Conditioning-strength dependent involvement of NMDA NR2B subtype receptor in the basolateral nucleus of amygdala in acquisition of auditory fear memory

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It is known that N-methyl-D-aspartate (NMDA) receptor in the basolateral nucleus of amygdala (BLA) is essential for fear memory formation. NMDA NR2B and NR2A subtype receptors exhibit difference in electrophysiological and signaling properties. However, it is unclear whether these two subtype receptors have different roles in fear memory formation. Here, we provide evidence, using pharmacological blockade and genetic interference, that NR2B is involved in acquisition of auditory fear memory in a conditioning-strength dependent way. Pre-conditioning intra-BLA infusion of the NR2B selective antagonist ifenprodil or Ro25-6981 impaired 48-h auditory fear memory (AFM) induced by five but not one CS–US pairing protocol, while similar treatment with the NR2A antagonist NVP-AAM077 disrupted memory for both protocols. Consistently, genetic over-expression of NR2B C-terminal in the BLA, which interferes with the C-terminal mediated intracellular signaling, produced a severe deficit in 48-h AFM for five but not one CS–US pairing protocol, whereas over-expression of NR2A C-terminal impaired memory for both protocols. Furthermore, pre-conditioning infusion of ifenprodil down-regulated the elevated phosphorylation level of extracellular signal-regulated kinase (ERK) induced by five CS–US pairing protocol. Thus, the involvement of BLA NR2B in AFM acquisition depends on conditioning strength.

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1. Introduction

Fear conditioning is a form of associative learning, where animals come to express fear responses to a neutral stimulus (conditioned stimulus, CS) that is paired with an aversive stimulus (unconditioned stimulus, US). The basolateral nucleus of the amygdala (BLA) plays an essential role in fear conditioning. Pharmacological blockade of neural activity and its biochemical concomitants in the BLA interferes with formation of fear memory, and lesion to the BLA prevents fear memory from acquisition (Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 1999, 2003; Sah et al., 2003). Long-term potentiation (LTP), a synaptic model of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993), occurs at input synapses of CS in the BLA (Chapman et al., 1990; Huang and Kandel, 1998; Rogan and LeDoux, 1995; Weisskopf et al., 1999), and fear conditioning induces associative LTP-like changes in BLA neurons (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997).

Electrophysiological and behavioral pharmacological studies have established that N-methyl-D-aspartate receptor (NMDAR) in the BLA plays an important role in synaptic plasticity and fear conditioning (Blair et al., 2001). For example, intra-BLA blockade of NMDAR disrupts induction of LTP and interferes with acquisition of auditory fear memory (Bauer et al., 2002; Campeau et al., 1992; Fanselow and Kim, 1994; Fendt, 2001; Gewirtz and Davis, 1997; Lee and Kim, 1998; Miserendino et al., 1990). Evidence also shows that NMDAR protein and current are down-regulated in the amygdala during maintenance of fear memory (Zinebi et al., 2003).

NMDAR is a heteromeric complexes composed of the obligatory NR1 subunit in combination with NR2 (A–D) and NR3 (A–B) subunits (Laube et al., 1998; Perez-Otano et al., 2001). NR2B and NR2A exhibit several important differences that could influence NMDAR-mediated synaptic plasticity and behavioral memory. For example, NR2B has longer current duration than NR2A (Laurie and Seeburg, 1994; Monyer et al., 1992; Priestley et al., 1995; Vicini et al., 1998) and carries more calcium charge per unit current than NR2A (Sobczyk et al., 2005). NR2B and NR2A have distinct intracellular binding partners (Barria and Malinow, 2005; Husi et al., 2000; Sans et al., 2000; Steigerwald et al., 2000; Vissel et al., 2002). However, it
is poorly understood whether or not NR2B and NR2A in the BLA contribute differently to formation of behavioral memory.

The present study examined the roles of NR2B and NR2A in the BLA in acquisition of auditory fear memory, using pharmacological blockade and acute genetic delivery technique that interfere with the intracellular signaling mediated by NR2B and NR2A C-terminals, respectively. We used two conditioning protocols with different conditioning strength, one of which included one CS–US pairing and the other five CS–US pairings.

2. Materials and methods

2.1. Subjects

Subjects were adult male Sprague Dawley rats (Shanghai Laboratory Animal Center, Chinese Academy of Sciences). They were housed in the plastic cages (1–2 per cage) and placed on a 12 h light/dark cycle. Food and water were provided ad libitum throughout the experiment. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health, USA (1996), were approved and monitored by the Ethical Committee of Animal Experiments at the Fudan University Institute of Neurobiology (Shanghai, China), and have complied with all the guidelines to minimize animal suffering and to reduce the number of animals used.

