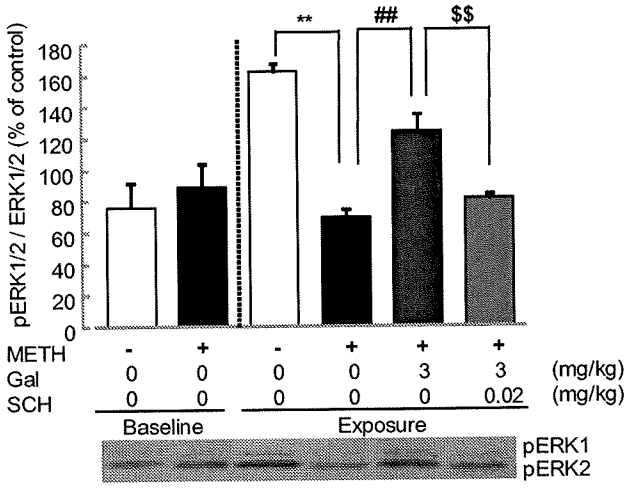

Fig. 6. Attenuative effect of galatantamine on impairmement of learning-associated ERKI phosphorylation is mediated by D1 receptors. METH (1 mg/kg, s.c.) was injected for 7 days. Control groups were treated with same volume of saline. Galantamine (3 mg/kg, p.o.) and SCH 23390 (0.02 mg/kg, s.c.) were administered 1hr and 30 min before the training trial, respectively. Immediately after the training trial, mice were sacrificed by decapitation and ERK phosphorylation and expression in the prefrontal cortex were detected by Western blotting. The phosphorylation ratio was calculated as ERK phosphorylation vs ERK expression. Values indicate the mean ± S.E. (n = 5). **p< 0.01 compared with (saline + saline/saline)-treated group that was exposed to novel objects (exposure) (Bonferroni test). ##p<0.01 compared with (METH + saline/saline)-treated group (exposure) (Bonferroni test). $$p<0.01 compared with (METH + galantamine/saline)-treated group (exposure) (Bonferroni test). METH: Methamphetamine, Gal: Galantamine, SCH: SCH23390

6. METH 連続投与マウスにおけるガランタミンの認知障害緩解作用に対する PD98059 の作用

METH 連続投与マウスに認められる認知障害に対するガランタミンの緩解作用は，PD98059 によって有意に拮抗された (Fig. 7A). 一方，saline 連続投与マウスにおいて，PD98059 単独投与は，NORT における探索嗜好率に有意な影響を及ぼさなかった (Fig. 7C). また，PD98059 は，訓練試行および保持試行における総探索時間に影響を及ぼさなかった (Fig. 7B, 7D).

