3. Results

3.1. NK cell depletion enhanced EAE in SJL/J mice

3.1.1. In vivo NK cell depletion

Before and after treatment of naïve SJL/J mice with NK1.1 mAb, $CD_3^-NK1.1^+$ cells were 2.51%, 0.65%; $CD_{19}^-DX_5^+$ cells were 1.29%, 0.33%; and $DX_5^+NK1.1^+$ cells were 1.02%, 0.15% in the spleen, respectively. Thus, anti-NK1.1 mAb treatment depleted about 75% of NK cells in vivo.

Isotype-matched control mAb did not induce depletion of NK cells, which was proved both by previous work in our lab (Zhang et al., 1997) and by present work (data not show).

3.1.2. NK cell depletion enhanced EAE in SJL/J mice

Immunization with the used dose of $PLP_{136-150}$ induced a relatively mild form of EAE in untreated SJL/J mice and the mice injected with PBS. On the contrary, administration of anti-NK1.1 mAb 1 day before and 14 days after immunization resulted in aggravation of EAE, although the onset time of the two groups was not different discernibly. By the end of observation, the mice from the control group recovered almost completely, whereas the anti-NK1.1 mAb-treated mice did not show any recovery signs (Fig. 1). The clinical score of isotype-matched control mAb-treated mice was essentially the same as that of PBS-treated controls (data not show).

3.1.3. Low NKT cell levels in SJL/J mice

Anti-NK1.1 mAb depleted $NK1.1^+$ population which include both NK cells and NKT cells, NKT cells have also been suspected to be involved in the regulation of autoimmune processes (Mieza et al., 1996; Sumida et al., 1995; Fritz and Zhao, 2001). Interestingly, NKT cell population is quite low in SJL/J mice, much lower than that in C57BL/6J mice (Fig. 2).

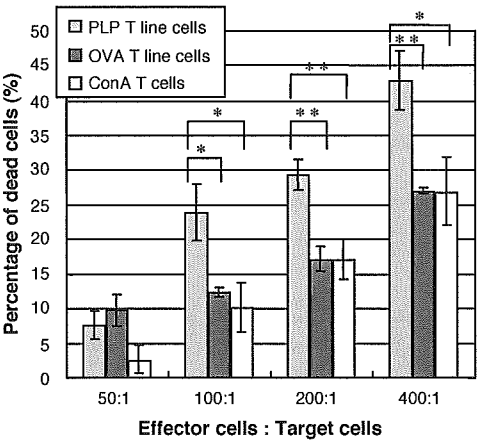

Fig. 4. Specificity of NK cell cytotoxicity. Cytotoxic effect of naïve mice splenic NK cells to PLP-specific T cell lines, OVA-specific T cell lines, and ConA-stimulated T cells were examined and compared by using the same method as described in the legend to Fig. 3. The final results were calculated by subtracting the background and expressed as mean ± S.E. of three samples at each $E:T$ ratio. $^*P<0.05$; $^{**}P<0.01$.

3.2. NK cells exert direct cytotoxic effect on myelin antigen-specific, encephalitogenic T cells

The cultured PLP-specific, encephalitogenic T line cells were used as the target. The cytotoxicity of naïve mice splenic NK cells to the target was assayed by the flow cytometric method.

PKH 2-labeled cells showed a uniform mean fluorescence intensity of 10^2. If target cells were incubated alone without effector cells for 4 h, most of the target cells (94.38%) were still living (Fig. 3A). However, if the target cells were incubated with naïve mice spleen cells, after 4 h of incubation, the dead target cells, which were shown as PKH 2, PI double positive cells, increased obviously (Fig. 3B). Further more, the percentage of dead target cells

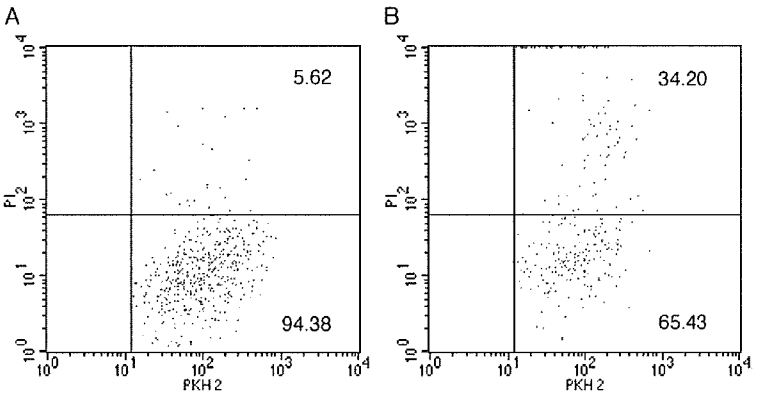

Fig. 3. NK cells exert direct cytotoxic effect on PLP-specific encephalitogenic T cell lines. The target T line cells were labeled with PKH 2, then labeled target cells were incubated with nonlabeled naïve mice spleen cells at the ratio of 50:1, 100:1, 200:1, 400:1 (Effector:Target). After 4 h of incubation, cells were harvested and stained with PI. Dead target T cells were double positive for both PKH 2 and PI, while living target cells were only positive for PKH 2. (A) without effector cells; (B) with effector cells at the ratio of 200:1. One representative experiment out of three is shown.

increased when the effector cells (SJL/J mice spleen cells) were increased in the culture in a dose-dependent manner (Figs. 4 and 6). This result implied that NK cells in the spleen exerted direct cytotoxic effect on PLP-specific, encephalitogenic target T cells.

3.3. Cytotoxicity to PLP-specific, encephalitogenic T line cells were enhanced by using enriched NK cells as effector cells

Before and after NK cell enrichment, $NK1.1^+$ cells were 3.99%, 29.19%; DX_5^+ cells were 2.38%, 30.56%; $NK1.1^+/DX_5^+$ cells were 1.93%, 25.97% respectively (Fig. 5).

Cytotoxicity to PLP-specific, encephalitogenic T line cells was enhanced by using enriched NK cells as effector cells (Fig. 6). This result further confirmed that the cytotoxicity to myelin antigen-specific T cells was due to the NK cells in the spleen.

3.4. NK cells also exert direct cytotoxic effect on OVA-specific T cell line and ConA-stimulated T cells

To investigate the specificity of NK cell cytotoxic effect on their targets, OVA-specific T cell line and ConA-stimulated T cells were also examined as target cells by exactly the same method as described above. We found that NK cells also exert direct cytotoxic effect on OVA-specific T line cells and ConA-stimulated T cells (Fig. 4). However, PLP-specific T line cells tended to be killed more efficiently.

4. Discussion

4.1. NK cells play a regulatory role in EAE

The present work demonstrated the inhibitory role of NK cells in EAE in SJL/J mice by in vivo treatment with

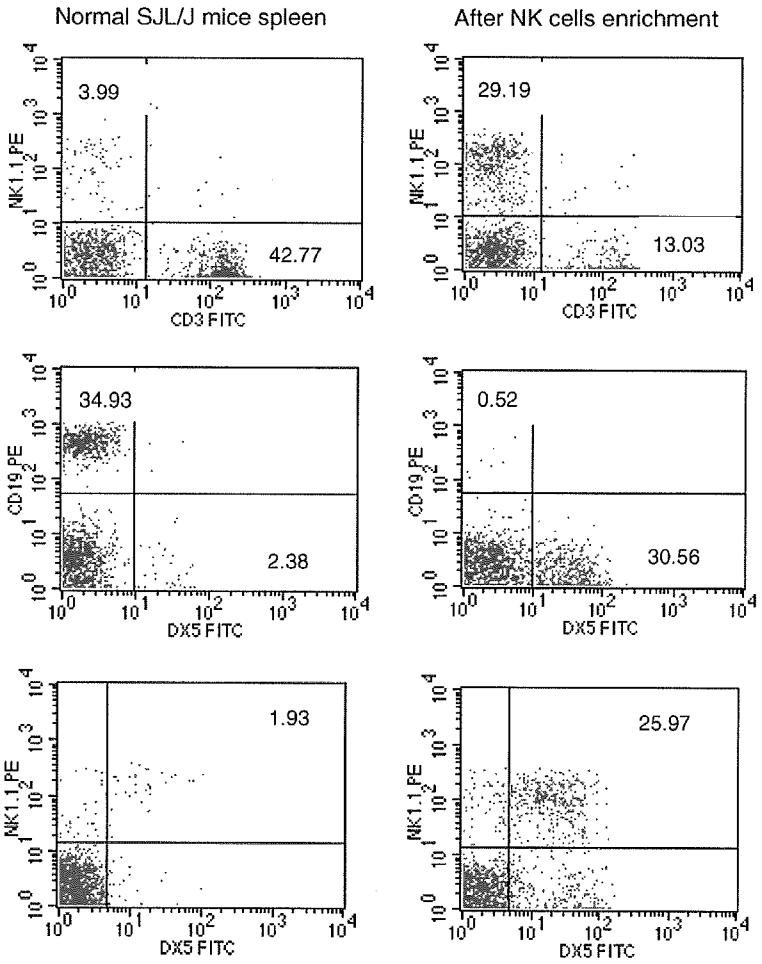

Fig. 5. NK cell enrichment. Nonadherent spleen cells which were prepared from naïve SJL/J mice were incubated with anti-mouse CD_{19} Microbeads and anti-mouse CD_5 Microbeads for 15 min. Then NK cells were negatively selected by passing through a MACS column. Then the purity of NK cells was examined and compared with that of normal SJL/J mice spleen cells.