2.2. Surgery

Rats (200–240 g) were anesthetized with sodium pentobarbital (40 mg/kg ip). Stainless steel guide cannulae (23-gauge) were bilaterally positioned just above the basolateral nucleus of amygdala (BLA) based on the coordinates from Paxinos and Watson (1986): 2.8 mm posterior to bregma, 5.0 mm lateral to the midline, and 6.5 mm ventral to the skull surface. The guide cannulae were fixed to the skull with dental cement. Dummy cannulae, cut 0.5 mm longer than the guide cannulae, were inserted into the guide cannulae to prevent clogging and reduce the risk of infection. Rats were given at least 5 days to recover before experimental procedures.

2.3. Drug administration

For NR2A, the relatively selective antagonist NVP-AAM077 was used to dissect NR2A function. For NR2B, two antagonists were used: the non-competitive and selective NR2B antagonist ifenprodil tartrate salt (Williams, 2001) and its derivative Ro25-6981 hydrochloride. All drugs were diluted in 0.01 M phosphate-buffered saline (PBS, pH 7.4). A new sealed vial of drug was used each time, and all solutions were prepared at the same day and stored at 37 ºC and 5% CO2 in an incubator. In vitro transcribed RNA molecules from pSFV(-)-EGFP, pSFV(-)-NVP-AAM077, and pSFV(-)-Ro25-6981 were cotransfected with pSFV-helper2 RNA (a gift from Dr. Kenneth Lundstrom) into BHK-21 cells by electroporation (GenePulsifer; Bio-Rad, Hercules, CA). All virus production was performed at 31 ºC. Forty-eight hours after electroporation, virus stocks were harvested, filtered sterilized, and activated with chymotrypsin (Invitrogen). The reaction was terminated with the trypsin inhibitor aprotonin (Invitrogen). Virus was concentrated by centrifugation for 4 h at 20,000 g at 4 ºC and preserved in PBS. Final virus titers (× 106 infectious units/ml) were determined by infection of BHK-21 cells with serial dilutions of virus stocks, followed by fluorescence microscopy examination at 3 d after infection. Viral expression was visualized as green fluorescent protein (GFP) in cells infected with the virus.

2.4. DNA constructs, virus packaging and viral delivery

DNA constructs and virus packaging were the same as described by Chen et al. (2007). In brief, a CDNA encoding eCFP (Clontech) was amplified by PCR to generate 5’ Xhol and 3’ Spel sites and inserted into the Xhol and Spel sites of the noncotransfected Semiliki Forest virus (SFV) vector (a mutant form of pSFV1 vector, a gift from Dr. Kenneth Lundstrom, Basel, Switzerland) to produce pSFV(-)-eCFP construct. The SFV vector has its specific promoter (sub-genomic promoter; 26s promoter). SFV construct contains a transgene other gene delivery approaches, including a rapid and high-level transgenic expression. SFV can efficiently and preferentially infect neurons but not non-neuronal cells (Ehrengruber and Lundstrom, 2002; Lundstrom et al., 2003). The CDNAs encoding the carboxyl cytoplasmic tails (C-tails) of NR2A (B83–1464 amino acids (aa)) and NR2B (B393–1462 aa) were amplified by PCR to generate 5’ Spel and 3’ NotI sites inserted into the Spel and NotI sites of the pSFV(-)-eCFP vector to produce pSFV(-)-NVP-AAM077 and pSFV(-)-Ro25-6981 constructs. The sequences of all constructs were verified by DNA sequencing. Baby hamster kidney (BHK-21) cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 ºC and 5% CO2 in an incubator.

2.5. Fear conditioning

Fear conditioning took place in a transparent plexiglass chamber (36 cm × 23 cm × 18 cm, LWH) with a metal grid floor and a speaker (San Diego Instruments, USA). Infrared equipment was installed on the walls of the chamber to monitor behavior of rats. Rats were given 5 min to accustom to the chamber pre-conditioning. Two training protocols were employed. For one CS–US pairing protocol, rats were presented with one tone conditioned stimulus (CS; 2.2 kHz and 96 dB for 30 s) that co-terminated with a foot shock unconditioned stimulus (US: 10 mA, 2 s); for five CS–US pairing protocol, rats were given five CS–US paired presentations (0.8 mA, 0.5 s for each US, with inter-presentation interval of 90–120 s) (Rodrigues et al., 2001). After conditioning, rats were returned to home cages.

Auditory fear memory was tested 48 h post-conditioning. Rats were placed into a novel chamber for 90 s and were then given three CS presentations, each lasting 30 s with inter-CS interval of 20 s. Freezing response during the CS presentations was used as a measure for auditory fear memory (Jin et al., 2007).