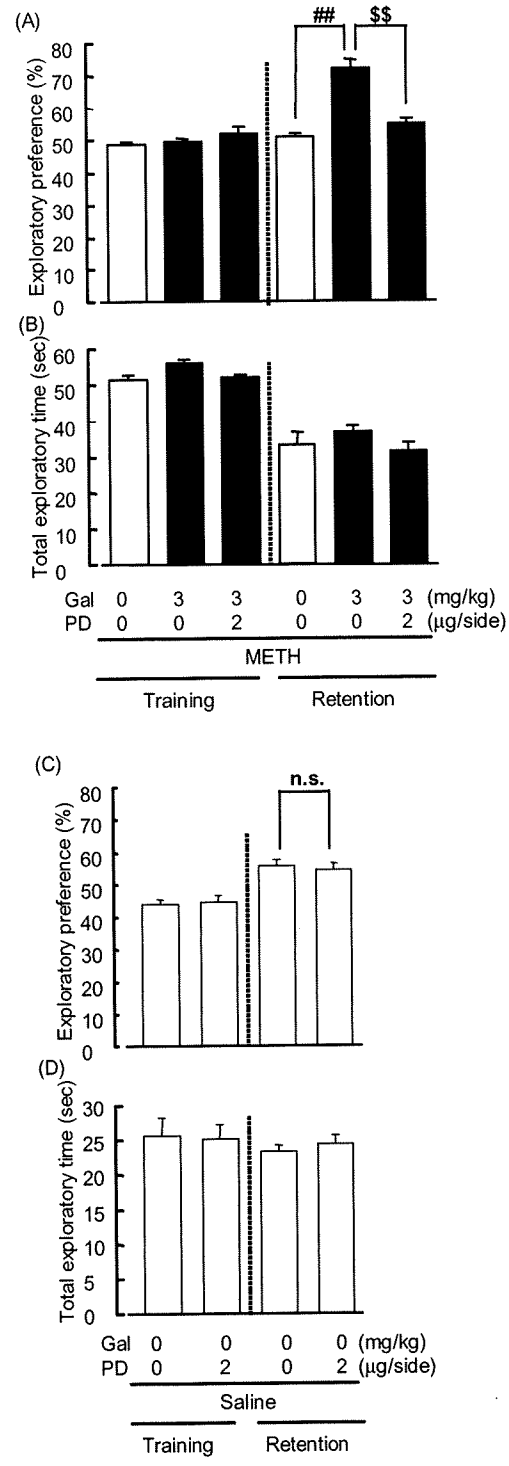

Fig. 7. Attenuative effect of galantamine is mediated by ERK signaling. (A, C): Exploratory preference, (B, D): Total approach time. METH (1 mg/kg, s.c.) was injected for 7 days. Control groups were treated with same volume of saline. Galantamine (3 mg/kg, p.o.) was administered 1 hr before the training trial. PD98059 was infused into the prefrontal cortex (2 μg /side) 30 min before the training trial. A: Values indicate the mean ± S.E. (n = 6-8). ##p< 0.01 compared with (METH + saline/vehicle)-treated group (Bonferroni test). $$p< 0.01 compared with (METH + galantamine/vehicle)-treated group (Bonferroni test). C: Values indicate the mean ± SE (n = 10-11). n.s.: not significant. METH: Methamphetamine, Gal: Galantamine, PD: PD98059

D. 考察

METH 依存者において，幻覚・妄想や認知障害など覚せい剤精神病が発症する[7]. METH 依存者に認められる PFC の機能障害は認知障害と関連していることが機能的磁気共鳴画像法を用いた研究により報告されている[8]. また，METH 連続投与マウスに認められる認知障害には，PFC におけるドパミン D1 受容体が関係していることが報告されている[9]. 一方，ガランタミンは，nAchR に対するアロステリックモデュレーターとして働き，また弱いコリンエステラーゼ阻害作用を併せ持つアルツハイマー病の治療薬である[3]. また，本研究室において，ガランタミンはアルツハイマー病モデル動物であるアミロイドベータ注入マウスにおいて，ニコチン型アセチルコリン受容体を介して海馬内の細胞外ドパミン遊離量を増加し，学習障害を緩解することを報告している[10]. 本発表においてガランタミンの投与は METH 連続投与マウスに認められる認知障害に対する緩解効果を示し，前頭皮質のドパミンの遊離を亢進させた．このような効果は nAChR 拮抗薬であるメカミラミンにより拮抗された．そのため，ガランタミンのアロステリック作用による nAchR の活性化により，細胞外ドパミン遊離量が増加されたものと考えられる．また，ドパミン D1 受容体拮抗薬である SCH23390 はガランタミンの投与による METH 連続投与マウスに認められる認知障害に対する緩解効果を抑制した. 従ってガランタミンの投与による METH 連続投与マウスに認められる認知障害に対する緩解効果はドパミン D1 受容体が刺激された結果であると考えられる.

マウスの PFC 両側へ MEK 阻害剤である PD98059 を微量注入すると，NORT において認知障害が惹起されること，METH 連続投与により生じる認知障害の機序について，PFC における学習に関連した ERK1/2 の活性化の低下が関与していることが示唆されている[9]. 本研究において，METH 連続投与マウスに認められた ERK1/2 活性化の障害はガランタミン投与により有意に改善され，この改善作用は SCH23390 によって拮抗された．さらに，NORT において，ガランタミンの認知障害改善作用は，PD98059 によって拮抗された．したがって，ガランタミンはドパミン D1 受容体を介して，PFC における ERK1/2 のリン酸化を増加させ，このドパミン D1 受容体-ERK1/2 シグナル経路の活性化が認知障害の改善に重要な役割を果たしていることが示唆された.

