

Long-Lasting Impairment of Associative Learning Is Correlated with a Dysfunction of *N*-Methyl-D-aspartate–Extracellular Signaling-Regulated Kinase Signaling in Mice after Withdrawal from Repeated Administration of Phencyclidine

Takeshi Enomoto, Yukihiro Noda, Akihiro Mouri, Eun-Joo Shin, Dayong Wang, Rina Murai, Kazuo Hotta, Hiroshi Furukawa, Atsumi Nitta, Hyoung-Chun Kim, and Toshitaka Nabeshima

Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan (T.E., Y.N., A.M., E.-J.S., D.W., R.M., K.H., A.N., T.N.); Division of Clinical Science in Clinical Pharmacy Practice, Management and Research, Faculty of Pharmacy, Meijo University, Nagoya, Japan (Y.N.); Neurotoxicology Program, College of Pharmacy, Kangwon National University, Chunchon, South Korea (E.-J.S., H.-C.K.); and Department of Medical Chemistry, Faculty of Pharmacy, Meijo University, Nagoya, Japan (H.F.)

Received January 21, 2005; accepted September 7, 2005

ABSTRACT

In humans, the administration of phencyclidine causes schizophrenic-like symptoms that persist for several weeks after withdrawal from phencyclidine use. We demonstrated here that mice pretreated with phencyclidine (10 mg/kg/day s.c. for 14 days) showed an enduring impairment of associative in a Pavlovian fear conditioning 8 days after cessation of phencyclidine treatment. Extracellular signaling-regulated kinase (ERK) was transiently activated in the amygdalae and hippocampi of saline-treated mice after conditioning. In the phencyclidine-treated mice, the basal level of ERK activation was elevated in the hippocampus, whereas the activation was impaired in the amygdala and hippocampus after conditioning. Exogenous *N*-methyl-D-aspartate (NMDA), glycine, and spermidine-induced

ERK activation was not observed in slices of hippocampus and amygdala prepared from phencyclidine-treated mice. Repeated olanzapine (3 mg/kg/day p.o. for 7 days), but not haloperidol (1 mg/kg/day p.o. for 7 days), treatment reversed the impairment of associative learning and of fear conditioning-induced ERK activation in repeated phencyclidine-treated mice. Our findings suggest an involvement of abnormal ERK signaling via NMDA receptors in repeated phencyclidine treatment-induced cognitive dysfunction. Furthermore, our phencyclidine-treated mice would be a useful model for studying the effect of antipsychotics on cognitive dysfunction in schizophrenia.

In humans, phencyclidine, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, has been shown to induce schizophrenia-like psychosis representing positive

(e.g., hallucination and paranoia) and negative symptoms (e.g., emotional withdrawal and motor retardation) and cognitive dysfunction (Javitt and Zukin, 1991). It is interesting that such schizophrenia-like symptoms persisted for several weeks after withdrawal from long-term phencyclidine use (Rainey and Crowder, 1975; Allen and Young, 1978; Jentsch and Roth, 1999). Phencyclidine psychosis has been demonstrated to represent a drug-induced model of schizophrenia (Javitt and Zukin, 1991). We have reported previously that repeated phencyclidine treatment induces persistent enhancement of immobility in a forced swimming test (Noda et al., 1995, 2000) and social behavior deficit in mice (Qiao et al., 2001), which would be an animal model of negative symp-

This work was supported, in part, by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (14370031, 15922139, 16922036, and 17390018), for Scientific Research on Priority Areas on “elucidation of glia-neuron network mediated information processing systems” from the Ministry of Education, Culture, Sports, Science and Technology (16047214), from a Funds from Integrated Molecular Medicine for Neuronal and Neoplastic Disorders (21st Century COE program), from the Japan Brain Foundation, from the Mitsubishi Pharma Research Foundation, and from an SRF Grant for Biomedical Research.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.105.011304.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; ERK, extracellular signaling-regulated kinase; NR1 subunit; *N*-methyl-D-aspartate receptor ζ subunit; MK-801, dizocilpine maleate; VEH, vehicle; OLZ, olanzapine; HAL, haloperidol; PCP, phencyclidine; ANOVA, analysis of variance; TBS, Tris-buffered saline; BDNF, brain-derived neurotrophic factor; PCP, phencyclidine.

toms. In cognitive function, it is well known that one-time administration of phencyclidine induces a transient cognitive dysfunction (Nabeshima et al., 1986; Noda et al., 2001). Jentsch and colleagues (1997a,b) have reported that repeated phencyclidine treatment induces an enduring working memory impairment in the T-maze of rats and memory impairment in an "object retrieval with a detour" task of monkeys. However, there were contradictory results about the enduring impairment of working memory in rats (Stefani and Moghaddam, 2002; Li et al., 2003). Although it has reported recently that spatial learning is impaired 1 day after repeated phencyclidine treatment in object recognition test of mice (Mandillo et al., 2003), it is not clear whether long-lasting cognitive dysfunction occurred in other types of learning and memory tasks.

Pavlovian fear conditioning is a useful tool for investigating associative learning, because the neuronal circuits underlying this task have been mapped (Maren, 2001). The cued fear conditioning depends on the amygdala, whereas the contextual fear conditioning depends on both the hippocampus and amygdala. Recent studies have identified the molecular basis of learning in this task. Activation of extracellular signaling-regulated kinase (ERK) via NMDA receptors in the hippocampus and amygdala is required for associative learning in fear conditioning (Atkins et al., 1998; Schafe et al., 2000; Athos et al., 2002).

The present study was designed to test the hypothesis that phencyclidine-pretreated mice develop an impairment of associative learning in Pavlovian fear conditioning after withdrawal from phencyclidine, and such impairment is mediated via a dysfunction of NMDA-ERK signaling. We attempted to investigate whether an impairment of associative learning is produced after repeated administration of phencyclidine and the changes of ERK activation in phencyclidine-treated mice after fear conditioning and NMDA receptor stimulation. Finally, for determining whether our model could be used as a model of the cognitive dysfunction in schizophrenia, the effects of haloperidol (a typical antipsychotic) and olanzapine (an atypical antipsychotic) on the associative learning in phencyclidine-treated mice were investigated.

Materials and Methods

Animals. Male mice of the ddY strain (Japan SLC Inc., Shizuoka, Japan), weighing 25 to 27 g at the beginning of experiments, were used. They were housed in plastic cages, received food (CE2; Clea Japan Inc., Tokyo, Japan) and water ad libitum, and were maintained on a 12/12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM). All experiments were performed in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

Cued and Contextual Fear Conditioning. Cued and contextual fear conditioning was performed according to a previous report (Nagai et al., 2003) with a minor modification. For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in a neutral cage (17 × 27 × 12.5 cm) for 1 min and then in the conditioning cage (25 × 31 × 11 cm) for 2 min. For training (conditioning phase), mice were placed in the conditioning cage, and then a 15-s tone (80 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.8 mA was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea Co. Ltd., Osaka, Japan). This proce-

dures was repeated four times with 15-s intervals. Cued and contextual tests were carried out 1 to 2 h or 1 day after fear conditioning. For the cued test, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical with the conditioned stimulus. For the contextual test, mice were placed in the conditioning cage, and the freezing response was measured for 2 min in the absence of the conditioned stimulus.

Drugs. Phencyclidine HCl [1-(1-phenylcyclohexyl) piperidine hydrochloride] was synthesized by the authors according to the method of Maddox and colleagues (1965) and was checked for purity. Haloperidol (Sigma Chemical, St. Louis, MO) was purchased commercially. Olanzapine was supplied by Eli Lilly & Co. (Indianapolis, IN). Phencyclidine was dissolved in saline. Haloperidol and olanzapine were suspended in saline containing 0.1% (w/v) carboxymethyl cellulose sodium salt.