2.6. Western blot analysis

Ten minutes after conditioning, rats were overdose anesthetized with sodium pentobarbital and decapitated. Amygdalar tissues were collected quickly for protein isolation. The tissues were homogenized in an ice-cold lysis buffer containing 1 Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM NaF, 1 mM Na3VO4, 1 mM PMSF and 0.5% protease inhibitors (Protease Inhibitor Cocktail, Roche Diagnostics Corporation, USA). The homogenates were centrifuged at 10,000 rpm for 10 min at 4 ºC. An aliquot of the supernatant was taken as the protein assay, and the remaining supernatant was added to the equal volume of SDS-PAGE sample buffer.

Samples (20 μg protein per lane), after being quantified, were loaded and subjected to preparative 10% SDS-PAGE, and transferred electrophoretically onto PVDF membranes (Roche Diagnostics Corporation) using an electrophoresis system (Bio-Rad, Hercules, CA, USA) and a mini-trans blot electrotransfer system (Bio-Rad). The membranes were blocked in 5% non-fat dried milk for 2 h at room temperature to block non-specific binding and then incubated with the mouse monoclonal anti-phospho-ERK1/2 antibody (diluted 1:1000; Cell Signalling Technology, Beverly, MA) at 4 ºC overnight. The membranes were washed three times for 10 min each in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20) and incubated for 2 h with the horseradish peroxidase-conjugated secondary antibody, goat anti-mouse IgG (1:1000 dilution; Pierce Biotechnology, Inc., Rockford, IL). The membranes were washed again three times for 10 min each in TBST and the signals were detected using the enhanced ECL system (Pierce Biotechnology). X-ray films were exposed to the membranes for several seconds and developed for visualization of the immunoreactive bands. Multiple exposures of each membrane were taken to ensure the linearity of the immunoreactive bands. The membranes were then stripped by washing them twice for 10 min each in β-mercaptoethanol-containing stripping buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) at 80 ºC. The stripped membranes were washed thoroughly in TBST, re-blocked for 2 h with 5% non-fat dried milk at room temperature, and then probed for total ERK using anti-total ERK antibody (1:2500 dilution; Cell Signalling Technology). The intensities of the immunoreactive bands were quantified using Quantity One software (Bio-Rad). The ratio of phospho-ERK1/2 intensity to total ERK intensity for each lane in the same membrane was presented for phospho-ERK level. The ratios of each lane were normalized to the one of control groups (five CS only) in the same membrane.

Slice stimulation was performed as described previously (Ennomo et al., 2005; Maniya et al., 2003) with a minor modification. Naïve rats were decapitated under pentobarbital anesthesia and their brains were immediately removed. The coronal slices containing the amygdala were dissected at a thickness of 300 μm using
vibratome (Ted Pella INC., USA). After incubated for 45 min at room temperature in ACSF (in mM: 124 NaCl, 2.5 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1 NaH₂PO₄, and 10 glucose; saturated with 95% O₂ and 5% CO₂), brain slices were stimulated with 20 μM NMDA and 10 μM glycine for 15 min in the presence of 0.3 μM NVP-AAM077, respectively. The brain slices were then washed with ACSF three times and the amygdala was dissected quickly with a blade. The dissected tissues were homogenized as described above for western blot analysis.

2.7. Immunohistochemistry

Five minutes after conditioning, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After cryoprotected in 10, 20, 30% (w/v) sucrose in 0.1 M PB for 24 h each at 4 °C, brains were sectioned at 20-μm thickness with a cryostat (Leica CM900, Q4 Germany). We collected one brain section from five continuous sections. Brain sections were blocked in 6% Donkey Serum (v/v) in PBS plus 1% bovine serum albumin (BSA) (w/v) and 0.2% Triton X-100 overnight at 4 °C.

For double immunofluorescence labeling, brain sections were incubated first in a mixture of two primary antibodies, and then in a mixture of two secondary antibodies. Phospho-ERK1/2 was detected by using rabbit monoclonal antibody against phospho-ERK1/2 (1:400 dilution; Cell Signaling Technology, Beverly, MA) and revealed with donkey anti-rabbit IgG tagged with fluorescein isothiocyanate (1:200 dilution; Jackson Immuno Research). Mouse anti-neuronal nuclei (NeuN) monoclonal antibody (1:1000 dilution, Chemicon, Temecula, CA) was used to label neurons and the labeling was revealed with Texas Red dye-conjugated donkey anti-mouse IgG (1:200 dilution; Jackson Immuno Research, West Grove, PA).

Fluorescently labeled brain sections were visualized under a confocal laser scanning microscope (Leica SP2, Mannheim, Germany) using 10× objective lens. Dye filter, pinhole aperture, detector gain, and offset were initially set to obtain pixel densities within a linear range, and were then kept constant for experimental comparisons. Signals were acquired using sequential line scanning. We focused on fluorescence localization in the cell body, including the cytoplasm and nucleus. The pERK1/2 positive cells in the BLA were quantified using a computerized image analysis system (Leica Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). The average number of positive cells from each rat was calculated average of three for four brain sections on different levels throughout the BLA.

