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.

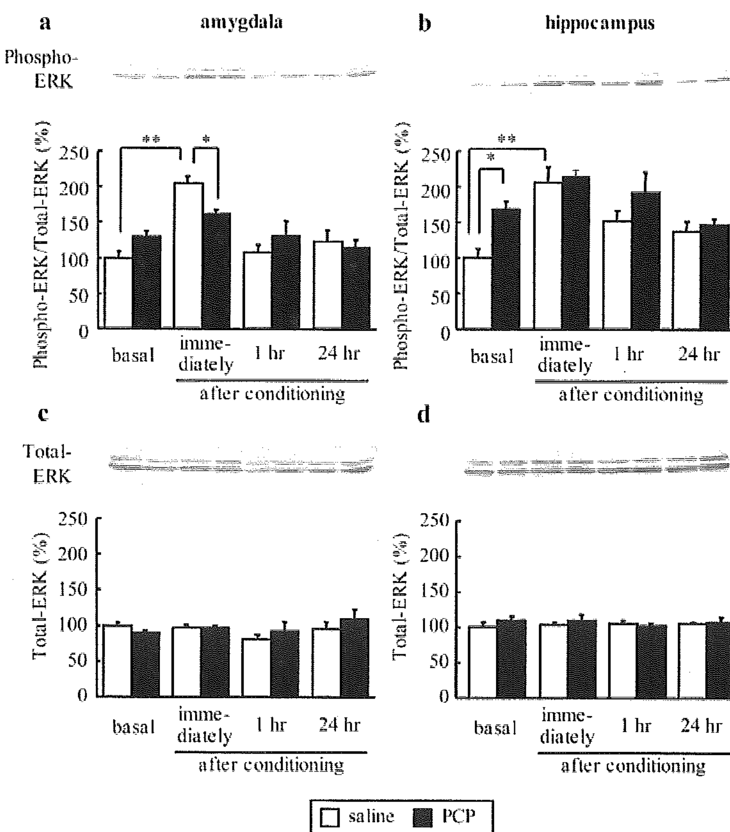

Fig. 2. ERK activation in the amygdala and hippocampus after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Representative Western blots and phospho-ERK/total ERK immunoreactivity in the amygdalae (a) ($n = 10$ in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

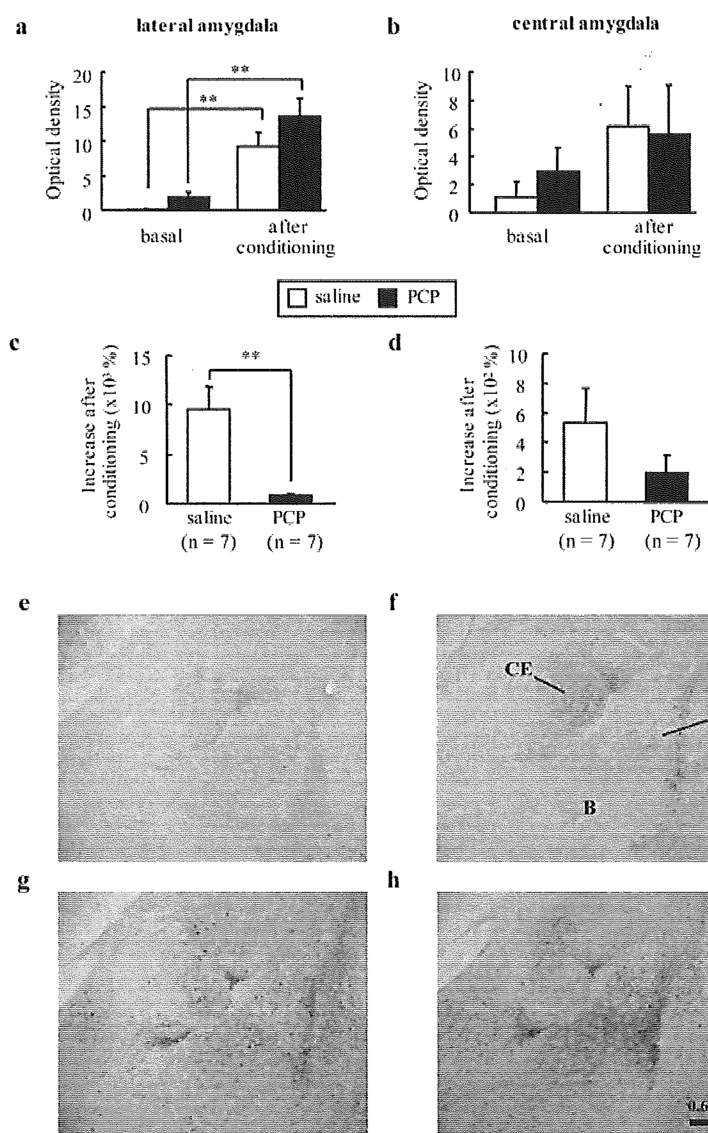

Fig. 3. ERK activation in the lateral amygdala and central amygdala after fear conditioning. Fear conditioning was performed 8 days after cessation of phencyclidine treatment (10 mg/kg/day s.c. for 14 days). Phospho-ERK levels in the lateral (a) and central amygdalae (b) of saline- and phencyclidine-treated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