Drug Treatment. Phencyclidine (10 mg/kg/day s.c.) or saline was administered once a day for 14 consecutive days. Fear conditioning was performed 8 days after the withdrawal of repeated phencyclidine treatment. For investigating the effects of repeated antipsychotic treatment, mice were administered haloperidol (1 mg/kg/day p.o.), olanzapine (3 mg/kg/day p.o.), or vehicle once a day for 7 consecutive days from 1 day after the final phencyclidine treatment. On the day after the final haloperidol or olanzapine treatment, fear conditioning was performed. On the basis of our previous studies in a forced swimming test of mice (Noda et al., 1995, 2000), the doses of phencyclidine, olanzapine, and haloperidol were chosen. All compounds were administered in a volume of 0.1 ml/10 g body weight.

Western Blotting Analysis. Western blotting analysis was performed as described previously (Mizuno et al., 2002) with a minor modification. Before (basal) and immediately, 1 and 24 h after fear conditioning, the mice were killed by decapitation, and the brain was immediately removed. Hippocampi and amygdalae were rapidly dissected out, frozen, and stored at -80°C until used. The brain samples were homogenized in ice-cold buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin, 4 μg/ml aprotinin, and 4 μg/ml leupeptin for measuring ERK; or 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium orthovanadate, 20 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin for measuring the NMDA receptor ζ subunit (NR1) and actin]. The lysate was centrifuged at 8000g for 10 min at 4°C. The protein concentration of the supernatant was determined by a Bradford assay (Bio-Rad, Hercules, CA). Sample buffer was added to the supernatant, and the mixture was boiled at 95°C for 5 min. Equivalent amounts of protein (50 μg) were electrophoresed on SDS-polyacrylamide gel, transferred to PVDF membranes (Millipore Corporation, Billerica, MA), and blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The membranes were incubated with anti-phospho-ERK antibody (1:1000; New England Biolabs, Beverly, MA), anti-NR1, CT antibody (1:1000; Upstate Biotechnology, Lake Placid, NY), or anti-actin antibody (C-11) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The membranes were washed with the washing buffer (50 mM Tris-HCl, pH 7.4, 0.05% Tween 20, and 150 mM NaCl) three times for 10 min each. After incubation with secondary antibodies conjugated to horseradish peroxidase, the membranes were washed with the washing buffer three times for 10 min each. The immune complex was detected by an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and exposed to X-ray film. Images on X-ray film were captured using charge-coupled device camera (Atto Bioscience, Tokyo, Japan). The band intensities were quantitatively analyzed using the Atto Densitograph Software Library Lane Analyzer. To measure total (phospho- and nonphospho-) ERK, membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 10 min and incubated with anti-ERK antibody (1:1000; New

England Biolabs) at 4°C overnight. The immune complex was detected as described above. To evaluate the ERK activation, the phospho-ERK levels were normalized to the total ERK levels in the same membranes, and then each phospho-ERK/total ERK level was normalized to basal phospho-ERK/total ERK level in saline-treated mice. To evaluate the NR1 levels, the NR1 levels were normalized to the actin levels in the same membranes, and then each NR1/actin level was normalized to NR1/actin level in saline-treated mice.

Immunohistochemical Analysis. Immunohistochemical analysis was performed as described previously (Schafe et al., 2000; Kim et al., 2003) with a minor modification. Before and immediately after fear conditioning, mice were anesthetized with chloral hydrate (200 mg/kg i.p.) and then transcardially perfused with ice-cold 20 mM Tris-buffered saline (TBS) followed by 4% paraformaldehyde in 0.1 M Tris-HCl buffer, pH 7.4. Perfused brains were fixed at 4°C for 24 h in the same fixative and then immersed in 30% sucrose in TBS until the brain sank to the bottom. Immersed brains were cut into 30- μ m transverse free-floating sections using a horizontal sliding microtome. For immunohistochemistry, sections were blocked in 0.3% hydrogen peroxide in TBS for 30 min and then followed by 30-min incubation in 0.4% Triton X-100, 4% normal goat serum, and 0.25% bovine serum albumin in TBS. After incubation in primary antibody against phospho-ERK (1:200) at 4°C for 48 h, sections were incubated in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at room temperature for 2 h, and then followed by 1 h incubation in Avidin-Biotin complex (Vector Laboratories) at room temperature. 3,3'-Diaminobenzidine was used as a chromogen. According to a previous article (Sananbenesi et al., 2002), the density of phospho-ERK-stained cells were analyzed from the standardized area of 0.72 mm² in lateral and central amygdala, 1.58 to 1.70 mm posterior to bregma, using computer-based image analysis system (C. Imaging System; Compic, Mars, PA) attached to a light microscope (Olympus BX60-FLB-3; Olympus, Tokyo, Japan). To evaluate the ERK activation, the phospho-ERK levels after fear conditioning were normalized to the mean basal phospho-ERK level in each treatment.

Slice Stimulation. Slice stimulation was essentially performed as described previously (Mamiya et al., 2003) with a minor modification. Eight days after the final phencyclidine treatment, the mice were killed by decapitation, and the brain was immediately removed. Amygdalae and hippocampi were dissected. Hippocampi were sliced at a thickness of 300 μ m in a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, UK). Amygdalae was sliced manually with a blade. After preincubation at 37°C in Ringer's buffer (10 mM HEPES-NaOH, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 10 mM glucose; gassed with 95% O₂ and 5% CO₂) for 5 min, each slice was stimulated with NMDA (100 μ M), glycine (10 μ M), and spermidine (1 mM) for 5 min. After NMDA receptor stimulation, the slices were washed with ice-cold Ringer's buffer two times. The slices were homogenized as described above for Western blotting analysis.

Statistical Analysis. Statistical analysis was performed by one-way or two-way analysis of variance (ANOVA) using Bonferroni's test. The unpaired *t* test was used to compare two sets of data. A value of *p* < 0.05 was considered statistically significant. Data were expressed as the mean \pm S.E.M.

Results

Effect of Repeated Phencyclidine Treatment on Fear Conditioning in Mice. In the preconditioning phase, both saline- and phencyclidine-treated mice hardly showed the freezing response. There were no differences in basal levels of freezing response between the two groups (data not shown). In cued (amygdala-dependent but hippocampus-independent) and contextual (amygdala- and hippocampus-dependent) fear conditioning, animals learned to fear tone and

context associated with the room for foot shock. Both saline- and phencyclidine-treated mice showed marked cued and contextual freezing response 1 to 2 h after fear conditioning, and there was no significant difference between the two groups (Fig. 1, a and b). However, when cued and contextual freezing response was measured 1 day later, the phencyclidine-treated mice exhibited less freezing response (cued test: *p* < 0.01; contextual test: *p* < 0.05; Fig. 1, c and d). No alterations of nociceptive response were found in the phencyclidine-treated mice: the minimal current required to elicit flinching/running, jumping, or vocalization in the phencyclidine-treated mice was the same as that in the saline-treated mice (data not shown).

ERK Activation in the Amygdala and Hippocampus after Fear Conditioning. Phospho-ERK levels in the amygdala and hippocampus of the saline-treated mice were significantly increased immediately after fear conditioning, compared with basal levels (before conditioning) (*p* < 0.01, Fig. 2, a and b). The increase in phospho-ERK levels was transient and returned to basal levels within 1 h in both regions. In the amygdalae of the phencyclidine-treated mice, fear conditioning did not significantly increase the phospho-ERK levels compared with the basal level (Fig. 2a), and the phospho-ERK level immediately after conditioning in the phencyclidine-treated mice was significantly lower than that in the saline-treated mice (*p* < 0.05, Fig. 2a).

