Amygdala depotentiation *ex vivo* requires mitogen-activated protein kinases and protein synthesis

Jeongyeon Kim, Sungmo Park, Sukwon Lee and Sukwoo Choi

We have recently characterized a form of *ex vivo* depotentiation (depotentiation$_{ex\ vivo}$), which correlates tightly with fear extinction, at thalamic input synapses onto the lateral amygdala. Here, we examined the effects of learning-attenuating drugs, reported to impair fear extinction when microinjected into the basolateral amygdala, on depotentiation$_{ex\ vivo}$. U0126, a mitogen-activated protein kinase inhibitor, and cycloheximide, a protein synthesis inhibitor, blocked depotentiation$_{ex\ vivo}$. However, ifenprodil, an NR2B-containing NMDA receptor inhibitor, did not alter depotentiation$_{ex\ vivo}$, although it blocked amygdala long-term potentiation. These findings indicate that amygdala depotentiation shares some molecular processes with learning and further suggest that different forms of synaptic plasticity in the basolateral amygdala mediate fear extinction. *NeuroReport* 20:517–520 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

NeuroReport 2009, **20**:517–520

Keywords: depotentiation, fear extinction, mitogen-activated kinase, NR2B-containing NMDA receptors, protein synthesis

School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, Republic of Korea

Correspondence to Dr Sukwoo Choi, School of Biological Sciences, College of Natural Sciences, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Republic of Korea
Tel: +82 2 880 6700; fax: +82 2 872 1933; e-mail: sukwoo12@snu.ac.kr

Received 19 December 2008 **accepted** 13 January 2009

Introduction

Fear extinction is the phenomenon in which conditioned fear responses disappear when conditioned animals are exposed repeatedly to conditioned stimuli without aversive unconditioned stimuli [1,2]. There is considerable evidence that the basolateral amygdala has a critical role in the extinction of auditory-cued fear memory; pharmacological blockade of NMDA receptors, NR2B-containing NMDA receptors, mitogen-activated protein kinases, neuropeptide Y Y_1 receptors, cannabinoid receptor 1, metabotropic glutamate receptors, or protein synthesis in the basolateral amygdala has been shown to attenuate fear extinction [3–11]. Most of these drugs have also been shown to attenuate learning, which is consistent with the prevailing view that fear extinction involves new learning [1,2]. However, it is also possible that these molecules are involved in amygdala depotentiation. In this study, we have determined whether an antagonist for mitogen-activated protein kinases, protein synthesis, or NR2B-containing NMDA receptors inhibits paired pulse low-frequency stimulation (pp-LFS)-induced depotentiation$_{ex\ vivo}$, the reversal of fear conditioning-induced potentiation, which has been proposed as a cellular mechanism for fear extinction [9].

Methods

To avoid possible bias, our experiments were performed in a blinded manner. All behavioral procedures were approved by the Institute of Laboratory Animal Resources of Seoul National University. Male Sprague–Dawley rats (4–5 weeks old) were given free access to food and water and housed under an inverted 12 h/12 h light/dark cycle. Behavioral training was performed during the dark portion of the cycle. For the depotentiation$_{ex\ vivo}$ experiments, rats were trained using auditory-cued fear conditioning, and amygdala slices were prepared 72 h after conditioning. For fear conditioning, rats were placed in a conditioning chamber for 2 min. A neutral tone (30 s, 2.8 kHz, 85 dB) coterminating with an electrical foot shock (1.0 mA, 1 s) was then presented three times at an average interval of 100 s. The rats returned to their cages 1 min after the application of last shock.