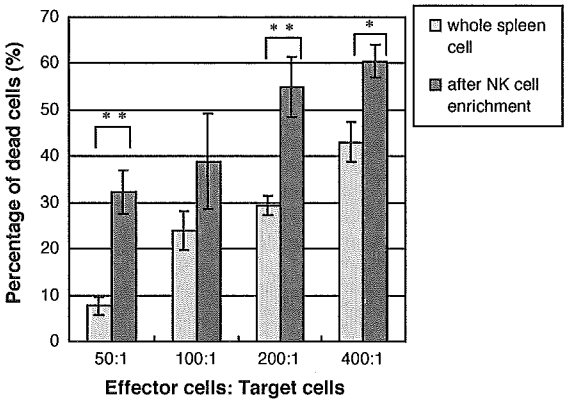

Fig. 6. Cytotoxicity to PLP-specific, encephalitogenic T line cells was enhanced by using enriched NK cells as effector cells. Cytotoxic effect to PLP-specific, encephalitogenic T cells by using naïve mice splenic NK cells and enriched NK cells as effector cells was examined and compared by using the same method as described in the legend to Fig. 3. The final results were calculated by subtracting the background and expressed as mean ± S.E. of three samples at each *E*:*T* ratio. $*P<0.05$; $**P<0.01$.

NK1.1 mAb (Fig. 1). Although we did not entirely exclude the involvement of NKT cells from the experiment, our previous paper shows that aggravation of EAE by NK cell deletion was also seen in β2-microglobulin$^{-/-}$ (β2m$^{-/-}$) mice, indicating that NK cells can play a regulatory role in a manner independent of CD8$^+$ T cells or NK1.1$^+$ T cells (NKT cells). Furthermore, EAE passively induced by the MOG $_{35-55}$-specific T cell line was also enhanced by NK cell deletion in B6, β2m$^{-/-}$, and recombination activation gene 2 (RAG-2)$^{-/-}$ mice, indicating that the regulation by NK cells can be independent of T, B, or NKT cells (Zhang et al., 1997). We found that the number of NKT cells is quite low in SJL/J mice, much lower than that of B6 mice (Fig. 2). Moreover, it has been reported that NKT cells in SJL/J mice are functionally deficient (Yoshimoto et al., 1995).

4.2. Mechanism of NK cell regulatory roles in EAE

What we are most interested in is the mechanism by which NK cells could mediate immune regulation. To date, the major effector activities of NK cells which have been identified are cytokine production and the ability of inducing target lysis. In our experiment, we proved the induction of target lysis by NK cells, although we cannot exclude that cytokines produced by NK cells also involved in the regulation of the disease. We found that NK cells exerted direct cytotoxic effect on newly stimulated myelin antigen-specific, encephalitogenic T cells, as well as OVA-specific T cells and ConA-stimulated T cells (Figs. 3 and 4). Although encephalitogenic T cells tended to be killed more efficiently, we conclude that NK cells play a regulatory role in EAE through killing of syngeneic T cells which include myelin antigen-specific, encephalitogenic T cells, thus ameliorate EAE. It has been reported that activated, but not resting T cells can be recognized and killed by syngeneic NK cells (Rabinovich et al., 2003), which may coincide with our observation. Although CD8$^+$ T cells could also exert cytotoxic effect on target cells, CD8$^+$ T cells are needed to be activated before they play their functional role. So we think it is unlikely that CD8$^+$ T cells could play an important role on cytotoxicity within 4 h of incubation. Furthermore, when using enriched NK cells in which CD_3^+ T cells were greatly decreased as effector cells (Fig. 5), we found that the cytotoxicity to myelin antigen-specific T cells were enhanced (Fig. 6). This result further indicated that NK cells in the spleen exerted cytotoxic effect on encephalitogenic T cells.

It has been known that NK cells induce target lysis via perforin (Kagi et al., 1994)- and/or TRAIL (Takeda et al., 2001)-dependent mechanisms. Although we have not yet examined the molecular mechanism of NK cell-mediated target lysis, it has been reported that blockade of TRAIL with its soluble receptor exacerbated EAE (Hilliard et al., 2001). We need to work further to identify the target molecule which may be expressed on the surface of the encephalitogenic T cells.

As to the therapy for MS, it has been accepted that type I interferon, especially IFN-β, is one of the standard therapy for relapsing–remitting and secondary progressive MS, and it is probably the most effective non-immunosuppressive therapy available now. And it is also well known that IFN-α/β increases cytotoxicity of NK cells. This is consistent with our conclusion that NK cells play a regulatory role in EAE through killing of syngeneic T cells which include myelin antigen-specific, encephalitogenic T cells, thus ameliorate EAE. Furthermore, on examining the effect of treating patients with relapsing–remitting MS with a high dose of IFN-β-1B, a strong inverse relationship between NK cell lytic activity and the number of active lesions identified by MRI was found in patients (Kastrukoff et al., 1999). It is interesting to know that TRAIL is a potential response marker for interferon-β treatment in multiple sclerosis (Wandinger et al., 2003). Other NK cell enhancer, such as Linomide, has also been reported to be able to suppress EAE (Karussis et al., 1993).

In summary, our results suggested that boosting NK cells in number and activity may be a promising immunotherapeutic strategy for multiple sclerosis and some other autoimmune diseases.

Acknowledgements

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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.)

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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-

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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 procedure 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 8000*g* 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-melcaptoethanol, 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-μm 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 mm^2 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 preformed 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 μm 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 $CaCl_2$, 10 mM glucose; gassed with 95% O_2 and 5% CO_2) for 5 min, each slice was stimulated with NMDA (100 μM), glycine (10 μM), 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 ± 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

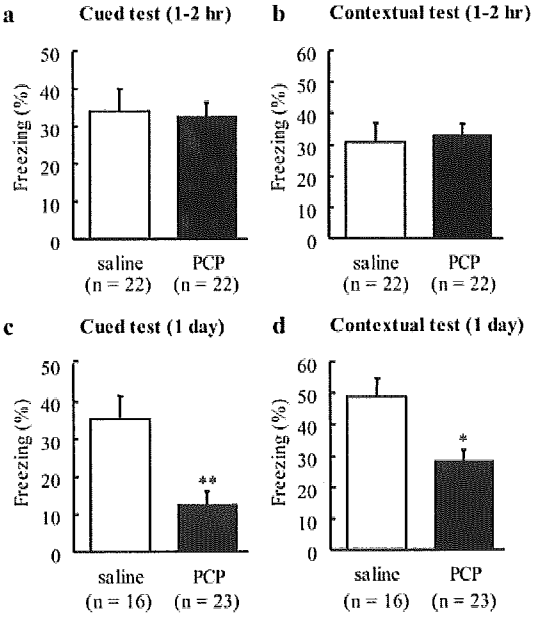

Fig. 1. Effect of repeated phencyclidine treatment on performance of fear conditioning in mice. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Cued and contextual tests were performed 1 to 2 h (a, cued test; b, contextual test) and 1 day after fear conditioning (c, cued test; d, contextual test). Values correspond to mean ± 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 μM), glycine (10 μM), 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.

