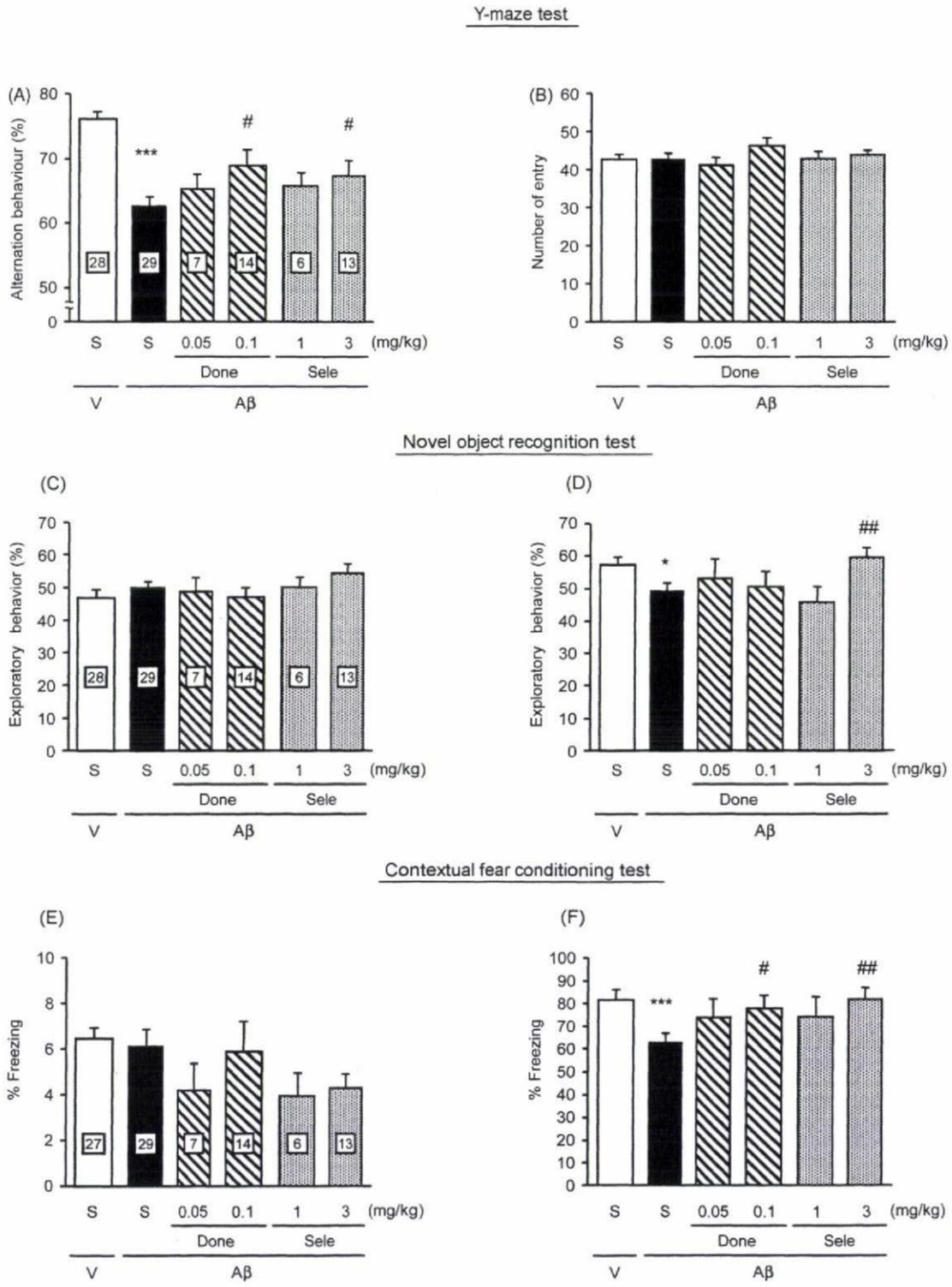


**Fig. 2.** Effects of administration of selegiline or donepezil alone on $A\beta_{(25-35)}$-induced memory impairment. Six, seven and nine days after i.c.v. injection of $A\beta_{(25-35)}$, the mice were subcutaneously administered donepezil (0.05 and 0.1 mg/kg), selegiline (1 and 3 mg/kg) or saline 30 min before each behavioral test. Panels A and B show the result of alternation behavior (A) and number of entries (B) in the Y-maze test. Panels C and D show the results of the training trial (C) and retention trial (D) in the novel objective test. Panels E and F show the results of the pre-conditioning phase (E) and retention session (F) in the contextual fear conditioning test. S, saline; V, vehicle (distilled water); Aβ, $A\beta_{(25-35)}$; Done, donepezil; Sele, selegiline. Values represent means ± S.E.M. The number of mice used in each group is shown in the column. $^{*}P<0.05$, $^{***}P<0.001$ vs. saline-treated, vehicle-injected mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. saline-treated, $A\beta_{(25-35)}$-injected mice.

of mice had the same levels of motivation, curiosity and motor function.

### 3.1.2. *Novel object recognition test*

During the training session, there were no significant differences in exploratory preference for two objects (Fig. 2C), and thus there was no biased exploratory preference in six groups without affecting total spent time in the exploration of objects. In the retention session, there were no differences in the total exploratory time among all the groups (data not shown). Exploratory preference for the novel object of vehicle-injected mice was significantly increased in the retention session compared to that in the training

session (one sample $t$-test, $P=0.0105$) or above chance set at 50% (one sample $t$-test, $P=0.0257$). However, a significant difference between vehicle- and $A\beta_{(25-35)}$-injected mice in the exploratory preference for the novel object was observed during the retention session [Kruskal–Wallis, $H=11.78$, d.f. = 5, $P=0.0379$; Dunnett, $P=0.0251$], indicating impairment of visual recognition memory. The administration of selegiline (3 mg/kg) alone significantly ameliorated $A\beta_{(25-35)}$-induced impairment of the exploratory behavior in the retention session, but this effect was not seen with donepezil [Kruskal–Wallis, $H=11.78$, d.f. = 5, $P=0.0379$; Dunnett, $P=0.0069$] (Fig. 2D).

#### 3.1.3. Contextual fear conditioning task

In the preconditioning phase, mice hardly showed a freezing response. There were no differences in basal levels of freezing response among all the groups (Fig. 2E). In the retention test, the vehicle-injected mice showed a marked contextual freezing response 24 h after fear conditioning (Fig. 2F), whereas the $A\beta_{(25-35)}$-injected mice presented less freezing responses in the contextual tests [Kruskal–Wallis, $H=17.43$, d.f. = 5, $P=0.0037$; Dunnett, $P=0.0005$]. The performance of $A\beta_{(25-35)}$-injected mice was completely restored by treatment with donepezil (0.1 mg/kg) or selegiline (3 mg/kg) [Kruskal–Wallis, $H=17.43$, d.f. = 5, $P=0.0037$; Dunnett, $P=0.0159$ (donepezil), $P=0.0053$ (selegiline)] (Fig. 2F). Since the low doses of donepezil (0.05 mg/kg) and selegiline (1 mg/kg) failed to improve $A\beta_{(25-35)}$-induced cognitive impairment, their conditions were used in all subsequent experiments. In the conditioning phase, there was no difference in the levels of flinching, running and jumping responses or vocalization by a foot shock among all the groups (data not shown), indicating no changes in nociceptive response, because we excluded the animals that did not represent normal nociceptive response in the conditioning phase from the contextual fear conditioning test.

### 3.2. Effects of co-administration of selegiline and donepezil on $A\beta_{(25-35)}$-induced memory impairment

We investigated whether co-administration of low-dose selegiline and donepezil attenuated $A\beta_{(25-35)}$-induced cognitive impairment.

In the Y-maze test, $A\beta_{(25-35)}$-induced impairment of alternation behavior was significantly improved by combined administration of donepezil (0.05 mg/kg) and selegiline (1 mg/kg) [Kruskal–Wallis, $H=47.36$, d.f. = 4, $P<0.0001$; Dunnett, $P<0.0001$], at doses that were not effective individually (Fig. 3A). The number of arm entries was not changed by any treatments (data not shown).

In the novel object recognition test, there were no significant differences in exploratory preference for two objects (the training session), or total exploratory time (the training and retention sessions), among all the groups (data not shown). The combined administration of donepezil (0.05 mg/kg) and selegiline (1 mg/kg) significantly improved $A\beta_{(25-35)}$-induced impairment of visual recognition memory [Kruskal–Wallis, $H=12.25$, d.f. = 4, $P=0.0156$; Dunnett, $P=0.0209$] (Fig. 3B).

In the contextual fear conditioning test, there were no differences in basal levels of freezing response among all the groups (data not shown). The combined administration of donepezil (0.05 mg/kg) and selegiline (1 mg/kg) significantly improved $A\beta_{(25-35)}$-induced impairment of the contextual freezing response [Kruskal–Wallis, $H=17.08$, d.f. = 4, $P=0.0019$; Dunnett, $P=0.008$] (Fig. 3C). In the conditioning phase, there was no difference in the levels of flinching, running and jumping responses or vocalization by a foot shock among all the groups (data not shown), indicating no changes in nociceptive response, because we excluded the animals that did not represent normal nociceptive response in the conditioning phase from the contextual fear conditioning test.

### 3.3. Antagonistic effects of scopolamine and haloperidol against the synergistic effect of selegiline and donepezil on $A\beta_{(25-35)}$-induced cognitive impairment

To determine whether the improving effect of co-administration of selegiline and donepezil on $A\beta_{(25-35)}$-induced cognitive impairment is mediated via muscarinic and/or dopamine receptors, we examined its antagonism by a muscarinic receptor antagonist scopolamine and a dopamine receptor antagonist haloperidol. We preliminarily confirmed that administration of scopolamine (0.1 mg/kg) and haloperidol (0.1 mg/kg) alone had no effect on the cognitive impairment in $A\beta_{(25-35)}$-injected mice in all behavioral tests, while in the contextual fear conditioning test, haloperidol (0.1 mg/kg)-treated, $A\beta_{(25-35)}$-injected mice did not represent less freezing responses compared to vehicle-injected mice during the retention session (data not shown). Therefore, we evaluated antagonistic effect of haloperidol at the dose of 0.03 mg/kg that did not change freezing responses in $A\beta_{(25-35)}$-injected mice in the contextual fear conditioning test.

Pre-administration of scopolamine (0.1 mg/kg) or haloperidol (0.1 mg/kg) significantly antagonized the improving effect of co-administration of selegiline and donepezil on $A\beta_{(25-35)}$-induced impairment of spontaneous alternation in the Y-maze task [Kruskal–Wallis, $H=23.37$, d.f. = 4, $P<0.0001$; Dunnett, $P=0.0111$ (scopolamine), $P=0.0495$ (haloperidol)] and novel object recognition test [Kruskal–Wallis, $H=16.30$, d.f. = 4, $P=0.0026$; Dunnett, $P=0.0027$ (scopolamine), $P=0.0243$ (haloperidol)] (Fig. 4A and B). In the novel object recognition test, there were no significant differences in exploratory preference for two objects (the training session), or total exploratory time (the training and retention sessions), among all the groups (data not shown). These results indicate that all groups of mice have the same levels of motivation, curiosity and motor activity.

In the contextual fear conditioning tests, the improving effects of co-administration of selegiline and donepezil on $A\beta_{(25-35)}$-induced cognitive impairment were significantly antagonized by both scopolamine (0.1 mg/kg) and haloperidol (0.03 mg/kg) [Kruskal–Wallis, $H=18.93$, d.f. = 4, $P=0.0008$; Dunnett, $P=0.0138$ (scopolamine), $P=0.0069$ (haloperidol)] (Fig. 4C). There were no differences in basal levels of freezing response among all the groups (data not shown), indicating no changes in motor function. In the conditioning phase, there was no difference in the levels of flinching, running and jumping responses or vocalization by a foot shock among all the groups (data not shown), indicating no changes in nociceptive response, because we excluded the animals that did not represent normal nociceptive response in the conditioning phase from the contextual fear conditioning test.

