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Ex vivo depotentiation of conditioning-induced potentiation at thalamic input synapses onto the lateral amygdala requires GluN2B-containing NMDA receptors

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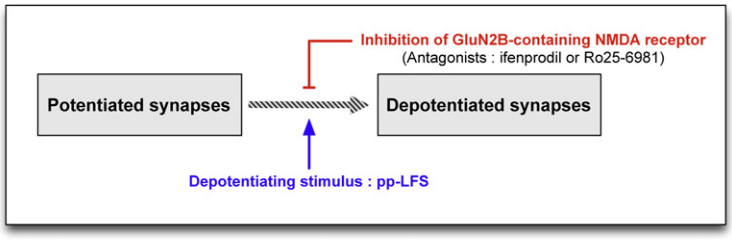
HIGHLIGHTS

- We examined a role of GluN2B-containing NMDARs in depotentiation at T-LA synapses.
- Ro25-6981, a GluN2B-containing NMDAR inhibitor blocked depotentiation and L-LTP.
- 3 μM ifenprodil, another GluN2B-containing NMDAR inhibitor, impaired depotentiation.
- 10 μM ifenprodil failed to inhibit depotentiation.

GRAPHICAL ABSTRACT

T-LA synapses in slices prepared from fear-conditioned rat.



T-LA synapses in slices prepared from fear-conditioned rat

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ABSTRACT

We have previously characterized the *ex vivo* depotentiation (depotentiation$_{ex\ vivo}$) of conditioning-induced synaptic potentiation at thalamic input synapses onto the lateral amygdala (T-LA synapses) as a potential cellular substrate for fear extinction: both depotentiation$_{ex\ vivo}$ and fear extinction require NMDA receptors, mitogen-activated protein kinases, metabotropic glutamate receptor 1, *de novo* protein synthesis and AMPA receptor internalization in the amygdala. Surprisingly, as shown in our and other previous studies, ifenprodil, an antagonist of GluN2B-containing NMDA receptors, fails to inhibit depotentiation$_{ex\ vivo}$ at a saturating concentration (10 μM), although it has been suggested that GluN2B-containing NMDA receptors are required for fear extinction. Because ifenprodil is also known to act on other molecular targets in addition to GluN2B-containing NMDA receptors, especially at high concentrations (*i.e.*, ≥10 μM), the ineffectiveness of 10 μM of ifenprodil may be due to its side effects. Therefore, in the present study, we tested Ro25-6981, a more specific antagonist of GluN2B-containing NMDA receptors, and a lower concentration (3 μM) of ifenprodil, which may reduce any possible side effects. Ro25-6981 (3 μM) blocked both depotentiation$_{ex\ vivo}$ and late-phase long-term potentiation at T-LA synapses. While 10 μM ifenprodil failed to inhibit depotentiation$_{ex\ vivo}$, a lower concentration (3 μM) of ifenprodil blocked depotentiation$_{ex\ vivo}$. Together, our findings suggest that depotentiation$_{ex\ vivo}$ requires GluN2B-containing NMDA receptors.

1. Introduction

Auditory fear memory is known to be encoded as conditioning-induced synaptic potentiation at thalamic and cortical input synapses onto the lateral amygdala [30,37,39], whereas it is widely held that fear extinction, the lessening of conditioned fear responses with repetitive presentation of the conditioned stimuli (CS) alone, is mediated by an inhibitory influence on the central amygdala (a major output structure of the amygdaloid complex). This inhibitory influence is thought to be exerted by both basal amygdala neurons and amygdala intercalated neurons, which are controlled by the prefrontal cortex [1,11,22,27,28,32,33]; see also [5,25]. In addition, there is sound evidence that depotentiation (or

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weakening) of the conditioning-induced synaptic potentiation at thalamic and cortical input synapses onto the lateral amygdala underlies fear extinction [7,8,14,13,17–19,23].

We have reported a new form of synaptic depotentiation at thalamic input synapses onto the lateral amygdala (T-LA synapses) [17]. Specifically, fear conditioning induces synaptic potentiation *in vivo*, and subsequently, the conditioning-induced synaptic potentiation can be depotentiated *ex vivo* in brain slices prepared from conditioned animals [17]. In that previous study, we found that depotentiating stimuli (paired-pulse low-frequency stimulation; pp-LFS) selectively produce synaptic depression in slices prepared from conditioned animals but not in slices from naïve or unpaired controls. Perhaps more importantly, this *ex vivo* depotentiation (depotentiation$_{ex\ vivo}$) is occluded by fear extinction, suggesting that these two events share some mechanisms. Depotentiation$_{ex\ vivo}$ requires *de novo* protein synthesis, mitogen-activated protein kinases, NMDA receptors, metabotropic glutamate receptor 1 (mGluR1) and AMPA receptor internalization [17,18]. Interestingly, fear extinction has also been shown to be attenuated by the intra-amygdala infusion of inhibitors of these molecules and processes [16–18,24,26], demonstrating a tight correlation between depotentiation$_{ex\ vivo}$ and fear extinction.

An exception to this tight correlation comes from our previous study using ifenprodil, an antagonist for NR2B-containing NMDA receptors [18]. Ifenprodil fails to inhibit depotentiation$_{ex\ vivo}$ at a saturating concentration (10 μM), although it blocks long-term potentiation at T-LA synapses. However, ifenprodil has been shown to attenuate fear extinction when microinfused into the LA [38], although it is not feasible to estimate the ifenprodil concentration achieved within the LA during extinction. Because ifenprodil has been shown to affect other molecular targets in addition to GluN2B-containing NMDA receptors, especially at high concentrations (≥10 μM) [2,4,6,9,12,29], it is possible that ifenprodil at high concentrations produces side effects on depotentiation$_{ex\ vivo}$ induction.