Brain slices were prepared using techniques described earlier [9,12]. Rats were anesthetized with isoflurane and decapitated. The isolated whole brains were placed in an ice-cold modified artificial cerebrospinal fluid (aCSF) solution containing 175 mM sucrose, 20 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, and 11 mM D-(+)-glucose, and gassed with 95% O_2/5% CO_2. Coronal slices (400 μm) including the lateral amygdala were cut using a vibroslicer (NVSL; World Precision Instruments, Sarasota, Florida, USA), incubated in normal aCSF containing 120 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, and 11 mM D-(+)-glucose, and continuously bubbled at room temperature with 95% O_2/5% CO_2. Immediately before transferring a slice to the recording chamber, the cortex overlying the lateral amygdala was cut away with a scalpel so that, in the presence of picrotoxin, cortical epileptic burst discharges would not invade the lateral amygdala.

Extracellular field potentials were recorded with parylene-insulated microelectrodes (573210; A-M Systems, Everett, Washington, USA) as described earlier [9,12].

0959-4965 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/WNR.0b013e328329412d

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Stimuli to thalamic pathways elicited simple negative field potentials that had a constant peak latency of approximately 5 ms and a duration of 3–15 ms. As shown by our own studies and those of others [9,12,13], field potentials (population spikes) at thalamic input synapses onto the lateral amygdala exhibited a constant and short latency of about 5 ms, followed by high-frequency stimulation, reliably and without failure, and could be blocked by kynurenic acid. This indicates that the field potentials measured in this study reflect glutamatergic and monosynaptic responses at thalamic input synapses onto the lateral amygdala. We included picrotoxin (10 μM) in the recording solution to isolate excitatory synaptic transmission and to block feedforward γ-aminobutyric acidergic inputs to principal neurons in the lateral amygdala. Baseline stimulation (0.017 Hz, 0.2 ms pulse duration) was delivered at an intensity (typically 0.1–0.25 mA) that evoked a response that was around half of the maximum evoked response. To induce depotentiation$_{ex\ vivo}$, pp-LFS (50 ms interstimulus interval) was delivered at 1 Hz for 15 min. Neuronal responses were amplified, filtered (low-pass filter, 1 kHz; high-pass filter, 1 Hz; Dam80; World Precision Instruments), and then digitized at 5 kHz (M-Series board; National Instruments, Austin, Texas, USA) or at 20 kHz (NAC 2.0 acquisition system, Thetaburst, Theta Burst Corp., Irvine, California, USA). The digitized signals were stored and analyzed on a computer using the WinLTP program (*www.winltp.com*) or NAC Gather software (Theta Burst Corp.). A submersion-type recording chamber (0.5 ml in volume) was continuously superfused with aCSF (33.5–34.5°C) at a constant flow rate of 1–2 ml/min. One or two slices were recorded per animal. To obtain stable, long-term recordings, we began recording at least 3.5 h after preparing the 400-μm-thick slices [14].

To improve the signal-to-noise ratio, data were averaged using a three-point running average in time-lapse experiments. A temporal average of the data points during the period of interest (0–30 min for baseline; 120–150 min for a post-pp-LFS period; 30–60 min for a post-high-frequency stimulation period) was used for statistical comparison of field potential results. Values of P less than 0.05 were considered significant. Drugs used were U0126, ifenprodil, and cycloheximide from Tocris Bioscience (Bristol, UK). Cycloheximide was dissolved in aCSF at a concentration of 300 μM. U0126 and ifenprodil were made up in stock solutions (dimethyl sulfoxide) and diluted more than 2000 times into aCSF.