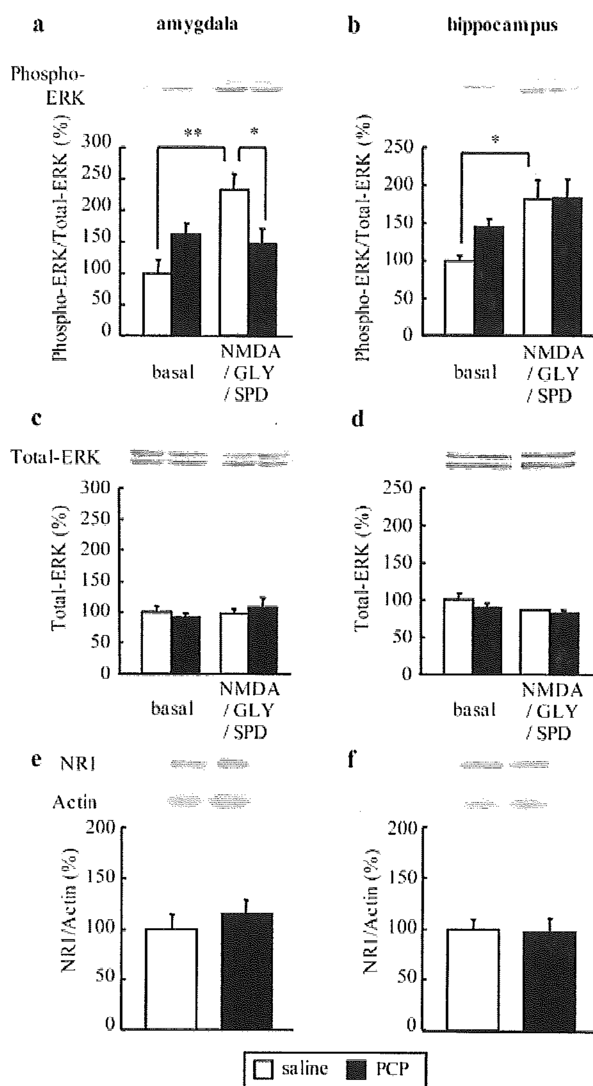

Fig. 4. ERK activation by NMDA receptor stimulation and the protein levels of NR1 in the amygdala and hippocampus. Slices of amygdala or hippocampus were stimulated with NMDA (100 μM), glycine (GLY; 10 μM), and spermidine (SPD; 1 mM) 8 days after the cessation of phencyclidine treatment (10 mg/kg/day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

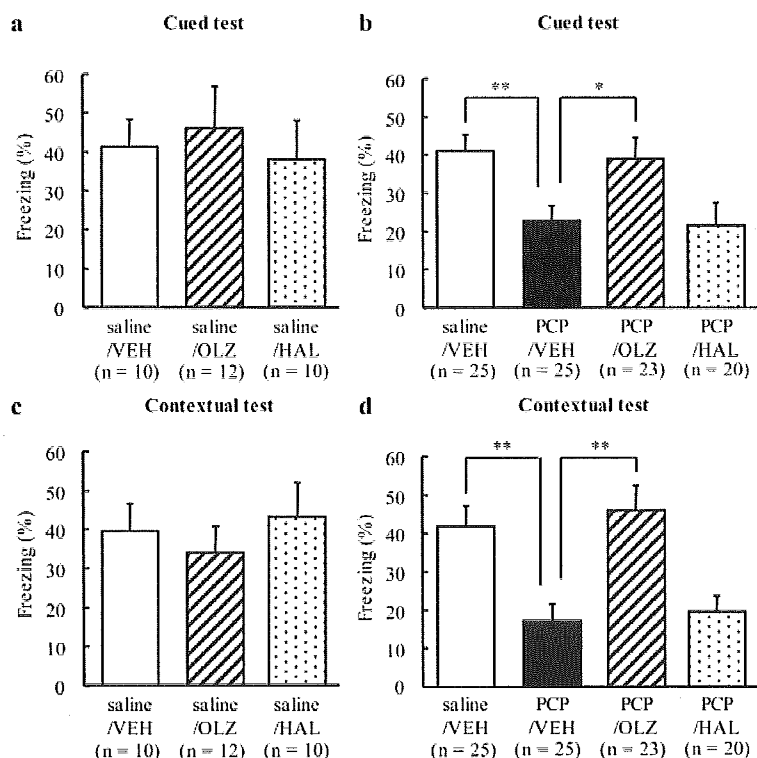

Fig. 5. Effects of repeated antipsychotic treatment on the impairment of associative learning induced by repeated phencyclidine treatment. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine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