E. 結論

ガランタミンは, nAChR のアロステリックな調節を介して PFC におけるドパミン作動性神経系の伝達を亢進させ，学習に関連した ERK1/2 シグナルの活性化を増強することにより，METH 連続投与マウスに認められる認知障害を改善するものと示唆される．また，ガランタミンはアルツハイマー病のみならず，統合失調症や覚せい剤精神病における認知障害に対しても有用な治療薬となる可能性が考えられる．今後，覚せい剤による薬物依存に対するガランタミンの効果について検討していく予定である.

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Research report

Synergistic effects of selegiline and donepezil on cognitive impairment induced by amyloid beta (25–35)

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ABSTRACT

Selegiline, an irreversible inhibitor of monoamine oxidase B used in the treatment of Parkinson's disease, has been demonstrated to have a potential cognition-improving effect in patients with Alzheimer's disease (AD) undergoing treatment with an acetylcholinesterase inhibitor donepezil. To confirm such clinical events, we investigated whether co-administration of donepezil with selegiline had a synergistic cognition-improving effect in an animal model of AD. Intracerebroventricular injection of amyloid beta protein fragment 25–35 [$A\beta_{(25-35)}$] induced impairment of learning and memory in a Y-maze, novel object recognition and contextual fear conditioning tests. Either donepezil or selegiline alone improved the cognitive impairments in the Y-maze and conditioned fear learning tasks in $A\beta_{(25-35)}$-injected mice, whereas donepezil, but not selegiline, failed to improve the impairment in a novel object recognition task. Co-administration of donepezil with selegiline, at doses that do not exert efficacy individually, significantly improved the deficits in all three tests, indicating a synergistic cognition-improving effect. These alleviating effects were antagonized by pretreatment with a muscarinic receptor antagonist scopolamine and a dopamine receptor antagonist haloperidol. These results suggest that selegiline potentiates the effect of donepezil on the cognitive impairment, and that the synergistic effect may be mediated through both the cholinergic and dopaminergic systems.

1. Introduction

Alzheimer's disease (AD), the most common neurodegenerative disorder in humans, is characterized by the deterioration of cognitive and mental functions, including learning and memory. The formation of extracellular deposits of amyloid beta peptide (Aβ), leading to the formation of neuritic plaques and neurofibrillary tangles in the cortex and hippocampus, is a prominent pathological feature of AD [32]. Aβ, a spontaneously aggregating peptide of 39–43 amino acids, is the primary protein component of senile plaques, the pathological hallmark of AD in the brain [29]. In particular, Aβ fragment 25–35 [$A\beta_{(25-35)}$] seems to be responsible for toxic and oxidative events leading to brain damage, such as oxidative stress-mediated changes in hippocampal long-term potentiation [37], and protein oxidation in fibroblasts derived from AD patients [4]. In animal experiments, it has been reported that intrahippocampal or intracerebroventricular (i.c.v.) injections of $A\beta_{(25-35)}$ induce histological and biochemical changes, learning deficits [20,21,25] and dysfunction of the cholinergic system, which play an important role in the cognitive deficits associated with aging and neurodegenerative diseases [12,36]. Thus, $A\beta_{(25-35)}$-injected animals are useful models for understanding the pathogenesis and progression of AD, and for evaluating new therapeutic agents for AD [12,20].

Cholinergic neurons degenerate in patients with AD and Alzheimer's type senile dementia, and the degree of degeneration correlates well with functional loss in these disorders [26]. Based on a cholinergic hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity through the cholinomimetic use of acetylcholinesterase inhibitors (AchEIs), acetylcholine precursors and cholinergic receptor agonists. In fact, an AchEI donepezil, has been approved for treatment of cognitive impairment in AD. However, acetylcholine-enhancing drugs can

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compensate for only part of the neuronal dysfunction in AD, and the enhancement of cognition by AchEIs is only transient [13].