2.8. Histology

To verify locations of infusions, rats were anesthetized with an overdose of pentobarbital sodium after the behavioral procedures were completed. Rats were transcardially perfused with saline, followed by 4% (v/v) formaldehyde solution, and then decapitated. Rat brains were then put in 30% (w/v) sucrose solution, and subsequently cut into sections of 40 μm with a cryostat (Leica CM900, Germany). Brain sections were mounted on gelatin-subbed glass slides and stained with neutral red (1% in ddH₂O).

To verify the spread of SFV infection, we also conducted histological examination 64–72 h after virus infusion. Brain sections were collected continuously. Virus infection was identified under a fluorescent microscope (Leica DMRXA Q500 W). We estimated the infection spread by counting the number of brain sections (40 μm in thickness) displaying green fluorescence.

2.9. Data analysis

Data were expressed as means ± SEM. Behavioral data among groups were statistically compared using one-way Analysis of Variance (ANOVA) with planned comparisons as post hoc analysis. Western-blots and immunohistochemical results were compared using Mann–Whitney U-test. p < 0.05 was considered significant. ANOVA was conducted using STATISTICA (StatSoft Inc., Chicago, IL, USA).

3. Results

We first conducted pilot experiments to evaluate the effects of the two conditioning protocols on auditory fear memory: Protocol-1 included one CS–US pairing with 1.0 mA US lasting 2.0 s, and Protocol-2 five CS–US pairings with 0.8-mA US lasting 0.5 s. Memory retention was tested 48 h post-conditioning. Learned fear was calculated using the formula (Cue–PreCue)/(Cue + PreCue) > 100 (Amorapanth et al., 1999). As shown in Table 1, rats trained with the two protocols showed comparable freezing scores before conditioning (PreCue). When exposed to the CS 48 h post-conditioning, rats trained with Protocol-2 exhibited significantly stronger fear response than those trained with Protocol-1, as expressed by freezing score and ‘learned fear’ (also see Figs. 1–3). Protocol-2 conditioning induced stronger fear memory than Protocol-1 conditioning, indicating that these two protocols have different conditioning strength. Thus, we selected these two protocols for the following experiments.

3.1. Pharmacological blockade of NR2A and NR2B subtype receptors

We first examined the effects of intra-BLA infusion of NR2A- or NR2B-antagonists on acquisition of auditory fear memory induced by one CS–US pairing protocol (Fig. 1). The NR2A antagonist NVP-AAM077 (0.006 μg in 0.5 μl PBS, n = 6; or 0.06 μg in 0.5 μl PBS, n = 7), the NR2B antagonists ifenprodil (0.1 μg in 0.5 μl PBS, n = 9; 1.0 μg in 0.5 μl PBS, n = 6) and Ro25-6981 (2.5 μg in 0.5 μl PBS, n = 6), or PBS (0.5 μl, n = 9) was bilaterally infused into the BLA 15 min pre-conditioning. Auditory fear memory was tested 48 h post-conditioning. An ANOVA revealed no group effect in freezing score before cue delivery (F(5,35) = 0.69, n.s.). As shown, rats treated with NVP-AAM077 showed a severe amnesia for the auditory cue (Fig. 1A; F(2,19) = 10.44, p < 0.01) and post hoc revealed that both 0.006 μg and 0.06 μg NVP-AAM077 produced a significant decrease in freezing behavior (p < 0.01) compared with vehicle controls, whereas rats treated with ifenprodil or Ro25-6981 exhibited no amnesia for the auditory cue (Fig. 1A; F(2,21) = 0.57, n.s. for ifenprodil; F(1,12) = 0.33, n.s. for Ro25-6981). It seems that acquisition of auditory fear memory induced by one CS–US pairing involves NR2A but not NR2B in the BLA.

It has been reported that pharmacological blockade of NR2B in the amygdala impairs acquisition of fear memory induced by five CS–US pairings (Rodrigues et al., 2001). Thus, it would be possible that the recruitment of NR2B in fear memory formation depends on training strength. To test this possibility, we repeated the experiments by Rodrigues et al. (2001) with a five CS–US pairing protocol. NVP-AAM077 (0.006 μg in 0.5 μl PBS, n = 6), ifenprodil (1.0 μg in 0.5 μl PBS, n = 7), Ro25-6981 (2.5 μg in 0.5 μl PBS, n = 6), or PBS (0.5 μl, n = 6) was bilaterally infused into the BLA 15 min pre-conditioning. An ANOVA revealed no group effect in freezing score before cue delivery (F(4,26) = 0.06, p < 0.01). As shown in Fig. 2, rats treated with NVP-AAM077, ifenprodil or Ro25-6981 all exhibited a serious deficit in 48-h memories for the auditory cue (Fig. 2A; F(1,10) = 26.97, p < 0.01 for NVP-AAM077; F(1,11) = 12.02, p < 0.01 for ifenprodil; F(1,10) = 12.07, p < 0.01 for Ro25-6981), which were induced by the five CS–US pairing protocol.