Results

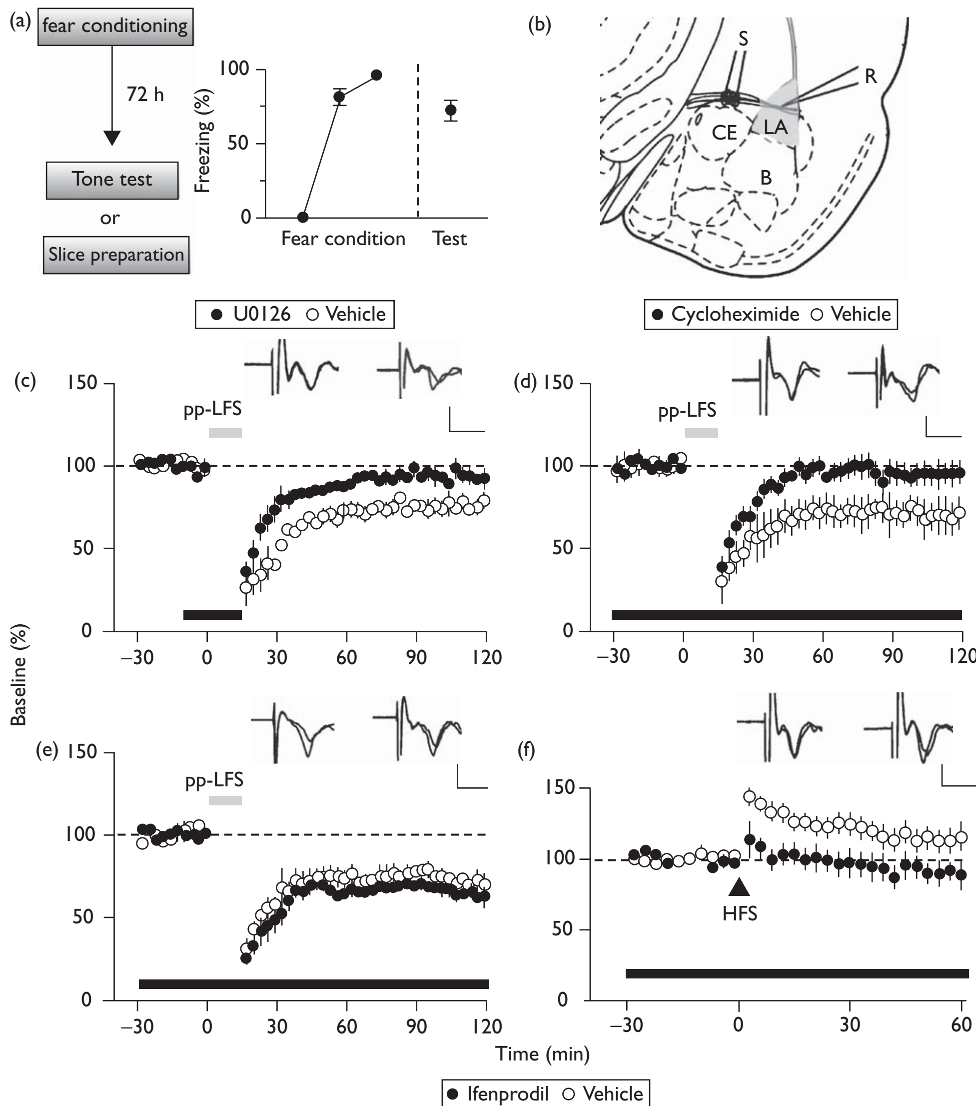
Fear-conditioned rats exhibited strong and consistent freezing when exposed to a tone (conditioned stimuli) (72.14 ± 13.78%, $n = 4$), indicating that our conditioning protocol was effective in establishing fear memory (Fig. 1a). Pp-LFS produced synaptic depression in amygdala slices prepared from fear-conditioned rats (Fig. 1). This depression seems to represent depotentiation$_{ex\ vivo}$, the reversal of *in vivo* synaptic potentiation preserved in amygdala slices, because pp-LFS produces synaptic depression in amygdala slices prepared only from fear-conditioned rats, but not from naive and unpaired controls, as reported in our earlier study [9]. In addition, U0126 and ifenprodil at concentrations used herein have been shown to have no significant effects on basal synaptic transmission at thalamic input synapses onto the lateral amygdala in naive rats [16,17]. We found little effects of cycloheximide (300 μM) on baseline responses of field potentials in the same pathways from naive rats (data not shown).