## 4. Discussion

A number of studies have demonstrated that acute or continuous injections of Aβ into the brain cause neurodegeneration and impairment of learning and memory [20,40]. $A\beta_{(25-35)}$ containing the 11-amino acid sequence (25–35) of Aβ is neurotoxic *in vitro* [43] and *in vivo* [20], and its neurotoxicity may more likely mimic the oligometric Aβ which is believed to be a key factor influencing cognitive function in AD [22]. A single i.c.v. injection of $A\beta_{(25-35)}$ induces marked deficiencies in both short- and long-term memory in mice, and increases deposition and dissemination of Aβ in the cortex and hippocampus of mice, which is consistent with the clinicopathological picture of AD [20,23]. In the present study, we found that $A\beta_{(25-35)}$-injected mice showed impairments of spatial working memory in the Y-maze test, visual recognition memory in the novel object recognition test, and associative fear memory in the contextual fear

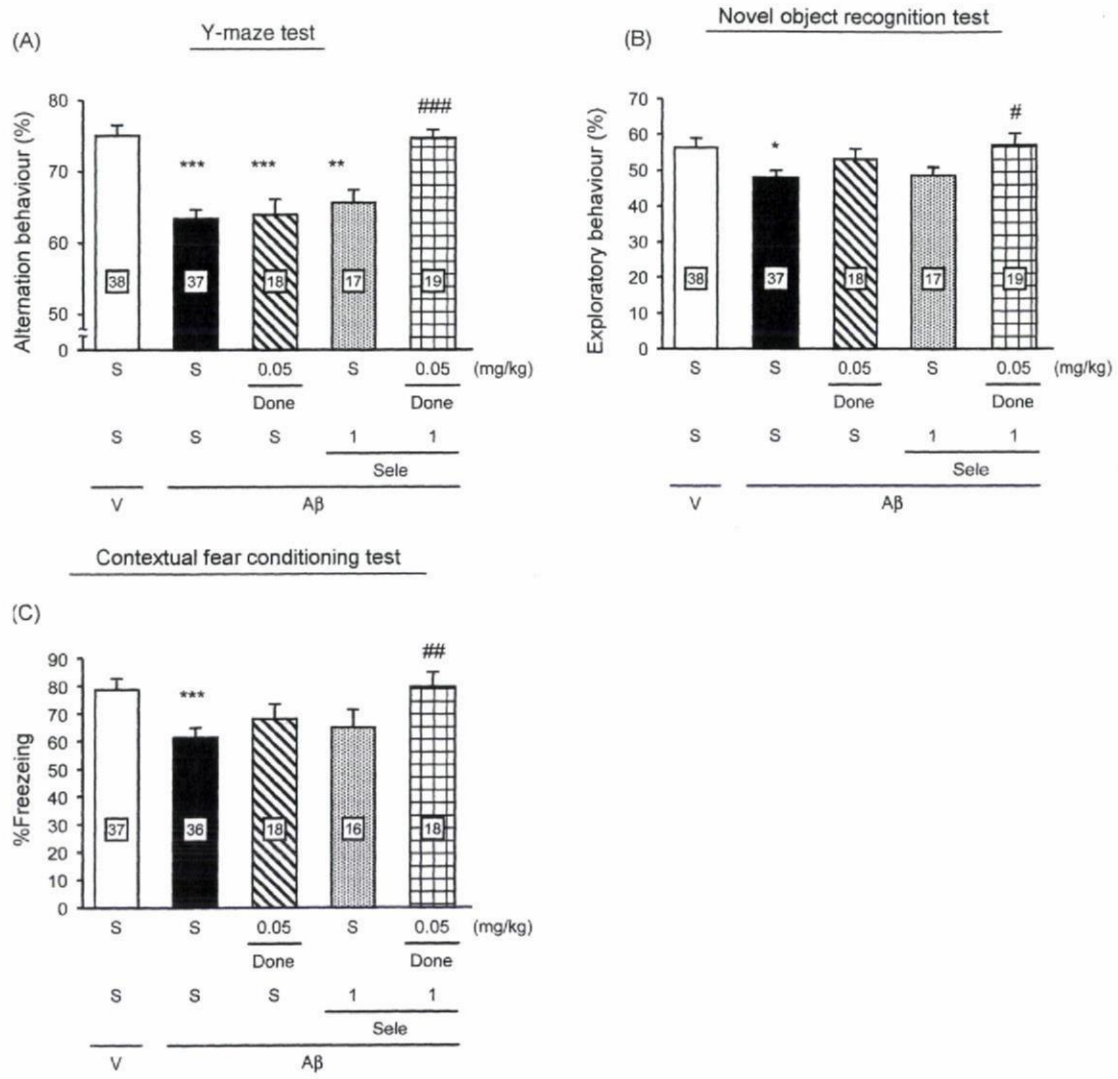


**Fig. 3.** Effects of co-administration of selegiline and donepezil on $A\beta_{(25-35)}$-induced memory impairment. Six, seven and nine days after i.c.v. injection of $A\beta_{(25-35)}$, the mice were subcutaneously administered donepezil (0.05 mg/kg), selegiline (1 mg/kg) or saline 30 min before each behavioral test. Panels A, B and C show the result of alternation behavior (A) in the Y-maze test, retention trial (B) in the novel objective test, and retention session (C) in the contextual fear conditioning test, respectively. S, saline; V, vehicle (distilled water); Aβ, $A\beta_{(25-35)}$; Done, donepezil; Sele, selegiline. Values represent means ± S.E.M. The number of mice used in each group is shown in the column. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. saline-treated, vehicle-injected mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. saline-treated, $A\beta_{(25-35)}$-injected mice.

conditioning test, which are known to require the control function of the hippocampus. $A\beta_{(25-35)}$-injected mice did not show any significant differences in motivation and movements, as evidenced by the number of arm entries in the Y-maze test, exploratory preference found during the training session, the total amount of time spent exploring two objects in the novel object recognition test, and the freezing time during the preconditioning phase in the contextual fear conditioning test. From these results, it is likely that impairment of performance in the $A\beta_{(25-35)}$-injected mice is due to learning and memory deficits associated with hippocampal functions.

The mechanism of memory impairment in the $A\beta_{(25-35)}$-infused mice is still unknown. However, previous reports [20,38] have demonstrated that histological examination of Cresyl violet-stained brain sections indicates a moderate but significant cell loss within the frontoparietal cortex and the hippocampal formation of mice treated with aged $A\beta_{(25-35)}$ (9 nmol) and that examination of Congo red-stained sections in the same animals exhibits the presence of numerous amyloid deposits throughout these brain areas. Although we did not perform histochemical experiments in the $A\beta_{(25-35)}$-injected mice in the present study, we consider the $A\beta_{(25-35)}$-injected mice as the animal model of AD in the incipient stage.

Single administration of donepezil at 3 mg/kg improved memory impairment induced by $A\beta_{(25-35)}$ in the Y-maze and contextual fear conditioning tests (Fig. 2). Our findings were consistent with previous reports that donepezil significantly improves alternation deficits in Y-maze and impairment of memory in step-through type passive avoidance tests in the $A\beta_{(25-35)}$-injected mice [21] and deficits of spatial learning in a water T-maze, and contextual and cued memory in fear conditioning tests in the Tg2576 transgenic mouse, which overexpresses human amyloid precursor protein linked to AD [5]. Another AchEI, tacrine, recovers memory impairment induced by i.c.v. injection of $A\beta_{(25-35)}$ [20]. Therefore, it is suggested that $A\beta_{(25-35)}$ induces hypofunction of the cholinergic system in the hippocampus.

The hippocampal formation plays a central role in learning and memory in the mammalian brain. The hippocampus also receives dopaminergic input, particularly from the ventral tegmental area [24]. A functional role of the hippocampal dopaminergic system has been indicated by behavioral studies

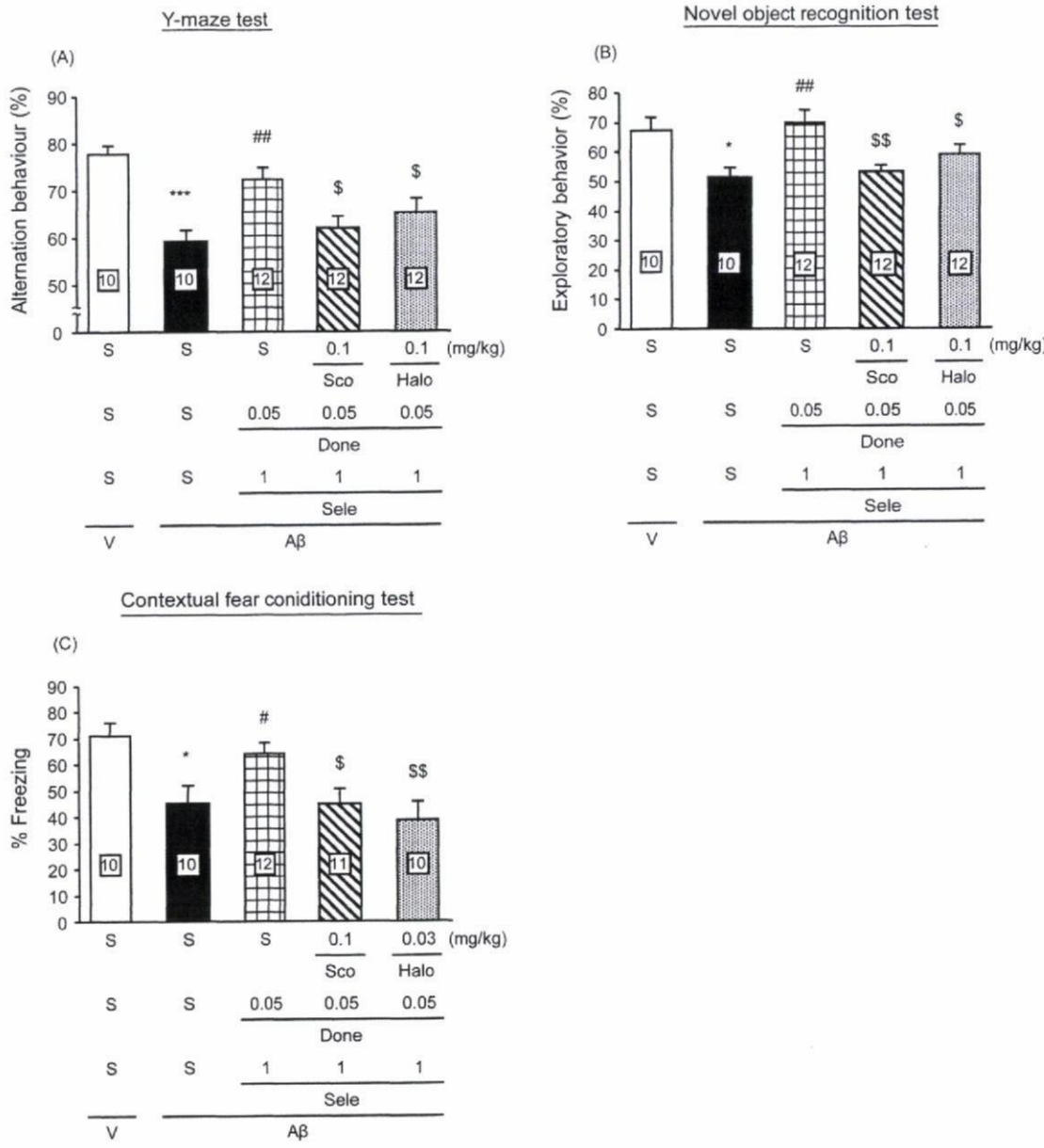


**Fig. 4.** Antagonistic effects of scopolamine and haloperidol against the synergistic cognition-improving effect of co-administration of selegiline and donepezil in $A\beta_{(25-35)}$-injected mice. Six, seven and nine days after i.c.v. injection of $A\beta_{(25-35)}$, the mice were subcutaneously administered donepezil, selegiline or saline 30 min before each behavioral test. The mice were subcutaneously administered scopolamine, haloperidol or saline 45 and 60 min before each behavioral test, respectively. Panels A, B and C show the result of alternation behavior (A) in the Y-maze test, retention trial (B) in the novel objective test, and retention session (C) in the contextual fear conditioning test, respectively. S, saline; V, vehicle (distilled water); Aβ, $A\beta_{(25-35)}$; Done, donepezil; Sele, selegiline; Sco, scopolamine; Halo, haloperidol. Values represent means ± S.E.M. The number of mice used in each group is shown in the column. $^{*}P<0.05$, $^{***}P<0.001$ vs. (saline + saline + saline)-treated, vehicle-injected mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. (saline + saline + saline)-treated, $A\beta_{(25-35)}$-injected mice. $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs. (Done + Sele + saline)-treated, $A\beta_{(25-35)}$-injected mice.

demonstrating enhancement of positive reinforcement learning, visual discrimination, and passive avoidance behavior after intrahippocampal injections of dopamine receptor agonists, as well as impairment of spatial navigation after depletion of hippocampal dopamine [19]. Thus, the dopaminergic system is implicated in cognitive processes in a variety of brain regions, including the hippocampus. Monoamine oxidase B is localized in various regions of the human brain including the hippocampus. In the present study, single administration of selegiline also improved memory impairment induced by $A\beta_{(25-35)}$ in the Y-maze, novel object recognition and contextual fear conditioning tests. These effects might be mediated by the increased level of dopamine in the hippocampus. In several clinical trials, selegiline improved episodic memory and learning in patients with AD [35].