The basal phospho-ERK level in the hippocampi of the phencyclidine-treated unconditioned mice was significantly elevated compared with that in the saline-treated unconditioned mice (*p* < 0.05, Fig. 2b). However, fear conditioning did not cause a further increase compared with the basal level (Fig. 2b). Fear conditioning did not cause any changes in

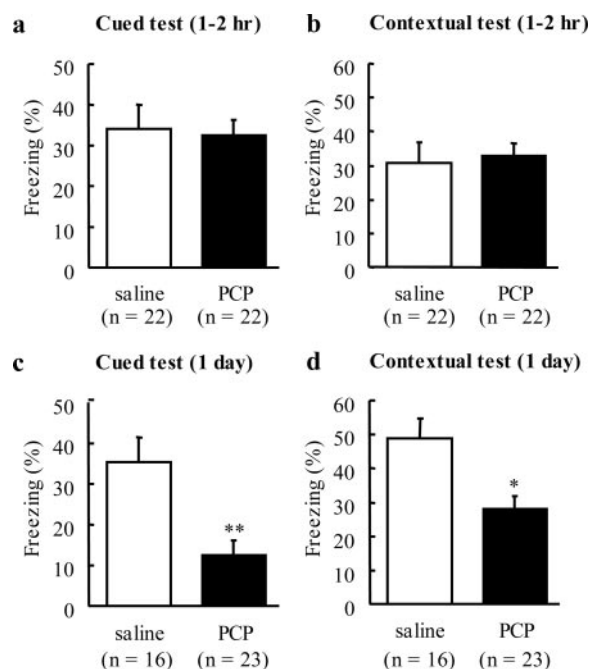


Fig. 1. Effect of repeated phencyclidine treatment on performance of fear conditioning in mice. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Cued and contextual tests were performed 1 to 2 h (a, cued test; b, contextual test) and 1 day after fear conditioning (c, cued test; d, contextual test). Values correspond to mean \pm S.E.M. *, *p* < 0.05 and **, *p* < 0.01 versus saline-treated mice (unpaired *t* test).

total ERK levels in the amygdalae (Fig. 2c) and hippocampi (Fig. 2d) of the saline- or phencyclidine-treated mice.

Because amygdala consists of heterogeneous nuclei, we measured phospho-ERK levels in the lateral amygdala, which is an essential region for fear conditioning, and the central amygdala using immunohistochemical methods (Fig. 3, a–h). Repeated treatment with phencyclidine induced an increase in the basal phospho-ERK levels in the lateral and central amygdalae compared with those in saline-treated mice, but not significantly (Fig. 3a). Although the phospho-ERK levels in the lateral amygdalae of both saline- and phencyclidine-treated mice were significantly increased immediately after fear conditioning compared with the basal levels ($p < 0.01$, Fig. 3a), these changes were more pronounced in saline-treated mice than in phencyclidine-treated mice ($p < 0.01$, Fig. 3c). There were no significant changes in the central amygdala, but fear conditioning tended to increase the phospho-ERK levels in the central amygdalae of saline- and phencyclidine-treated mice (Fig. 3b). ERK activation in the central amygdalae of saline-treated mice tended to be intense compared with that of phencyclidine-treated mice (Fig. 3d).

ERK Activation in the Slices of Amygdala and Hippocampus by NMDA Receptor Stimulation. To confirm that ERK activation is facilitated after NMDA receptor stimulation, we measured the phospho-ERK levels in slices of amygdala or hippocampus stimulated with NMDA (100 μ M), glycine (10 μ M), and spermidine (1 mM). Under our experimental conditions, the increase in phospho-ERK levels was detected 5 min after stimulation with NMDA, glycine, and

spermidine compared with the basal level (without stimulation) in the amygdalae ($p < 0.01$, Fig. 4a) and hippocampi ($p < 0.05$, Fig. 4b) prepared from saline-treated mice. The basal levels of phospho-ERK in the amygdalae (Fig. 4a) and hippocampi (Fig. 4b) of phencyclidine-treated mice tended to be increased compared with those of saline-treated mice. However, stimulation with NMDA, glycine, and spermidine did not cause a further increase in phospho-ERK levels of the amygdalae (Fig. 4a) or hippocampi (Fig. 4b) prepared from phencyclidine-treated mice. After stimulation, phospho-ERK level in the amygdalae of phencyclidine-treated mice was significantly lower than that of saline-treated mice ($p < 0.05$, Fig. 4a). There was no significant difference in total ERK levels in amygdala (Fig. 4c) and hippocampus (Fig. 4d) between saline- and phencyclidine-treated mice.

Effect of Repeated Phencyclidine Treatment on the Protein Levels of NR1 in the Amygdala and Hippocampus. We measured the protein levels of NR1, which is the obligatory subunit of NMDA receptors, in the amygdala and hippocampus using the same schedule as fear conditioning. However, there was no significant difference in NR1 levels of the amygdala (Fig. 4e) and hippocampus (Fig. 4f) between saline- and phencyclidine-treated mice.

Effects of Antipsychotics on the Impairment of Associative Learning Induced by Repeated Phencyclidine Treatment. We investigated the effects of repeated treatment with olanzapine and haloperidol on associative learning in saline- or phencyclidine-treated mice. Fear conditioning was performed 1 day after repeated olanzapine (3 mg/kg/day p.o. for 7 days) or haloperidol (1 mg/kg/day p.o.