We first examined the effects of U0126, an antagonist of mitogen-activated kinases, on depotentiation$_{ex\ vivo}$. We applied 20 μM U0126 for a total of 25 min, first for 10 min before pp-LFS and then for an additional 15 min during pp-LFS. Application of U0126 impaired depotentiation$_{ex\ vivo}$ compared with vehicle groups (U0126-treated = 93.61 ± 3.72%, $n = 5$; vehicle groups = 75.54 ± 4.79%, $n = 4$; $P < 0.05$, unpaired t-test, Fig. 1c). Next, to determine whether protein synthesis is critical for depotentiation$_{ex\ vivo}$, we used cycloheximide, a protein synthesis inhibitor. Application of cycloheximide (300 μM) blocked depotentiation$_{ex\ vivo}$ compared with vehicle groups (cycloheximide-treated = 95.06 ± 5.67%, $n = 5$; vehicle groups = 70.58 ± 3.06%, $n = 6$; $P < 0.01$, unpaired t-test, Fig. 1d). Cycloheximide itself had no significant effects on baseline synaptic responses (96.73 ± 3.38% of baseline after a 30 min treatment, $n = 5$, $P > 0.3$; paired t-test). These findings suggest that both mitogen-activated kinase activity and protein synthesis are required for pp-LFS-induced depotentiation$_{ex\ vivo}$.

We then determined whether NR2B-containing NMDA receptors are required for pp-LFS-induced depotentiation$_{ex\ vivo}$. Application of 10 μM ifenprodil, an antagonist of NR2B-containing NMDA receptors, had no significant effects on ppLFS-induced depotentiation$_{ex\ vivo}$ compared with vehicle groups (ifenprodil-treated = 66.03 ± 3.2%, $n = 5$; vehicle groups = 73.68 ± 2.12%, $n = 4$; $P > 0.1$, unpaired t-test, Fig. 1e). Ifenprodil had no significant effects on baseline responses (99.61 ± 3.01% of baseline after a 30-min treatment, $n = 5$, $P > 0.9$; paired t-test). To test whether ifenprodil at a concentration used herein is effective in blocking NR2B-containing NMDA receptors under our experimental conditions, we examined the effects of ifenprodil on long-term potentiation induced by two trains of high-frequency stimulation (at 100 Hz for 1 s) at thalamic input synapses onto the lateral amygdala. Consistent with a previous observation that ifenprodil impaired long-term potentiation in this pathway [17], we found that the application of ifenprodil impaired long-term potentiation compared with vehicle groups

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Fig. 1



Blockade of mitogen-activated protein kinases or of protein synthesis resulted in inhibition of depotentiation$_{ex\ vivo}$, whereas inhibition of NR2B-containing NMDA receptors had no significant effects. (a) Left, the behavioral procedure for the experiments. Right, behavioral results. To avoid possible changes in synaptic properties because of the test stimuli, one set of rats was killed to prepare brain slices, whereas another set was used to monitor conditioned freezing. (b) Schematic illustration of a brain slice containing amygdala [15]. A stimulating electrode was placed in the fibers from the internal capsule. B, basal nucleus of amygdala; CE, central nucleus of amygdala; LA, lateral nucleus of amygdale; R, recording electrode; S, stimulating electrode. (c) U0126 inhibited depotentiation$_{ex\ vivo}$. (d) Cycloheximide inhibited depotentiation$_{ex\ vivo}$. (e) Ifenprodil did not show any significant effects on *depotentiation*$_{ex\ vivo}$. (f) Ifenprodil blocked amygdala long-term potentiation. All representative traces are an average of five traces. Scale bars, 0.2 mV and 5 ms. HFS, high-frequency stimulation; pp-LFS, paired pulse low-frequency stimulation.

(ifenprodil-treated = 102.0 ± 7.63%, $n = 4$; vehicle groups = 129.4 ± 5.93%, $n = 5$; $P < 0.05$, unpaired *t*-test, Fig. 1f).