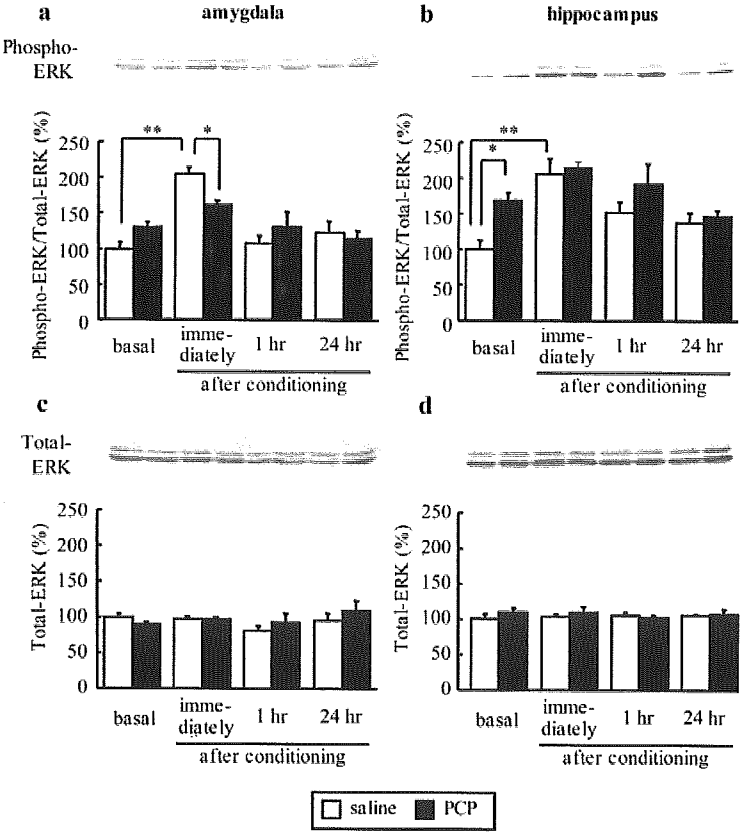

Fig. 2. ERK activation in the amygdala and hippocampus after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae (a) ($n = 10$ in 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 ± 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

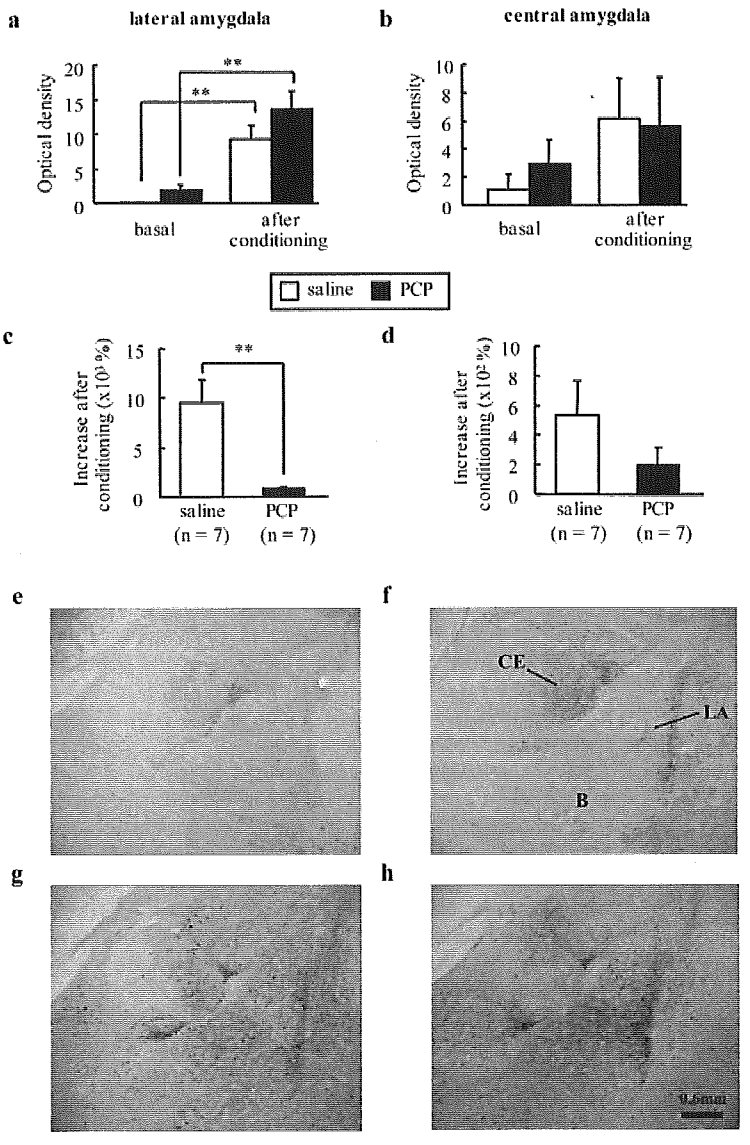

Fig. 3. ERK activation in the lateral amygdala and central amygdala after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Phospho-ERK levels in the lateral (a) and central amygdalae (b) of saline- and phencyclidine-treated 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 ± 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 n) 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.

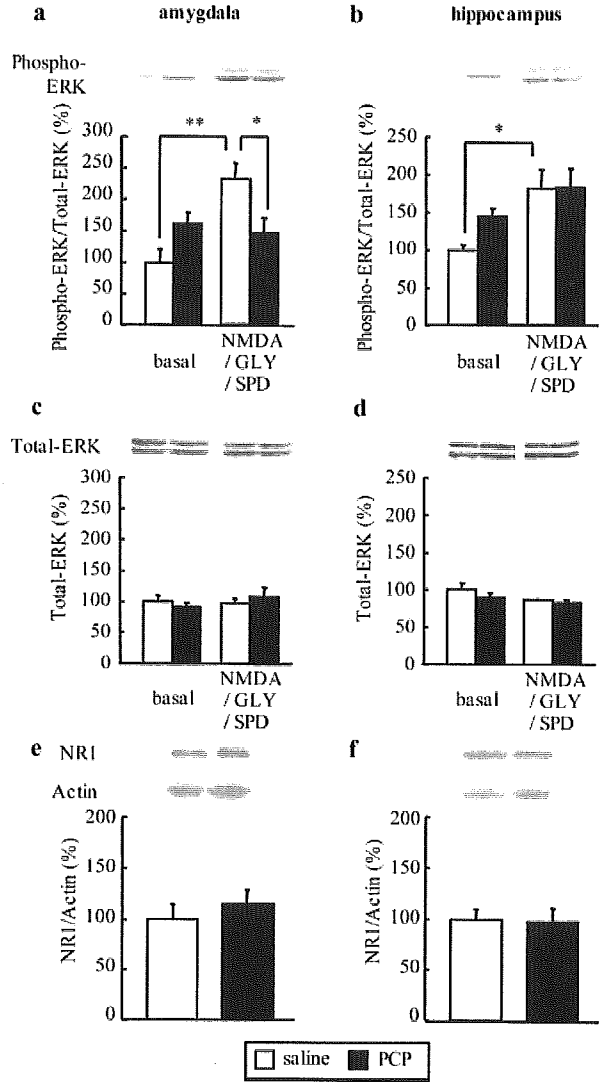

Fig. 4. ERK activation by NMDA receptor stimulation and the protein levels of NR1 in the amygdala and hippocampus. Slices of amygdala or hippocampus were stimulated with NMDA (100 μM), glycine (GLY; 10 μM), and spermidine (SPD; 1 mM) 8 days after the cessation of phencyclidine treatment (10 mg/kg/day 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 ± 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).

Discussion

There are many reports that a single administration of phencyclidine or ketamine alters cognition in healthy volunteers or patients with schizophrenia via gutamatergic 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

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 immunohistochemichal 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, immunohistochemichal 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 simulation. 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

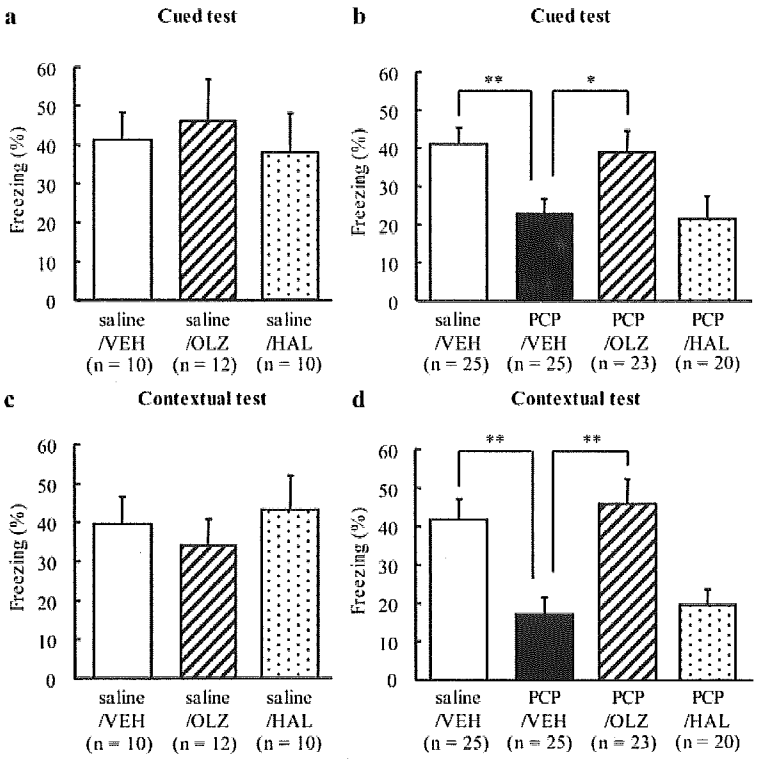

Fig. 5. Effects of repeated antipsychotic treatment on the impairment of associative learning induced by repeated phencyclidine treatment. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine 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 ± 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 conditioning-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