Co-administration of selegiline and donepezil at subthreshold doses significantly ameliorated memory impairment in $A\beta_{(25-35)}$-injected mice in all of the behavioral tests, which was consistent with the finding that selegiline and tacrine improve performance in scopolamine + *p*-chlorophenylalanine-treated rats in a water maze task [6]. It is considered that the interaction of selegiline and donepezil is synergistic in nature, because the acting sites are different between both drugs.

In the present study, synergistic effects of co-administration of selegiline and donepezil on memory impairment induced by $A\beta_{(25-35)}$ were antagonized by pretreatment with dopamine receptor antagonist haloperidol, as well as muscarinic receptor antagonist scopolamine. These findings indicate that the dopaminergic–cholinergic interaction is partly involved in the synergistic effects of selegiline and donepezil. Pathological abnor-

malities in monoaminergic innervations in the forebrain of AD patients are known to exist in addition to abnormal cholinergic innervations. Previous studies have reported that: (1) the forebrain dopaminergic and cholinergic systems in humans are related to cognitive function [27]; (2) increases in hippocampal levels of dopamine and acetylcholine are associated with the learning process [41]; and (3) dopamine modulates acetylcholine release at cholinergic [11] and glutamatergic [42] synapses in the hippocampus. Selegiline can enhance dopaminergic neurotransmission due to its monoamine oxidase B inhibitory action. Shimazu et al. [30] have shown that selegiline increases acetylcholine release in the frontal cortex, and that such an effect is mimicked by dopamine D1 receptor agonists and blocked by dopamine D1 receptor antagonists. Thus, it is possible that selegiline enhances the level of dopamine in the hippocampus, followed by increasing the level of acetylcholine in the hippocampus, and remission of memory impairment. It is unlikely that the synergistic effects of co-administration of selegiline and donepezil or tacrine on memory impairment are due to pharmacokinetic mechanisms related to metabolism by cytochrome P450 (CYP), because donepezil, tacrine and selegiline are mainly metabolized through CYP2D6/3A4, CYP1A2 and CYP2B6, respectively [14,28].

It is reported that donepezil interacts with the sigma 1 receptor [16] and its anti-amnesic effects against $A\beta_{(25-35)}$-induced toxicity involve its sigma 1 agonistic property as well as cholinergic agonistic property [21]. Furthermore, haloperidol, used as dopamine receptor antagonist in this study, also has affinity for sigma 1 receptor. Therefore, it is possible that sigma 1 receptor is involved in the synergistic effects of co-administration of selegiline and donepezil in $A\beta_{(25-35)}$-injected mice and further investigation would be needed into this point.

Oxidative stress plays an important role in AD, and is induced by several processes related to Aβ, including toxic inflammatory responses [39]. One major index of oxidative stress is the level of glutathione (GSH). The GSH system is responsible for removing hydrogen peroxide from mitochondria and the cytosol, and therefore, constitutes an important protective mechanism for minimizing oxidative damage during energy metabolism. Reduction in GSH levels has been observed in specific regions of the central nervous system affected by AD [10]. Furthermore, $A\beta_{(25-35)}$ used in the present study are known to deplete endogenous GSH levels in neurons and astrocytes in a calcium-dependent manner [1]. In our preliminary experiment, we found that i.c.v. injection of $A\beta_{(25-35)}$ caused a reduction in GSH levels in the frontal cortex and hippocampus in mice, and co-administration of selegiline and donepezil tended to alleviate the $A\beta_{(25-35)}$-induced reduction in GSH level in the frontal cortex (data not shown). Selegiline has been reported to produce a significant increase in GSH levels and activities of superoxide dismutase (SOD) 1 and SOD2 in mesencephalic slice cultures [34]. Donepezil has been also reported to attenuate $A\beta_{(25-35)}$-induced toxicity in PC12 cells [31]. Therefore, neuroprotective action through antioxidant effects induced by co-administration of selegiline and donepezil may be involved in amelioration of cognitive deficits.

In conclusion, selegiline, as well as donepezil, improved memory impairment in $A\beta_{(25-35)}$-injected mice. Co-administration of selegiline and donepezil, at doses that do not exert efficacy individually, ameliorated memory impairment induced by $A\beta_{(25-35)}$ in a battery of learning and memory behavioral tests. These results suggest that selegiline can synergistically potentiate the improving effects of donepezil on the memory and cognitive deficits, and that the synergistic effects may be partly mediated through both the cholinergic and dopaminergic systems. Thus, selegiline may be a new drug for therapy of AD, in combination with AchEIs.

## Acknowledgements

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Research report

# The long-lasting effects of cross-fostering on the emotional behavior in ICR mice

Lingling Lu[a,b], Takayoshi Mamiya[a], Ping Lu[a,b], Minae Niwa[a,e], Akihiro Mouri[a,c], Li-Bo Zou[b], Taku Nagai[d], Masayuki Hiramatsu[a], Toshitaka Nabeshima[a,c,*]

[a] Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Japan
[b] Department of Pharmacology, School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, China
[c] Japanese Drug Organization of Appropriate Use and Research, Japan
[d] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Japan
[e] Japan Society for the Promotion of Science, Japan

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ABSTRACT

Early-life stress during the postnatal period could precipitate long-lasting alterations in the functional properties underlying emotional expression in humans, but how the psychological stress of cross-fostering affects emotional behavior during adulthood in mice remains primarily unknown. The purpose of the present study was to examine the long-term effects of cross-fostering on the emotional behavior and cognitive functions of ICR offspring in adulthood. Cross-fostering was performed from postnatal day 7 for 3 weeks. Mice were divided into three groups: (1) biological group: pups born from ICR dams fostered by their original mothers; (2) in-foster group: pups born from ICR dams but adopted by other ICR dams and (3) cross-foster group: ICR pups adopted by C57 dams. ICR mice were subjected to behavioral experiments at the age of 8 weeks. Emotional behaviors in the cross-fostered mice were significantly altered in the open-field, elevated plus maze and forced swimming tests, as well as social interaction tests. However, the cross-fostered mice showed normal memory function in the Y-maze and novel object recognition tests. The contents of serotonin metabolisms were decreased in the prefrontal cortex and hippocampus indicated the deficit of serotoninergic neuronal function by cross-fostering. These findings suggested that the early-life stress of cross-fostering induced long-lasting emotional abnormalities, which might be possibly related to alterations of serotonin metabolisms.



## 1. Introduction

Stressful events have been implicated in the onset or exacerbation of psychological disturbances in humans [2]. Exposure to adverse events early in life, such as childhood neglect and physical or sexual abuse is regarded as one of the most prominent environmental factors associated with the increased risk of emotional disorders [13]. Evidence is mounting to support the hypothesis that adverse early environments underlie vulnerability to a variety of psychological disorders, such as anxiety, depression and schizophrenia [6,7].

Early-life stress, during the prenatal or postnatal period, exerts lasting effects on neural development thus affecting behavior in rodents [1,33]. For instance, rats exposed to prenatal stress in utero exhibit increases in anxiety or depression-like behaviors [20,36] and impaired cognitive function with aging [33]. Prenatal stress also induces long-term changes in neurobiological systems, including hyperactivity of the hypothalamo-pituitary-adrenal (HPA) axis in response to later stress [20]. Changes of postnatal interactions with pups and dams could profoundly affect the emotionality as well as cognition of offspring in rats too [3]. It has been reported that maternal deprivation for long periods during the first 3 weeks of life impairs emotional behavior and affects pyramidal dendritic outgrowth in the prefrontal cortex [29]. In contrast, some postnatal manipulations have opposite effects on the development of offspring. Repeated maternal separation for a period of 15 min each day for 3 weeks, known as postnatal handling, has anxiolytic properties, which reduces anxiety-like behavior in adult rats [36], improves the performance of aged offspring in cognitive tasks [17], and attenuates stress-induced secretion of corticosterone.

Cross-fostering as a kind of postnatal psychological stress, could modify the mother–infants relationship early in life and mimic the psychology of childhood adoption which has frequently happened recently. Clinical researchers have reported that adopted children with a history of prenatal substance exposure [4,28] or placed relatively late in their adoptive home are at heightened

* Corresponding author at: Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan. Tel.: +81 52 839 2735; fax: +81 52 839 2738.
*E-mail address:* tnabeshi@ccmfs.meijo-u.ac.jp (T. Nabeshima).

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risk of social, intellectual, and emotional problems [32]. Animal studies have also shown different responses of pups to adoption caused by differences in maternal care: BALB/cByJ dams displayed less nursing and licking/grooming of pups and spent less time in the nest than C57BL/6ByJ dams [10,11,30]. Other researchers have examined the influence of the interaction between genetic susceptibility and environmental factors on emotional behavior and reported that cross-fostering affects the level of anxiety in rats [12,39]. These findings indicate that cross-fostering may affect the behavior of offspring. However, most researchers have focused on the differences in maternal care between several species. It remains to be determined whether cross-fostering has long-lasting effects on emotionality as well as cognition in offspring during adulthood in ICR mice.

In the present study, to systematically investigate long-term effects of cross-fostering on emotional and cognitive functions in offspring, we examined emotional behavior, response to stress, social interaction, and cognitive function in adult ICR mice which had experienced cross-fostering for 3 weeks.

## 2. Materials and methods

### 2.1. Animals

Pregnant ICR and C57BL/6JJms Slc dams (E12) obtained from SLC Japan (Shizuoka, Japan) were maintained on a 12/12-h light/dark cycle (lights on from 08:00 to 20:00) with free access to food (CE2; Clea Japan Inc., Tokyo, Japan) and water. Dams were housed individually till parturition. ICR pups were weaned at 28 days of age and housed by sex in each group. Male pups were used for behavioral analyses at the age of 8–9 weeks. All of the behavioral experiments were carried out in a sound-attenuated and air-conditioned experimental room (23 ± 1 °C, 50 ± 5% humidity). The mice were habituated for at least 30 min before the tests and all behavioral tests were recorded by DVD camera to reconsider these results. The experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (2007).

### 2.2. Cross-fostering procedure

Cross-fostering was performed at the postnatal day 7 (PD7) and completed within 5 min. Litters which consisted of 10 pups per dam with an equal number of males and females were used when possible. In this way, mice were divided into three different groups: (1) biological group: pups born from ICR dams and fostered by their original mothers; (2) in-foster group: pups born from ICR dams but adopted by other ICR dams and (3) cross-foster group: ICR pups adopted by C57 dams. All of the pups in each litter were taken out from their original cages and shortly separated from their original dams. Then, for the biological group, pups in the same litters were returned to their own dams one by one; for the in-foster group, the pups were put into their adopted dams within the same strain; while for the cross-fostered pups, they were put into other cages of C57 dams. All of the litters were composed of pups with same development history. Each group for each time contained more than three litters and were repeated more than 3 times. All of the pups were weighed once a week from birth to 8 weeks old. Male offspring were randomly used to check the behavioral changes and biochemical analyses.