The dopaminergic system has been implicated in cognitive processes in a variety of brain regions, including the mesolimbic system, since dopamine modulates transmitter release at cholinergic [11] and glutamatergic [42] synapses in the hippocampus. Previous study has shown that disturbances in the dopaminergic system induce learning and memory impairment [9]. Meanwhile, pathological and clinical evidences reported to date are suggestive of the involvement of the dopaminergic system in dementia. For example, there is a correlation between loss of hippocampal dopamine D_2 receptors and memory impairment in AD [17]. In addition, it has been shown that memory impairment induced by intraperitoneal injection of scopolamine is ameliorated by the injection of dopamine D_2 receptor agonists into the ventral hippocampus [8], suggesting that dopaminergic agents could have therapeutic potential in patients with cholinergic deficits, e.g. those with AD and dementia with Lewy bodies.

Selegiline, a selective monoamine oxidase B inhibitor (MAO-BI), is used worldwide for the treatment of Parkinson's disease [2]. Previous study has shown that selegiline improves episodic memory and learning in patients with AD [35], and spatial memory in aged or a cholinotoxin AF64A-treated rats [18,33]. The increase in dopaminergic activity consequent to the inhibition of monoamine oxidase B activity is often considered to be the neurochemical mechanism involved in the improvement of cognitive performance caused by selegiline in aged rats [3] and individuals affected by Alzheimer's type dementia [35]. Furthermore, selegiline has also been demonstrated to have potential cognition-improving efficacy in AD patients treated with the AchEI donepezil [33].

To confirm such clinical events, the present study was designed to test the hypothesis that co-administration of donepezil with selegiline improves cognitive impairment in an $A\beta_{(25-35)}$-injected animal model of AD, and that the synergistic cognition-improving effects of selegiline and donepezil are mediated via activation of the cholinergic and dopaminergic systems. We attempted to investigate: (1) the effects of single or concurrent administration of selegiline and donepezil on memory impairment induced by $A\beta_{(25-35)}$, and (2) that the cognition-improving effects of selegiline are antagonized by acetylcholine and/or dopamine antagonists in $A\beta_{(25-35)}$-injected mice.

2. Materials and methods

2.1. Animals

Male ICR mice (5-week-old, Nihon SLC, Shizuoka, Japan) were housed in plastic cages, received food (CE2, Clea Japan, Tokyo, Japan) and water *ad libitum*, and were maintained on a 12-h light:12-h dark cycle (lights on at 8.00 a.m.). All experiments were performed in a blind manner and in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine (Japan). The procedures involving animals and their care conformed to the institutional guidelines set out in "Principles of Laboratory Animal Care" (NIH publication No. 85–23, revised 1985).

2.2. Drugs and treatment

Selegiline hydrochloride (FP Pharmaceutical, Osaka, Japan), donepezil hydrochloride (Eisai, Ibaraki, Japan), scopolamine hydrobromide (Nacalai Tesque, Kyoto, Japan) and haloperidol (Sigma, St. Louis, MO, USA) were dissolved in saline. The doses of all drugs are expressed as those of the salt. $A\beta_{(25-35)}$ (Bachem, Torrance, CA, USA) was dissolved in distilled water (vehicle) at a concentration of 1 mg/mL (0.9375 mM) and stored at −20 °C.

Mice were intracerebroventricularly injected vehicle or $A\beta_{(25-35)}$ that had undergone incubation for 4 days at 37 °C, a procedure that permits aggregation. Vehicle- and $A\beta_{(25-35)}$-injected mice were subcutaneously administered selegiline, donepezil, scopolamine and haloperidol 30, 30, 45 and 60 min before each behavioral test (Y-maze, novel object recognition and contextual fear conditioning tests), respectively on 3 separate occasions. Three different approaches for drug administration were used as shown in Table 1: (1) selegiline and donepezil by themselves (Fig. 2); (2) co-administration of selegiline and donepezil (Fig. 3) and (3) scopolamine or haloperidol + co-administration of donepezil and selegiline. We preliminarily confirmed that single administrations of donepezil, selegiline, scopolamine and haloperidol, at doses used in this study, had no effect on cognitive function of the vehicle-injected mice in all performed tests.