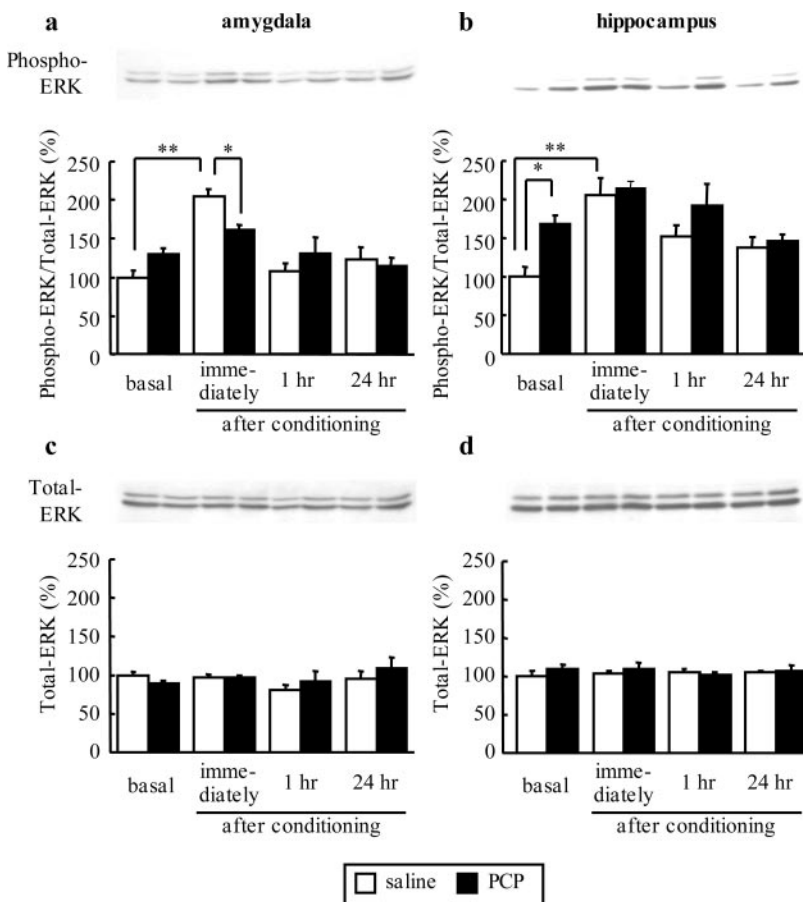


Fig. 2. ERK activation in the amygdala and hippocampus after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae (a) ($n = 10$ in each group before or immediately after fear conditioning, $n = 8$ in each group 1 or 24 h after fear conditioning) and hippocampi (b) ($n = 7$ in each group) of saline- and phencyclidine-treated mice after fear conditioning. Representative Western blots and total ERK immunoreactivity in the amygdalae (c) and hippocampi (d) of saline- and phencyclidine-treated mice after fear conditioning. Values correspond to the mean \pm S.E.M. Results with two-way ANOVA were: a, treatment, $F_{1,64} = 0.000007$ ($p = 0.998$); time, $F_{3,64} = 17.09$ ($p < 0.01$); treatment-by-time interaction, $F_{3,64} = 4.56$ ($p < 0.01$); b, treatment, $F_{1,48} = 7.32$ ($p < 0.01$); time, $F_{3,48} = 9.29$ ($p < 0.01$); treatment-by-time interaction, $F_{3,48} = 1.51$ ($p = 0.22$); c, treatment, $F_{1,64} = 0.47$ ($p = 0.50$); time, $F_{3,64} = 1.45$ ($p = 0.24$); treatment-by-time interaction, $F_{3,64} = 0.84$ ($p = 0.48$); d, treatment, $F_{1,48} = 1.01$ ($p = 0.32$); time, $F_{3,48} = 0.14$ ($p = 0.94$); treatment-by-time interaction, $F_{3,48} = 0.59$ ($p = 0.62$). Basal, before conditioning; *, $p < 0.05$; **, $p < 0.01$ (Bonferroni's test).

for 7 days) treatment. Repeated olanzapine and haloperidol treatment did not affect the cued (Fig. 5a) and contextual (Fig. 5c) freezing response in saline-treated mice. On the other hand, repeated olanzapine treatment reversed the impairment of associative learning produced by repeated phencyclidine treatment in both cued ($p < 0.05$, Fig. 5b) and contextual tests ($p < 0.01$, Fig. 5d). Repeated haloperidol treatment did not reverse the phencyclidine-induced impairment of associative learning in either test (Fig. 5, b and d).

Effects of Repeated Antipsychotic Treatments on the Impairment of ERK Activation Induced by Repeated Phencyclidine Treatment. Repeated treatment with olanzapine or haloperidol did not affect the phospho-ERK levels in the amygdalae of saline-treated mice (Fig. 6a). When olanzapine was repeatedly administered to phencyclidine-treated mice, fear conditioning significantly increased the phospho-ERK level in the amygdala ($p < 0.01$, Fig. 6b). Fear conditioning did not increase the phospho-ERK levels in

the amygdalae of phencyclidine/haloperidol-treated mice (Fig. 6c). Total ERK levels in the amygdalae of saline- and phencyclidine-treated mice were not changed by repeated olanzapine (Fig. 6, d and e) and haloperidol (Fig. 6, d and f) treatment.

Olanzapine treatment significantly increased the basal phospho-ERK level in the hippocampi of saline-treated mice ($p < 0.05$, Fig. 7a) and tended to increase the basal phospho-ERK level in the hippocampi of phencyclidine-treated mice (Fig. 7b). Haloperidol treatment significantly increased the basal phospho-ERK levels in the hippocampi of both saline- ($p < 0.05$, Fig. 7a) and phencyclidine-treated mice ($p < 0.05$, Fig. 7c). A further increase in phospho-ERK levels was caused by fear conditioning in saline/vehicle- ($p < 0.01$, Fig. 7a), saline/olanzapine- ($p < 0.05$, Fig. 7a), saline/haloperidol- ($p < 0.05$, Fig. 7a), and phencyclidine/olanzapine-treated mice ($p < 0.05$, Fig. 7b), but not in phencyclidine/vehicle- (Fig. 7, b and c) and phencyclidine/haloperidol-treated mice (Fig. 7c). Total-ERK levels in the hippocampi of saline- and