### 2.3. Open-field test

As previously described [23], the apparatus for the open-field test consisted of a square area with black walls (*L* 40 cm × *W* 40 cm × *H* 40 cm) and was set in the experimental room. The floor of the field was divided into 64 identical squares (16 center squares, 16 corner squares and 32 other squares) so that the ambulation of mice could be measured. A light (100 W bulbs) was positioned 100 cm above the center. Each mouse was placed in the same corner square of the apparatus and allowed to explore freely for 5 min. During this period, the latency to cross the center squares, the time spent in the center and the corner squares, the numbers of ambulation, rearing and grooming events, and the frequency of defecation as well as urination were counted [30]. To count ambulation or rearing, entry into a square was defined as all four legs being inside the square. At the end of the test, the mouse was returned to its home cage and the apparatus was thoroughly cleaned with 70% ethanol.

### 2.4. Elevated plus maze test

The elevated plus maze was made of wood and consisted of two open arms (*L* 25 cm × *W* 8 cm) and two closed arms (*L* 25 cm × *W* 8 cm × *H* 20 cm) emanating from a common central platform (8 cm × 8 cm) to form a plus shape. The entire apparatus was elevated to a height of 50 cm above the floor. Testing commenced by placing a mouse on the central platform of the maze facing an open arm, and the standard 5-min test duration was employed. An entry was defined by all four legs entered into the arm. The open arm entries (%) and the time spent in open arms (%), and the total arm entries were calculated. After each test, the apparatus was thoroughly cleaned with 70% ethanol as previously described [40].

### 2.5. Forced swimming test

Mice were placed individually in a transparent polycarbonate cylinder (ϕ 8 cm × *H* 20 cm) containing water at 22 °C to a depth of 11.5 cm, and forced to swim for a 5-min period. The duration of immobility behavior was measured automatically by a SCANET MV-20 (Melquest Co. Ltd., Toyama, Japan), as described previously [27].

### 2.6. Social interaction test

The apparatus for the social interaction test was made of a gray polycarbonate (*L* 30 cm × *W* 25 cm × *H* 25 cm) [31]. Lighting in the experimental room consisted only of a dark light (25 W bulbs) and was diffused to minimize shadows in the arena. Before the test, each mouse was habituated alone in the apparatus for 10 min on two consecutive days. On the test day, the mice were randomly assigned according to gender to an unfamiliar partner in each group. The pairs of unfamiliar mice were placed in the apparatus for 10 min and the total amount of time spent in active social interaction, such as sniffing, grooming, following and mounting as well as crawling over or under the partner, was recorded. Passive contact (sitting or lying with bodies in contact) was not included in social interaction. At the end of the test, all the boluses were removed and the apparatus was cleaned with 70% ethanol.

### 2.7. Y-maze test

The Y-maze apparatus was made of wood and consisted of three arms (*L* 40 cm × *W* 12 cm × *H* 3 cm at bottom, *L* 40 cm × *W* 12 cm × *H* 10 cm at top) which converged at equal angles. Mice were placed at the center of the apparatus and allowed to move freely through the maze during the 8-min session. The series of arm entries was recorded visually. Alternation was defined as successive entry into the three arms on overlapping triplet sets. Alternative behavior (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two) multiplied by 100 [25].

### 2.8. Novel object recognition test

The novel object recognition test was performed following previous reports [21]. The test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box (*L* 30 cm × *W* 30 cm × *H* 30 cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed in the back corner of the box. A mouse was then placed midway at the front of the box and the total time spent exploring the two objects was recorded for 10 min. During the retention session, animals were placed back into the same box 24 h after the training session, in which one of the familiar objects used during training was replaced with a novel object. The animals were then allowed to explore freely for 5 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, the ratio of time spent exploring either of the two objects (training session) or the novel object (retention session) over the total amount of time spent exploring both the objects, was used to assess cognitive function.

### 2.9. Determination of monoamine and its metabolite levels in the brain

The prefrontal cortex and hippocampus were dissected out from the brains on an ice-cold plate immediately after the mice were decapitated. Each part of brain sample was quickly frozen and stored in a deep freezer at −80 °C until assayed. The contents of norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) were determined using high-performance liquid chromatography with electrochemical detection [41].

### 2.10. Statistical analysis

All data were expressed as the means ± S.E.M. The analysis of body weight during the period of cross-fostering was conducted with a two-way analysis of variance (ANOVA), followed by Bonferroni's test as a *post hoc* comparison. Other statistical differences were tested using a one-way ANOVA followed by Bonferroni's test. A probability level of $P < 0.05$ was regarded as statistically significant.

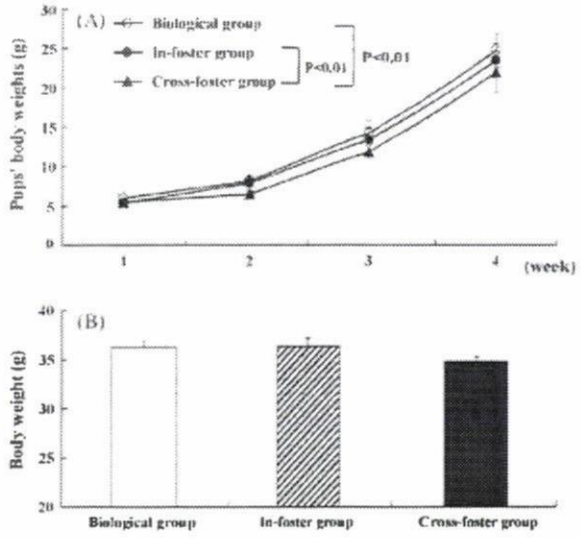


**Fig. 1.** Effect of cross-fostering on body weight. The body weight of pups during the cross-fostering period from 1 to 4 weeks (A) and at the age of 8 weeks (B). Data are expressed as the mean ± S.E.M. for 10–18 mice (Bonferroni's test).

## 3. Results

### 3.1. Effect of cross-fostering on body weight

As reported previously, body weight was considered as an assessment of cross-fostering, since it was sensitive to a change of rearing conditions [12]. To confirm the effect of cross-fostering, the weight of pups was measured during the period of cross-fostering and throughout their development. As shown in Fig. 1, the pups in the in-foster group gained weight as observed in the biological group. However, body weight was significantly lower in the cross-foster group than the biological or in-foster group during the period of cross-fostering ($F_{group(2,168)} = 22.40$, $P < 0.01$; $F_{week(3,168)} = 1394.66$, $P < 0.01$; $F_{group \times week(6,168)} = 2.13$, $P > 0.05$, Fig. 1A). No significant differences were observed among the three groups at the age of 8 weeks when all of the behavioral tests were started ($F_{(2,44)} = 1.80$, $P > 0.05$, Fig. 1B).

### 3.2. Effect of cross-fostering on behavior in the open-field test

An open-field test under mild stressful conditions is commonly used to detect emotional changes in mice [40]. In-fostered mice showed no changes of behavior in the open-field test compared with biological mice (Fig. 2). Meanwhile, the mice in the cross-foster group spent less time in the center squares ($F_{(2,43)} = 11.87$, $P < 0.01$, Fig. 2B) but longer in the corners ($F_{(2,43)} = 3.61$, $P < 0.05$, Fig. 2C) than either the biological or in-foster group when exposed to a novel environment under mild stressful conditions in the open-field. Furthermore, cross-fostered mice showed significant decreases in ambulation ($F_{(2,43)} = 5.26$, $P < 0.01$, Fig. 2D) and rearing ($F_{(2,43)} = 7.03$, $P < 0.01$, Fig. 2E) compared with the in-foster group. There were no significant differences in the latency to the center squares ($F_{(2,43)} = 0.37$, $P > 0.05$, Fig. 2A), the number of grooming events ($F_{(2,43)} = 0.55$, $P > 0.05$, Fig. 2F), and the frequency of defecation ($F_{(2,43)} = 0.12$, $P > 0.05$, Fig. 2G) as well as urination ($F_{(2,43)} = 0.72$, $P > 0.05$, Fig. 2H) in the cross-foster group (Fig. 2).

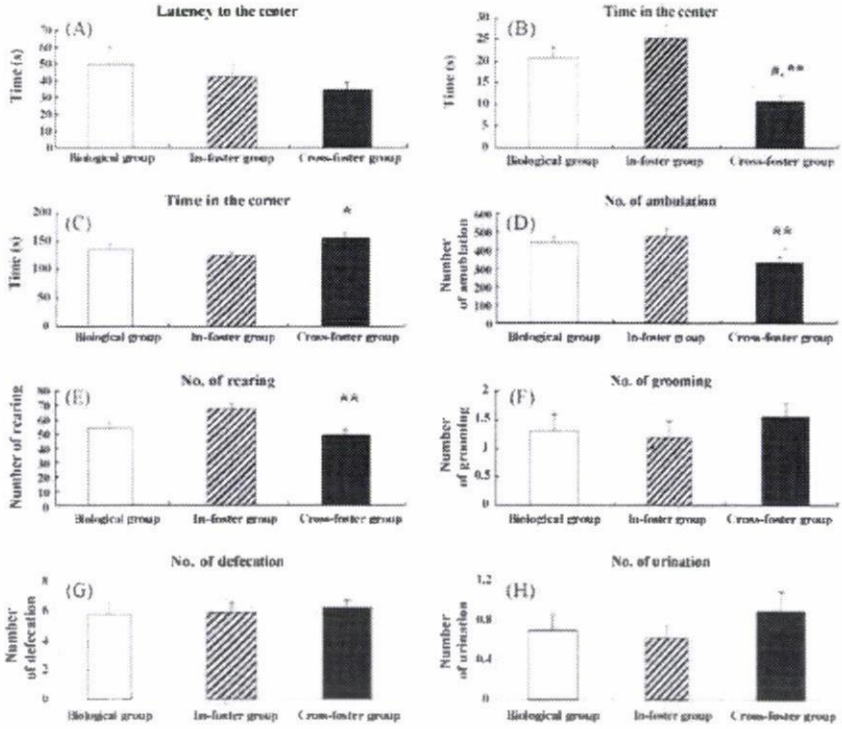


**Fig. 2.** Effect of cross-fostering on behavior in the open-field test. Time of latency to the center (A). Time spent in the center (B). Time spent in the corner (C). The number of times ambulation occurred (D). The number of times rearing occurred (E). The number of times grooming occurred (F). The number of times defecation occurred (G). The number of times urination occurred (H). Data are expressed as the mean ± S.E.M. for 10–18 mice. *$P < 0.05$, **$P < 0.01$ vs. in-foster group; #$P < 0.05$ vs. biological group (Bonferroni's test).

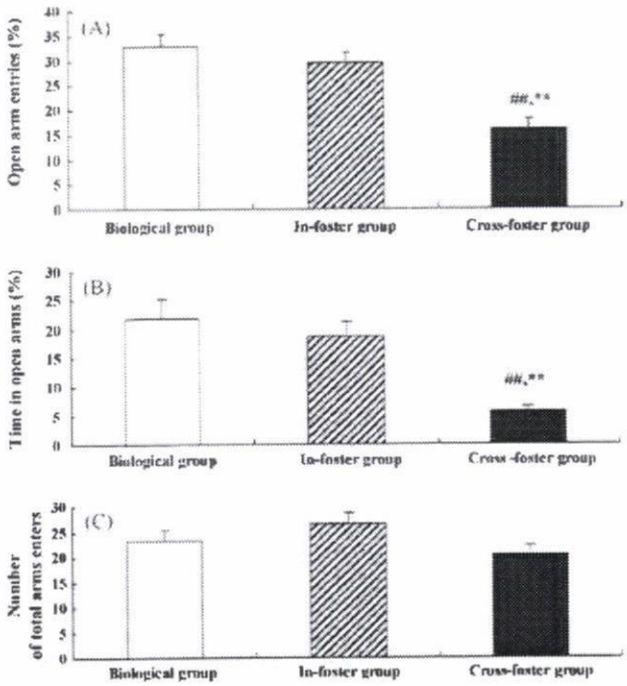


**Fig. 3.** Effect of cross-fostering on the behavior in the elevated plus maze test. Relative percentage of open arm entries (A). Relative percentage of time spent in open arms (B). Total number of arm entries (C). Data are expressed as the mean ± S.E.M. for 10–18 mice. $^{**}P < 0.01$ vs. in-foster group mice; $^{\#\#}P < 0.01$ vs. biological group (Bonferroni's test).