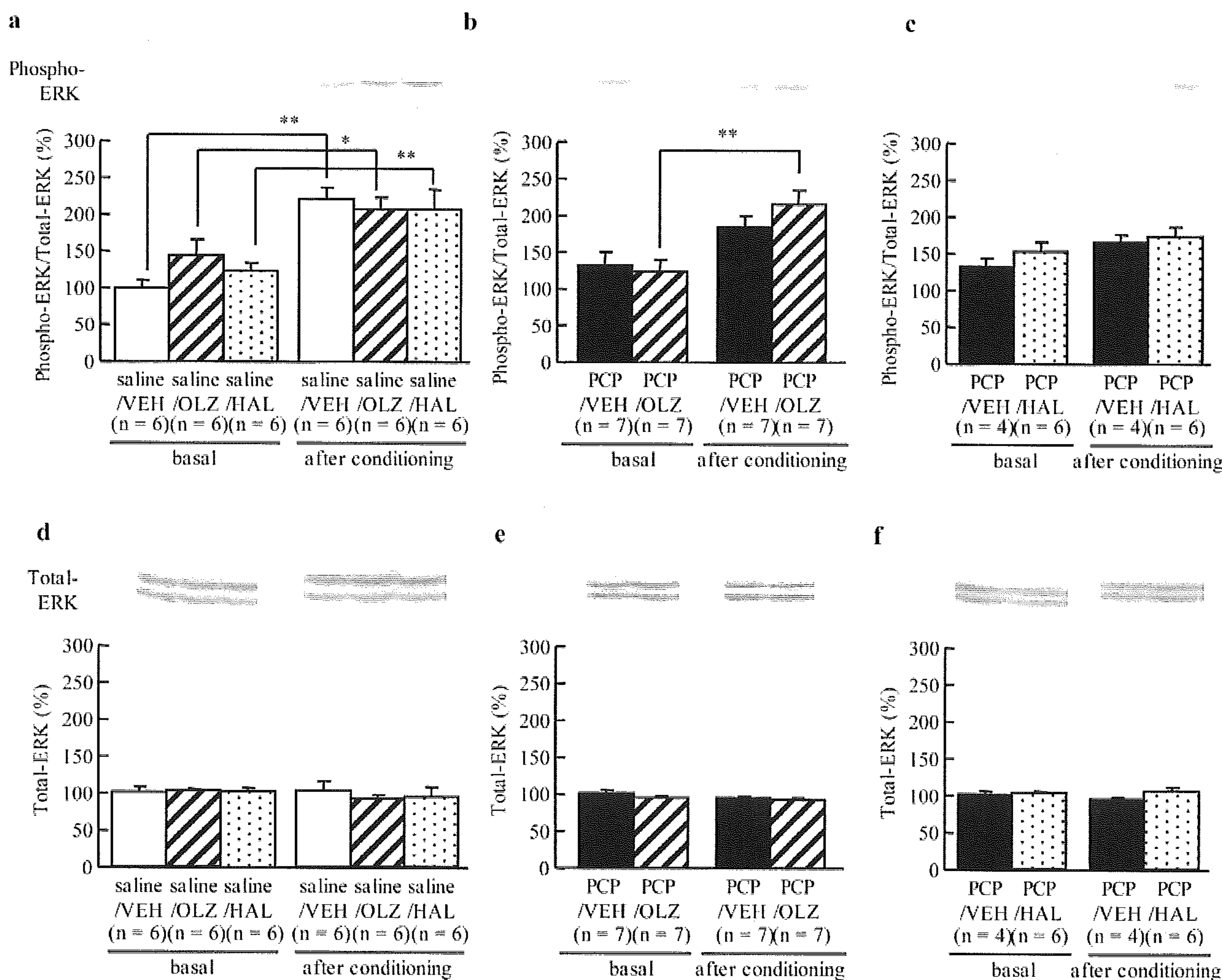

Fig. 6. Effects of repeated antipsychotic treatment on ERK activation in the amygdala after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine-treatment (10 mg/kg/day 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-

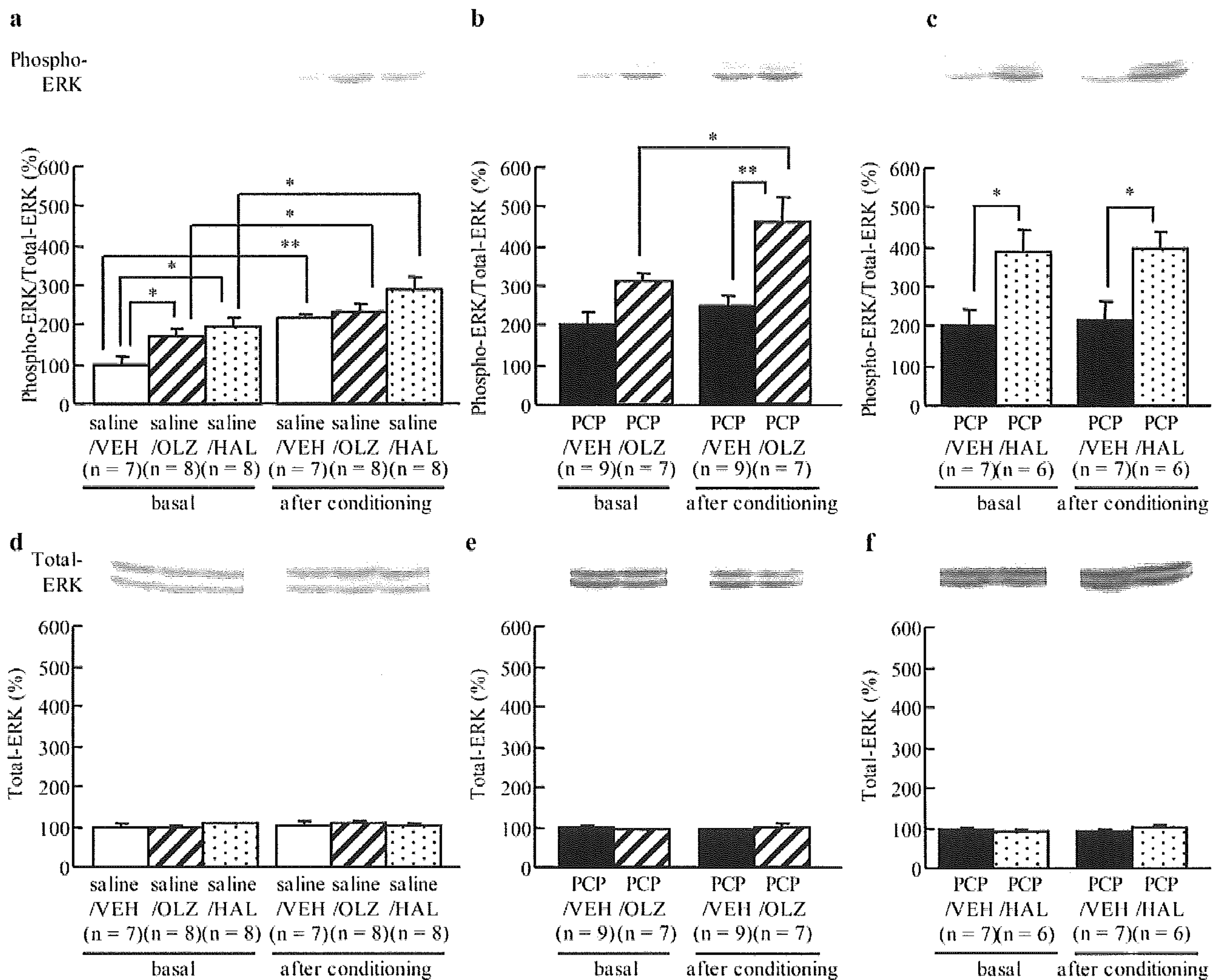

Fig. 7. Effects of repeated antipsychotic treatment on ERK activation in the hippocampus after fear conditioning. Mice were administered olanzapine (OLZ; 3 mg/kg/day p.o.), haloperidol (HAL; 1 mg/kg/day p.o.), or vehicle (VEH) for 7 days from 1 day after the cessation of phencyclidine treatment (10 mg/kg/day 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.