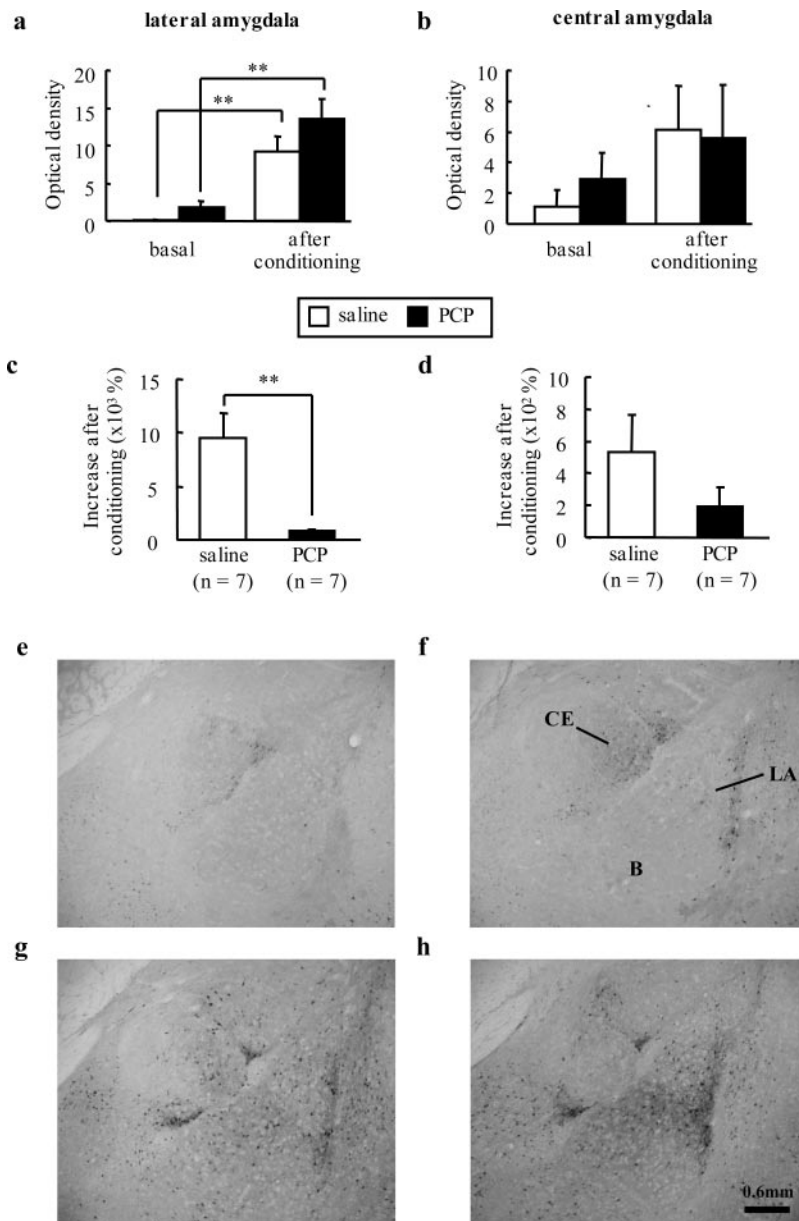


Fig. 3. ERK activation in the lateral amygdala and central amygdala after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Phospho-ERK levels in the lateral (a) and central amygdalae (b) of saline- and phencyclidine-treated mice before (basal) and immediately after fear conditioning ($n = 6$ in saline-treated mice before fear conditioning, $n = 8$ in phencyclidine-treated mice before fear conditioning, $n = 7$ in each group immediately after fear conditioning). The increasing percentage of phospho-ERK expression immediately after fear conditioning in the lateral (c) and central amygdalae (d) of saline-treated and phencyclidine-treated mice. Values correspond to the mean \pm S.E.M. of 7 mice. Representative photomicrographs of phospho-ERK expression in the amygdalae of saline-treated (e and g) and phencyclidine-treated (f and h) mice. Phospho-ERK was markedly increased in the amygdala immediately after fear conditioning (g and h), compared with basal levels (e and f). Results with two-way ANOVA were the following: a: treatment, $F_{1,24} = 2.72$ ($p = 0.11$); time, $F_{1,24} = 32.38$ ($p < 0.01$); treatment-by-time interaction, $F_{1,24} = 0.59$ ($p = 0.45$); b: treatment $F_{1,24} = 0.06$ ($p = 0.81$); time, $F_{1,24} = 2.31$ ($p = 0.14$); treatment-by-time interaction, $F_{1,24} = 0.20$ ($p = 0.66$). **, $p < 0.01$ [Bonferroni's test (a) or unpaired t test (c)]. B, basolateral amygdala; CE, central amygdala; LA, lateral amygdala.

phencyclidine-treated mice were not changed by repeated olanzapine (Fig. 7, d and e) or haloperidol (Fig. 7, d and f) treatment.

Discussion

There are many reports that a single administration of phencyclidine or ketamine alters cognition in healthy volunteers or patients with schizophrenia via glutamatergic hypofunction caused by blockade of NMDA receptors (Javitt and Zukin, 1991; Adler et al., 1998; Tamminga, 1998). Because this cognitive impairment recovers quickly, the ability of a single phencyclidine administration to impair memory in human is caused, at least in part, by phencyclidine-induced transient confusion (Javitt and Zukin, 1991; Ellison, 1995). The study of short-term phencyclidine administration to rodents may be relevant to some of the short-lasting cognitive effects of phencyclidine in humans (e.g., disorientation and dissociative states), whereas long-term phencyclidine model would be more relevant to cognitive deficits in schizophrenia because cognitive deficits in schizophrenia is enduring and is not usually accompanied by impairment of orientation to time, place, and person. In the present study, the impairment of associative learning, which depends on the amygdala and hippocampus, was observed 8 days after withdrawal from repeated phencyclidine treatment. This impairment of associative learning was detected 24 h, but not 1 to 2 h, after fear conditioning, suggesting a failure of memory consolidation. As far as we know, this is the first report of long-lasting impairment of associative learning after withdrawal from repeated phencyclidine treatment. This persistent impairment of learning was consistent with reports of enduring working memory impairment in a T-maze of rats and memory impairment in an "object retrieval with a detour" task of monkeys (Jentsch et al., 1997a,b). It was also consistent with the clinical observation that schizophrenia-like psychosis persisted for several weeks after withdrawal from long-term phencyclidine use in humans (Rainey and Crowder, 1975; Allen and Young, 1978; Jentsch and Roth, 1999). Furthermore, associative learning is disrupted in patients with schizophrenia (Rushe et al., 1999), and dysfunction of the amygdala and hippocampus contributes to the pathophysiology of schizophrenia (Heckers et al., 1998; Edwards et al., 2001). A single dose of phencyclidine may produce a reversible neurotoxic effect related to the cognitive deficits, whereas repeated phencyclidine treatment could produce long-lasting structural changes in cerebral cortex and hippocampus (Ellison, 1995; Olney and Farber, 1995; Olney et al., 1999). These results suggest that repeated phencyclidine treatment causes the long-lasting impairment of associative learning and the neuronal and/or signal circuit needed to perform associative learning task.

Recent studies have demonstrated a critical role of ERK activation in associative learning in cued and/or contextual fear conditioning (Atkins et al., 1998; Schafe et al., 2000; Athos et al., 2002). Our finding, that phencyclidine-treated mice failed to activate ERK, was consistent with the impairment of associative learning in fear conditioning. In the amygdalae and hippocampi of saline-treated mice, phospho-ERK levels were transiently increased immediately after fear conditioning and returned to basal levels within 1 h. The time course of ERK activation in our experiments was consistent with that in long-term potentiation experiments (Davis et al., 2000), whereas it was different from reports that showed ERK activation 1 h after fear conditioning in rats (Atkins et al., 1998; Schafe et al., 2000). The reason for