### 3.3. Effect of cross-fostering on behavior in the elevated plus maze

In the in-foster group, no significant changes in behavior in the elevated plus maze were observed compared with that in the biological group (Fig. 3). The percentage of open arm entries was significantly decreased in the cross-foster group compared with both the biological and the in-foster group ($F_{(2,44)} = 15.90$, $P < 0.01$, Fig. 3A). Notably, the percentage of time spent in the open arms was remarkably reduced in the cross-foster group compared with the other two control groups ($F_{(2,44)} = 14.06$, $P < 0.01$, Fig. 3B) whereas total arm entries was unaffected ($F_{(2,44)} = 2.79$, $P > 0.05$, Fig. 3C).

### 3.4. Effect of cross-fostering on immobility time in the forced swimming test

To further investigate the emotional response to stress, we examined the effect of cross-fostering on forced swimming-induced immobility. As shown in Fig. 4, the cross-fostered mice showed a significant increase of immobility time in the first 5 min

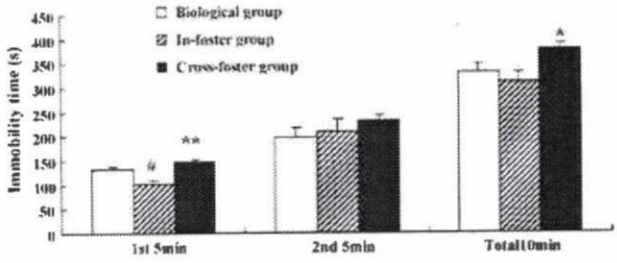


**Fig. 4.** Effect of cross-fostering on immobility time in the forced swimming test. Data are expressed as the mean ± S.E.M. for 10–17 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. in-foster group; $^{\#}P < 0.05$ vs. biological group (Bonferroni's test).

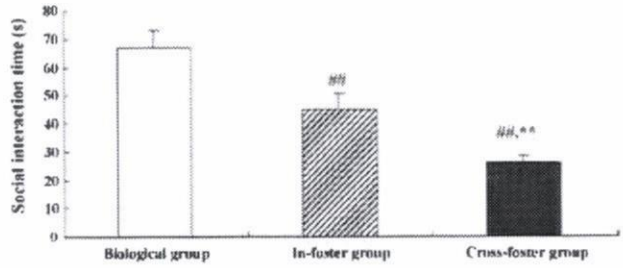


**Fig. 5.** Effect of cross-fostering on social interaction. Active social interaction behaviors, such as sniffing and grooming the partner, following, mounting, and crawling under or over the partner were recorded as the time of interaction during this period. Data are expressed as the mean ± S.E.M. for 10–17 mice. $^{**}P < 0.01$ vs. in-foster group; $^{\#\#}P < 0.01$ vs. biological group (Bonferroni's test).

and total 10 min but not the second 5 min (first 5 min: $F_{(2,42)} = 9.06$, $P < 0.01$; second 5 min: $F_{(2,42)} = 0.74$, $P = 0.48$, $P > 0.05$; total 10 min: $F_{(2,43)} = 4.88$, $P < 0.05$, Fig. 4), compared with the in-fostered mice, which implied a state of increased depression which affected adaptation to stress. On the contrary, in-fostered mice showed a significant reduction of immobility time compared with biological control mice in the first 5 min, but not the second 5 min and total 10 min ($P < 0.05$, Fig. 4).

### 3.5. Effect of cross-fostering on social interaction

The in-foster group showed a significant decrease in social interaction behavior compared with the biological group (Fig. 5). In addition, social interaction time was significantly shorter in the cross-foster group than the biological and in-foster groups ($F_{(2,42)} = 17.84$, $P < 0.01$, Fig. 5).

### 3.6. Effect of cross-fostering on behavior in the Y-maze test

There were no significant differences in spontaneous alternation behavior among the biological, in-fostered and cross-fostered mice in the Y-maze test (Fig. 6). The total number of arm entries was also unchanged among the three groups ($F_{(2,44)} = 0.40$, $P > 0.05$; $F_{(2,44)} = 2.75$, $P > 0.05$, respectively, Fig. 6).

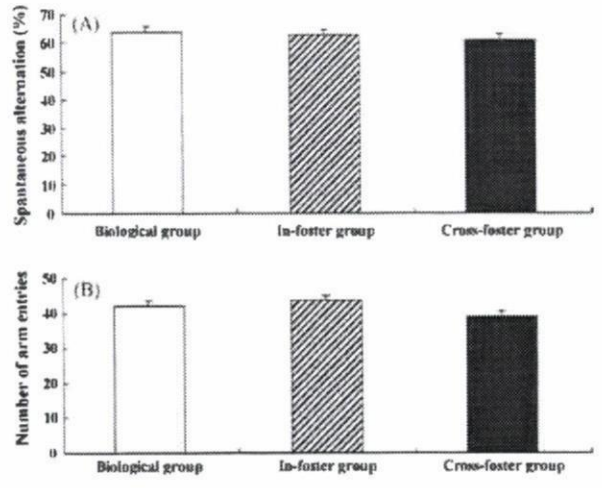


**Fig. 6.** Effect of cross-fostering on behavior in the Y-maze test. The percentage of spontaneous alternation behavior (A). The number of arm entries (B). Data are expressed as the mean ± S.E.M. for 10–18 mice. There were no significant differences among the groups (Bonferroni's test).

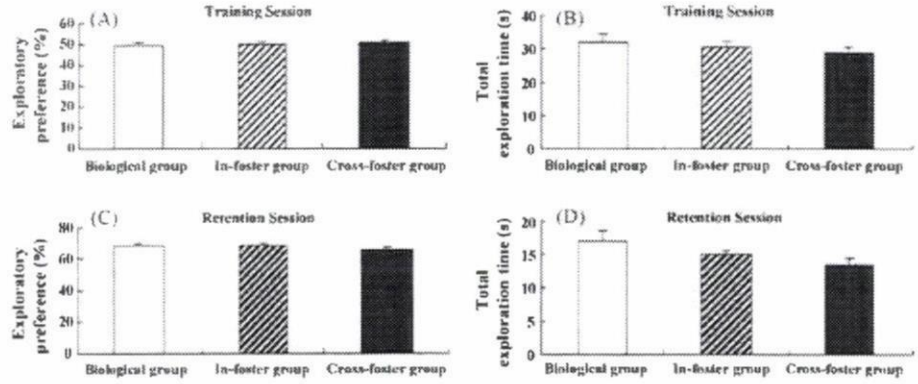


Fig. 7. Effect of cross-fostering on performance in the novel object recognition test. The percentage of exploratory preference in the training session (A). Total exploratory time in the training session (B). The percentage of exploratory preference in the retention session (C). Total exploratory time in the retention session (D). Data are expressed as the mean ± S.E.M. for 10–18 mice. There were no significant differences among the groups (Bonferroni's test).

### 3.7. Effect of cross-fostering on performance in the novel object recognition test

In the training session, the mice in the biological, in-foster and cross-foster groups spent equal amounts of time exploring either of the two objects ($F_{(2,44)} = 0.21$, $P > 0.05$, Fig. 7A), and thus there was no biased exploratory preference in either group of animals. In addition, total time spent in the exploration of objects in the training session did not differ among the three groups ($F_{(2,44)} = 0.55$, $P > 0.05$, Fig. 7B).

When retention performance was tested 24 h after the training session, three were no differences in the level of exploratory preference for the novel objects among the three groups ($F_{(2,44)} = 0.93$, $P > 0.05$, Fig. 7C). The total exploration time did not differ among the three groups in the retention session either ($F_{(2,44)} = 2.55$, $P > 0.05$, Fig. 7D).

### 3.8. Alteration of monoamine metabolism in the prefrontal cortex and hippocampus

To clarify the neurochemical basis of altered emotional behavior in cross-fostered mice, the amount of monoamines and their metabolites in the prefrontal cortex and hippocampus were determined. As shown in Fig. 8A, a significant decease of NE, 5-HT and its metabolites 5-HIAA as well as DA in the prefrontal cortex in cross-foster group was observed, compared with those in biological group (NE: $F_{(2,22)} = 3.93$, $P < 0.05$; 5-HT: $F_{(2,22)} = 3.80$, $P < 0.05$; 5-HIAA: $F_{(2,22)} = 6.07$, $P < 0.01$; DA: $F_{(2,22)} = 4.48$, $P < 0.05$, Fig. 8A). But, there were no differences for the contents of dopamine metabolites among groups, including DOPAC and HVA (DOPAC: $F_{(2,22)} = 1.16$, $P > 0.05$; HVA: $F_{(2,22)} = 0.94$, $P > 0.05$, Fig. 8A). In the hippocampus, compared with the biological group, the mice in cross-foster group also showed significant reduction of 5-HIAA ($F_{(2,25)} = 7.00$, $P < 0.01$, Fig. 8B), but no changes in NE, 5-HT, DA, DOPAC and HVA (NE: $F_{(2,25)} = 0.88$, $P > 0.05$; 5-HT: $F_{(2,25)} = 2.06$, $P > 0.05$; DA: $F_{(2,22)} = 2.92$, $P > 0.05$; DOPAC: $F_{(2,25)} = 0.63$, $P > 0.05$; HVA: $F_{(2,25)} = 1.87$, $P > 0.05$, Fig. 8B). For the in-foster group, the contents of NE in the prefrontal cortex and 5-HIAA in the hippocampus were also reduced compared with biological group (Fig. 8A and B). Whereas, the turnovers of monoaminergic neuronal systems were not affected by in- and cross-fosterings (data were not showed).

## 4. Discussion

Cross-fostering as a kind of early-life stress in rodents could mimic the psychology of children adopted as babies or suffering neglect as well as physical abuse [34]. Although most adopted individuals are well adjusted, population-based studies have reported an elevated risk for psychological maladjustment in adopted children compared with representative samples of nonadopted children [15]. A meta-analysis of findings from more than 25,000 adoptees, revealed significantly more behavioral and emotional problems among adoptees than nonadoptees [38]. Studies in animal models have found that cross-fostering within 24 h after birth affects the maternal behavior and pups' responses [11,30]. But, few articles have systemically examined its long-lasting effects on emotional or cognitive functions in ICR mice.

Clinical researches have reported that approximately 120,000 children in the USA are adopted annually, and adopted individuals constitute about 1.5 million children at young age [26]. However, the face of adoption is changing from decreasing domestic adoptions to a sharp increasing of international ones. Worldwide, approximately 40,000 children per year are moved between more than 100 countries through adoption [14]. Therefore, there is a persistent concern that adopted children may be at heightened risk for mental health or adjustment problems [16]. To clarify this concern, we designed the in- and cross-fostering groups to be equivalent to

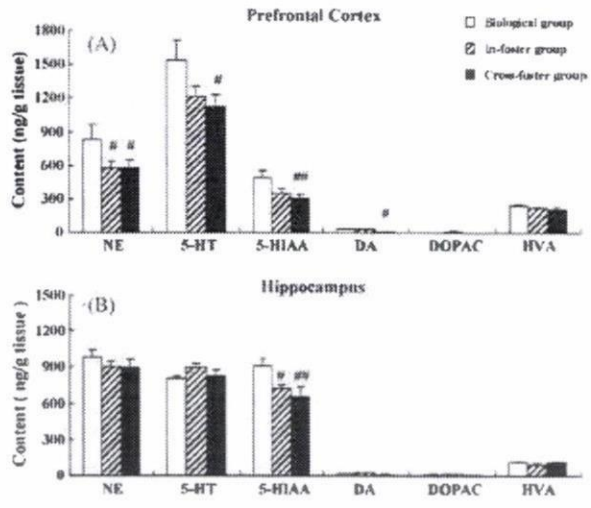


Fig. 8. Monoamines and their metabolite contents in the prefrontal cortex and hippocampus. The contents of monoamines and their metabolites in the prefrontal cortex (A). The monoamines and their metabolite contents in the hippocampus (B). Data are expressed as the mean ± S.E.M. for 8–10 mice. $^{**}P < 0.01$ vs. in-foster group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. biological group (Bonferroni's test).

'the domestic' and 'international adoptions', respectively. Furthermore, we also think the degree of stress induced by adoption might be different for both pups and their adopted dams from in-fostering and biological groups. Therefore, it might produce different effects on long-term behavioral abnormality in each group. Some of our results showed these differences in behaviors and neurochemical parameters among them.