Table 1
Drugs, doses of drugs and numbers of animals in each experiment

Group No.	N	Treatment			
		Aβ	Drug (mg/kg)		
Experiment 1 (Fig. 2)					
1	27–28	Vehicle	Saline		
2	29	Aβ	Saline		
3	7	Aβ	Donepezil (0.05)		
4	14	Aβ	Donepezil (0.1)		
5	6	Aβ	Selegiline (1.0)		
6	13	Aβ	Selegiline (3.0)		
Group No.	N	Treatment			
		Aβ	Drug 1 (mg/kg)	Drug 2 (mg/kg)	
Experiment 2 (Fig. 3)					
1	37–38	Vehicle	Saline	Saline	
2	36–37	Aβ	Saline	Saline	
3	18	Aβ	Donepezil (0.05)	Saline	
4	16–17	Aβ	Saline	Selegiline (1.0)	
5	18–19	Aβ	Donepezil (0.05)	Selegiline (1.0)	
Group No.	N	Treatment			
		Aβ	Drug 1 (mg/kg)	Drug 2 (mg/kg)	Antagonist (mg/kg)
Experiment 3 (Fig. 4)					
1	10	Vehicle	Saline	Saline	Saline
2	10	Aβ	Saline	Saline	Saline
3	12	Aβ	Donepezil (0.05)	Selegiline (1.0)	Saline
4	11–12	Aβ	Donepezil (0.05)	Selegiline (1.0)	Scopolamine (0.1)
5	10–12	Aβ	Donepezil (0.05)	Selegiline (1.0)	Haloperidol (0.1 or 0.03)

Three different approaches for drug administration were used: (1) selegiline and donepezil separately (Fig. 2); (2) co-administration of selegiline and donepezil (Fig. 3) and (3) scopolamine or haloperidol + co-administration of selegiline and donepezil. Aβ: $A\beta_{(25-35)}$.

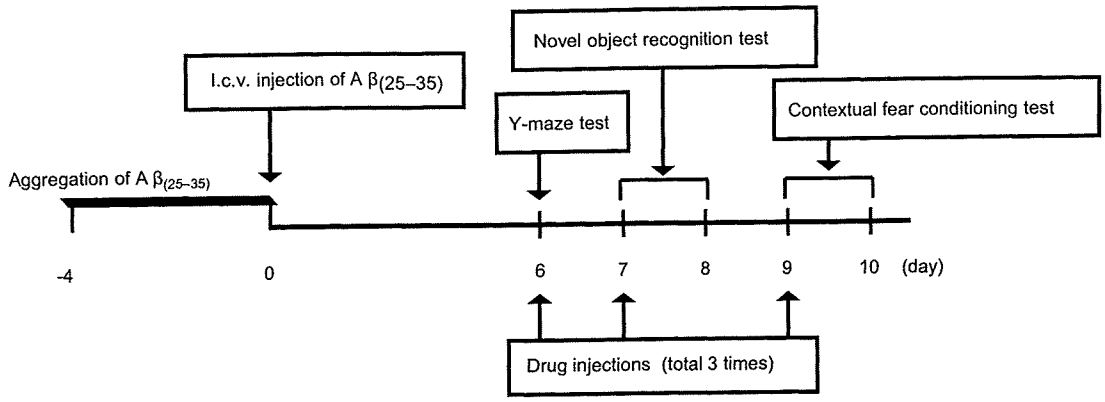

Fig. 1. Behavioral experimental schedule. i.c.v., intracerebroventricular.

2.3. $A\beta_{(25-35)}$-injected mouse model

I.c.v. injections of $A\beta_{(25-35)}$ were performed as described previously [20]. Briefly, a microsyringe with a 28-gauge stainless-steel needle bended an angle of 90° at the point 3 mm far from the tip of the needle was used for i.c.v. injections. Mice were anesthetized lightly with ether to be free from distress and stabilized with nose cone until i.c.v. injection had finished, and the needle was unilaterally inserted by hand 1 mm to the right of the midline point, equidistant from each eye, at an equal distance between the eyes and the ears, and perpendicular to the skull. The skulls of mice were not exposed to perform the injections to save the time and to be free from stress. The i.c.v. injection of $A\beta_{(25-35)}$ (3 nmol/3.2 μL) or vehicle (3.2 μL) was performed slowly over a period of 2 min. Mice exhibited normal behavior within 1 min after injection. We used vehicle as control of $A\beta_{(25-35)}$ in accordance with a previous report [20]. Neither insertion of the needle nor injection of the vehicle had any influence on survival, behavioral responses or cognitive function in consistent with a previous report [20]. The injection site of each mouse was confirmed by injecting Indian ink in preliminary experiments and dissecting the brain after all experiments.

