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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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ABSTRACT

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.

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

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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 Watsons (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 −20 °C. We used two doses of NVP-AAM077 (0.006 and 0.06 μg, in 0.5 μl PBS), two doses of ifenprodil (0.1 and 1.0 μg, in 0.5 μl PBS) and one dose of Ro25-6981 (2.5 μg, in 0.5 μl PBS) for intra-BLA infusion. Equal volume of PBS was infused as vehicle control (0.5 μl).

Rodrigues et al. (2001) reported that intra-amygdala infusion of 0.1 or 1.0 μg ifenprodil (in 0.5 μl vehicle) produced a deficit in fear memory. Based on this, the present study also selected the 0.1 and 1.0 μg doses of ifenprodil for intra-BLA infusion. The 0.06 μg dose of NVP-AAM077 was chosen as it was equal to the 0.1 μg dose of ifenprodil in molarity. We also used a 10-fold lower dose of NVP-AAM077 (0.006 μg) for intra-BLA infusion.

For drug administration, rats were held gently while the dummy cannulae were exchanged with 30-gauge infusion cannulae. The tip of the injection needle was 2.0 mm beyond that of the guide cannulae, yielding a total distance of 8.5 mm from the skull surface. The infusion cannulae were connected to 10-μl Hamilton syringes via polyurethane tubing. The tubing was back-filled with PBS, with a small air bubble separating the PBS from drug solution. Drug solution was infused bilaterally with an infusion pump at a rate of 0.2 μl/min. Each side of the BLA was infused with a total volume of 0.5 μl drug solution. After infusion, the infusion cannulae were left in place for an additional 2 min to allow drug solution to diffuse away from the tip of the cannulae. The dummy cannulae were then re-placed and rats were returned to home cages. Infusions were performed 15 min pre-conditioning.

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 EGFP (Clontech) was amplified by PCR to generate 5′ XhoI and 3′ SpeI sites and inserted into the XhoI and SpeI sites of the noncytotoxic Semliki Forest virus (SFV) vector (a mutant form of pSFV1 vector, a gift from Dr. Kenneth Lundstrom, Basal, Switzerland) to produce pSFV(pd)-EGFP construct. The SFV vector has its specific promoter (sub-genomic promoter: 26s promoter). SFV confers several advantages over 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 [838–1464 amino acids (aa)] and NR2B (839–1482 aa) were amplified by PCR to generate 5′ SpeI and 3′ NotI sites inserted into the SpeI and NotI sites of the pSFV(pd)-EGFP vector to produce pSFV(pd)-NR2Atail-EGFP and pSFV(pd)-NR2Btail-EGFP 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/streptomycin. Cultures were maintained at 37 °C and 5% CO_2 in an incubator. *In vitro* transcribed RNA molecules from pSFV(pd)-EGFP, pSFV(pd)-NR2Atail-EGFP, and pSFV(pd)-NR2Btail-EGFP were cotransfected with pSFV-helper2 RNA (a gift from Dr. Kenneth Lundstrom) into BHK-21 cells by electroporation (GenePulserII; Bio-Rad, Hercules, CA). All virus production was performed at 31 °C. Forty-eight hours after electroporation, virus stocks were harvested, filter sterilized, and activated with chymotrypsin (Invitrogen). The reaction was terminated with the trypsin inhibitor aprotinin (Invitrogen). Virus was concentrated by centrifugation for 4 h at 20,000 × *g* at 4 °C and dissolved in PBS. Final virus titers ($\geq 10^{10}$ 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.

Virus was bilaterally infused into the BLA on anesthetized rats at a rate of 0.1 μl/min using a pump. After infusion, the infusion cannulae were left in place for an additional 5–10 min to allow the solution to diffuse away from the cannula tip. The infusion volume was 0.6 μl for each BLA. Behavioral experiments were performed 64–72 h after virus infusion, as SFV demonstrated the best infection *in vivo* 64–72 h post-infusion.

2.5. Fear conditioning

Fear conditioning took place in a transparent plexiglas 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 freezing behavior of rats.

Rats were given 5 min to acclimate 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; 1.0 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 r.p.m. for 10 min at 4 °C. An aliquot of the supernatant was taken for 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 a electrophoresis system (Bio-Rad, Hercules, CA, USA) and a mini trans-blot electrophoretic transfer 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 Signaling Technology, Beverly, MA) at 4 °C 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 immunoreactivity bands. Multiple exposures of each membrane were taken to ensure the linearity of the immunoreactive bands. The membranes were then stripped by washing them two times 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 60 °C. 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 Signaling 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 (Enomoto et al., 2005; Mamiya 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