Previous studies have shown that adoption at different points in postnatal period affect the responses of pups and dams in rats: Early adoption on PD1 prevents the stress-induced secretion of corticosterone which is observed in offspring separated early, reduces locomotor activity in a novel environment, and improves spatial recognitive function [3,8]. In contract, later adoptions (PD5 and PD12) prolong the stress-induced secretion of corticosterone, increase locomotor response to novelty, and disrupt spatial recognition [3,8]. Furthermore, the second postnatal week has been reported as critical to establish the proper responses to later stress in adolescence [22]. Therefore, cross-fostering was carried out from PD7 to P28 in the present study to focus on the emotional behavior of offspring in adulthood.

Body weight during development has been considered as an indicator of maternal rearing [12]. In this study, we measured the body weight of pups throughout their development. No significant differences were observed at the age of 8 weeks when all of the behavioral tests were started. Therefore, it is unlikely that the behavioral changes in cross-fostered mice are due to malnutrition in adulthood. On the contrary, the body weight of ICR pups was reduced by cross-fostering during the period of adoption, indicating the maternal rearing by C57BL/6 dams is impaired by the adoption of a different strain of pups and the ICR pups is sensitive to adoption by C57BL/6 dams. Other possible explanations for the decrease in pups' body weight include the number of litters, the quality of adopted maternal care and the nourishment in milk, etc. [37], since we have used different strain of mice and alternated the relationship of dam and pups during their developing period. In this paper, we merely want to show the final co-effects of these factors in the first step of experiments. Therefore, we did not separate the exact role for each factor. Further researches are needed to clarify whether each factor differently influences on body weight in cross-fostering.

The changes in emotional behavior of mice were examined by the open-field, elevated plus maze and forced swimming as well as social interaction tests. In the open-field test, the mice are exposed to aversive stimuli (novelty, lit, and open area) to avoid and new places to explore. The internal conflict is measured by the duration of stay in peripheral areas and avoidance of the center area, called thigmotaxis, and the number of ambulation or rearing events is thought to reflect the exploratory tendencies in mice [35]. In the present study, the cross-fostered mice showed an increase in thigmotaxis and decrease in exploratory tendencies. The results were consistent with the previous report that C57BL/6By] mice raised by BALB/cBy] dams spent less time in the center of the open-field area [30]. Furthermore, in the elevated plus maze test, the cross-fostered mice showed a decrease in the both of the number of open arm entries and the time spent in open arms, parameters of potential anxiety in mice [18]. Taken together, these behavioral studies suggested the cross-fostered mice are in a state of increased anxiety. Immobility time in forced swimming test is used to evaluate the state of stress or depression since it can be increased by stressors and reversed by some antidepressants [9]. The cross-fostered mice showed a significant enhancement of immobility time during the first 5 min in the forced swimming test which suggests increased sensitivity to stress or inappropriate coping responses when facing severely stressful situations. Social withdrawal is regarded as a feature of emotional disorders [19]. We examined the social behavior of offspring in adulthood. The social interaction time in the cross-foster group was remarkably shorter than that in the in-foster group. Interestingly, the mice not only in cross-foster group but also in-foster group showed a significant decrease in social interaction behavior compared with those in the biological control group, suggesting the social interaction test may be more sensitive for detecting the behavioral changes of fostering. Taken together, all of the behavioral results indicate that the cross-fostered mice were in a state of high emotionality and so failed to adapt to other stressors.

Cognitive function was also evaluated by measuring the spontaneous alternation behavior of mice in the Y-maze test (an index of spatial memory) and exploratory preference in the novel object recognition test (an index of visual recognition memory) [25]. There were no significant differences in cognitive function among the different fostering groups suggesting that the cross-fostered mice were normal in cognitive function including short-term memory and visual recognition memory in ICR mice. However, the data supporting unaffected memory are inconclusive: later adoption from PD5 or PD12 has been reported to impair memory in the Y-maze test for male adult offspring in rats, whereas early adoption from PD1 has the opposite effect [3]. The differences may be partly due to either the period of cross-fostering or the strains of mice in each study. Therefore, we cannot make such conclusion that the stress of cross-fostering might not be strong enough to impair learning or memory in ICR mice. Other types of memory should be tested such as spatial memory, contextual memory, latent learning associated with selective attention and motor learning.

In the present study, we used the same offspring for behavioral tests, repeatedly, to measure various emotional and cognitive functions. It is unlikely that the carry-over effects of previous behavioral tests would affect the following behavioral tests, since (1) each behavioral test consists of different parameters which affect motivation and curiosity, etc., (2) all groups have the same influences from previous behavioral tests and (3) we compared them with the control one. The control group in the present study did not show any behavioral abnormality compared with that in our previous studies [23,25,40].

To investigate the neurochemical basis of these emotional abnormalities, we measured the levels of monoamine neurotransmitters and their metabolites in the prefrontal cortex and hippocampus, which related to emotional and cognitive function [40]. We found the contents of 5-HT and 5-HIAA were reduced in cross-fostered mice, indicating the deficit of serotoninergic neuronal function by cross-fostering. It is well known the serotonergic system plays a critical role in regulation of emotional stress during development, and the dysfunction of serotonergic system has been implicated in the etiology of emotional disorders [16,22]. Therefore, the aberrant serotonergic system or its receptors induced by cross-fostering may lead to these emotional abnormalities. Furthermore, since the dysfunctions of noradrenergic and dopaminergic system are also related to some stress-induced disorders, such as depression [5,24], the reduction of NE and DA in the prefrontal cortex might partly contribute to these emotional abnormalities of cross-fostered mice in the present study. However, further research is needed to determine the precise mechanisms of cross-fostering on impaired emotional behavior in ICR mice.

## 5. Conclusion

In conclusion, the present study demonstrated that cross-fostering of ICR pups with C57BL/6 dams from PD7 for 3 weeks affected the emotionality, but not memory, of offspring in adulthood which could mimic the psychology of adoption in humans. Furthermore, stress-related psychological diseases are known to

involve both genetic susceptibility and environmental factors, but the interactions of genes and the environment in the susceptibility to stress are still unclear [7]. The early-life stress induced by cross-fostering has important implications for research into vulnerability to stress or the interactions of genetic and environmental factors using stress or psychiatric disease-related genetic animal models.

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Behavioural Pharmacology

# $GABA_B$ receptor agonist baclofen improves methamphetamine-induced cognitive deficit in mice

Sawako Arai [a,1], Kazuhiro Takuma [a,1], Hiroyuki Mizoguchi [a,b], Daisuke Ibi [a], Taku Nagai [c], Hiroyuki Kamei [d], Hyoung-Chun Kim [e], Kiyofumi Yamada [a,c,f,*]

[a] Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan
[b] Futuristic Environmental Simulation Center, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-8601, Japan
[c] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
[d] Laboratory of Clinical Pharmacy Practice and Health Care Management, Faculty of Pharmacy, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya 468-8503, Japan
[e] Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon 200-701, South Korea
[f] CREST, JST, Nagoya 466-8560, Japan

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ABSTRACT

In this study, we investigated the effects of $GABA_A$ and $GABA_B$ receptor agonists on the methamphetamine-induced impairment of recognition memory in mice. Repeated treatment with methamphetamine at a dose of 1 mg/kg for 7 days induced an impairment of recognition memory. Baclofen, a $GABA_B$ receptor agonist, ameliorated the repeated methamphetamine-induced cognitive impairment, although gaboxadol, a $GABA_A$ receptor agonist, had no significant effect. $GABA_B$ receptors may constitute a putative new target in treating cognitive deficits in patients suffering from schizophrenia, as well as methamphetamine psychosis.



## 1. Introduction

Methamphetamine is a highly addictive drug of abuse, and addiction to methamphetamine has increased to epidemic proportions worldwide (Cretzmeyer et al., 2003; Rawson et al., 2002). Chronic use of methamphetamine causes psychiatric symptoms, such as hallucination and delusions, and long-term cognitive deficits (Simon et al., 2000; Kalechstein et al., 2003; Nordahl et al., 2003; Srisurapanont et al., 2003), which are indistinguishable from paranoid schizophrenia (Yui et al., 2002; Srisurapanont et al., 2003). In a previous study, we demonstrated that repeated methamphetamine treatment caused an enduring impairment of recognition memory in a novel object recognition test in mice, and that methamphetamine-induced cognitive impairment was reversed by an atypical antipsychotic, clozapine, but not haloperidol (Kamei et al., 2006). Furthermore, the same treatment in rats resulted in a significant impairment of spatial working memory, which was ameliorated by clozapine but not haloperidol (Nagai et al., 2007). Thus, methamphetamine-induced memory impairment in rodents may be a useful model for cognitive deficits in methamphetamine abusers and schizophrenic patients.

The GABA receptor system is known to play a significant role in modulating the dopamine system (Tepper and Lee, 2007). Several studies have demonstrated that GABA receptor agonists can inhibit the effects of drugs of abuse. For example, baclofen has been shown to attenuate amphetamine-induced increase in dopamine levels in the nucleus accumbens (Brebner et al., 2005), and $GABA_A$ receptors on ventral tegmental area dopamine neurons play a significant role in attenuating the effects of drugs of abuse in a similar manner to that of $GABA_B$ receptors (Westerink et al., 1996). Although many studies have examined the effects of GABA receptor agonists on hyperdopaminergic conditions induced by psychostimulant drugs, few studies have investigated the effects of GABA receptors on cognitive deficits induced by drugs of abuse.

Recent studies suggest that alterations of GABA systems are related to the pathophysiology of schizophrenia (Lewis, 2000; Benes and Berretta, 2001). Moreover, it is suggested that impairment in GABA-mediated inhibition in the prefrontal cortex may provide a mechanism of disturbance in cognitive processes, such as working memory, in individuals with schizophrenia (Lewis, 2000; Benes and Berretta, 2001). Cognitive dysfunction is considered a core feature of schizophrenia (Elvevåg and Goldberg, 2000), and the degree of cognitive deficit may be the best predictor of long-term functional outcome for individuals with schizophrenia (Green, 1996). Despite the clinical

* Corresponding author. Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan. Tel.: +81 52 744 2674; fax: +81 52 744 2682.
*E-mail address:* kyamada@med.nagoya-u.ac.jp (K. Yamada).
[1] S.A. and K.T. contributed equally to this work.

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importance of cognitive dysfunction in schizophrenia, there are no appropriate drug therapies.

In this study, to develop novel pharmacotherapy for cognitive deficits in schizophrenia patients and methamphetamine abusers, we examined the effects of $GABA_A$ and $GABA_B$ receptor agonists on methamphetamine-induced impairment of recognition memory in mice.

## 2. Materials and methods

Male ICR mice (7–8 weeks old) were obtained from Japan SLC Inc. (Shizuoka, 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–dark cycle (lights on at 9:00 am). Food and tap water were available ad libitum. 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.

Methamphetamine hydrochloride (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), *R*(+)-baclofen hydrochloride (Sigma-Aldrich Co., St Louis, MO) and gaboxadol hydrochloride (Sigma-Aldrich) were dissolved in saline. All drugs were administered in a volume of 0.1 ml/10 g body weight. Mice were given methamphetamine (1 mg/kg, s.c.) daily once for 7 days. One day after the last treatment of methamphetamine, novel object recognition test commenced as described below.

Novel object recognition test was carried out as described previously (Kamei et al., 2006; Mizoguchi et al., 2008). The experimental apparatus consisted of a Plexiglas box (30×30×35 cm high), with a sawdust-covered floor. The apparatus was located in a sound-attenuated room and was illuminated with a 20 W bulb.

The novel object recognition test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects for 3 consecutive days (habituation session, days 1–3). During the training session, two novel objects were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore the box for 10 min (day 4). The objects were a golf ball, a wooden column and a wall socket, which were different in shape and color but similar in size. The animals were considered to be exploring the object when the head of the animal was facing the object or the animal was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention sessions, the animals were placed back into the same box 24 h (day 5) after the training session, in which one of the familiar objects used during training was replaced by a novel object. The animals were then allowed to explore freely for 5 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index in the retention session, the ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as a ratio of the time spent exploring the object that was replaced by the novel object in the retention session, over the total exploring time.

Baclofen (1 and 2 mg/kg, s.c.) and gaboxadol (1 and 3 mg/kg, s.c.) were administered once 15 min before the training session in novel object recognition test (day 4). No drugs were given during the habituation (day 1–3) and the retention sessions (day 5) in the novel object recognition test.

