaced with NS. There were not detectable difference in the NR1, NR2A, and NR2B expression levels between both sides of the rACC, we therefore pooled the two sides of the rACC for the western blot experiments.

Consistent with immunohistochemical results, no obvious change in NR1 expression level was found following i.pl. formalin injection either with or without contextual conditioning exposure (One way ANOVA, $F_{3, 16} = 1.359$, $p > 0.05$) (Fig. 2B and 2C). Formalin nociceptive conditioning induced a robust increase in the levels of NR2A and NR2B (One way ANOVA, NR2A: $F_{3, 16} = 5.393$, $p < 0.05$; NR2B: $F_{3, 16} = 15.128$, $p < 0.01$) (Fig. 2D–2G). No significant differences in NR2A or NR2B levels were found between formalin injection with and without contextual conditioning exposure (Student's *t-test*, $p > 0.05$).

3.3 Enhancement of NMDA-evoked currents following formalin injection

To confirm whether up-regulated NR2A and NR2B subunits contribute to NMDA receptor-mediated response following formalin inflammation, we compared the NMDA-evoked currents in ACC neurons from naïve, NS- and formalin-injected rats.

Conventional whole-cell patch-clamp recording were performed from visually identified pyramidal cells in layer II/III of ACC slices. Application of NMDA (20 μ M) for 60 s by addition of this compound to a low Mg^{2+} (0.1 mM) and high Ca^{2+} (3.8 mM) perfusion solution in the presence of TTX (1 μ M), evoked a large sustained inward current. The mean amplitude of the

current was 335.62 ± 26.41 pA ($n=42$); holding potential -50 mV) in naive rats. Application of AP5 (50 μ M), but not CNQX (20 μ M), completely abolished the NMDA-evoked current (Fig. 3A). No significant difference in amplitude of NMDA-evoked currents was found between i.pl. NS and naive rats. However, as predicted, the amplitude of NMDA-evoked currents was significantly greater in ACC slices from formalin-injected rats (6 hrs after formalin: 499.63 ± 41.81 pA, $n=34$) compared with that of NS controls (6 hrs after i.pl. NS: 368.71 ± 13.46 pA, $n=46$; $p<0.01$) (Fig. 3B).

3.4 Blockade of NR2A or NR2B suppressed pain-related aversion but not acute pain behavior evoked by formalin injection

Our previous studies have demonstrated that NMDA receptors in the rACC mediate the acquisition of pain-related negative emotion [20,30]. Whether the upregulation of NMDA receptor NR2A and NR2B subunits in the rACC is associated with the acquisition of pain-related aversion was further addressed in this study. As previous observation, when unilateral i.pl. injection of formalin was paired with a arbitrarily given compartment in the place conditioning apparatus, the rats spent significant less time in this compartment on the post-conditioning day as compared with the pre-conditioning day (469 ± 45 s preconditioning vs. 201 ± 67 s post-conditioning; Paired *t*-test, $p<0.01$), which means that CPA was produced (Fig. 4A and 4B). I.pl. injection of NS did not produce CPA. The differences in the CPA scores (i.e. the time spent in the formalin-paired compartment on the pre-conditioning day minus that on the post-conditioning day) between NS-treated and formalin-treated groups were statistically significant (*t*-test, $p<0.05$, $n=7$) (Fig. 4C).

Microinjection of NR2B selective antagonist Ifenprodil (0.2 μ g/ μ l, 0.6 μ l per side) or Ro 25-6981 (2 μ g/ μ l, 0.6 μ l per side) into the rACC 20 min before formalin-paired conditioning completely blocked F-CPA acquisition (Fig. 4D-4F). When NR2A AS ODNs (2 nmol/per side) were microinjected into the rACC 6 hrs before F-CPA conditioning, rats also failed to produce CPA (Fig. 4D-4F). No significant difference was found in the time spent in the formalin-paired conditioning compartment between pre- and postconditioning day. In addition, F-CPA was blocked by NR2A selective antagonist NVP-AAM077 (0.012 μ g/ μ l, 0.6 μ l per side) (data not shown). Vehicle (NS) and MS ODNs had no effect on F-CPA (Fig. 4E and 4F). The differences in the CPA scores among NS, Ifenprodil, Ro 25-6981, NR2A AS and MS ODNs groups were statistically significant (One-way ANOVA, $p<0.01$) (Fig. 4F). Western blot analysis showed that intra-rACC of NR2A AS but not NR2A MS ODNs significantly suppressed formalin-induced up-regulation of NR2A protein level, confirming the effect of specificity of NR2A AS in blocking NR2A gene expression (Fig. 4G and 4H).