Acknowledgments

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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

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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.
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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

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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

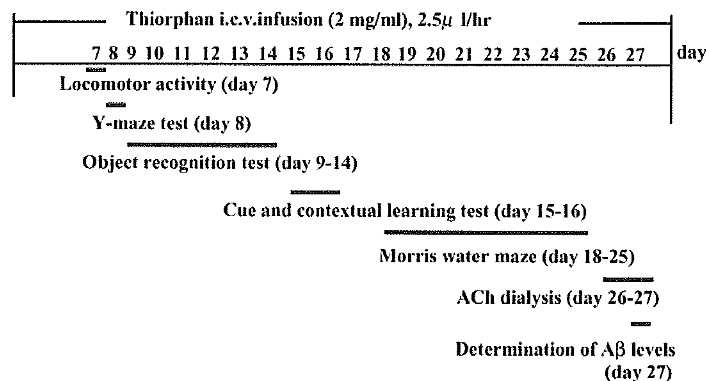

Fig. 1. Experimental schedule. i.c.v.: intracerebroventricular; Aβ: amyloid β peptide; ACh: acetylcholine.

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 Bonferroni 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.

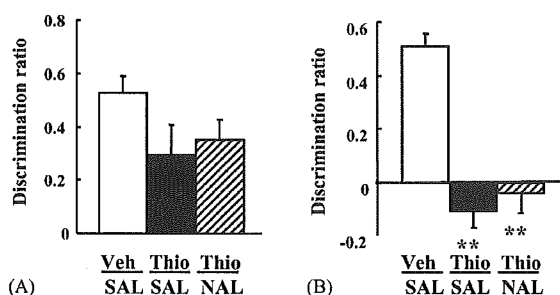

Fig. 2. Effect of continuous infusion of thiorphan into the cerebral ventricle on the performance of rats in a spontaneous object recognition test of: standard (A) and configural (B) conditions. The experiments were carried out on the days 9–14 after the start of thiorphan infusion. Values indicate the 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

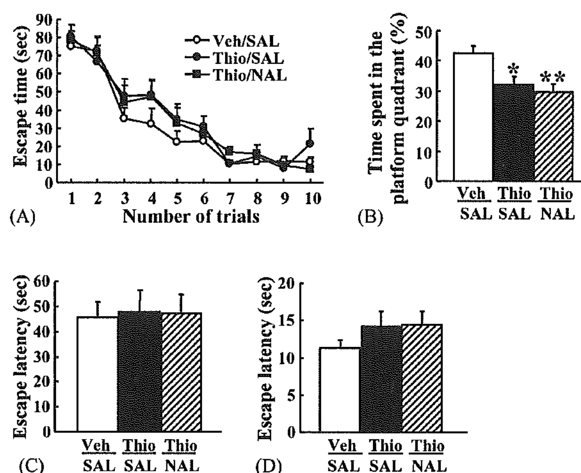

Fig. 3. Effect of continuous infusion of thiorphan into the cerebral ventricle on the performance of rats in the training (A) and probe (B) trials of reference memory, and the sample (C) and test (D) trials of working memory in the water maze task. The experiments were carried out on the days 18–25 after 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.

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%).

The escape latencies in the first trials (sample trial) and in the second to fifth trials (test trials) of the working memory test are shown in Fig. 3C and D, respectively. There was no significant difference in the sample trials among the three groups (Fig. 3C). In the test trial, the Thio/SAL- and Thio/NAL-treated rats exhibited a prolonged escape latency, compared to Veh/SAL-treated rats, but there was no significant difference among the three groups (Fig. 3D). In the reference and working memory test of water maze task, there was no significant difference in the swimming speed among the three groups (data not shown).

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

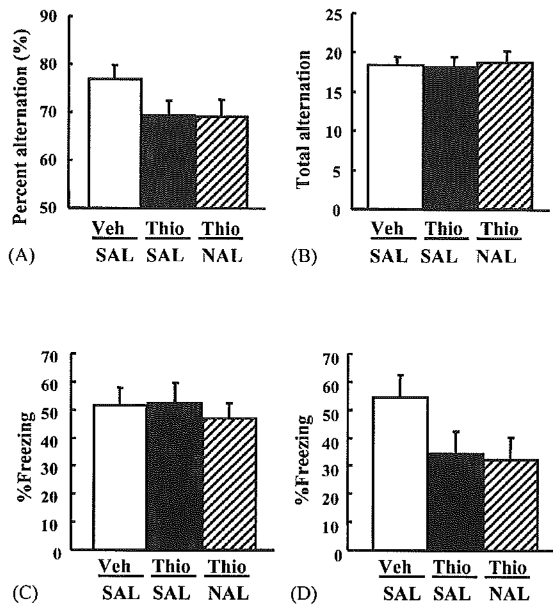

Fig. 4. Effect of continuous infusion of thiorphan into the cerebral ventricle on the spontaneous alternation behavior (A) and the number of arm entries (B) during an 8-min session in the Y-maze task, and on performance in tone cue (C) and context (D) fear-conditioned-learning tests. The experiments 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).

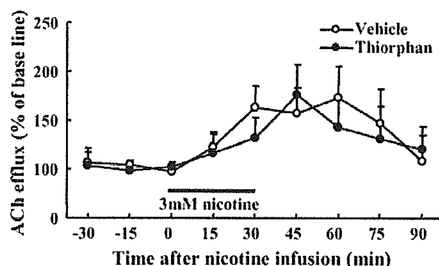

Fig. 5. Hippocampal extracellular ACh release stimulated by nicotine in vehicle- and thiorphan-treated rats on the days 26–28. Nicotine–Ringer was applied for 30 min, after which normal Ringer's solution was used. Values indicate the mean ± S.E.M. for seven to eight animals. Results with the two-way 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,

e.g. [Leu] enkephalin enhances and impairs the acquisition of a one-way step-through active avoidance response in a dose-dependent manner [24,25]. To exclude a possible involvement of enkephalins, of which metabolism may be affected by the inhibition of neprilysin, in memory learning tasks in the rat, we administered naloxone (1 mg/kg, i.p.) to thiorphan-treated rats before each experiment. However, naloxone treatment had no effect on cognitive performance in the thiorphan-infused rats. The genetic approach supports our finding, because neprilysin deficiency does not significantly elevate enkephalin levels in the brain [47]. Thus, the cognitive impairment in the thiorphan-infused rats is not due to the presence of redundant enkephalin.