Taken together, these results suggest that NMDA NR2B in the BLA is required for acquisition of auditory fear memory induced by strong but not weak conditioning, while NR2A is involved non-differentially.

3.2. Genetic interference with NR2A and NR2B C-terminal signaling

To further confirm the conditioning-strength dependent involvement of NR2B in fear memory formation, we used a gene interference technique to express the cytoplasmic C-terminal domain of NR2A or NR2B subtype receptor in the BLA to selectively interfere with C-terminal mediated intracellular signaling. It is reported that NR2A and NR2B C-terminals bind to intracellular adaptor proteins and/or signaling molecules that determine the specificity of NR2A- and NR2B-dependent synaptic signaling pathways (Barria and Malinow, 2005; Husi et al., 2000; Sheng, 2001; Vissel et al., 2002). The functional significance of the NR2A and NR2B C-terminals has been highlighted by investigations in mice bearing C-terminally truncated mutations (Kohr et al., 2003; Sprengel et al., 1998). These mice showed altered synaptic plasticity and deficient memory.

Here, we used a Semliki Forest virus (SFV) to in vivo over-express the cytoplasmic carboxyl tail (C-tail) of NR2A (838–1464 aa) or of NR2B (839–1482 aa) fused with EGFP. A previous study showed that these C-tail peptides do not perturb the channel opening of NMDAR (see Supplementary Figure 2 of Chen et al., 2007). SFV
Rats received fear conditioning 3 days post-infusion of the virus into the BLA. Memory test was carried out 48 h post-conditioning. An ANOVA for freezing scores during the pre-conditioning period showed that there was no significant difference in freezing score among the normal control rats ($n = 6$) and rats treated with SFV(pd)-EGFP, SFV(pd)-NR2A$^{tail}$-EGFP, and SFV(pd)-NR2B$^{tail}$-EGFP ($F(3,22) = 0.989$, n.s.; figure not shown). As shown in Fig. 3, rats treated with SFV(pd)-NR2A$^{tail}$-EGFP (NR2A$^{tail}$ group) but not SFV(pd)-NR2B$^{tail}$-EGFP (NR2B$^{tail}$ group) showed deficient auditory fear memory, as compared with rats treated with SFV(pd)-EGFP (EGFP group, $n = 7$) (Fig. 3A; $F(1,11) = 13.43$, $p < 0.01$ for NR2A$^{tail}$, $n = 6$; $F(1,12) = 0.51$, n.s. for NR2B$^{tail}$, $n = 7$), which was induced by one CS–US pairing protocol. However, both NR2A$^{tail}$ and NR2B$^{tail}$ groups exhibited a severe deficit in auditory fear memory (Fig. 3A; $F(1,8) = 16.85$, $p < 0.01$ for NR2A$^{tail}$, $n = 5$; $F(1,9) = 16.15$, $p < 0.01$ for NR2B$^{tail}$, $n = 5$), which was induced by five CS–US pairing protocol.

Histological examination showed that the NR2A$^{tail}$ and NR2B$^{tail}$ groups of rats had great many cells in the BLA demonstrating green fluorescence of EGFP 64–72 h post-infusion of virus (Fig. 3E), indicating that the BLA cells expressed the C-tail peptide of NR2A or NR2B.

Table 1

<table>
<thead>
<tr>
<th>Protocol</th>
<th>PreCue</th>
<th>Cue</th>
<th>Learned fear</th>
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<tr>
<td>Protocol-1: one CS–US</td>
<td>32.5 ± 3.5</td>
<td>66.5 ± 7.5*</td>
<td>33.9 ± 4.5*</td>
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<td>(1.0 mA, 2.0 s)</td>
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<tr>
<td>Protocol-2: five CS–US</td>
<td>31.8 ± 1.7</td>
<td>90.0 ± 5.9</td>
<td>47.3 ± 3.7</td>
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<td>(0.8 mA, 0.5 s)</td>
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Data is expressed as the percentage of freezing (mean ± SEM). Learned fear is calculated as (Cue–PreCue)/(Cue + PreCue) × 100. Protocol-1: $n = 7$; Protocol-2: $n = 6$; *, $p < 0.05$ for Protocol-2 vs. Protocol-1, $t$-test.

Fig. 1. NMDA NR2B subtype receptor is not required for acquisition of auditory fear memory induced by one CS–US pairing. (A) Pre-conditioning inhibition of NR2A, but not NR2B, impaired 48-h auditory fear memory. Shown in inset are the effects of NR2A and NR2B blockade on ‘learned fear’, as calculated using the formula (Cue–PreCue)/(Cue + PreCue) × 100. **$p < 0.01$ vs. vehicle group, one-way ANOVA. (B) Reconstruction of the infusion sites in the BLA. Vehicle: filled squares; 0.006 µg NVP: open circles; 0.06 µg NVP: filled circles; 0.1 µg ifen: open triangles; 1.0 µg ifen: open squares; Ro25: black squares (left). A representative coronal section showing an infusion site of 0.006 µg NVP in the BLA, as indicated by the arrow (right); BLA: basolateral nucleus of amygdala; NVP: NVP-AAM077; ifen: ifenprodil; Ro25: Ro25-6981.