Discussion

Our findings suggest that both mitogen-activated kinase activity and protein synthesis are critical for ppLFS-induced depotentiation$_{ex\ vivo}$. In contrast, NR2B-containing NMDA receptors do not seem to be involved in pp-LFS-induced depotentiation$_{ex\ vivo}$. Our present findings, along with those of previous studies [3,5–8, 10], indicate that pp-LFS-induced depotentiation$_{ex\ vivo}$ is blocked by four of the five drugs (D-AP5, CPCCOEt, U0126, anisomycin/cycloheximide, and ifenprodil) which have been shown to attenuate fear extinction when microinjected into the basolateral amygdala, thereby supporting the strong association between pp-LFS-induced depotentiation$_{ex\ vivo}$ and fear extinction. Li *et al.* [18] have recently predicted that fear extinction involves multiple forms of synaptic plasticity at different types of synapses in the lateral amygdala. Consistent with this prediction, our results suggest another plastic mechanism

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

that depends on NR2B-containing NMDA receptors in the basolateral amygdala, supported by earlier studies [10,19].

Considering the inhibitory effect of D-AP5 on pp-LFS-induced depotentiation$_{ex\ vivo}$ [9], it may be odd that ifenprodil did not have a significant effect on pp-LFS-induced depotentiation$_{ex\ vivo}$. There may be several explanations for this. First, NR2A-containing NMDA receptors may be involved in pp-LFS-induced depotentiation$_{ex\ vivo}$. If this were the case, it would be interesting to determine whether an NR2A-containing NMDA receptor inhibitor impairs fear extinction. Second, no subtype specificity of NMDA receptors may exist for pp-LFS-induced depotentiation$_{ex\ vivo}$. In this case, an important condition that ensures a successful induction of depotentiation would be whether summed calcium currents through NR2A-containing and NR2B-containing NMDA receptors go beyond a threshold for depotentiation induction. Therefore, one prediction on the basis of the latter possibility is that higher concentrations of ifenprodil, which can also at least partially inhibit NR2A-containing receptors, would impair pp-LFS-induced depotentiation$_{ex\ vivo}$.

Synaptic plasticity in amygdala interneurons has been proposed as a critical mechanism for fear extinction [6,20–22]. NR2B-containing receptors may participate in plastic mechanisms at excitatory input synapses onto interneurons in the amygdala. At the same time, our data do not eliminate the possibility that mitogen-activated protein kinases, protein synthesis, and metabotropic glutamate receptors are involved in the interneuronal plasticity in the amygdala (see Ref. [23]). Irrespective of these mechanisms, ppLFS-induced depotentiation$_{ex\ vivo}$ serves as a viable model system in which extinction-modulating drugs can be prescreened.

Conclusion

In this study, we have shown that mitogen-activated protein kinases and protein synthesis are required for depotentiation$_{ex\ vivo}$ at thalamic input synapses onto the lateral amygdala. NR2B-containing NMDA receptors do not seem to be involved in the amygdala depotentiation.

Acknowledgements

This work was supported by Korea Research Foundation Grants KRF-2005-041-C00422 and 2007-313-C00679 funded by the Korean Ministry of Education and Human Resource Development, Korea Science and Engineering Foundation Grant R01-2007-000-11323-0 funded by the Korean Ministry of Science and Technology, and Brain Research Center of the 21st Century Frontier Research Program Grant M103KV010027-07K2201-02710 funded by the Korean Ministry of Science and Technology. S.L., J.K., and S.P. were supported by Brain Korea 21 Research Fellowships from the Korean Ministry of Education.