Previous reports have shown that continuous infusion of Aβ40 into the rat cerebral ventricle impairs several learning and memory tasks [33,34,49,52,53] and nicotine-stimulated extracellular ACh release [17,50]. In the present study, however, the rats continuously infused with thiorphan showed only an impairment of novelty discrimination in a configural version of object recognition behavior, and spatial memory in a probe trial of the water maze task, but no difference in the nicotine-stimulated ACh release, compared to the vehicle-treated rats. Such an inconsistency in the results obtained from between Aβ40- and thiorphan infusion model may be due to differences in the regions, where Aβ40 is accumulated and involved in cognitive functions; a significant accumulation of Aβ is observed in the cerebral cortex and hippocampus of Aβ40-infused rats, whereas only in the cerebral cortex of the thiorphan-infused rats. Regarding a region-specific accumulation of Aβ, Newell et al. [32] also reported that the intracerebroventricular infusion of thiorphan elevates only cerebral Aβ40 level in the rabbits, consistent with our observation. Therefore, this inconsistency may be explained by a region-specific Aβ40 accumulation derived from the distribution of thiorphan infused into the cerebral ventricle and different neuronal sensibility for the accumulation between the cerebral cortex and hippocampus. Furthermore, for Aβ40 infusion experiments human-type Aβ is generally used, whereas in the thiorphan infusion experiments accumulation of endogenous rodent-type Aβ is expected. As another possibility, it may be pointed out a difference of Aβ sequence between human and rodents. Because human-type Aβ shows higher self-assembly than rodent-type Aβ [4,38], Aβ accumulated by the Aβ40 infusion and thiorphan infusion may present different status for oligomerization and fibrilization each other.

Some cortex regions are necessary for the acquisition and retention of hippocampus-dependent memory such as the performance of object recognition task and Morris water maze task, and such cognition is disrupted by cortical lesions [3,12,57]. Our results suggest that the cortex played a critical role in novelty discrimination in configural version of object recognition behavior and spatial memory in a probe trial of water maze tasks. However, we need to further investigate neurochemically and neuropharmacologically whether the cerebral accumulation of Aβ induces dysfunctions of the cortex in the thiorphan-infused rats. In addition, Iwata et al. [21] have reported that direct infusion of thiorphan into the hippocampus of rats elevates the hippocampal Aβ levels. To investigate a role of neprilysin in the metabolism of Aβ and cognitive function in the hippocampus, we need to employ this model.

In conclusion, we demonstrated that the inhibition of endogenous neprilysin activity in vivo by intracerebroventricular infusion of thiorphan increases concentration of Aβ in the cortex and impairs some cognitive functions. Recently, some gene analyses have disclosed that single nucleotide or dinucleotide-repeated polymorphisms on the neprilysin gene increase susceptibility to AD [2,16,46]. These studies suggest not only that the polymorphisms of neprilysin gene could be a risk factor for the development of AD, and but also that the loss of brain neprilysin activity could be a pathogenic mechanism leading to the age-related deposition of Aβ and development of AD.

Acknowledgements

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Research report

Effects of memantine and donepezil on amyloid β-induced memory impairment in a delayed-matching to position task in rats

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Abstract

We investigated the effects of memantine and donepezil on amyloid β (Aβ)-induced memory impairment in rats, which was assessed by a delayed-matching to position (DMPT) paradigm in three-lever operant chambers. Aggregated Aβ1-40 was microinjected bilaterally (1 nmol/side) into both CA1 and CA3 subfields of the hippocampus in rats that had previously performed the DMTP task. Memantine (20 mg/(kg day), s.c.) was continuously infused by an osmotic minipump for 4 weeks from 3 days before the microinjection of Aβ. Donepezil (2.5 mg/kg, p.o.) was administered 60 min before the DMTP test session. Bilateral microinjections of Aβ1-40 into the hippocampus resulted in a delayed, but persistent impairment of DMTP performance, which appeared more than 50 days after the injection. Memantine prevented the development of Aβ-induced memory impairment, while donepezil symptomatically alleviated the deficits. Because of a ceiling effect, the combination of donepezil with memantine failed to produce any additive or synergic effects. These results support the clinical data showing that memantine and donepezil are effective for the treatment of Alzheimer's disease. Moreover, it is suggested that memantine is effective for preventing Aβ-induced short-term memory impairment.
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Keywords: Memantine; Donepezil; Alzheimer's disease; Amyloid β; Working memory; Hippocampus; Cholinesterase inhibitor; NMDA receptor

1. Introduction

Alzheimer's disease (AD) is the most common cause of the progressive decline of cognitive function in aged humans, and is characterized by the presence of numerous senile plaques and neurofibrillary tangles accompanied by neuronal loss. Although the exact pathogenesis of neuronal degeneration and cognitive impairment in AD remains to be fully defined, several pharmacological strategies have been proposed for the treatment of the disease [39].

Because a remarkable dysfunction of the cholinergic system is present in the brains of AD patients, and has been shown to be correlated with the severity of the cognitive impairment [28], it has been proposed that enhancement of cholinergic neurotransmission may ameliorate cognitive impairment in AD [3]. In fact, cholinesterase inhibitors, including tacrine, donepezil, rivastigmine and galantamine, have been successfully developed and approved for the treatment of moderate to severe AD [16]. Donepezil, a potent and selective inhibitor of brain cholinesterase [15], showed encouraging results in palliative therapy for AD [32].

The other hypothesis regarding the mechanism of neurodegeneration in AD is that excessive activation of glutamate receptors might be responsible for part of the

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neuronal damage observed in AD [9,27,38]. Although it is unlikely that glutamate-mediated exicitotoxicity is the primary etiopathological factor in AD, it may significantly contribute to the development of neurodegeneration in AD and it also has been suggested that impaired glutamatergic neurotransmission (overactivation of *N*-methyl-D-aspartate (NMDA) receptors) plays a role in the cognitive deficit [4,25]. In fact, recent clinical studies have demonstrated that memantine, a moderate affinity and uncompetitive NMDA receptor antagonist [25], is effective for the treatment of AD [30,35]. In preclinical studies, apart from experiments showing improvement of learning, it has been demonstrated that memantine protects against neuronal and behavioral deficits in rats treated with quinolinic acid [21,44] or ibotenic acid [1]. At present, memantine has been approved in Europe and USA for the treatment of moderate to severe AD. According to the excitotoxic hypothesis in chronic neurodegenerative diseases including AD, it should be emphasized that memantine as a preventive therapy may be more effective in early stage AD. Therefore, the neuroprotective effects of memantine should be investigated in an animal model of early stage AD.