Fig. 2. NMDA NR2B subtype receptor is required for acquisition of auditory fear memory induced by five CS–US pairings. (A) Pre-conditioning inhibition of either NR2A or NR2B impaired 48-h auditory fear memory. Shown in inset are the effects of NR2A and NR2B blockade on ‘learned fear’, as calculated using the formula (Cue–PreCue)/(Cue + PreCue) × 100. **$p < 0.01$ vs. vehicle group, one-way ANOVA. (B) Reconstruction of the infusion sites in the BLA. Vehicle: filled squares; 0.006 µg NVP: open circles; 1.0 µg ifen: open squares; Ro25: open triangles (left). A representative coronal section showing an infusion site of ifenprodil in the BLA, as indicated by the arrow (right); BLA: basolateral nucleus of amygdala; NVP: NVP-AAM077; ifen: ifenprodil; Ro25: Ro25-6981.
NR2B. Meanwhile, we found that there were relatively many fluorescence-labeled cells outside the BLA in some rats, with the spread of virus infection larger than 0.8 mm. These rats were excluded from the database although they exhibited a deficient fear memory. Histological examination also identified a very few cases with infusion of the virus outside the BLA. These animals with offsite infusion of the virus exhibited no deficit in fear memory (figure not shown).

A pilot experiment showed that green fluorescence of EGFP in the BLA couldn’t be observed 20 days after virus infusion. Thus, we retrained the EGFP, NR2Atail and NR2Btail groups of rats on the five CS–US pairing protocol 16 days after the retention test (i.e., 21 days post-infusion of virus). As shown in Fig. 3C, the NR2Btail and NR2Btail groups could establish the fear memories again (Fig. 3C, auditory memory, $F(2,12) = 0.58$, n.s.; contextual memory, $F(2,12) = 0.26$, n.s.), indicating that physiological function of the BLA was intact.

To confirm that the deficient memory in the NR2Atail and NR2Btail groups was not due to non-specific effects of virus infusion, we conducted an additional experiment with rats that were infused...
into the BLA with SFV(pd)-EGFP, SFV(pd)-NR2Abrid-EGFP or SFV(pd)-NR2Bbrid-EGFP and sham trained with five unpaired presentations of CS and US 3 days post-infection of virus. As shown in Fig. 3D, the three groups of rats did not establish 48-h fear memory for the auditory cue ($F(2,12) = 2.019$, n.s.).

Therefore, the genetic interference experiment further indicates that NMDA NR2B in the BLA is essential for acquisition of fear memory triggered by strong but not weak conditioning, whereas NR2A is involved non-differentially.

3.3. Up-regulation of phospho-ERK upon NR2B stimulation and fear conditioning

It has been shown that activation of extracellular signaling-regulated kinase (ERK) in the amygdala and hippocampus is facilitated by NMDAR stimulation (Enomoto et al., 2005). Recent studies indicate that NR2B is coupled to ERK1/2 by Ras-guanynucleotide releasing factor 1 (Hardingham et al., 2001; Krapivinsky et al., 2003). To see if NR2B stimulation could trigger ERK phosphorylation in the BLA, we measured, using western blot analysis, the level of phospho-ERK in amygdalar slices incubated with NMDA (20 μM) and glycine (10 μM). As shown in Fig. 4A, treatment with NMDA significantly elevated the level of phospho-ERK1/2 (Mann–Whitney U-test, $p < 0.05$). The NMDA-induced enhancement was blocked by the NR2B antagonist ifenprodil (3 μM; Mann–Whitney U-test, $p > 0.05$ vs. control), but not by the NR2A antagonist NVP-AAM077 (0.3 μM; Mann–Whitney U-test, $p < 0.05$ vs. control), confirming that ERK is a downstream target of NR2B but not NR2A.

It is known that activation of ERK in the amygdala and hippocampus is required for fear conditioning (Athos et al., 2002; Atkins et al., 1998; Enomoto et al., 2005; Krapivinsky et al., 2003; Schafe et al., 2000). Considering that BLA NR2B is involved in acquisition of auditory fear memory induced by the five CS–US pairing protocol, we attempted to know if this conditioning protocol could trigger phosphorylation of ERK in the BLA, and if so, whether blockade of NR2B could down-regulate phospho-ERK1/2 level. To do this, we infused ifenprodil (10 μg in 0.5 μl) into the BLA 10 min pre-conditioning. As shown in Fig. 4B and C, the number of phospho-ERK1/2 positive cells in the BLA significantly increased upon the conditioning, and this increase was blocked when ifenprodil was infused.