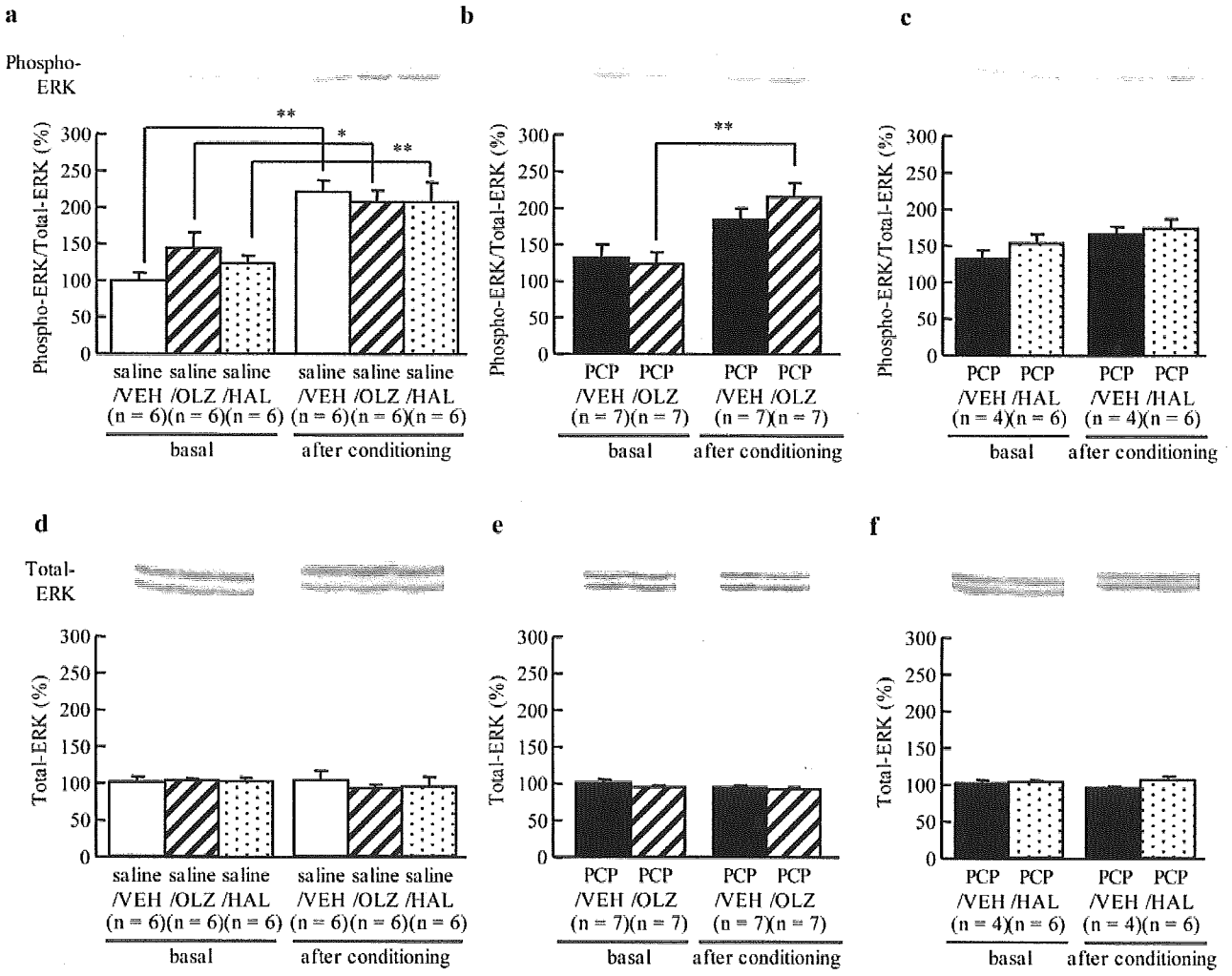

Fig. 6. Effects of repeated antipsychotic treatment on ERK activation in the amygdala after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine-treatment (10 mg/kg/day 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 (b), phencyclidine/vehicle, and 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), phen-cyclidine/vehicle, and phencyclidine/haloperidol (f). Values correspond to the mean ± 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 $F_{2,30} = 1.33$ ($p = 0.28$); b, treatment, $F_{1,24} = 0.57$ ($p = 0.46$); time, $F_{1,24} = 18.51$ ($p < 0.01$), treatment-by-time interaction, $F_{1,24} = 1.36$ ($p = 0.26$); c, treatment, $F_{1,16} = 1.18$ ($p = 0.29$); time, $F_{1,16} = 4.08$ ($p = 0.06$); treatment-by-time interaction, $F_{1,16} = 0.20$ ($p = 0.66$); 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-

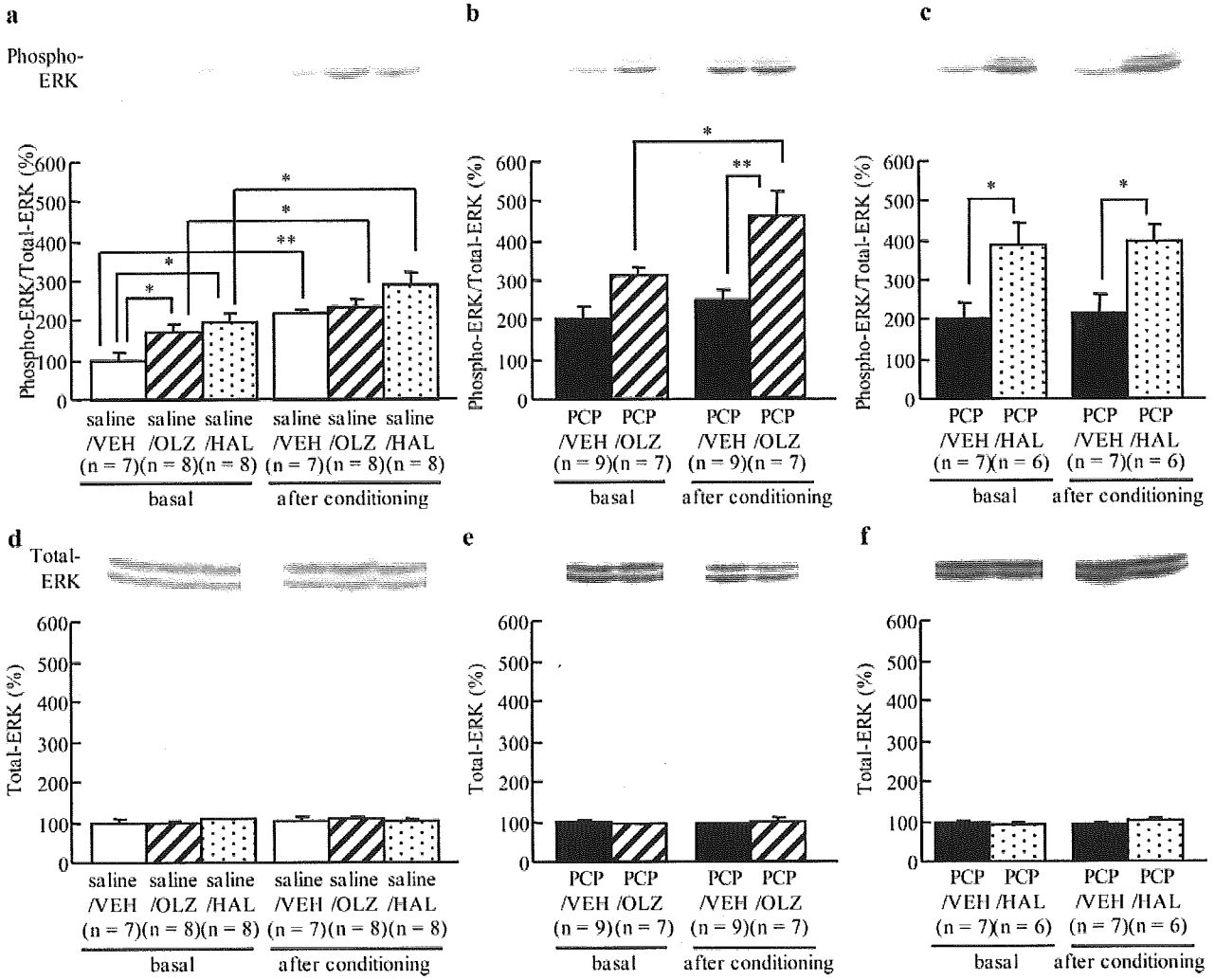

Fig. 7. Effects of repeated antipsychotic treatment on ERK activation in the hippocampus after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine treatment (10 mg/kg/day 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), phen-cyclidine/vehicle, and phencyclidine/haloperidol-treated mice (f). Values correspond to the mean ± 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.