References

1 Myers KM, Davis M. Behavioral and neural analysis of extinction. *Neuron* 2002; **36**:567–584.
2 Maren S, Quirk GJ. Neuronal signaling of fear memory. *Nat Rev Neurosci* 2004; **5**:844–852.
3 Lu KT, Walker DL, Davis M. Mitogen-activated protein kinase cascade in the basolateral nucleus of amygdala is involved in extinction of fear-potentiated startle. *J Neurosci* 2001; **21** (**RC162**):15.
4 Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, *et al*. The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 2002; **418**:530–534.
5 Lin CH, Yeh SH, Lu HY, Gean PW. The similarities and diversities of signal pathways leading to consolidation of conditioning and consolidation of extinction of fear memory. *J Neurosci* 2003; **23**:8310–8317.
6 Barad M, Gean PW, Lutz B. The role of the amygdala in the extinction of conditioned fear. *Biol Psychiatry* 2006; **60**:322–328.
7 Herry C, Trifilieff P, Micheau J, Lüthi A, Mons N. Extinction of auditory fear conditioning requires MAPK/ERK activation in the basolateral amygdala. *Eur J Neurosci* 2006; **24**:261–269.
8 Kim J, Lee S, Park H, Song B, Hong I, Geum D, *et al*. Blockade of amygdala metabotropic glutamate receptor subtype 1 impairs fear extinction. *Biochem Biophys Res Commun* 2007; **355**:188–193.
9 Kim J, Lee S, Park K, Hong I, Song B, Son G, *et al*. Amygdala depotentiation and fear extinction. *Proc Natl Acad Sci U S A* 2007; **104**:20955–20960.
10 Sotres-Bayon F, Bush DE, LeDoux JE. Acquisition of fear extinction requires activation of NR2B containing NMDA receptors in the lateral amygdala. *Neuropsychopharmacology* 2007; **32**:1929–1940.
11 Gutman AR, Yang Y, Ressler KJ, Davis M. The role of neuropeptide Y in the expression and extinction of fear-potentiated startle. *J Neurosci* 2008; **28**:12682–12690.
12 Lee O, Lee CJ, Choi S. Induction mechanisms for L-LTP at thalamic input synapses to the lateral amygdala: requirement of mGluR5 activation. *Neuroreport* 2002; **13**:685–691.
13 Huang YY, Martin KC, Kandel ER. Both protein kinase A, mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. *J Neurosci* 2000; **20**:6317–6325.
14 Sajikumar S, Frey JU. Late-associativity, synaptic tagging, and the role of dopamine during LTP and LTD. *Neurobiol Learn Mem* 2004; **82**:12–25.
15 Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. San Diego: Academic; 1998.
16 Schafe GE, Atkins CM, Swank MW, Bauer EP, Sweatt JD, LeDoux JE. Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *J Neurosci* 2000; **20**: 8177–8187.
17 Bauer EP, Schafe GE, LeDoux JE. NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. *J Neurosci* 2002; **22**:5239–5249.
18 Li G, Nair SS, Quirk GJ. A biological realistic network model of acquisition and extinction of conditioned fear associations in lateral amygdala neurons. *J Neurophysiol* 2008; [in press].
19 Myers KM, Ressler KJ, Davis M. Different mechanisms of fear extinction dependent on length of time since fear acquisition. *Learn Mem* 2006; **13**:216–223.
20 Royer S, Paré D. Bidirectional synaptic plasticity in intercalated amygdala neurons and the extinction of conditioned fear responses. *Neuroscience* 2002; **115**:455–462.
21 Paré D, Quirk GJ, Ledoux JE. New vistas on amygdala networks in conditioned fear. *J Neurophysiol* 2004; **92**:1–9.
22 Likhtik E, Popa D, Apergis-Schoute J, Fidacaro GA, Paré D. Amygdala intercalated neurons are required for expression of fear extinction. *Nature* 2008; **454**:642–645.
23 Azad SC, Monory K, Marsicano G, Cravatt BF, Lutz B, Zieglgänsberger W, Rammes G. Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. *J Neurosci* 2004; **24**:9953–9961.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.