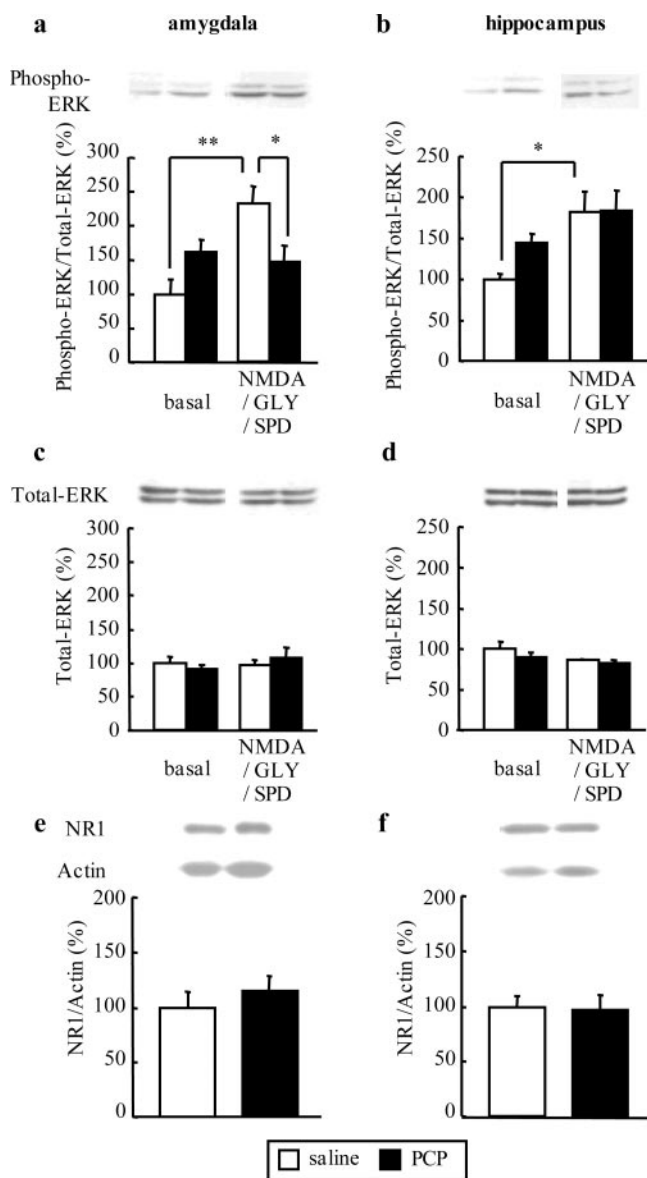


Fig. 4. ERK activation by NMDA receptor stimulation and the protein levels of NR1 in the amygdala and hippocampus. Slices of amygdala or hippocampus were stimulated with NMDA (100 μ M), glycine (GLY; 10 μ M), and spermidine (SPD; 1 mM) 8 days after the cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae (a) and hippocampi (b) of saline- and phencyclidine-treated mice after the stimulation ($n = 6$). Representative Western blots and total ERK immunoreactivity in the amygdalae (c) and hippocampi (d) of saline- and phencyclidine-treated mice after the stimulation ($n = 6$). The NR1 levels were measured 8 days after the cessation of phencyclidine treatment. Representative Western blots and NR1/actin immunoreactivity in the amygdala (e) ($n = 5$) and hippocampus (f) ($n = 8$) of saline- and phencyclidine-treated mice. Values correspond to the mean \pm S.E.M. Results with two-way ANOVA were: a, treatment, $F_{1,20} = 0.25$ ($p = 0.62$); stimulation, $F_{1,20} = 6.34$ ($p < 0.05$); treatment-by-stimulation interaction, $F_{1,20} = 10.36$ ($p < 0.01$); b, treatment, $F_{1,20} = 1.40$ ($p = 0.25$); stimulation, $F_{1,20} = 9.80$ ($p < 0.01$); treatment-by-stimulation interaction, $F_{1,20} = 1.19$ ($p = 0.29$); c, treatment, $F_{1,20} = 0.05$ ($p = 0.82$); stimulation, $F_{1,20} = 0.74$ ($p = 0.40$); treatment-by-stimulation interaction, $F_{1,20} = 0.04$ ($p = 0.84$); d, treatment, $F_{1,20} = 1.44$ ($p = 0.24$); stimulation, $F_{1,20} = 3.42$ ($p = 0.08$); treatment-by-stimulation interaction, $F_{1,20} = 0.33$ ($p = 0.57$). *, $p < 0.05$; **, $p < 0.01$ (Bonferroni's test).

this discrepancy is unknown. One possibility is the different duration of unconditioned stimulation (foot shock) between our condition (total duration, 20 s) and the previously reported condition (1 or 5 s) (Atkins et al., 1998; Schafe et al., 2000).

Although fear conditioning-induced ERK activation in the amygdalae of phencyclidine-treated mice was completely abolished in Western blotting, ERK activation in the lateral amygdalae of phencyclidine-treated mice was less pronounced but observed in immunohistochemical analysis. Although the reason for the difference is unknown, it might be caused by the difference of sensitivity in two methods. As it has been reported (Schafe et al., 2000), fear conditioning induced ERK activation most intensely in the lateral amygdala, whereas in the other nuclei of amygdala, ERK activation is weak and variable among individual mice. Therefore, immunohistochemical analysis would be more sensitive compared with Western blotting. However, an important finding in the present study is that fear conditioning-induced changes in the lateral amygdala were more pronounced in saline-treated mice than in phencyclidine-treated mice.

A previous report demonstrated that the activation of ERK is mediated via NMDA receptors in fear conditioning, because a single treatment with MK-801, a noncompetitive NMDA receptor antagonist, blocks both the activation of ERK and associative learning (Atkins et al., 1998). We investigated NMDA-ERK signaling after stimulation with exogenous NMDA, glycine, and spermidine in slices of the amygdala and hippocampus. In the amygdalae and hippocampi from saline-treated mice, phospho-ERK levels were increased after the stimulation. However, stimulation with NMDA, glycine, and spermidine failed to increase phospho-ERK levels in the amygdalae and hippocampi from phencyclidine-

treated mice. Our results clearly suggest that repeated phencyclidine treatment disrupts the activation of ERK mediated via NMDA receptors. The dysfunction of NMDA-ERK signaling is not accompanied by changes in the NR1 levels in the amygdala and hippocampus. It might be caused by the functional alterations to the NMDA receptor itself or alterations to the intracellular signaling via NMDA receptors.

Kyosseva and colleagues (2001) have reported that continuous phencyclidine infusion increases basal phospho-ERK levels in the cerebellum, but not in the hippocampus, brain stem, and frontal cortex of rats. It contrasts with our results that repeated phencyclidine treatment increased the basal level of phospho-ERK in the hippocampus. Although the reason for discrepancy as to the affected region is unknown, it might be caused by some differences of experimental protocol about species (mice versus rats), phencyclidine administration schedule (pulsatile injection for 14 days versus continuous infusion for 10 days), and duration of withdrawal from phencyclidine (8 days versus 1 day). The increase in basal ERK activation might be caused by adapted response to alterations of neurotransmitters or intracellular signaling as a result of repeated phencyclidine treatment. However, the adapted responses might be insufficient to mediate further ERK activation in associative learning or NMDA receptor stimulation. The most important finding in our study is the disruption of NMDA-ERK signaling during learning in vivo as well as on stimulation with exogenous NMDA, glycine, and/or spermidine ex vivo. The dysfunction of ERK signaling via NMDA receptors in phencyclidine-treated mice is consistent with the hypothesis of dysfunction of the glutamatergic system in schizophrenia (Carlsson et al., 1997; Olney et al., 1999).

We measured phospho-ERK levels in the mice that were

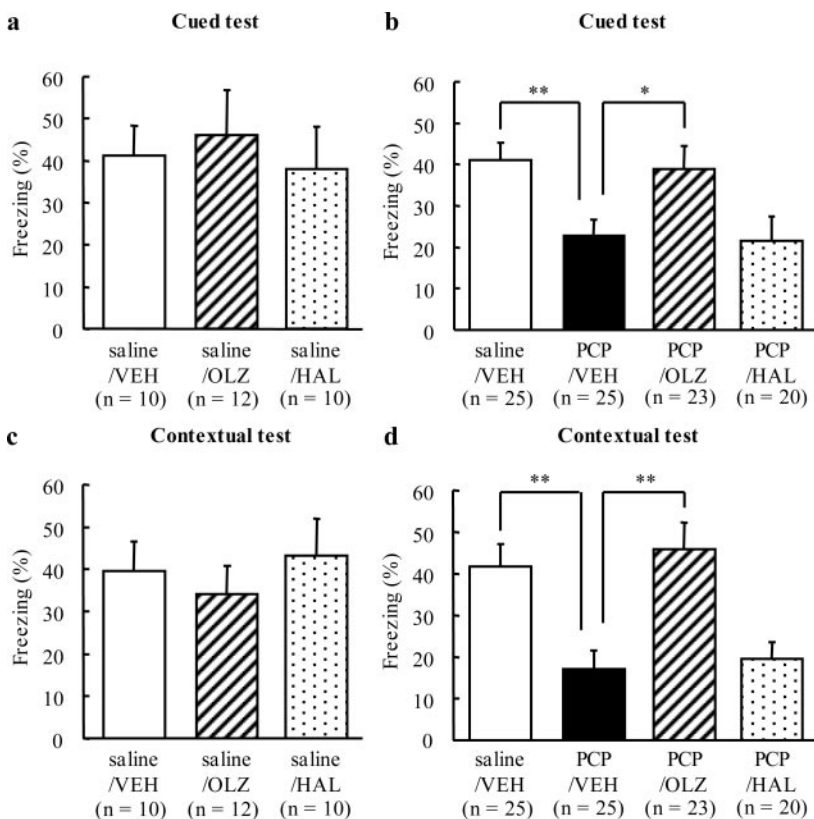


Fig. 5. Effects of repeated antipsychotic treatment on the impairment of associative learning induced by repeated phencyclidine treatment. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Fear conditioning was performed 1 day after the final antipsychotic treatment. Cued (a and b) and contextual tests (c and d) were performed 1 day after fear conditioning. Values correspond to the mean \pm S.E.M. Results with one-way ANOVA were: a, $F_{2,29} = 0.38$ ($p = 0.69$); b, $F_{3,89} = 4.72$ ($p < 0.01$); c, $F_{2,29} = 0.18$ ($p = 0.84$); d, $F_{3,89} = 8.14$ ($p < 0.01$). *, $p < 0.05$; **, $p < 0.01$ (Bonferroni's test).

treated repeatedly with antipsychotics. Phencyclidine/olanzapine-treated mice showed fear conditioning-induced ERK activation in the amygdalae and hippocampi but not phencyclidine/vehicle-treated mice. Although haloperidol and olanzapine increased the basal phospho-ERK levels in the hippocampi of saline- and phencyclidine-treated mice, haloperidol, unlike olanzapine, failed to recover fear conditioning-induced ERK activation in the amygdalae and hippocampi of phencyclidine-treated mice. Although the mechanism by which antipsychotics increased basal and postconditioning phospho-ERK levels is unknown, an important finding is that the improvement of associative learning on repeated olanzapine treatment accompanied fear condi-

tioning-induced ERK activation in the amygdala and hippocampus. Other signaling pathways might also process associative learning in the amygdalae of phencyclidine/olanzapine-treated mice, because the phospho-ERK level after conditioning in the amygdalae of phencyclidine/olanzapine-treated mice was not significantly different from that in phencyclidine/vehicle-treated mice.