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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

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Research report

Inhibition of neprilysin by thiorphan (i.c.v.) causes an accumulation of amyloid β and impairment of learning and memory

Akihiro Mouri[a], Li-Bo Zou[b], Nobuhisa Iwata[c], Takaomi C. Saido[c], Dayong Wang[a], Min-Wei Wang[b], Yukihiro Noda[a,d], Toshitaka Nabeshima[a,*]

[a] *Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8560, Japan*
[b] *Department of Pharmacology, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, China*
[c] *Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, Saitama, Japan*
[d] *Division of Clinical Science in Clinical Pharmacy Practice, Management and Research, Faculty of Pharmacy, Meijo University, Nagoya, Japan*

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Abstract

An accumulation of amyloid β peptide (Aβ) due to an imbalance between anabolism and catabolism triggers Alzheimer's disease (AD). Neprilysin is a rate-limiting peptidase, which participates in the catabolism of Aβ in brain. We investigated whether rats continuously infused with thiorphan, a specific inhibitor for neprilysin, into the cerebral ventricle cause cognitive dysfunction, with an accumulation of Aβ in the brain. Thiorphan-infused rats displayed significant cognitive dysfunction in the ability to discriminate in the object recognition test and spatial memory in the water maze test, but not in other hippocampus-dependent learning and memory tasks. Thiorphan infusion also elevated the Aβ40 level in the insoluble fraction of the cerebral cortex, but not that of the hippocampus. There was no significant difference in the nicotine-stimulated release of acetylcholine in the hippocampus between vehicle- and thiorphan-infused rats. These results indicate that continuous infusion of thiorphan into the cerebral ventricle causes cognitive dysfunction by raising the level of Aβ in the cerebral cortex, and suggest that a reduction of neprilysin activity contribute to the deposition of Aβ and development of AD.

Keywords: Alzheimer's disease; Amyloid β; Thiorphan; Neprilysin; Cognitive dysfunction; Rat

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by a progressive loss of cognitive, language and behavioral functions [1], affecting 20–30 million people worldwide [48]. An accumulation of amyloid β peptide (Aβ) is a triggering event that causes the decades-long pathological cascade leading to AD development [14]. Changes in the metabolic balance of Aβ are closely associated with the accumulation of Aβ [42,43]. Most cases of familial AD are caused by an increase in anabolic activity [13]. However, in sporadic cases of AD, where an increase in anabolism seems to be rare, a reduction in the catabolic activity of Aβ-degrading enzymes may account for the accumulation of Aβ. Reverse genetic studies have so far identified neprilysin [21], insulin-degrading enzyme [8] and endothelin converting enzymes 1 and 2 [6] as Aβ-degrading enzymes.

Neprilysin seems to play a major role in Aβ degradation in the brain among all of the Aβ-degrading enzyme candidates thus far examined, based on wide distribution in the cerebral cortex and the limbic region including the hippocampus, presynaptic and axonal localization [10], presence of the active site facing the extracellular side, and comparison of brain Aβ-elevating effects by deficiencies of the candidate peptidase genes [19,43,45]. To our knowledge, neprilysin is only one peptidase capable of degrading oligomeric forms as well as monomeric form of Aβ [19,26]. The expression levels of neprilysin in the specific regions, such as the hippocampus and cerebral cortex, has been demonstrated to be selectively reduced not only in aged rodents [22], but also during early stages in sporadic cases of AD [55,56]. Neprilysin gene-deficient mice have shown a gene dosage-dependent elevation of endogenous

* Corresponding author. Tel.: +81 52 744 2674; fax: +81 52 744 2682.
E-mail address: tnabeshi@med.nagoya-u.ac.jp (T. Nabeshima).

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Aβ levels in the brain [23]. In contrast, the exposure of amyloid precursor protein (APP) transgenic mice to an enriched environment results in a pronounced reduction in cerebral Aβ levels and amyloid deposits with an elevation of neprilysin activity [27]. The overexpression of neprilysin in the brains of APP transgenic mice decelerates Aβ deposition [20,28,29]. Recently, Saito et al. [44] have reported that somatostatin, a neuropeptide, decreases Aβ in the mouse brain though upregulation of neprilysin. These findings suggest that a reduction in neprilysin activity contributes to development of AD by the promoting deposition of Aβ and that an increase in neprilysin activity may be a potential therapeutic target in the treatment of AD.

Previous pharmacological experiments show that continuous infusion of a specific inhibitor for neprilysin, thiorphan, into the hippocampus of rats, raise the hippocampal levels of Aβ [21]. However, it is not clear whether inhibition of neprilysin activity by thiorphan causes cognitive dysfunction in several types of learning and memory tasks through an increase of Aβ contents in the brain. The present study was designed to test the hypothesis that rats, in which the effect of neprilysin was inhibited by thiorphan infusion into the brain, showed an impairment of learning and memory accompanying the elevated Aβ levels. We attempted to investigate effects of infusion of thiorphan into the cerebral ventricle on: (1) cognitive function using a battery of learning and memory tests, (2) the release of acetylcholine (ACh) stimulated by nicotine in the hippocampus and (3) change of endogenous Aβ levels in the hippocampus and cerebral cortex.

2. Materials and methods

2.1. Animals

Male 8-week-old Wistar rats (Oriental Bioservice, Kyoto, Japan), weighing 260–300 g at the beginning of the 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-h light:12-h dark cycle (lights on at 09:00 h, off at 21:00 h). All experiments were performed in accordance with 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 "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

2.2. Drugs

Thiorphan and naloxone were purchased from Sigma Co. (St. Louis, MO, USA). Thiorphan (2 mg/ml) was dissolved in saline containing 1 mM ascorbic acid (adjusted to pH 6.8 with NaOH). Continuous infusion (2.5 μl/h) of thiorphan was continued for 4 weeks by attaching an infusion cannula to a mini-osmotic pump (Alzet MODEL 2ML4; DURECT Co. Ltd., Cupertino, CA, USA). Neprilysin is also known as enkephalinase because of its ability to cleave enkephalins [30,41,51]. To exclude a possible involvement of enkephalins, of which metabolism may be affected by the inhibition of neprilysin, in behavioral changes in the rat, naloxone (1 mg/kg, i.p.) or saline (1 ml/kg, i.p.) was administered 30 min before each experiment.

2.3. Surgery

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and fixed on a stereotactic instrument (Narishige, Tokyo, Japan). The cannula for thiorphan infusion was implanted into the right ventricle (coordinates—anteroposterior (AP): −0.3 mm; mediolateral (ML): 1.2 mm from the bregma; dorsoventral (DV): 4 mm from the skull), according to the atlas of Paxinos and Watson [39]. As a control, rats were infused with the vehicle only (saline containing 1 mM ascorbic acid; adjusted to pH 6.8 with NaOH). We have confirmed that the vehicle itself failed to induce any behavioral and neurochemical changes at this flow rate (data not shown).

2.4. Experimental design

Previous report, 5 days continuous intracerebroventricular infusion of thiorphan increased both cortical and cerebrospinal fluid concentration of Aβ [32]. Thus, the tests started on the day 7 after thiorphan infusion, and were carried out sequentially according to the experimental schedule shown in Fig. 1.

2.5. Measurement of spontaneous locomotor activity

The measurement of locomotor activity in a novel environment was carried out on the day 7 after the start of thiorphan infusion. Rats were placed individually in a transparent acrylic cage with a black frosting Plexiglas floor (45 cm × 26 cm × 40 cm), and locomotor activity was measured for 15 min using digital counters with an infrared sensor (Scanet SV-10; MELQUEST Co. Ltd., Toyama, Japan). The system was equipped with photosensor frames in the side walls. Locomotor activity was defined as the total number of beam breaks due to horizontal movement measured by photosensors. The acrylic cage was wiped with paper towel between animals and it kept clean.