In the present study, to re-examine whether depotentiation$_{ex\ vivo}$ induction requires GluN2B-containing NMDA receptors, we used a more specific antagonist for GluN2B-containing NMDA receptors, Ro25-6981, in the micromolar range [10,31,42]. We also tested a lower concentration of ifenprodil to reduce any side effects. To avoid any bias, all experiments were performed in a blinded manner.

2. Materials and methods

The subjects were male Sprague–Dawley rats (4–5 weeks old) obtained from a commercial supplier (Samtaco, Osan, Korea). Fear conditioning and slice electrophysiology were performed as previously described [17,18,21] under the guidelines of the Institute of Laboratory Animal Resources of Seoul National University.

For fear conditioning, the rats were placed in a conditioning chamber (context A) and left undisturbed for 2 min. A neutral tone (30 s, 2.8 kHz, 85 dB) co-terminating with an electrical foot shock (1.0 mA, 1 s) was presented three times at an average interval of 100 s. The rats were returned to their home cage 60 s after the last shock was applied. On day 3, a single conditioned tone stimulus (30 s, 2.8 kHz, 85 dB) was presented in context B for tone testing 4 min after placement of the rats. Conditioned freezing was defined as immobility, with the exception of respiratory movements. Freezing was quantified as the percentage of freezing time during the tone test (30 s).

For slice electrophysiology, the rats were anesthetized with isoflurane and decapitated. The isolated whole brains were placed in an ice-cold modified artificial cerebrospinal fluid (aCSF) solution containing (in mM) 175 sucrose, 20 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, and 11 D-(+)-glucose and gassed with 95% O_2/5% CO_2. Coronal slices (400 μm) that included the LA were cut using a vibroslicer (HA752, Campden Instruments, Loughborough, UK or VT1200, Leica, Germany), incubated in normal aCSF containing (in mM) 120 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 2 $CaCl_2$, and 11 D-(+)-glucose and continuously bubbled at room temperature with 95% O_2/5% CO_2. Prior to transferring a slice to the recording chamber, the cortex overlying the LA was cut away with a scalpel such that cortical epileptic burst discharges would not invade the LA in the presence of picrotoxin. A submersion-type chamber (approximately 0.5 ml) was continuously superfused with aCSF (33.0–34.5 °C) at a constant flow rate of 1–2 ml/min maintained by a peristaltic pump (REGLO digital, Ismatec, Switzerland). Picrotoxin (10 μM) was included in the recording solution to isolate excitatory synaptic transmission and block feed-forward GABAergic inputs to principal neurons in the LA [15,17,18].

We chose brain slices containing a well-isolated, sharply defined trunk (containing thalamic afferents) innervating the dorsolateral division of the LA, in which somatosensory and auditory inputs are known to converge [34]. Thalamic afferents were stimulated using a concentric bipolar electrode (MCE-100, Rhodes Medical Instruments, CA) placed on the midpoint of the trunk between the internal capsule and medial boundary of the LA. Extracellular field potential recordings were made using parylene-insulated microelectrodes (573,210, A-M Systems, Carlsborg, WA) placed beneath the midpoint of the trunk horizontally spanning the LA. As shown by our own studies and others [15,17,18,21], field potentials at T-LA synapses exhibited a constant and short latency of approximately 4 ms, followed high-frequency stimulation (HFS) reliably and without failure, and could be blocked by kynurenic acid. Baseline stimulation (0.017 Hz, 0.2 ms pulse duration) was delivered at an intensity (typically 10–25 μA) that evoked a response that was approximately 50% of the maximum evoked response. To induce depotentiation $_{ex\ vivo}$, paired-pulse low-frequency stimulation (pp-LFS) was delivered at 1 Hz for 15 min (50 ms inter-stimulus interval). Late-phase long-term potentiation (L-LTP) was elicited by six trains of HFS (100 Hz, 1 s in duration, 1-min interval) with the same intensity and pulse duration as the test stimuli. One or two slices were recorded per animal. CPCCOEt, Ro25-6981 and ifenprodil were purchased from Tocris Bioscience (Bristol, UK). Ro25-6981 was dissolved in aCSF at a concentration of 3 μM. The stock solutions of ifenprodil and CPCCOEt were constituted in dimethyl sulfoxide and diluted more than 1000 times in aCSF. A probability value of $p < 0.05$ was considered indicative of statistical significance.

3. Results

We used an auditory fear conditioning protocol that has been shown to produce persistent synaptic potentiation at T-LA synapses [17,30,37]. Conditioned rats exhibited strong and consistent freezing behavior when exposed to the CS (80.0 ± 4.8%, $n = 5$) but little freezing to the context during a 4 min acclimation period before tone presentation (8.5 ± 5.3%, $n = 5$; Fig. 1A). As shown in Fig. 1B, pp-LFS successfully induced synaptic depression at T-LA synapses in brain slices prepared from vehicle controls (77.8 ± 2.8%, $n = 5$, $p = 0.0013$, paired t-test). This depression appears to represent the reversal of the *in vivo* synaptic potentiation that was preserved in amygdala slices (depotentiation$_{ex\ vivo}$) because pp-LFS produces synaptic depression only in amygdala slices from conditioned rats but not in slices from naïve or unpaired controls, as shown in our previous studies [17]. To determine whether depotentiation$_{ex\ vivo}$ requires GluN2B-containing NMDA receptors, we used Ro25-6981, a specific antagonist for GluN2B-containing NMDA receptors [10].