The effect of olanzapine would not be caused by prevention of phencyclidine-induced neurodegeneration, because olanzapine treatment was initiated after the cessation of repeated phencyclidine treatment. It is possible that olanzapine restores normal function in the fear conditioning test as a result of complex changes in the interaction of various neural

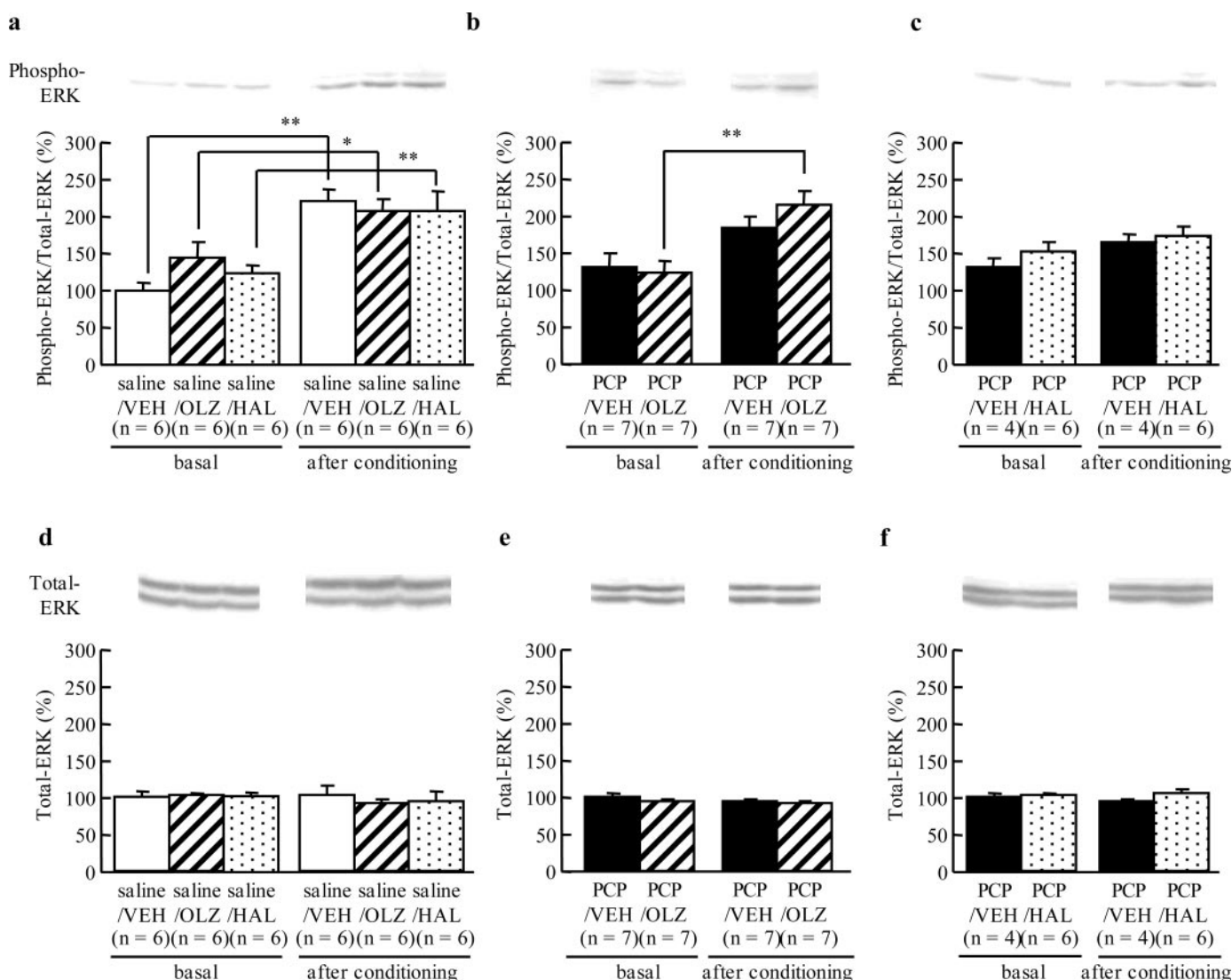


Fig. 6. Effects of repeated antipsychotic treatment on ERK activation in the amygdala after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine-treatment (10 mg/kg/day s.c. for 14 days). Fear conditioning was performed at 1 day after the final antipsychotic treatment. Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae of mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (a), phencyclidine/vehicle, phencyclidine/olanzapine, phencyclidine/haloperidol (b), phencyclidine/vehicle, phencyclidine/olanzapine, phencyclidine/haloperidol (c). Representative Western blots and total ERK immunoreactivity in the amygdalae of mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (d), phencyclidine/vehicle, phencyclidine/olanzapine (e), phencyclidine/vehicle, and phencyclidine/haloperidol (f). Values correspond to the mean \pm S.E.M. Results with two-way ANOVA were: a, treatment, $F_{2,30} = 0.38$ ($p = 0.68$); time, $F_{1,30} = 36.34$ ($p < 0.01$); treatment-by-time interaction, $F_{1,24} = 1.36$ ($p = 0.26$); b, treatment, $F_{1,16} = 1.18$ ($p = 0.29$); time, $F_{1,16} = 4.08$ ($p = 0.06$); treatment-by-time interaction, $F_{1,16} = 0.20$ ($p = 0.66$); c, treatment, $F_{2,30} = 0.16$ ($p = 0.86$); time, $F_{1,30} = 0.65$ ($p = 0.42$); treatment-by-time interaction, $F_{2,30} = 0.28$ ($p = 0.76$); d, treatment, $F_{2,30} = 0.16$ ($p = 0.86$); time, $F_{1,30} = 0.65$ ($p = 0.42$); treatment-by-time interaction, $F_{2,30} = 0.28$ ($p = 0.76$); e, treatment, $F_{1,24} = 0.78$ ($p = 0.38$); time, $F_{1,24} = 1.48$ ($p = 0.24$); treatment-by-time interaction, $F_{1,24} = 0.17$ ($p = 0.68$); f, treatment, $F_{1,16} = 2.08$ ($p = 0.17$); time, $F_{1,16} = 0.06$ ($p = 0.82$); treatment-by-time interaction, $F_{1,16} = 0.65$ ($p = 0.43$). *, $p < 0.05$; **, $p < 0.01$ (Bonferroni's test).