NMDA receptors in the spinal cord have been shown to contribute to carrageenan-induced inflammatory pain [43] and partial chronic constriction injury (pCCI)-induced neuropathic sensitization [41]. To determine whether NMDA receptor NR2A and NR2B subunits in the rACC are also important for formalin-induced spontaneous pain, we examined formalin-induced biphasic nociceptive responses. As shown in Fig. 4I and 4J, intraplantar (i.pl.) injection of 5 % formalin elicited characteristic biphasic nociceptive responses (including lifting, licking, shaking and biting). An early response (phase 1) lasting for about 5 min was followed by a 5–10 min period of decreased activity, and then a late response (phase 2) that lasted for about 40 min. Intra-rACC microinjection of NS, Ifenprodil, NR2A AS or MS ODNs failed to change formalin-induced biphasic nociceptive behaviors within 45 min (Fig. 4I and 4J). Two-way ANOVA revealed no significant effect of intra-rACC treatment ($F_{3,23}=5.369$, $p>0.05$) and no significant interaction between intra-rACC treatment and time ($F_{24,184}=0.31$, $p>0.05$). No obvious motor dysfunction (such as circling behavior, head weaving or limb paralysis) was produced by intra-rACC Ifenprodil, NR2A AS or MS ODNs at the doses tested.

3.5 Blockade of NR2A or NR2B failed to affect fear conditioning formation

To address whether formalin-induced NR2A or NR2B up-regulation in the rACC is specific for pain-related aversion, we also assessed the effects of NR2A AS ODNs or NR2B antagonists on fear conditioning learning. For this purpose, we examined the effects of Ifenprodil, Ro 25-6981, NR2A AS or MS ODNs on mild foot-shock-induced fear conditioning (S-CPA). Like formalin injection, a low intensity electric foot-shock (0.5 mA, 2s), which has been demonstrated failing to evoke nociceptive responses in spinal dorsal horn neurons [6], produced CPA (Fig. 5A–5C). Interestingly, S-CPA was also NMDA-independent, because microinjections of Ifenprodil, Ro 25-6981 or NR2A AS ODNs into bilateral rACC had not effect on S-CPA (Fig. 5D and 5F).

3.6 Blockade of NR2A or NR2B attenuated formalin nociceptive conditioning-induced Fos expression in the rACC

Previous studies have shown that *c-fos* was induced in the rACC by formalin injection and retrieval of nociceptive experience [20–22]. Consistently, in the present study, formalin injection with contextual conditioning exposure triggered a similar Fos expression on the both sides of the rACC (Fig. 6A and 6B). The formalin nociceptive conditioning-induced Fos expression of the rACC was further confirmed by western blot analysis (Fig. 6D and 6E). Preadministration of NR2B antagonists Ifenprodil and Ro 25-6981, or NR2A AS ODNs significantly attenuated Fos expression in the rACC. Similarly, intra-rACC of NVP-AAM077, a NR2A selective antagonist, can also suppress Fos expression in the rACC (data not shown). Either NS or MS ODNs into the bilateral rACC had no effect on formalin-induced Fos expression (One-way ANOVA, $F_{6,21} = 5.026$, $p < 0.01$) (Fig. 6C–6E).

4. Discussion

An important finding of the present study is that both NMDA receptor subunits NR2A and NR2B expressions in the rACC were upregulated with formalin nociceptive conditioning, which may contribute to enhanced NMDA receptor-mediated currents in rACC neurons, and induce the formation of pain-related negative aversion when paired with a distinct context.

The formalin-test was introduced as an animal model of persistent pain in 1977, and has been used extensively in rats and mice. It is clear that s.c. formalin injection is painful and unpleasant to humans [4]. In rats, hindpaw formalin injection produces both acute nociceptive behaviors and CPA, indicating that formalin is aversive to the animal in a manner resembling the response to noxious stimuli to humans. In the present study, we observed that formalin nociceptive conditioning induced a significant increase in both NR2A and NR2B expression levels in the rACC. This result is inconsistent with the previous reports from Wu et al [42] showing that only NR2B, but not NR2A expression in the ACC was upregulated by CFA-induced peripheral inflammation. This discrepancy is hard to explain at present, but the differences in animal species (mice vs. rats) and inflammatory pain models (CFA-induced chronic persistent pain vs. formalin-induced acute tonic pain) may be primary. In agreement with the previous observation on different model [42], the present results showed that the expression of NR1 subunit remained unchanged on the rACC after formalin inflammation. Given the fact that NR1 is basely synthesized in considerable excess (estimated to be ~10-fold) compared with NR2 [12,40], it is reasonable that NR1 subunit level in the rACC did not change with formalin inflammation. The upregulated NR2A and NR2B subunits may stimulate the trafficking of pre-existing pools of NR1 subunit to the synaptic membrane, forming functional NMDA receptors [9].