The senile plaques are composed of amyloid β (Aβ), a 39–43 amino acid peptide fragment of the amyloid β precursor protein [33]. Aβ is cytotoxic to neurons [43] and renders neurons vulnerable to various insults including excitotoxicity [18,39]. The amyloid cascade hypothesis has been proposed in the etiopathology of AD [10], and accumulating evidence supports the hypothesis [33,39]. We have previously demonstrated that a continuous intracerebroventricular infusion of Aβ1-40 or Aβ1-42, but not Aβ40-1, causes learning and memory impairment, which was accompanied by cholinergic dysfunction [12,23,40], overproduction of nitric oxide [36,37] and oxidative stress [14,41]. The Aβ-induced memory impairment was exaggerated by ovariectomy [42], but ameliorated by antioxidants such as α-tocopherol [41] and inducible NO synthase inhibitors [36,37]. Acute injections of Aβ into the cortex or hippocampus also produce neurodegeneration and memory impairment in rodents [2,8,20,24,31], although the results are somewhat controversial. The Aβ-induced learning and memory impairment is a valuable model to assess the effects of novel antidementia drugs [39].

In the present study, we examined the effects of memantine on Aβ-induced memory impairment in rats and compared them with those of donepezil. We used a delayed-matching to position (DMPT) paradigm in three-lever operant chambers to investigate short-term memory [5,6,22], the cognitive domain being impaired in early stage AD [11,17].

2. Materials and methods

2.1. Animals

Male Fischer344 rats, 11–12 weeks old and weighing 230–260 g at the start of experiment, were obtained from Charles River Japan (Yokohama, Japan). The animals were housed in plastic cages and kept in a regulated environment (23 ± 1 °C, 50 ± 5% humidity), with a 12-h light:12-h dark cycle (lights on at 9:00 a.m.). All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Kanazawa University.

2.2. DMTP task

The animals were subjected to a food deprivation regime that reduces body weight to 75–80% of the initial weight for 7 days before the start of training. The operant chambers used and the training and test procedures for the DMTP task in the present study were the same as previously described by Miyamoto et al. [20]. Two operant chambers (San Diego Instruments, San Diego, CA, USA) were used, and each chamber had three retractable levers and three panel lights (4 cm × 4 cm) for sample stimulation above the retractable levers. The feeder connected to the food dispenser was located below the center lever, and a house light was located above the center panel light.

In the DMTP task, one of the three panel lights was illuminated for 5 s, and then the three levers were presented into the box, one of four delays (0, 8, 16, 32 or 64 s) being randomly enforced between the time the panel light was turned off and the presentation of the three levels. The sequence of the position of the panel light illuminated was randomly selected. If the rat pressed the correct lever, which was located just below the lit panel light, the rat was rewarded with a food pellet (45 mg) and all the levers were retracted. The next trial started 30 s after food reinforcement. If the rat failed to press the correct lever within 10 s or pressed one of the wrong levers, the levers were withdrawn with no reinforcement, and there was an intertrial interval of 30 s before the onset of the next trial. Each daily session consisted of 54 trials with four delays: 18 trials for 0-s delay, 12 trials for 8-s delay, 9 trials for 16-s delay, 9 trials for 32-s delay and 6 trials for 64-s delay presented randomly. The position of the correct lever for each delay was balanced within each session and presented in a different random order each day. The correct response percentage [(correct responses/total responses) × 100] on each delay was calculated. The number of trials completed (lever pressing within 10 s of the presentation of the three levers) was also recorded, and the percentage of trials completed [(trials completed/total trials) × 100] was calculated. Eleven rats whose correct response rate was more than 80% at the 0-s delay for 2 consecutive days were used for the microinjection of Aβ1-40.

2.3. Microinjection of Aβ1-40 into the hippocampus

Aβ1-40 (Bachem, Feinchemikalien AG, Switzerland) was dissolved in distilled water at a concentration of 1 nmol/μl, and incubated at 37 °C for 7 days to promote the aggregation [29]. The rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and restrained in a stereotaxis apparatus. Eight rats received bilateral microinjections of aggregated Aβ1-40 (injection volume: 1 μl/site) into both CA1 (A: −4.3, L: ±2.0, V: 2.6) and CA3 (A: −3.3, L: ±2.6, V: 3.5) subfields of the hippocampus, according to the atlas of Paxinos and Watson [26]. The vehicle (distilled water) was microinjected bilaterally into the hippocampal CA1 and CA3 in control rats ($n = 3$).

2.4. Measurement of locomotor activity

Each rat was placed in a standard transparent rectangular cage (50 cm × 50 cm × 50 cm high), and the locomotor activity was measured for a period of 30 or 60 min using an infrared detector (Neuroscience, Tokyo, Japan) placed over the cage.

2.5. Drug treatment and experimental design

Memantine HCL (Batch: R8825) and donepezil (Lot: 13031705) were kindly donated by Merz Pharmaceuticals (Frankfurt, Germany) and Eisai Co. Ltd. (Tsukuba, Japan), respectively. Memantine was dissolved in saline, and was continuously infused s.c. at a dose of 20 mg/(kg day) with an Alzet osmotic pump (model 2ML2, Alza, Palo Alto, CA) for 4 weeks from 3 days before to 25 days after the intrahippocampal injections of Aβ1-40 ($n = 4$). The dose of memantine (20 mg/(kg day)) used in the present study has been reported to yield pseudo steady-state serum levels close to the therapeutic range (1.2 μM) [21]. Some Aβ-treated rats ($n = 4$) and control rats ($n = 3$) received implantations of water-filled osmotic pumps. The osmotic pumps were renewed on day 11 to maintain the infusion of memantine or the vehicle until day 25. Accordingly, three groups with different treatments were prepared: control ($n = 3$), Aβ-vehicle ($n = 4$) and Aβ-memantine groups ($n = 4$).