4. Discussion

The present results suggest that: (1) NMDA NR2A and NR2B are both required for acquisition of auditory fear memory, but NR2B is recruited in a conditioning-strength-dependent way and (2) NR2B-ERK signaling might underlie the role of NR2B in formation of fear memory.

The 0.006 and 0.06 μg doses of NVP-AAM077 significantly impaired fear memory acquisition, while the 0.1 μg dose of ifenprodil did not. As NVP-AAM077 is a relatively selective antagonist for NR2A–NMDARs, with 10-fold higher selectivity for NR2A- than for NR2B–NMDARs. Thus, the deficient memory induced by NVP-AAM077 was most likely to be mediated via inhibition of NR2A instead of NR2B–NMDARs.

As known, NR2A and NR2B have distinct electrophysiological and signaling properties (Barria and Malinow, 2005; Husi et al., 2000; Laurie and Seeburg, 1994; Monyer et al., 1992; Priestley et al., 1993; Sans et al., 2000; Steigerwald et al., 2000; Vicini et al., 1998; Vissel et al., 2002). Dissection of their functions will promote our understanding of the role of NMDAR in learning and memory. However, as specific antagonists for NR2A are not well developed, the role of NR2A in learning and memory is still poorly understood. Recently, a relatively selective NR1/NR2A antagonist, NVP-AAM077, has been developed. However, its selectivity has been much debated. Some previous studies argue that NVP-AAM077 is not sufficient to discriminate between NR2A and NR2B, with about 10-fold selectivity (Frizelle et al., 2006; Neyton and Paoletti, 2006). In the present study, we used NVP-AAM077 for pharmacological blockade of NR2A in the BLA and found that NVP-AAM077 treatment non-differentially impaired acquisition of fear memory induced by the two conditioning protocols, one of which included a single CS–US pairing and the other five CS–US pairings. Although NVP-AAM077 could act at NR2B as well, it is unlikely that the NVP-AAM077 effect was mediated partly by NR2B, because similar treatment with the selective NR2B antagonist ifenprodil or Ro25-6981 produced no deficit in acquisition of fear memory induced by the single CS–US pairing protocol. More importantly, we over-expressed the C-terminal peptide of NR2A in the BLA to interfere with the intracellular signaling mediated by the C-tail of NR2A and found that rats under this condition showed a severe deficit in acquisition of fear memory for the single CS–US conditioning, well consistent with the previous study by Sprengel et al. (1998), who showed that gene-targeted mice without the intracellular C-terminal domain of NR2A in the hippocampus showed a deficient contextual fear memory. Thus, these two lines of evidence together indicate an essential role of NR2A in the BLA in fear memory formation.

Interestingly, our results suggest the importance of NR2B in the BLA for fear memory formation depends on conditioning strength. Intra-BLA blockade of NR2B using ifenprodil or Ro25-6981 caused a severe deficit in acquisition of fear memory induced by the five CS–US pairing protocol, while similar treatment with the drugs had no impact on acquisition of fear memory triggered by the single CS–US pairing protocol. In parallel, rats with over-expression of the C-terminal peptide of NR2B in the BLA exhibited a deficient fear memory induced by the five but not one CS–US pairing protocol. It is well documented that NR2B is important for synaptic plasticity and fear memory formation in the amygdala (Bauer et al., 2002; Nakazawa et al., 2006; Rodrigues et al., 2001), hippocampus (Tang et al., 1999) and cortex (Zhao et al., 2005). Several lines of evidence have showed that NR2B in the amygdala is essential for fear conditioning. For example, intra-amygdala blockade of NR2B using ifenprodil, or interference with the NR2B-mediated signaling using polyamine inhibitor or with the phosphorylation site of NR2B (Tyr-1472) through knock-in mutation, impairs fear memory formation and fear memory extinction (Nakazawa et al., 2006; Rodrigues et al., 2001; Sotres-Bayon et al., 2007; Zinebi et al., 2003). These previous studies employed three to five CS–US pairing protocols for conditioning (but see Nakazawa et al., 2006). Especially, Rodrigues et al. (2001) showed that pre-conditioning blockade of NR2B in the lateral nucleus of amygdala (LA) impaired 24-h long-term fear memory. These authors used a similar conditioning protocol with five CS–US pairings: each CS was paired with a 0.5-mA US lasting 0.5 s. However, they did not compare the effects of intra-BLA blockade of NR2B on strong- vs. weak-conditioning induced fear memories.