Consistent with the fact that ACC neurons receive inputs from both sides of the body, and dense connecting fibers exist between the two sides of the ACC [14,31,34,38], our data showed

that all the NMDA receptor subunits NR1, NR2A, and NR2B were bilaterally distributed, and unilateral injection of formalin-induced increase in the expressions of NR2A and NR2B subunits as well as Fos in the rACC were also bilateral. The significant of peripheral inflammation-induced upregulation of NR2A and NR2B in the rACC might consist in facilitating nociceptive transmission, and enhancing excitability of rACC neurons. Our previous study showed that following peripheral inflammation, the enhancement of NMDA currents induced by D-serine was more pronounced in rACC neurons [8]. The present study further demonstrated that NMDA-evoked currents were significantly enhanced at 6 hrs after formalin injection. Indeed, ACC neuronal activity has been demonstrated to be necessary and sufficient for noxious stimuli to produce an aversive teaching signal [16]. The present result that either the acquisition of F-CPA or formalin nociceptive conditioning-induced Fos expression was blocked by NR2A AS ODNs or NR2B antagonists into the rACC further supports the proposal.

Our data showed that blockade of NR2A or NR2B in the rACC did not suppress formalin-induced spontaneous pain, in support of earlier studies from our and other groups showing that either bilateral lesions of the ACC or blockade of glutamate receptors in the rACC did not inhibit formalin-induced acute nociceptive responses [6,15,16,20,30]. In contrast, Wu et al. [42] reported that bilateral microinjections of a selective NR2B antagonist, Ro25-6981, into the ACC significantly reduced CFA-induced mechanical allodynia in mice, suggesting an involvement of inflammatory pain. Besides the difference in animal species (mice vs. rats), it appears that the NR2B in the rACC does not modulate acute pain, such as tail-flick reflex evoked by radiant heat and hot plate response [42], as well as formalin-induced two phases acute pain. Indeed, Wei et al. [39] also reported that within the first 55 min after hindpaw formalin injection (phase 1 and phase 2), nociceptive behavioral responses were similar between wild type and NR2B transgenic mice. Only in phase three (55–120 min after formalin), NR2B transgenic mice exhibited more pronounced responses compared to wild type mice.

Given that F-CPA paradigm is based on the associative learning between painful stimulus-induced aversive emotion and neutral environmental context, one possibility argue is whether the blockade of NR2A or NR2B subunit antagonist on F-CPA was due to blocking neural processing relating to learning and memory. To address this issue, we examined the effects of intra-rACC NR2A AS ODNs and Ifenprodil or Ro 25,6981 on fear conditioning induced by a low-intensity electric foot shock. Although we can not completely rule out this possibility, it is important to point out that neither NR2A AS ODNs nor NR2B antagonists can block the animal's ability of S-CPA formation. Together with previous findings from our and other groups that CPA induced by foot-shock [6] and κ -opioid receptor agonist U69,593 [15] or morphine and cocaine-induced CPP (conditioned place preference) [35,36] was not blocked by the ACC lesions, it is reasonable to conclude that the NR2A and NR2B in the rACC may specifically contribute to pain-related negative affect or pain-related aversive learning rather than general learning memory. Thus, a new strategy targeted at NMDA NR2A or NR2B subunit might be raised for the prevention of pain-related emotional disturbance.

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Fig. 1. Immunohistochemistry for NMDA receptor NR1, NR2A, and NR2B subunits on the rACC
 A, Photomicrographs showing expression of NR1 on the contralateral rACC at 6 hrs after i.pl. injection of NS and formalin. Low, double immunofluorescence showing that NR1 (green) is co-localized with NeuN (neuronal marker, red), but not with GFAP (astrocytic marker, red), and OX-42 (microglial marker, red) in the rACC. B, Quantification of NR1-positive cells on both sides of the rACC. C,E, Photomicrographs showing expression of NR2A (C) and NR2B (E) on the contralateral rACC at 6 hrs after i.pl. injection of NS and formalin. Right, double immunofluorescence showing that either NR2A (C, green) or NR2B (E, green) is co-localized with NeuN (red) in the rACC. D,F, Quantification of NR2A-(D) and NR2B-(F) IP cells on both sides of the rACC. * $p < 0.05$, ** $p < 0.01$ vs. respective NS control.

Fig. 2. Western blot for NMDA receptor NR1, NR2A, and NR2B subunits from the rACC representative of 6 hrs after i.pl. injection of NS and formalin with or without contextual conditioning exposure

A, Schematic of experimental designed in subsequent studies. B–G Western blot analysis revealing a significant increase in NR2A (D,E) and NR2B (F,G), but not NR1 (B,C) in the rACC following i.pl. formalin injection with or without contextual conditioning exposure. Two sides of the rACC were pooled in this experiment. Tubulin served as loading control. * $p < 0.05$, ** $p < 0.01$ vs. respective NS control.