2.6. Y-maze test

The task was carried out on the day 8 after the start of thiorphan infusion under the same condition as described previously [37]. The maze was made of

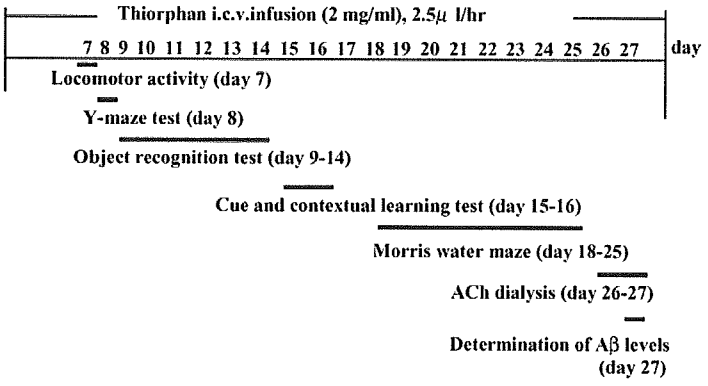

Fig. 1. Experimental schedule. i.c.v.: intracerebroventricular; Aβ: amyloid β peptide; ACh: acetylcholine.

gray painted wood. Each arm was 35 cm long, 25 cm high and 10 cm wide. The arms converged in an equilateral triangular central area that was 10 cm at its longest axis. Each rat, naive to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded visually. An entry was considered completed when the hind paws of the rat had been completely placed in the arm. Alternation was defined as successive entries into the three arms, on overlapping triplet sets. The percent alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two), multiplied by 100.

2.7. Novel-object recognition test

The experiments were carried out on the days 9–14 after the start of thiorphan infusion and according to the method of Ennaceur et al. [7] with a minor modification. Namely the apparatus consisted of an open box (80 cm × 80 cm × 20 cm) made of wood, the inside of which was painted gray. Triplicate copies were made of the objects to be discriminated, which were made of glass, plastic or metal. The weight of the objects ensured that they could not be displaced by the rats. The apparatus was placed in a sound-isolated room. A light bulb fastened in the upper part of the room provided a constant illumination of about 40 lux at the level of the task apparatus.

All rats were given two habituation sessions on the days 9 and 10 where they were allowed 3 min to explore the apparatus (with no object present). The rats carried out a test session. On the day 11, the rats were firstly tested on the standard version of the task with retention delays of 1 h. After that they were tested on the configural version using retention delays of 1 h (on the day 14: the interval between sessions was 3 days). Each rat received one session of each version. A session consisted of a sample phase and a choice phase with an intervening delay period. The duration of each phase was 3 min.

At the start of each sample phase, two identical objects (A1 and A2) were placed in the back corner of the box, 10 cm from the side wall. A rat was then placed in the middle front of the box and the total time spent exploring the two objects was recorded by the experimenter with two stop watches. Exploration of an object was defined as directing the nose to the object at a distance of less than 2 cm and/or touching it with the nose. The rat was put back in its home cage after 3 min had elapsed. After a delay, the rat was reintroduced into the open field for 3 min. In the standard version of the object recognition task, the open field now contained a third identical copy of the familiar object (A3) and a new object (B).

The conditions in the configural version were the same as those used in the standard version of the task, except that now a third identical copy of the sample stimulus (A) was re-configured for the choice phase. A re-configured stimulus (A^*) consisted of a different spatial arrangement of the elements of the original sample (A). This meant that although each constituent part of the sample was familiar, the overall appearance was novel.

Comparisons focused on the total time spent exploring objects during the sample and choice phases. Analyses of variance were performed on the following measures: (1) e_1, which is the total time spent exploring the two identical objects in the sample phase; (2) e_2, which is the total time spent in exploring the two objects in the choice phase; (3) d_1, the discrimination index, which is the difference in time spent exploring the two objects in the choice phase (e.g. B − A in the standard condition or $A^* - A$ in the configural conditions); (4) d_2, the discrimination ratio, which is the difference in exploration time (d_1) divided by the total time spent exploring the two objects in the choice phase (e.g. (B − A)/(B + A) in the standard condition and $(A^* - A)/(A^* + A)$ in the configural conditions).

2.8. Cue and contextual fear conditioning tests

The experiments were carried out on the days 15 and 16 after the start of thiorphan infusion. On the first day, for measuring basal levels of the freezing response (preconditioning phase), the rats were individually placed in a neutral cage (40 cm × 30 cm × 40 cm) and the freezing time was determined for 1 min. The rats were removed from the neutral cage to a conditioning cage equipped with a metal wire floor, and the freezing time was determined for 2 min. For training (conditioning phase), rats were placed in the conditioning cage, and then a 60 s tone (74 dB) was delivered as a conditioned stimulus. Immediately after the termination of the tone stimulus, a foot shock of 0.5 mA for 0.5 s was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea Co. Ltd., Osaka, Japan). Cued and contextual tasks were carried out 1 day after the fear conditioning. For the cued task, the freezing response was measured in the neutral cage for 1 min in the presence of a continuous-tone stimulus identical to the conditioning stimulus. For the contextual task, the freezing response was measured in the conditioning cage for 2 min in the absence of the conditioned stimulus. The freezing response was defined as the all paws of the rat stayed still and stooped down with fear.

2.9. Morris water maze test

The experiments were carried out on the days 18–25 after the start of thiorphan infusion. The Morris water maze task [31] was performed as previous reports with a minor modification [18,35,36,54]. A circular water tank (140 cm in diameter and 45 cm high) was consisted with four equally spaced quadrants (north, south, east and west). A transparent platform was set at the east quadrant of the tank, 40 cm from the wall (10 cm in diameter, surface 2 cm below the surface of the water) in reference memory task. The pool was located in a large room, in which there were some cues external to the maze. The positions of these cues were left unchanged throughout the task.

2.9.1. Reference memory task

The task was conducted twice a day for 5 consecutive days, one session consisting of 2 trials (2 trials × 5 days; inter-trial interval: 3 h). In each trial, the rat was placed in the water at one of five starting positions (that were spaced equally around the rim of the tank), with the sequence of the positions being selected randomly. The latency to escape onto the platform was measured. If the rat found the platform, it was allowed to remain there for 15 s and was then returned to its home cage. If the rat could not find the platform within 90 s, the trial was terminated and the animal was put on the platform for 15 s. Escape latency was assigned using the Target/2 system (Neuroscience Idea Co. Ltd.).

2.9.2. Probe task

After 10th training trial of the reference memory task on the day 22, the platform was removed from the pool and animals underwent a 90 s spatial probe trial. The time spent in the quadrant where the platform had been located during training was measured using the Target/2 system.

2.9.3. Working memory (repeated acquisition) task

The Working memory task was conducted for 3 consecutive days from days 23 to 25 after the start of thiorphan infusion, and consisted of five trials (one session) per day. The procedure was similar to the training for the standard water maze task, except that the location of the platform was changed for each session. Since the platform's position was changed daily, this task evaluates working memory [9]. For each trial, the rat was put into the pool at one of five starting positions, the sequence of the positions being selected randomly. The first trial of each session was an informative sample trial in which the rat was allowed to swim to the platform in its new location and to remain there for 15 s. The rat was placed in a home cage for an interval of 1 min. The platform remained in the same location throughout the remaining four trials of the day. Spatial working memory was regarded as the mean escape latency of the second to fifth trials. The working memory of each rat was assessed based on the mean performance for 3 consecutive days.

2.10. Determination of extracellular ACh release

On the day 26 after starting the infusion of thiorphan, the cannula was removed and a dialysis probe was implanted to measure the release of ACh [17,50]. Briefly, rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic apparatus (Narishige). A guide cannula (EICOM, Kyoto, Japan) was implanted into the hippocampus (AP: −3.8 mm; ML: 2.2 mm from bregma; DV: 2.0 mm from the skull). On the day 27 (24 h after the implantation of the guide cannula), the dialysis probe (A-I-8-03; membrane length 3 mm, EICOM) was implanted into the hippocampus and Ringer's solution (147 mM NaCl, 4 mM KCl and 2.3 mM $CaCl_2$) containing 10^{-5} M eserine was perfused at a flow rate of 2.0 μl/min. Under the free moving condition, the dialysate

was collected every 15 min and the amount of ACh in the dialysate was determined using an HPLC system with electrochemical detection (ECD). Details of the measurement of ACh by HPLC–ECD have been described previously [15]. After the basal release of ACh had been reached, nicotine (free base, 3 mM) was infused for 30 min.