2.4. Behavioral analysis

Previous reports have shown that acute exposure of aged $A\beta_{(25-35)}$ to hippocampal cultures induces apoptosis-mediated neuronal toxicity during 6 days incubation, and cognitive dysfunction in several learning and memory tests in mice [20]. The behavioral tests started on day 6 after $A\beta_{(25-35)}$ injection, and were carried out sequentially according to the experimental schedule shown in Fig. 1. The present study was conducted in a blind manner.

2.5. Spontaneous alternation in Y-maze test

The Y-maze task was carried out on day 6 after $A\beta_{(25-35)}$ injection, as described in previous reports [20]. The maze was made of black painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at the center of the apparatus, and allowed to move freely through the maze during an 8 min session. The number of arm entries was recorded visually. Alternation was defined as successive entry into the three arms, on overlapping triplet sets. The alternation behavior (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two), multiplied by 100.

2.6. Novel object recognition test

The task was carried out on days 7–8 after $A\beta_{(25-35)}$ injection, according to the method of [15], with a minor modification. The novel object recognition test procedure consisted of three sessions: habituation, training and retention. Each mouse was individually habituated to the box (30 cm × 30 cm × 35 cm high), with 10 min of exploration in the absence of objects on days 5 and 6 after the Y-maze test (habituation session). During the training session on day 7, two novel objects (e.g. object A and B) were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore in the box for 10 min. The objects were constructed from a golf ball, wooden column and 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 8) after the training session, in which one of the familiar objects (e.g. object A) used during training was replaced by a novel object (object C). The mouse was then allowed to explore freely for 10 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 the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function [A or B/(B + A) × 100 (%) in the training session, and B or C/(B + C) × 100 (%) in the retention session].

2.7. Contextual fear conditioning test

The contextual fear conditioning task was carried out on days 9–10 after $A\beta_{(25-35)}$ injection, according to a previous report [7], with a minor modification. For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in the conditioning cage (25 cm × 31 cm × 11 cm high) for 2 min and the freezing response was continuously measured by experimenter using a stopwatch on day 9. For training (conditioning phase), mice were placed in the conditioning cage, and then a 15-s tone (85 dB) was delivered as a conditioned stimulus. During the last 5 s of the tone stimulus, a foot shock of 0.8 mA was delivered as an unconditioned stimulus through a shock generator (NeuroScience idea, Osaka, Japan). This procedure was repeated four times at 15-s intervals. We excluded the animals that did not represent normal nociceptive response in the conditioning phase from the contextual fear conditioning test. One day after fear conditioning, mice were placed in the conditioning cage, and the freezing response was continuously measured for 2 min (retention session). The freezing response was defined as none of the mouse paws moving.

2.8. Statistical analysis

Results were expressed as means ± S.E.M. for $n = 6$–37. A SAS program (ver. 5.0, SAS Institute, Cary, NC, USA) was used to perform all analyses. Statistical difference among the experimental groups was tested using Kruskal–Wallis analysis for behavioral tests, and Dunnett's test was employed for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of single administration of selegiline or donepezil on $A\beta_{(25-35)}$-induced memory impairment

3.1.1. Y-maze task

$A\beta_{(25-35)}$-injected mice showed significantly reduced spontaneous alternation behavior compared to vehicle-injected mice [Kruskal–Wallis, $H = 36.65$, d.f. = 5, $P < 0.0001$; Dunnett, $P < 0.0001$] (Fig. 2A), indicating impairment of spatial working memory. When $A\beta_{(25-35)}$-injected mice were administered donepezil (0.1 mg/kg) or selegiline (3 mg/kg) alone, the alternation behavior was significantly increased compared to that of vehicle-treated $A\beta_{(25-35)}$-injected mice [Kruskal–Wallis, $H = 36.65$, d.f. = 5, $P < 0.0001$; Dunnett, $P = 0.0211$ (donepezil), $P = 0.0316$ (selegiline)] (Fig. 2A). There was no significant difference in the number of arm entries among any of the groups (Fig. 2B), indicating that all groups