Donepezil was dissolved in distilled water and administered p.o. at a dose of 2.5 mg/kg. The dose of donepezil in the present study was selected based on our preliminary studies: donepezil at 2.5 mg/kg significantly ameliorated scopolamine (0.1 mg/kg)-induced impairment of DMTP performance, but had no effect on the performance in control animals (unpublished observations).

The experimental schedule is shown in Fig. 1. On days −1, 7, 9, 16, 23, 30, 37, 44, 51, 58, 65 and 72 after Aβ infusion, all animals in three groups were administered distilled water 1 h before the test session of the DMTP task. On the next day (days 10, 17, 24, 31, 38, 45, 52, 59, 66 and 73), they were administered donepezil 1 h before the test session. Locomotor activity of animals was measured on day 79 or 80 after Aβ infusion.

2.6. Histology

On day 82, the rats were anesthetized with pentobarbital and transcardially perfused with 250 ml of heparinized (0.1%, v/v) saline followed by 250 ml of phosphate-buffered saline (pH 7.4) containing 4% paraformaldehyde. Brains were removed and post-fixed in the same fixative overnight. After being cryoprotected with 30% sucrose, brains were cut into 40-μm-thick coronal sections. The sections throughout the hippocampus were mounted on gelatin-coated slides and stained with cresyl violet.

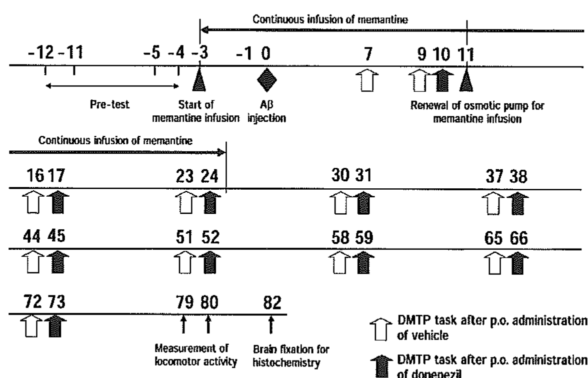

Fig. 1. Experimental schedule.

2.7. Statistical analysis

All data were expressed as the mean ± S.E. Statistical significance was determined with a one-way or two-way analysis of variance (ANOVA, repetitive measures), followed by the Student–Newman–Keuls test for multi-group comparisons when F-ratios were significant ($P < 0.05$).

3. Results

Bilateral injections of Aβ1-40 into both CA1 and CA3 subfields of the hippocampus in rats that had previously performed the DMTP task had little acute effect on DMTP performance. Moreover, continuous infusion of memantine had no effect on DMTP performance in Aβ-treated rats. Fig. 2 illustrates the changes in DMTP performance at the 0-s (A and B) and 8-s delay (C and D) in Aβ-injected rats with or without memantine treatment. Aβ1-40 and memantine had little effect on either the percentage of correct responses (Fig. 2A for 0-s delay: $F(2,8) = 0.187$, $P > 0.05$; Fig. 2C for 8-s delay: $F(2,8) = 0.424$, $P > 0.05$) or response time (Fig. 2B for 0-s delay: $F(2,8) = 0.486$, $P > 0.05$; Fig. 2D for 8-s delay: $F(2,8) = 0.646$, $P > 0.05$) by day 44 after Aβ1-40 injections. As shown in Fig. 3, Aβ1-40 had no effects on average DMTP performance from days 7 to 44. Moreover, continuous infusion of memantine for 4 weeks (from 3 days before to 25 days after the Aβ1-40 injections) did not affect the DMTP performance of Aβ1-40-treated rats. A two-way ANOVA revealed a significant effect of delay (Fig. 3A for choice accuracy: $F(4,296) = 97.667$, $P < 0.0001$; Fig. 3B for response time: $F(4,296) = 5.348$, $P < 0.001$), but not group (Fig. 3A for choice accuracy: $F(2,74) = 1.854$, $P > 0.05$; Fig. 3B for response time: $F(2,74) = 2.878$, $P > 0.05$).

On day 51 and thereafter, significant changes in DMTP performance were observed. A one-way ANOVA with repeated measures revealed a significant effect of group at the 0-s (Fig. 2A for choice accuracy: $F(2,8) = 0.082$, $P > 0.05$; Fig. 2B for response time: $F(2,8) = 6.817$, $P < 0.05$) and 8-s delay (Fig. 2C for choice accuracy: $F(2,8) = 14.180$, $P < 0.01$; Fig. 2D for response time: $F(2,8) = 10.345$, $P < 0.01$). Fig. 4 shows the average DMTP performance from days 51 to 72 following Aβ1-40 injections into the hippocampus. A two-way ANOVA revealed significant effects of delay (Fig. 4A for choice accuracy: $F(4,164) = 36.750$, $P < 0.0001$; Fig. 4B for response time: $F(4,164) = 4.737$, $P < 0.01$) and group (Fig. 4A for choice accuracy: $F(2,41) = 9.448$, $P < 0.001$; Fig. 4B for response time: $F(4,41) = 9.012$, $P < 0.001$), but not delay × group interaction (Fig. 4A for choice accuracy: $F(8,164) = 1.225$, $P > 0.05$; Fig. 4B for response time:

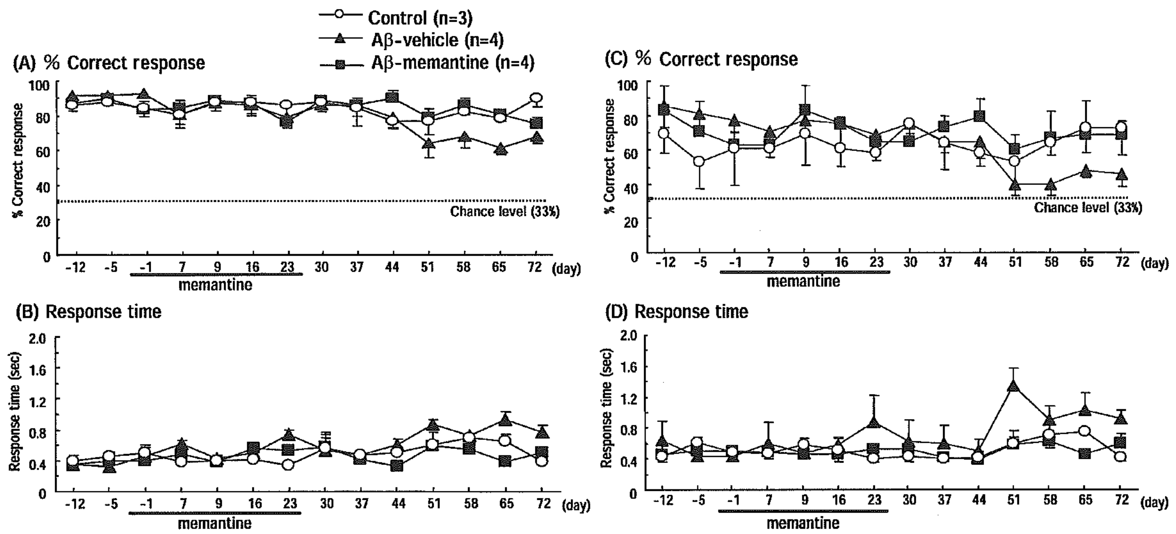

Fig. 2. Changes in DMTP performance at the 0-s (A and B) and 8-s delay (C and D) following bilateral microinjections of Aβ1-40 into the hippocampus in control, Aβ-vehicle and Aβ-memantine groups. Memantine (20 mg/(kg day)) was continuously infused with the Alzet osmotic pump for 4 weeks from 3 days before to 25 days after Aβ1-40 infusion. All animals were administered distilled water 1 h before the test sessions. (A and C) Percent correct response and (B and D) response time. Values indicate the mean ± S.E. ($n = 3$ for control group, $n = 4$ for Aβ-vehicle and Aβ-memantine groups).

$F(8,164) = 1.443$, $P > 0.05$). A post hoc analysis with the Student–Newman–Keuls test revealed that Aβ-vehicle group showed a significant decrease in choice accuracy ($P < 0.05$) and an increase in response time ($P < 0.05$) compared with the control group. Memantine significantly prevented the Aβ-induced decrease in choice accuracy ($P < 0.05$) and increase in response time ($P < 0.05$).

Fig. 5 shows the DMTP performance when the animals in the control, Aβ-vehicle and Aβ-memantine groups were administered donepezil (2.5 mg/kg, p.o.) 1 h before

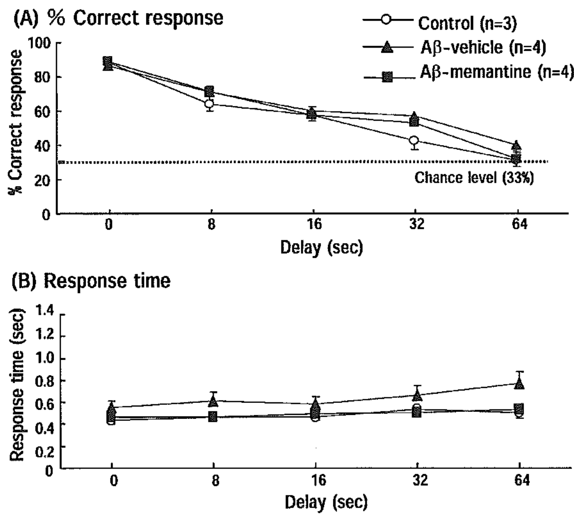

Fig. 3. Average DMTP performance from days 7 to 44 after bilateral microinjections of Aβ1-40 into the hippocampus in control, Aβ-vehicle and Aβ-memantine groups. (A) Percent correct response and (B) response time. Memantine (20 mg/(kg day)) was continuously infused with the Alzet osmotic pump for 4 weeks from 3 days before to 25 days after Aβ1-40 infusion. Values indicate the mean ± S.E. ($n = 3$ for control group, $n = 4$ for Aβ-vehicle and Aβ-memantine groups). A two-way ANOVA revealed no significant effect of group on the percent correct response [$F(2,74) = 1.854$, $P > 0.05$] or response time [$F(2,74) = 2.878$, $P > 0.05$].

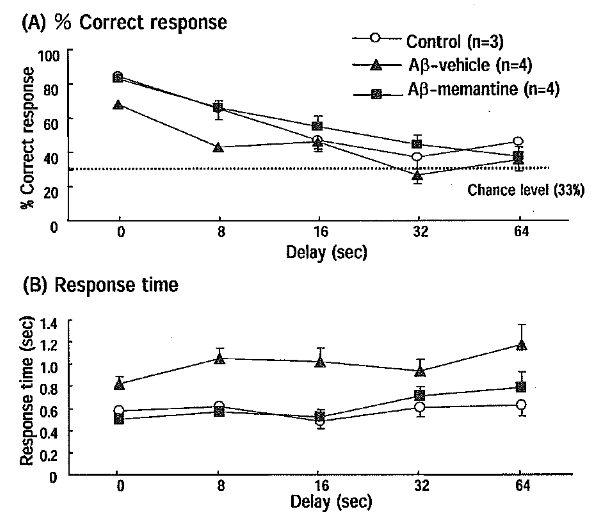

Fig. 4. Effect of memantine on Aβ-induced impairment of DMTP performance from days 51 to 72 following bilateral microinjections of Aβ1-40 into the hippocampus. (A) Percent correct response and (B) response time. Memantine was continuously infused at a dose of 20 mg/(kg day) with the Alzet osmotic pump for 4 weeks from 3 days before to 25 days after Aβ1-40 infusion. Values indicate the mean ± S.E. ($n = 3$ for control group, $n = 4$ for Aβ-vehicle and Aβ-memantine groups). A two-way ANOVA revealed a significant effect of group on the percent correct response [$F(2,41) = 9.448$, $P < 0.001$] and response time [$F(4,41) = 9.012$, $P < 0.001$].