To investigate effect of baclofen on motor function, locomotor activity was measured. Mice were given saline or methamphetamine at a dose of 1 mg/kg for 7 days. Mice were placed in home cage for 15 min following injection of saline or baclofen (2 mg/kg, s.c.) after the 3 day-withdrawal of repeated methamphetamine treatment, and then locomotor activity was measured for 10 min in a standard transparent rectangular rodent cage (25×30×18 high cm) using an infrared sensor (NS-AS01; Brainscience, Osaka, Japan) placed over the cage (Kamei et al., 2006; Mizoguchi et al., 2008).

All data were expressed as the mean±S.E.M.. Statistical analysis was carried out by one-way or two-way ANOVA, followed by Student–Newman–Keuls test for multigroup comparisons. *P* values less than 0.05 were taken to indicate significant differences.

## 3. Results

Repeated methamphetamine treatment (1 mg/kg, s.c.) for 7 days resulted in a significant reduction of the preference index in the retention session but not training session as compared with saline-treated control (Figs. 1A and 2A) although it had no effect on total exploratory time (Figs. 1B and 2B). The $GABA_A$ receptor agonist, gaboxadol, at doses of 1 mg/kg, failed to ameliorate the methamphetamine-induced reduction of exploratory preference to the novel object in the retention session of novel object recognition test (Fig. 1A). Although there was no difference between gaboxadol 1 mg/kg and 3 mg/kg in methamphetamine-treated animal ($P=0.10$), there was a tendency of recovery in gaboxadol-treated group at 3 mg/kg ($P=0.06$). Thus, we

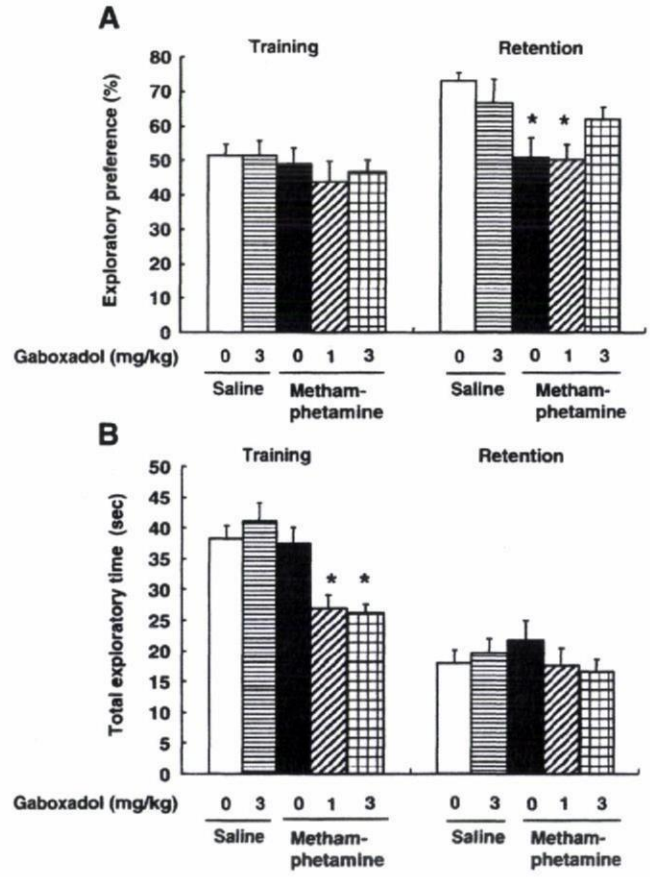


**Fig. 1.** Effect of gaboxadol on methamphetamine-induced impairment of recognition memory in mice. After the cessation of repeated methamphetamine (1 mg/kg, s.c.) treatment for 7 days, mice were subjected to the novel-object recognition test. Gaboxadol (1 and 3 mg/kg, s.c.), or saline was administered 15 min before the training session. (A) Exploratory preference. (B) Total exploration time. Values indicate the mean±S.E.M. (saline/saline, $n=13$; saline/Gaboxadol 3 mg/kg, $n=8$; methamphetamine/saline, $n=12$; methamphetamine/Gaboxadol 1 mg/kg, $n=7$; methamphetamine/Gaboxadol 3 mg/kg, $n=14$). ANOVA: (A, training) $F(4,49)=0.488$, $P=0.7448$; (A, retention) $F(4,49)=4.6$, $P<0.01$; (B, training) $F(4,49)=7.876$, $P<0.01$; (B, retention) $F(4,49)=0.637$, $P=0.6389$. *$P<0.05$ compared with the saline/saline-treated group (Student–Newman–Keuls test).

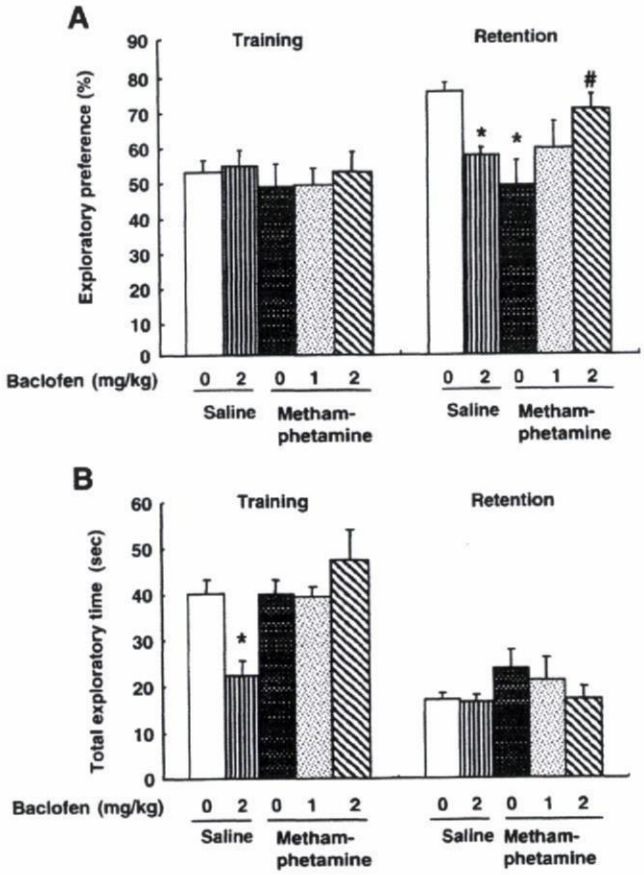


**Fig. 2.** Effect of baclofen on methamphetamine-induced impairment of recognition memory in mice. After the cessation of repeated methamphetamine (1 mg/kg) treatment for 7 days, mice were subjected to the novel-object recognition test. Baclofen (1 and 2 mg/kg, s.c.) or saline was administered 15 min before the training session. (A) Exploratory preference. (B) Total exploration time. Values indicate the mean ± S.E.M. (saline/saline, $n=12$; saline/Baclofen 2 mg/kg, $n=13$; methamphetamine/saline, $n=8$; methamphetamine/Baclofen 1 mg/kg, $n=8$; methamphetamine/Baclofen 2 mg/kg, $n=10$). ANOVA: (A, training) $F(4,46)=0.242$, $P=0.9133$; (A, retention) $F(4,46)=5.56$, $P<0.01$; (B, training) $F(4,46)=7.752$, $P<0.01$; (B, retention) $F(4,46)=1.2$, $P=0.3238$. *$P<0.05$ compared with the saline/saline-treated group. #$P<0.05$ compared with the methamphetamine/saline-treated group (Student–Newman–Keuls test).

also examined the effect of gaboxadol at 10 mg/kg. However, because high-dose gaboxadol at 10 mg/kg markedly reduced the exploratory activity of mice in the training session, they were not subjected to novel object recognition test (data not shown). Gaboxadol at 3 mg/kg had no effect on the exploratory preference (Fig. 1A) and total exploratory time (Fig. 1B) in both training and retention sessions in saline-treated control mice.

Next, we examined the effect of baclofen on methamphetamine-induced cognitive impairment. The $GABA_B$ receptor agonist dose-dependently improved the reduction of exploratory preference to the novel object in methamphetamine-treated mice (Fig. 2A). Baclofen at 2 mg/kg significantly ameliorated methamphetamine-induced cognitive impairment (Fig. 2A). Baclofen had no effect on the level of exploratory preference for the novel object in the training session or the total exploration time in both the training and retention sessions in methamphetamine-treated mice. Treatment with baclofen at 2 mg/kg in saline-treated control group resulted in a significant decrease in total exploratory time to novel objects in the training session (Fig. 2B), leading to a significant impairment of novel object recognition in the retention session (Fig. 2A). This is probably due to an insufficient exploratory behaviors in the training session, which could result in a poor discrimination of a novel object.

## 4. Discussion

We have previously demonstrated that repeated methamphetamine treatment in mice induces enduring recognition memory impairment, which is associated with dysfunction of the dopamine $D_1$ receptor-ERK1/2 pathway in the prefrontal cortex. Clozapine, but not haloperidol, completely restored the cognitive impairment induced by methamphetamine treatment when repeatedly administered for 7 days after withdrawal from methamphetamine, although acute treatment with these antipsychotics had no effect (Kamei et al., 2006). The data are consistent with clinical evidence that clozapine is superior to typical neuroleptics in improving cognitive deficits in schizophrenic patients (Lee et al., 1999). Thus, we propose that methamphetamine-induced cognitive impairment in mice may be a useful model for cognitive deficits in methamphetamine abusers and schizophrenic patients. In this study, we found that acute treatment with baclofen improved methamphetamine-induced cognitive deficit without affecting motor function, whereas repeated treatment was necessary for the effect of clozapine. These results suggest that $GABA_B$ receptor agonists may be more useful for the treatment of cognitive deficit in schizophrenia patients and methamphetamine abusers than clozapine and other antipsychotic drugs. In contrast, gaboxadol, a $GABA_A$ receptor agonist, had no effect on methamphetamine-induced cognitive deficits. However, gaboxadol is known to preferentially activate the $GABA_A$ receptor subtype containing the delta subunit, which mediated tonic inhibition. Therefore, gaboxadol may not be an ideal agonist for a global activation of $GABA_A$ receptors. Further studies are required to test this assumption.

Additionally, we think that the ameliorating effect of baclofen is not related to the effect on motor function. In fact, we examined the effect of baclofen at a dose of 2 mg/kg on locomotor activity of mice that had been treated with saline or methamphetamine (1 mg/kg) for 7 days. Baclofen had no effect on behavioral locomotion of repeated methamphetamine-treated group (saline/saline group ($n=7$), 399 ± 39.9 counts/10 min; saline/baclofen group ($n=7$), 343.7 ± 51.4 counts/10 min; methamphetamine/saline group ($n=7$), 429.1 ± 21.4 counts/10 min; methamphetamine/baclofen group ($n=7$), 346.3 ± 41.6 counts/10 min; $F(3,24)=1.08$, $P=0.37$). In Figs. 1 and 2, we showed the total exploratory time, which means locomotor activity in training and retention phase, respectively. Baclofen had no effect on the level of exploratory preference for the novel object in the training session or the total exploration time in both the training and retention sessions in methamphetamine-treated mice. These results suggest that baclofen has no effect on motor function in methamphetamine-treated mice. There was an apparent difference in sensitivity to baclofen between saline-treated control and methamphetamine-treated group: Baclofen at 2 mg/kg significantly reduced the total exploratory time in the training session in control mice, while the drug had no effect in the methamphetamine-treated mice. Regarding to this phenomenon, it is reported that repeated cocaine treatment decreases baclofen-stimulated [$^{35}$S]GTPγS binding to G protein in the nucleus accumbens, indicating desensitization of $GABA_B$ receptors (Xi et al., 2003). Thus, it is possibly that repeated methamphetamine treatment causes desensitization of $GABA_B$ receptor as does cocaine treatment.