Fig. 3. Effects of i.p.l. formalin injection on NMDA-evoked currents in rACC slices

A, an inward current was evoked by 20 μ M NMDA applied in low- Mg^{2+} ACSF in the presence of TTX (1 μ M) at -50 mV (upper); CNQX failed to affect the NMDA-evoked current (medial); AP5 completely blocked the NMDA-evoked current. Bar: applications of drugs. B, Quantitative results showing that the mean amplitude of NMDA-evoked currents was greater in formalin-injected rats than that in naïve and NS-injected controls. ACC pyramidal neurons were recorded in rACC slices obtained from normal, NS-injected, and formalin-injected rats 6 hrs after injection. ** $p < 0.01$ vs NS control.

Fig. 4. NMDA receptor NR2A and NR2B subunits contribute to the induction of F-CPA

A, Schematic of the protocol for behavioral testing. B,C, Histograms showing formalin-induced CPA. Mean $\pm$ SEM time spent in the treatment-paired compartment on pre- and post-conditioning day (B). Mean $\pm$ SEM F-CPA scores, i.e. the time spent in the treatment-paired compartment on the pre-conditioning day minus that on the post-conditioning day (C). D, Schematic of the behavioral procedure used in subsequent studies. E,F, Histograms showing effects of microinjection of NR2B antagonist Ifenprodil (0.2 μ g/ μ l, 0.6 μ l per side), Ro 25-6981 (2 μ g/ μ l, 0.6 μ l per side), or NR2A antisense (AS) ODNs (2 nmol/0.6 μ l per side) into the bilateral rACC on F-CPA. Values are mean $\pm$ SEM time in the formalin-paired compartment on pre-conditioning and post-conditioning days (E), and F-CPA scores (F). G,H, Western blot analysis showing that formalin-induced increase in level of NR2A receptor is blocked by intra-

rACC preadministration of NR2A AS but not NR2A MS ODNs. Tubulin serves as loading control. Both ipsilateral and contralateral sides of the rACC were pooled together in this experiment. I,J, Intra-rACC administration of NR2B antagonist Ifenprodil and NS (I), or NR2A AS and missense (MS) ODNs (J) has no effect on formalin-induced biphasic nociceptive responses. * $p < 0.05$, ** $p < 0.01$ vs. preconditioning day. + $p < 0.05$, ++ $p < 0.01$ vs. NS or MS ODNs control.

Fig. 5. NMDA receptor NR2A and NR2B subunits fail to affect the induction of S-CPA

A, Schematic of the protocol for behavioral testing. B,C, Histograms showing low-intensity (0.5 mA, 2 s) electric foot shock-induced CPA. Mean $\pm$ SEM time spent in the treatment-paired compartment on pre- and post-conditioning day (B). Mean $\pm$ SEM S-CPA scores, i.e. the time spent in the treatment-paired compartment on the pre-conditioning day minus that on the post-conditioning day (C). D, Schematic of the behavioral procedure used in subsequent studies. E,F, Histograms showing effects of microinjection of NR2B antagonist Ifenprodil (0.2 μ g/ μ l, 0.6 μ l per side), Ro 25-6981 (2 μ g/ μ l, 0.6 μ l per side), or NR2A AS ODNs (2 nmol/0.6 μ l per side) into the bilateral rACC on S-CPA. Values are mean $\pm$ SEM time in the formalin-paired compartment on pre-conditioning and post-conditioning days (E), and CPA scores (F).

*p<0.05, **p<0.01 vs. preconditioning day. ++p<0.01 vs. NS or MS control.

Fig. 6. Effects of blocking NMDA receptor NR2A or NR2B subunits on formalin nociceptive conditioning-induced Fos expression in the rACC

A, Schematic of experimental designed in subsequent studies. B, Photomicrographs showing expression of Fos on the rACC at 2 hrs after i.pl. injection of NS and formalin. C, Schematic of experimental designed in subsequent studies. D, Western blot for Fos from the rACC representative of 2 hrs after i.pl. injection of NS and formalin with contextual conditioning exposure. E, Densitometry analysis showing a significant increase in the Fos expression in the rACC following i.pl. injection of formalin with contextual conditioning exposure, which was significantly attenuated by intra-rACC injection of NR2B antagonists or NR2A AS ODNs. Two sides of the rACC were pooled in this experiment. Tubulin served as loading control. * $p < 0.05$, vs. naive; + $p < 0.05$ vs. intra-rACC NS or MS ODNs control.