2.11. Determination of Aβ40 and Aβ42 contents

The amounts of Aβ40 and Aβ42 in the soluble and insoluble fractions were determined by a sandwich ELISA using the combination of the monoclonal antibodies BNT77/BA27 and BNT77/BC05, respectively [11,21]. On the day 27 after starting the infusion of thiorphan, rats were sacrificed by decapitation, and brains were quickly removed and placed on an ice-cold glass plate. The hippocampus and cerebral cortex was rapidly dissected out, frozen and stored in a deep freezer at −80 °C until assayed. The frozen tissues were homogenized in four volumes of buffer A containing 50 mM Tris–HCl (pH 7.6), 150 mM NaCl and the protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany) with 10 strokes of a Teflon-glass homogenizer, and centrifuged at 200,000 × *g* for 20 min at 4 °C. The supernatant was used as the soluble fraction. The pellet was solubilized by sonication in the buffer A containing 6 M guanidine–HCl. The solubilized pellet was then centrifuged at 200,000 × *g* for 20 min at 4 °C, after which the supernatant was diluted 12-fold to reduce the concentration of guanidine–HCl and used as the insoluble fraction. The amounts of Aβ40 and Aβ42 in each fraction were determined by sandwich ELISA, respectively.

2.12. Statistical analysis

Statistical analysis was performed using the one- or two-way analysis of variance (ANOVA), followed by the Bonfferoni test. A value of $p < 0.05$ was considered statistically significant. Data were expressed as the mean ± S.E.M.

3. Results

3.1. Endogenous Aβ40 and Aβ42 in the hippocampus and cerebral cortex of the thiorphan-infused rats

Endogenous concentrations of Aβ40 and Aβ42 in the hippocampus and cerebral cortex were measured after a battery of behavior tests and measurement of ACh release (Table 1). The infusion of thiorphan tended to have increased levels of Aβ40 in the insoluble fraction of the hippocampus. In the insoluble fraction of the cerebral cortex, Aβ40 levels were significantly elevated in the Thio/SAL-treated rats. Naloxone pretreatment did not affect the elevation in levels of Aβ40 in the thiorphan-treated rats. However, there was no significant difference in Aβ42 content among the three groups in any fraction of the hippocampus or cerebral cortex.

3.2. Spontaneous locomotor activity in the thiorphan-infused rats

There was no difference in spontaneous locomotor activity among the three groups on the day 7 after the start of thiorphan infusion. The counts of locomotor activity for 15 min in the vehicle + saline (Veh/SAL)-, thiorphan + saline (Thio/SAL)- and thiorphan + naloxone (Thio/NAL)-treated rats were 4624 ± 276, 5033 ± 661 and 4411 ± 723, respectively (values indicate the mean ± S.E.M.). Results with the one-way ANOVA were not significantly different among the groups [$F_{(2,21)} = 0.56$, $p = 0.58$].

3.3. Novel-object recognition task in the thiorphan-infused rats

In the measurement of discrimination (d_2) between new and familiar objects in the standard experiment, Veh/SAL-treated rats spent more time exploring a new object than a familiar object 1 h after the training. The Thio/SAL- and Thio/NAL-treated rats tended to have shorter the extension time for a new object (Fig. 2A). In the configural experiment, however, the d_2 measures of the Thio/SAL-treated rats indicated that the rats did not discriminate between the objects (Fig. 2B). Naloxone administered the before sample phase did not change the measures of discrimination in the thiorphan-infused rats (Fig. 2B).

In the standard and configural conditions, there was no difference in the total spent time exploring the objects in e_1 or e_2 among any of the all sessions (data not shown).

Table 1
Effect of continuous infusion of thiorphan into the cerebral ventricle on endogenous Aβ40 and Aβ42 in the rat hippocampus and cerebral cortex

	Hippocampus		Cerebral cortex	
	Aβ40 (pmol/g)	Aβ42 (pmol/g)	Aβ40 (pmol/g)	Aβ42 (pmol/g)
Soluble Aβ				
Veh/SAL	0.020 ± 0.002	0.050 ± 0.003	n.d.	0.019 ± 0.002
Thio/SAL	0.021 ± 0.005	0.042 ± 0.006	n.d.	0.018 ± 0.003
Thio/NAL	0.019 ± 0.003	0.037 ± 0.004	n.d.	0.011 ± 0.002
Insoluble Aβ				
Veh/SAL	0.205 ± 0.021	0.258 ± 0.012	0.552 ± 0.042	0.529 ± 0.032
Thio/SAL	0.338 ± 0.082	0.339 ± 0.057	0.714 ± 0.049*	0.593 ± 0.038
Thio/NAL	0.303 ± 0.049	0.285 ± 0.034	0.723 ± 0.021*	0.494 ± 0.013

Values are expressed as pmol/g wet weight and are the means ± S.E.M. for four to five animals. The rats were decapitated on day 27 after the start of thiorphan infusion, and the amounts of Aβ40 and Aβ42 in the discrete brain regions were determined. Results with the one-way ANOVA on the Aβ40 and Aβ42 of the hippocampus and cortex were as follows. Hippocampus—soluble Aβ: Aβ40, $F_{(2,12)} = 0.16$, $p = 0.85$, and Aβ42, $F_{(2,12)} = 2.35$, $p = 0.14$; insoluble Aβ: Aβ40, $F_{(2,12)} = 1.56$, $p = 0.25$, and Aβ42, $F_{(2,12)} = 0.87$, $p = 0.44$. Cortex—soluble Aβ: Aβ42, $F_{(2,12)} = 3.91$, $p < 0.05$; insoluble Aβ: Aβ40, $F_{(2,9)} = 6.02$, $p < 0.05$, and Aβ42, $F_{(2,11)} = 0.96$, $p = 0.41$. Aβ: amyloid β; Veh: vehicle; Thio: thiorphan; SAL: saline; NAL: naloxone; n.d.: not detected.

* $p < 0.05$ compared to Veh/SAL rats.

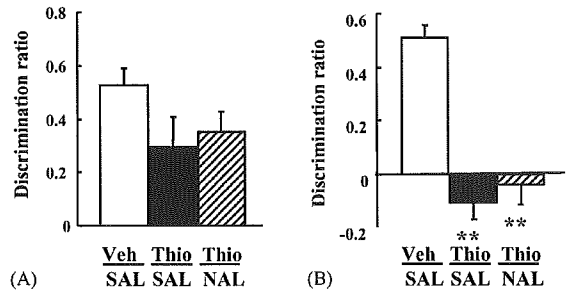

Fig. 2. Effect of continuous infusion of thiorphan into the cerebral ventricle on the performance of rats in a spontaneous object recognition test of: standard (A) and configural (B) conditions. The experiments were carried out on the days 9–14 after the start of thiorphan infusion. Values indicate the mean ± S.E.M. for 12–14 animals. Results with the one-way ANOVA were: standard version, no significant difference among the groups $F_{(2,37)} = 1.93$, $p = 0.16$; configural version, $F_{(2,37)} = 28.75$, $p < 0.01$. $^{**}p < 0.01$ compared to Veh/SAL-treated rats. Veh: vehicle; Thio: thiorphan; SAL: saline; NAL: naloxone.

3.4. Water maze task in the thiorphan-infused rats

Changes in escape latency, the time taken to find the hidden platform, in training trials in each group of rats are shown in Fig. 3A. The Thio/SAL- and Thio/NAL-treated rats exhibited a prolonged escape latency, compared to Veh/SAL-treated rats, but the difference was not significant among the three groups ($p = 0.07$; Fig. 3A). A 90 s spatial probe trial was carried out following the 10th training trial (Fig. 3B). The Thio/SAL-treated rats, compared to the Veh/SAL-treated rats, showed a significant decrease in the time spent in the quadrant in which the platform had been located during training. Naloxone administered the before probe trial did not change the amount of time spent in quadrant in the thiorphan-infused rats (Veh/SAL: 42.51 ± 2.38%; Thio/SAL: 32.04 ± 2.54%; Thio/NAL: 29.74 ± 2.51%).

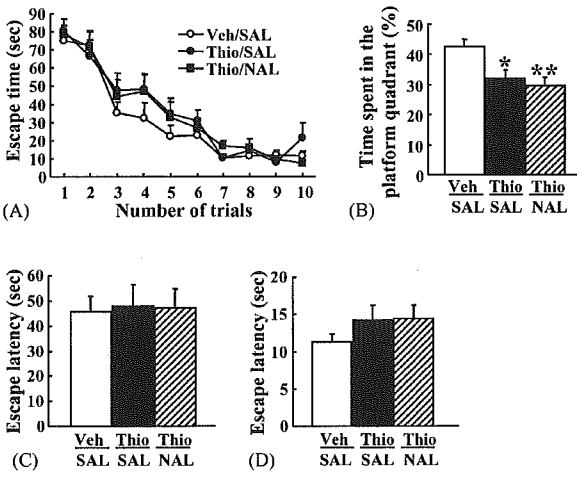

Fig. 3. Effect of continuous infusion of thiorphan into the cerebral ventricle on the performance of rats in the training (A) and probe (B) trials of reference memory, and the sample (C) and test (D) trials of working memory in the water maze task. The experiments were carried out on the days 18–25 after the start of thiorphan infusion. Values indicate the mean ± S.E.M. for 11–13 animals. Results with the two-way ANOVA were not significantly different among the groups: training trial, $F_{(2,335)} = 2.75$, $p = 0.07$ in reference memory. Results with the one-way ANOVA were: probe trial, $F_{(2,33)} = 7.07$, $p < 0.05$ in reference memory. Results with the two-way ANOVA were not significantly different among the groups: sample trial, $F_{(2,30)} = 0.03$, $p = 0.97$; test trial, $F_{(2,30)} = 1.21$, $p = 0.31$ in working memory. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to Veh/SAL-treated rats. Veh: vehicle; Thio: thiorphan; SAL: saline; NAL: naloxone.