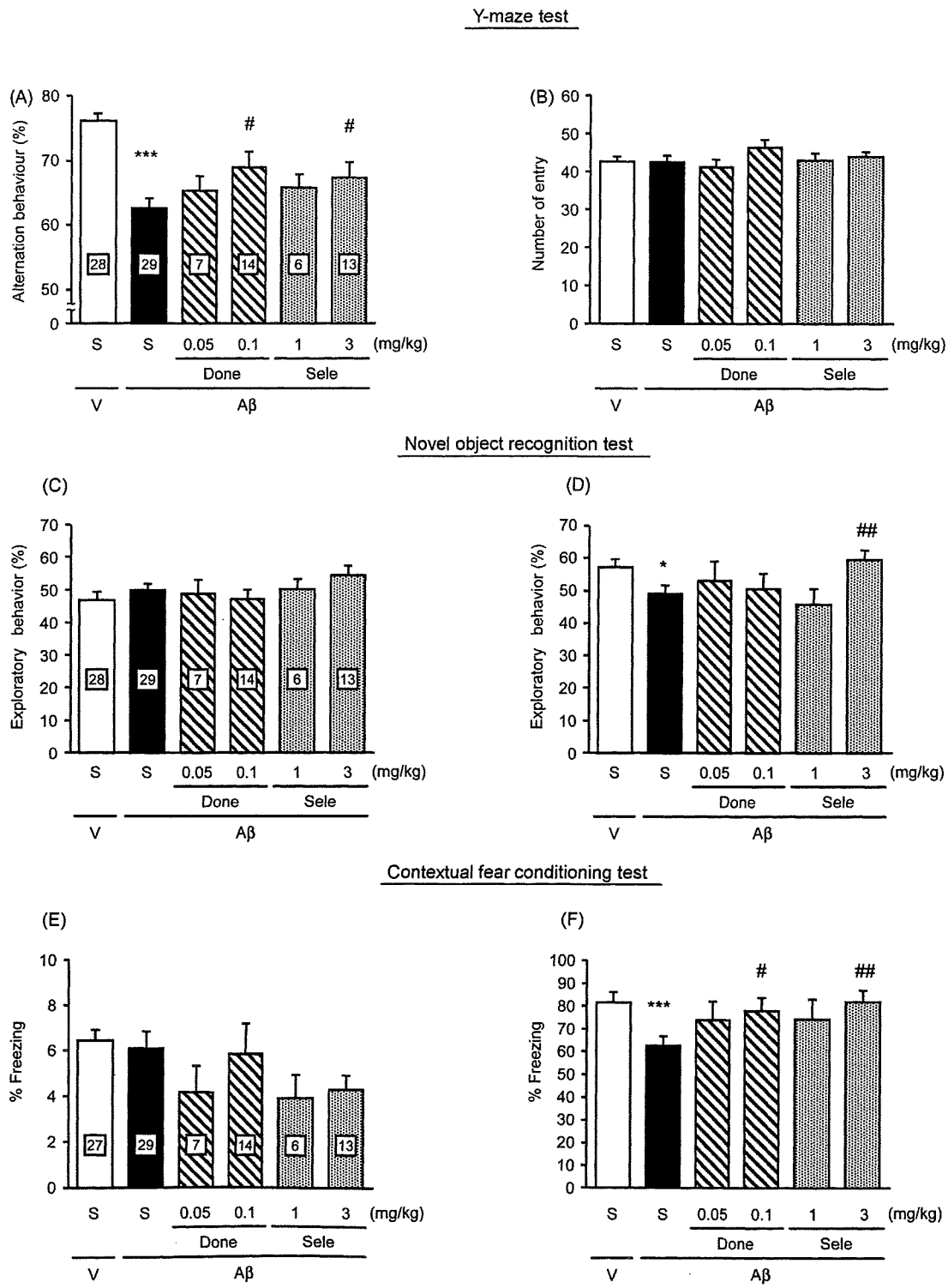

Fig. 2. Effects of administration of selegiline or donepezil alone on $A\beta_{(25-35)}$-induced memory impairment. Six, seven and nine days after i.c.v. injection of $A\beta_{(25-35)}$, the mice were subcutaneously administered donepezil (0.05 and 0.1 mg/kg), selegiline (1 and 3 mg/kg) or saline 30 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