circuits that were altered by repeated phencyclidine administration. The inability of haloperidol to reverse phencyclidine-induced impairment of associative learning suggests that the blocking of D_2 receptors alone is insufficient to reverse the impairment of learning in this model. Olanzapine would activate glutamate neurotransmission, because it induces the increase of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor binding in the rat hippocampus (Tascedda et al., 2001). It has been reported that olanzapine, but not haloperidol, enhances brain-derived neurotrophic factor (BDNF) mRNA expression (Bai et al., 2003) and antagonizes the MK-801-induced reduction of BDNF mRNA

expression in rat hippocampus (Fumagalli et al., 2003). We have shown previously that BDNF is involved in learning and memory by enhancing the phosphorylation of NMDA receptors (Mizuno et al., 2003). Therefore, it is possible that olanzapine might reverse the phencyclidine-induced impairment of associative learning and ERK activation by enhancing the activities of BDNF and NMDA receptors. These results are compatible with the clinical findings that olanzapine but not haloperidol improves cognitive dysfunction in patients with schizophrenia (Bhana et al., 2001). Because the pharmacological effects of these antipsychotics in this model would reflect their clinical effectiveness, phencyclidine-in-

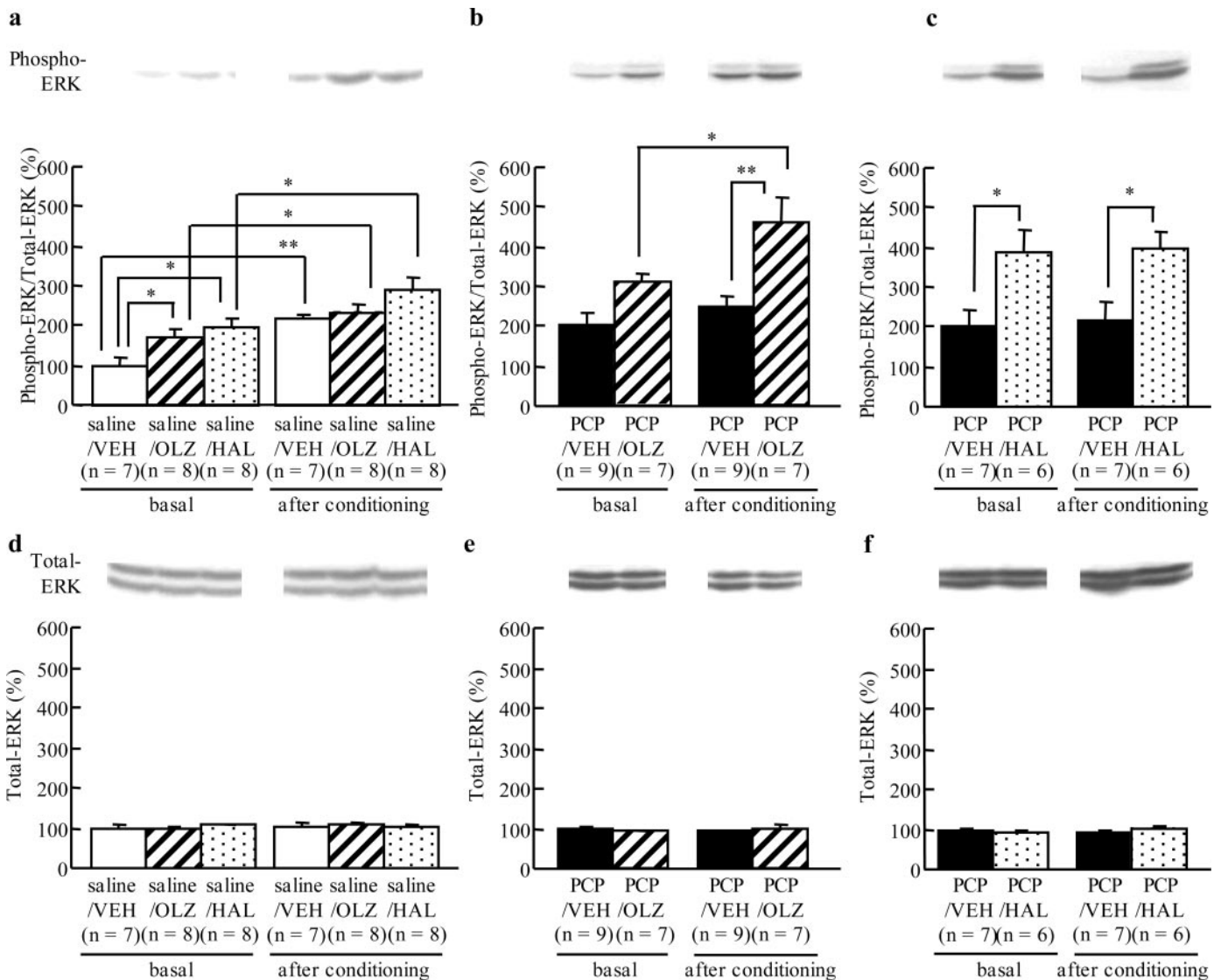


Fig. 7. Effects of repeated antipsychotic treatment on ERK activation in the hippocampus after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Fear conditioning was performed 1 day after the final antipsychotic treatment. Representative Western blots and phospho-ERK/total ERK immunoreactivity in the hippocampi of mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (a), phencyclidine/vehicle, phencyclidine/olanzapine (b), phencyclidine/vehicle, and phencyclidine/haloperidol (c). Representative Western blots and total ERK immunoreactivity in the hippocampi mice treated with saline/vehicle, saline/olanzapine, saline/haloperidol (d), phencyclidine/vehicle, phencyclidine/olanzapine (e), phencyclidine/vehicle, and phencyclidine/haloperidol-treated mice (f). Values correspond to the mean \pm S.E.M. Results with two-way ANOVA were the following: a, treatment, $F_{2,40} = 7.40$ ($p < 0.01$); time, $F_{1,40} = 25.08$ ($p < 0.01$); treatment-by-time interaction, $F_{2,40} = 0.77$ ($p = 0.47$); b, treatment, $F_{1,28} = 18.83$ ($p < 0.01$); time, $F_{1,28} = 6.88$ ($p < 0.05$); treatment-by-time interaction, $F_{1,28} = 2.21$ ($p = 0.15$); c, treatment, $F_{1,22} = 16.32$ ($p < 0.01$); time, $F_{1,22} = 0.10$ ($p = 0.76$); treatment-by-time interaction, $F_{1,22} = 0.0009$ ($p = 0.98$); d, treatment, $F_{2,40} = 0.18$ ($p = 0.84$); time, $F_{1,40} = 0.95$ ($p = 0.34$); treatment-by-time interaction, $F_{2,40} = 1.20$ ($p = 0.31$); e, treatment, $F_{1,28} = 0.21$ ($p = 0.65$); time, $F_{1,28} = 0.18$ ($p = 0.68$); treatment-by-time interaction, $F_{1,28} = 2.02$ ($p = 0.17$); f, treatment, $F_{1,22} = 0.53$ ($p = 0.47$); time, $F_{1,22} = 0.49$ ($p = 0.49$); treatment-by-time interaction, $F_{1,22} = 0.90$ ($p = 0.35$). *, $p < 0.05$; **, $p < 0.01$ (Bonferroni's test).

duced impairments of associative learning would be a useful model of cognitive dysfunction in schizophrenia.

Repeated phencyclidine treatment produces a long-lasting impairment of associative learning in mice. This impairment is accompanied by a dysfunction of NMDA-ERK signaling. This finding is the first step to understanding the mechanism of cognitive dysfunction in schizophrenic and/or phencyclidine psychoses. Furthermore, this animal model would provide a useful system for studying the effect of antipsychotics on the impairment of associative learning in schizophrenia, because the impairment was reversed by olanzapine but not by haloperidol.

Acknowledgments

We are grateful to the Eli Lilly Co. for the olanzapine.

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Address correspondence to: Drs. Toshitaka Nabeshima and Yukihiro Noda, Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsurum-cho, Showa-ku, Nagoya 466-8560, Japan. E-mail: tnabeshi@med.nagoya-u.ac.jp; y-noda@med.nagoya-u.ac.jp