3.5. Spontaneous alternation behavior in the thiorphan-infused rats

Spontaneous alternation behavior in the Y-maze test, a measure of immediate working memory, was tested on the day 8 after the start of thiorphan infusion. The percent alternation in the Thio/SAL- and Thio/NAL-treated rats was lower than that in the Veh/SAL-treated rats, but the difference among the three groups was not significant (Fig. 4A). There was no significant difference in the total number of arm entries among the three groups in the Y-maze task (Fig. 4B).

3.6. Cue and contextual fear conditioning tasks in the thiorphan-infused rats

In the preconditioning phase, the Veh/SAL-, Thio/SAL- and Thio/NAL-treated rats hardly showed a freezing response. There were no differences in the basal levels of the freezing response among the three groups (data not shown).

In cued and contextual fear conditioning, animals learned the tone and context associated with the foot shock. In the cued fear freezing response 24 h after fear conditioning, there was no significant difference between the three groups (Fig. 4C). When the contextual freezing response was measured 24 h after the fear conditioning, the Thio/SAL- and Thio/NAL-treated rats exhibited less of a response than the Veh/SAL-treated rats, but there was no significant among the three groups ($p = 0.09$; Fig. 4D).

3.7. Hippocampal extracellular ACh release stimulated by nicotine in the thiorphan-infused rats

There was no difference in the basal levels of ACh in the hippocampus between the vehicle-infused (1.330 ± 0.449 pmol/30 μl/15 min) and thiorphan-infused (1.349 ± 0.214 fmol/30 μl/15 min) rats. In the vehicle-infused rats, the extracellular

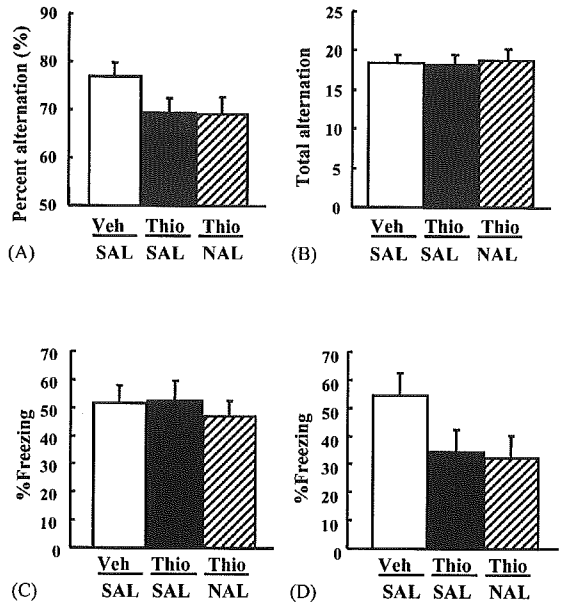

Fig. 4. Effect of continuous infusion of thiorphan into the cerebral ventricle on the spontaneous alternation behavior (A) and the number of arm entries (B) during an 8-min session in the Y-maze task, and on performance in tone cue (C) and context (D) fear-conditioned-learning tests. The experiments were carried out on the day 8 (Y-maze) and days 15–16 (fear-conditioned-learning tests) after the start of thiorphan infusion, respectively. Values indicate the mean ± S.E.M. for 12–14 animals. Results with the one-way ANOVA were not significantly different among the groups: percent alternation, $F_{(2,38)} = 1.96$, $p = 0.15$; total alternation number, $F_{(2,38)} = 0.04$, $p = 0.95$ in the Y-maze; cue dependent, $F_{(2,36)} = 0.21$, $p = 0.80$; context dependent, $F_{(2,36)} = 3.60$, $p = 0.09$ in fear-conditioned-learning test. Veh: vehicle; Thio: thiorphan; SAL: saline; NAL: naloxone.

level of ACh in the hippocampus was elevated about two-fold by perfusion of nicotine–Ringer for 30 min and returned to the basal level within 90 min (Fig. 5). There was no difference in ACh release stimulated by nicotine between the vehicle- and thiorphan-infused rats (Fig. 5).

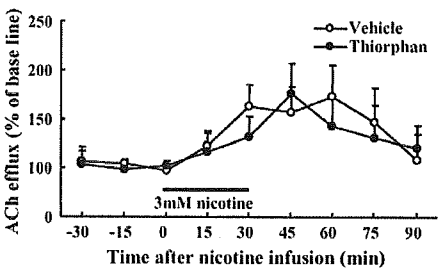

Fig. 5. Hippocampal extracellular ACh release stimulated by nicotine in vehicle- and thiorphan-treated rats on the days 26–28. Nicotine–Ringer was applied for 30 min, after which normal Ringer's solution was used. Values indicate the mean ± S.E.M. for seven to eight animals. Results with the two-way ANOVA were not significantly different between vehicle- and thiorphan-treated rats [$F_{(1,117)} = 0.96$, $p > 0.5$]. Basal levels of ACh in the hippocampus did not differ between vehicle-treated (1.330 ± 0.449 pmol/15 μl/30 min) and thiorphan-treated (1.349 ± 0.214 pmol/15 μl/30 min) rats ($p > 0.5$; Student's t-test). ACh: acetylcholine.

4. Discussion

Excessive deposition of Aβ in the brain is one of characteristics of AD patients. The accumulation of Aβ is caused by a breakdown in the balance between anabolism and catabolism in vivo [42]. Aβ-infused animals as well as APP transgenic mice and APP transgenic mice crossbred with familial AD-linked mutant presenilin 1 transgenic or knockin mice to enhance the anabolic activity, have been investigated using biochemical and behavioral approaches, and have contributed to the elucidation of AD-like neuronal dysfunction and development of new preventive and therapeutic approaches. However, there have been few studies with animal models of the diminution of the catabolic activity, as prepared by continuous infusion of thiorphan [5,21,32], nevertheless reduced levels of neprilysin, which could cause Aβ accumulation, have been observed in the brains of sporadic AD or of aged laboratory mice. Therefore, we attempted to investigate whether cognitive dysfunction is caused in thiorphan-infused rats. This study will be helpful for understanding a relationship of elevation in Aβ levels due to decreased activity of Aβ catabolism with cognitive dysfunction.

We found that the continuous intracerebroventricular infusion of thiorphan caused endogenous Aβ to accumulate in the insoluble fraction of the cortex, and induced impairments of both novelty discrimination in a configural version of object recognition behavior and a spatial memory in a probe trial of the water maze task in the rat. In other behavioral tasks, the rats also showed a tendency forward an impairment of memory. It is unlikely that the impairment in the performance cognitive dysfunction of the thiorphan-treated rats in learning and memory tasks is due to changes in motivation or sensorimotor function, since motivation difference among these behavioral tasks, and different skills are required for a good performance in each task. Actually, there was no difference in the locomotor activity and the number of total arm entries in the Y-maze task between the vehicle- and thiorphan-treated rats, indicating no changes in motor function and exploratory activity. Furthermore, there were also no differences between the vehicle- and thiorphan-infused rats in the escape latency onto the submerged platform in the first and sample trials of the working memory test in the water maze task. These results indicate no changes in motor function or vision.

Neprilysin plays an important role in Aβ degradation in the brain [21,23]. In the present experiments, continuous administration of thiorphan, a neprilysin inhibitor, increased the cerebral Aβ40 levels in the insoluble, but not soluble, fraction. Thiorphan infusion has been reported to produce Aβ plaques, as seen in AD brains, and appears to mimic the initial stage of amyloid deposition [21]. Aβ in an aqueous solution undergoes self-assembly leading to the transient appearance of soluble oligomers or protofibrils and ultimately to insoluble fibrils [40]. Thus, the impairment of memory may be related to the cerebral accumulation of insoluble Aβ induced by thiorphan. On the other hand, neprilysin, as previously termed enkephalinase, is also a potent enkephalin-degrading enzyme and has been suggested that it may be involved in peptidic neurotransmission [30,41,51]. Enkephalin is reported to affect cognitive function,