It has been shown that auditory fear conditioning is accompanied by an enhancement of synaptic plasticity at auditory input synapses in the BLA (Collins and Pare, 2000; McKernan and Shinnick-Gallagher, 1997; Repa et al., 2001; Rogan and LeDoux, 1995; Rogan et al., 1997). Indeed, the NR2B involvement in LTP induction depends on induction/training protocols. For example, in the CA1 region and the BLA, LTP induced by tetanus involves NR2B (Bauer et al., 2002; Berberich et al., 2007), whereas LTP induced by pairing low-frequency presynaptic stimulation with postsynaptic depolarization does not (Bauer et al., 2002; Liu et al., 2004). A previous study with single channel recording showed that NR2B has a lower opening probability than NR2A (Erreger et al., 2005). As NR2A is predominantly incorporated in synapses in mature neurons, whereas NR2B mainly in extrasynaptic membrane (Li et al., 1998; Rumbaugh and Vicini, 1999; Stocca and Vicini, 1998; Tovar...
and Westbrook, 1999), it is possible that glutamate molecules elicited by the single CS–US pairing protocol are not enough to activate NR2B. Increase in the conditioning strength, for example, conditioning with the five CS–US pairings, triggers release of enough glutamate molecules that could activate not only NR2A but NR2B as well.

It has been documented that fear conditioning induces, via NMDAR, the activation of extracellular signal-regulated kinase (ERK). Figure 4 shows the up-regulation of phospho-ERK upon NR2B stimulation and fear conditioning. (A) NMDA treatment significantly enhanced phospho-ERK1/2 level. This enhancement was blocked by ifenprodil (3 μM) but not by NVP-AAM077 (0.3 μM). BLA slices were incubated in ACSF containing NMDA (20 μM) and glycine (10 μM). *p < 0.05; **p < 0.01, Mann–Whitney U-test. n = 5 rats. (B) Phospho-ERK1/2 positive neurons in the BLA were increased upon the conditioning with five CS–US pairings (compare B2 with B1). This enhancement was blocked by pre-training intra-BLA infusion of ifenprodil (1 μg/0.5 μl each side) (B4), but not by vehicle infusion (B3). Phospho-ERK1/2 positive cells are labeled in green, and neurons are marked in red with NeuN. Scale bar, 50 μm. (C) Quantification of phospho-ERK1/2 positive neurons in the BLA. *p < 0.05 vs. normal with five CS training; *p < 0.05 vs. vehicle, Mann–Whitney U-test, n = 7 rats for normal group with five CS training, n = 7 rats for normal group with 5 CS–US conditioning, n = 5 rats for vehicle treatment, n = 7 rats for ifenprodil treatment.

Fig. 4. Up-regulation of phospho-ERK upon NR2B stimulation and fear conditioning. (A) NMDA treatment significantly enhanced phospho-ERK1/2 level. This enhancement was blocked by ifenprodil (3 μM) but not by NVP-AAM077 (0.3 μM). BLA slices were incubated in ACSF containing NMDA (20 μM) and glycine (10 μM). *p < 0.05; **p < 0.01, Mann–Whitney U-test. n = 5 rats. (B) Phospho-ERK1/2 positive neurons in the BLA were increased upon the conditioning with five CS–US pairings (compare B2 with B1). This enhancement was blocked by pre-training intra-BLA infusion of ifenprodil (1 μg/0.5 μl each side) (B4), but not by vehicle infusion (B3). Phospho-ERK1/2 positive cells are labeled in green, and neurons are marked in red with NeuN. Scale bar, 50 μm. (C) Quantification of phospho-ERK1/2 positive neurons in the BLA. *p < 0.05 vs. normal with five CS training; *p < 0.05 vs. vehicle, Mann–Whitney U-test, n = 7 rats for normal group with five CS training, n = 7 rats for normal group with 5 CS–US conditioning, n = 5 rats for vehicle treatment, n = 7 rats for ifenprodil treatment.
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Kohr, G., Jensen, V., Koeuster, H.J., Mihaljevic, A.L., Utvik, J.K., Kvello, A., Ottersen, O.P., 2007. Morphological blockade and genetic interference, that both NR2A and NR2B are coupled to activation of ERK signaling, whereas extrasynaptic NR2B–NMDARs-induced inhibition of ERK signaling (Ivanov et al., 2006; Kim et al., 2005). In the present study, the up-regulation of phospho-ERK following NR2B–NMDARs stimulation may be a balanced outcome of the synaptic NR2B-induced activation and the extrasynaptic NR2B-induced inhibition of ERK. However, as activation of extrasynaptic NR2B–NMDARs requires global release of glutamate which rarely occurs in physiological conditions but is usually seen in pathological conditions like hypoxic/ischaemic insults, the conditioning-induced up-regulation of phospho-ERK in the present study is mostly likely induced by synaptic NR2B–NMDARs.

In summary, the present study demonstrated, using pharmacological blockade and genetic interference, that both NR2A and NR2B in the BLA play an essential role in acquisition of auditory fear memory: while NR2A is required non-differentially, NR2B is involved in a conditioning-strength dependent way.

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