There are some studies suggesting that $GABA_B$ receptors play an important role in regulating dopamine neurons, while the role of $GABA_A$ receptors has been unclear. For example, previous studies showed that baclofen reduced the reinforcing effects of many substances of abuse, such as cocaine, nicotine, heroin, and alcohol (Cousins et al., 2002), possibly through $GABA_B$-mediated modulation of mesolimbic dopamine transmission (Bartholini, 1985). In fact, baclofen is known to stabilize the firing pattern of dopamine neurons (Erhardt et al., 2002). It was demonstrated that chronic coadministration of baclofen and amphetamine blocked the development of sensitization to the locomotor stimulation effect of amphetamine

(Bartoletti et al., 2005), and acute treatment with baclofen inhibited the expression of amphetamine-induced locomotor sensitization (Bartoletti et al., 2004). Moreover, a recent study showed that acute treatment with baclofen ameliorated ethanol-induced memory deficit in mice (Escher and Mittleman, 2004). Moreover, we have recently demonstrated that baclofen, but not gaboxadol, ameliorates methamphetamine- and MK-801-induced impairment of prepulse inhibition of the acoustic startle reflex in mice (Arai et al., 2008). These results support our findings that baclofen ameliorates repeated methamphetamine treatment-induced cognitive deficits. Taken together, the ameliorating effect of baclofen on cognitive impairment in methamphetamine-treated mice may be attributable to its effects on $GABA_B$ receptors in midbrain dopamine neurons.

In conclusion, we demonstrated that baclofen acutely ameliorated the cognitive deficit in repeated methamphetamine-treated mice, an animal model for cognitive deficits in methamphetamine abuse and schizophrenia. Our results suggest that baclofen may be superior to clozapine and other antipsychotic drugs that mainly affect dopamine $D_2$ and 5-$HT_2$ receptors. $GABA_B$ receptors may constitute a putative new target for treating cognitive deficits in patients suffering from schizophrenia, as well as methamphetamine psychosis. Further studies are necessary to clarify the molecular mechanisms of the action of baclofen.

## Conflict of interest

There are no conflicts of interest in this study.

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*Regular Article*

# Plasma amitriptyline level after acute administration, and driving performance in healthy volunteers

Kunihiro Iwamoto, MD,[1] Yukiko Kawamura, MS,[2] Masahiro Takahashi, MD,[1] Yuji Uchiyama, PhD,[3] Kazutoshi Ebe, ME,[3] Keizo Yoshida, MD, PhD,[1]* Tetsuya Iidaka, MD, PhD,[1] Yukihiro Noda, PhD[2] and Norio Ozaki, MD, PhD[1]

[1]*Department of Psychiatry, Nagoya University, Graduate School of Medicine,* [2]*Meijyo University, Graduate School of Pharmacy, Division of Clinical Science and Neuropsychopharmacology and* [3]*Toyota Central R&D Labs., Aichi, Japan*

*Aims:* Amitriptyline triggers the impairment of cognitive and motor functions and has been confirmed to have harmful effects on driving performance. Although interindividual differences in plasma concentration may cause variations in driving performance, the relationship between plasma amitriptyline concentration and its effect on driving performance has not been completely elucidated. Thus, the aim of the present study was to assess the influence of individual pharmacokinetic differences on driving performance and cognitive functions.

*Methods:* In this double-blinded study, 17 healthy male volunteers were given an acute, single, 25-mg dose of amitriptyline. The subjects were assigned three driving simulator tasks, three cognitive tasks, and the questionnaire of the Stanford Sleepiness Scale at the baseline and at 4 h after dosing. The plasma amitriptyline concentrations were measured on high-performance liquid chromatography.

*Results:* A significant positive correlation was observed between the plasma amitriptyline concentration and road-tracking performance ($r = 0.543$, $P < 0.05$). There was no significant correlation between the plasma amitriptyline concentration and other driving performance, cognitive functions, and subjective somnolence.

*Conclusions:* Amitriptyline produces a concentration-related impairment on road-tracking performance. Therapeutic monitoring of amitriptyline would be useful for predicting the difficulties involved while driving.

**Key words:** amitriptyline, antidepressants, automobile driving, cognition, drug monitoring.

INTERINDIVIDUAL DIFFERENCES IN drug responses occur even when the same dosage of a drug is prescribed to different individuals. Therapeutic drug monitoring (TDM) is one of the most valid tools utilized to minimize interindividual differences in drug responses. TDM enables a clinician to adjust the drug dosage and enhance efficacy and safety.[1] In the case of antidepressants, tricyclic antidepressants (TCA) are repeatedly recommended to be monitored for blood concentration[1–5] because these drugs have shown a fairly large interindividual variance in clinical response. The relationship between the blood TCA concentration and adverse effects, such as dropout rate, central nervous system toxicity, and cardiovascular toxicity has been reported.[4,6]

Among TCA, there is no consensus regarding the relationship between plasma amitriptyline concentration and therapeutic response, in contrast to that for imipramine, desipramine, and nortriptyline.[2,3] Previous studies reported different results regarding the relationship between plasma amitriptyline

*Correspondence: Keizo Yoshida, MD, PhD, Department of Psychiatry, Nagoya University, Graduate School of Medicine, 65 Tsurumai, Showa, Nagoya, Aichi 466-8550, Japan. Email: cxw01076@nifty.com*

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concentration and common adverse effects such as drowsiness and dry mouth. For example, although these adverse events were attributed to high plasma concentration of amitriptyline, correlation for low–moderate concentrations of amitriptyline was not observed.[7]

Epidemiological data indicate that TCA users are twice as likely to be involved in traffic accidents as compared to non-users.[8,9] Various studies have demonstrated the harmful effects of TCA on driving performance.[10] As for amitriptyline, impairment of road tracking performance and increase in brake reaction time have been reported.[11,12] Amitriptyline also has been linked to impairment of cognitive functions as well as driving performance. A single low dose of amitriptyline impaired cognitive functions as measured on cognitive tests such as auditory vigilance test, tapping test, arithmetic test, digit symbol substitution test, short term memory test, flicker-fusion test, and choice reaction time test.[13–19]

In our recent study we used simulator scenarios to examine car-following performance in the context of crowded urban roads and driving at relatively low speeds as well as other driving tasks routinely investigated in other previous studies. Furthermore, cognitive function was evaluated using the Wisconsin Card-Sorting Test (WCST), Continuous Performance Test (CPT), and N-back test. At 4 h after amitriptyline administration, road-tracking and car-following performance was significantly impaired, vigilance was reduced, and subjective somnolence was induced.[20]

Although the adverse effects of amitriptyline on driving performance and cognitive functions differ across individuals, to the best of our knowledge there have been no studies reported on the relationship between plasma amitriptyline concentration on the one hand, and driving performance and cognitive functions on the other. Considering the aforementioned factors, we examined the influence of individual pharmacokinetic differences on driving performance and cognitive functions using the same procedure as in our previous study.[20]

## METHODS

### Subjects

The sample consisted of 17 healthy male volunteers aged 30–42 years (mean ± SD, 35.8 ± 3.3 years). Based on health interviews and the Structured Clinical Interview for DSM-IV, the subjects were found to be free from any physical or psychiatric disorders and were not taking medication. All subjects had been in possession of a driving license for at least 10 years and had been driving a car daily (minimum, 5000 km/year). The study was approved by the ethics committee of the Nagoya University School of Medicine, and written informed consent was obtained from each subject prior to participation.

### Procedure

All subjects were tested at approximately 09.30 hours using the Stanford Sleepiness Scale (SSS),[21] driving tests, and cognitive tests. The entire testing lasted approximately 1 h for each person. Following baseline assessment, the subjects were given capsules containing 25 mg amitriptyline in a double-blind manner. The dose of 25 mg was selected because it is a recommended starting dose in Japan, and also because the higher dose of amitriptyline might cause severe side-effects, possibly interrupting the experiments. Blood samples (10 mL) were collected 4 h after administration, because that is when maximum plasma drug concentration occurs.[22] The patients were subjected to all the aforementioned tests again after blood drawing. The blood samples were immediately centrifuged at 1700 *g*. for 10 min, and the plasma was frozen at −30°C. Plasma amitriptyline concentrations were determined on high-performance liquid chromatography, as described previously.[23] Five-point calibration curves were set up for the range 2–200 ng/mL. A linear response function was obtained, and the limit of quantification was 2 ng/mL. The interday coefficient of variation for 4 days for plasma amitriptyline at 20 ng/mL was 11.2%. The intraday coefficients of variation were 1.1–1.2% ($n = 2$). Amitriptyline has an active metabolite, nortriptyline. Both amitriptyline and nortriptyline undergo benzylic hydroxylation, and the hydroxylated nortriptyline metabolites are still active.[24] Jiang *et al.* reported that the plasma concentration of nortriptyline was considerably lower than that of amitriptyline after a single dose of amitriptyline.[22] The plasma concentrations of nortriptyline and its metabolites were not analyzed because the present study used only single low dosing and a short sampling interval after administration.

The subjects received substantial training in driving and cognitive tests 1 or 2 weeks prior to the first testing; and in order to minimize the learning effects

the subjects were trained until they reached the plateau level. Furthermore, the subjects were prohibited from consuming alcohol or beverages containing caffeine for 12 h before taking the tests and were requested to sleep adequately on the previous evening. On the test days the subjects were also prohibited from ingesting substances that may induce wakefulness, such as caffeine, supplement drinks, chewing gum, or candies because these substances could exert a stimulating effect on their performance. During the intervals between the test series, the subjects were assigned certain light tasks to prevent them from taking short naps.

### Driving and cognitive tests

We used a driving simulator (Toyota Central R&D Labs, Nagakute, Japan) to examine three driving skills that appeared to be associated with the recent traffic accidents. The road-tracking test in the present study was based on a road-tracking test that was developed previously.[25,26] The subjects were instructed to drive at a constant speed of 100 km/h and stabilize their vehicles at the center of a gently winding road. The standard deviation of the lateral position (SDLP; cm), which indicates weaving, was considered a performance measure. The car-following test required the subjects to maintain a constant distance between the cars (targeted distance of 5 m) in the context of crowded urban roads driving at a speed of 40–60 km/h. The coefficient of variation (CV) was obtained by dividing the standard deviation of the car-following distance (m) between the cars by the mean value, and it was considered a performance measure.[27] Therefore, a smaller value of distance CV (DCV) would indicate a better performance. The harsh-braking test required the subjects to avoid crashing into the humanoid models that randomly ran on the road by harsh braking. The brake reaction time (BRT; ms) was considered a performance measure. Each test lasted for 5 min and the details have been described previously.[20]

The three cognitive tests were examined using a computer. In the WCST the performance was measured using the following indices: category achievement (CA), perseverative errors of Nelson (PEN), and difficulty of maintaining set (DMS).[28,29] In the CPT the performance was measured using the signal detection index d-prime (d'), which is a measure of discriminability computed from hits and false alarms.[30] In the N-back test a two-back condition was used, and the performance was measured as the percentage of correct responses (accuracy, %).[31,32]

### Statistical analysis

None of the outcome variables of the driving tests, cognitive tests, and subjective scales, except BRT (harsh-braking test) and d' (CPT), had a normal distribution. To clarify the correlations between plasma amitriptyline concentration and percent change in performance, the Spearman rank-order correlation coefficients (non-parametric) were calculated. PEN and DMS were analyzed as difference not percent change, because their baseline values could be 0 and percent change could not be calculated. BRT and d' were analyzed using the Pearson product-moment correlation. In order to analyze the drug effect, the baseline values were compared to that obtained at 4 h after dosing using the Wilcoxon signed-rank test. A paired $t$ test was used to analyze the BRT and d' data. All statistical tests were conducted using SPSS version 11 for Windows (SPSS Japan, Tokyo, Japan). Significance levels were set at 5% for all tests.

## RESULTS

### Correlations between plasma amitriptyline concentration and driving performance, cognitive function, and subjective assessments

The mean ± SD plasma amitriptyline concentration was 15.3 ± 6.4 ng/mL (range, 8.5–32.9 ng/mL). The relationships between the plasma amitriptyline concentration and driving performance, cognitive function, and subjective assessments are shown in Fig. 1. Data that indicate the coefficient of correlation of $-0.1 < r < 0.1$ are not shown. A significant correlation was observed between plasma amitriptyline concentration and percent change in SDLP (Fig. 1a). No significant correlations were detected between plasma amitriptyline concentration and the remaining driving, cognitive, and subjective variables (Fig. 1b–f). Percent change in CA, difference of PEN and percent change in SSS showed no significant correlations as follows: $r = -0.070$, $P = 0.789$ for CA, $r = 0.048$, $P = 0.855$ for PEN and $r = 0.035$, $P = 0.893$ for SSS; data not shown).