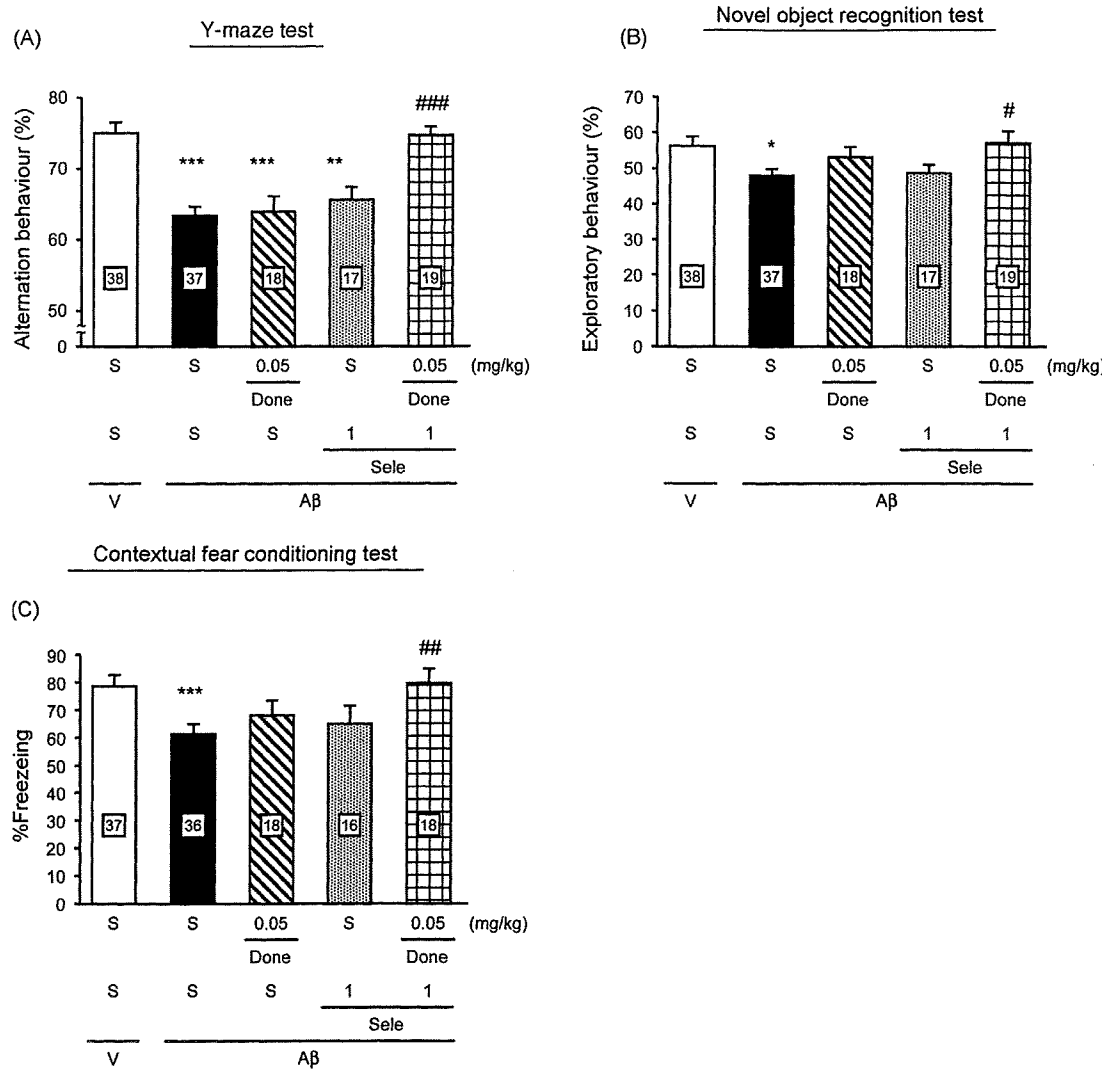

Fig. 3. Effects of co-administration of selegiline and donepezil on $A\beta_{(25-35)}$-induced memory impairment. Six, seven and nine days after i.c.v. injection of $A\beta_{(25-35)}$, the mice were subcutaneously administered donepezil (0.05 mg/kg), selegiline (1 mg/kg) or saline 30 min before each 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

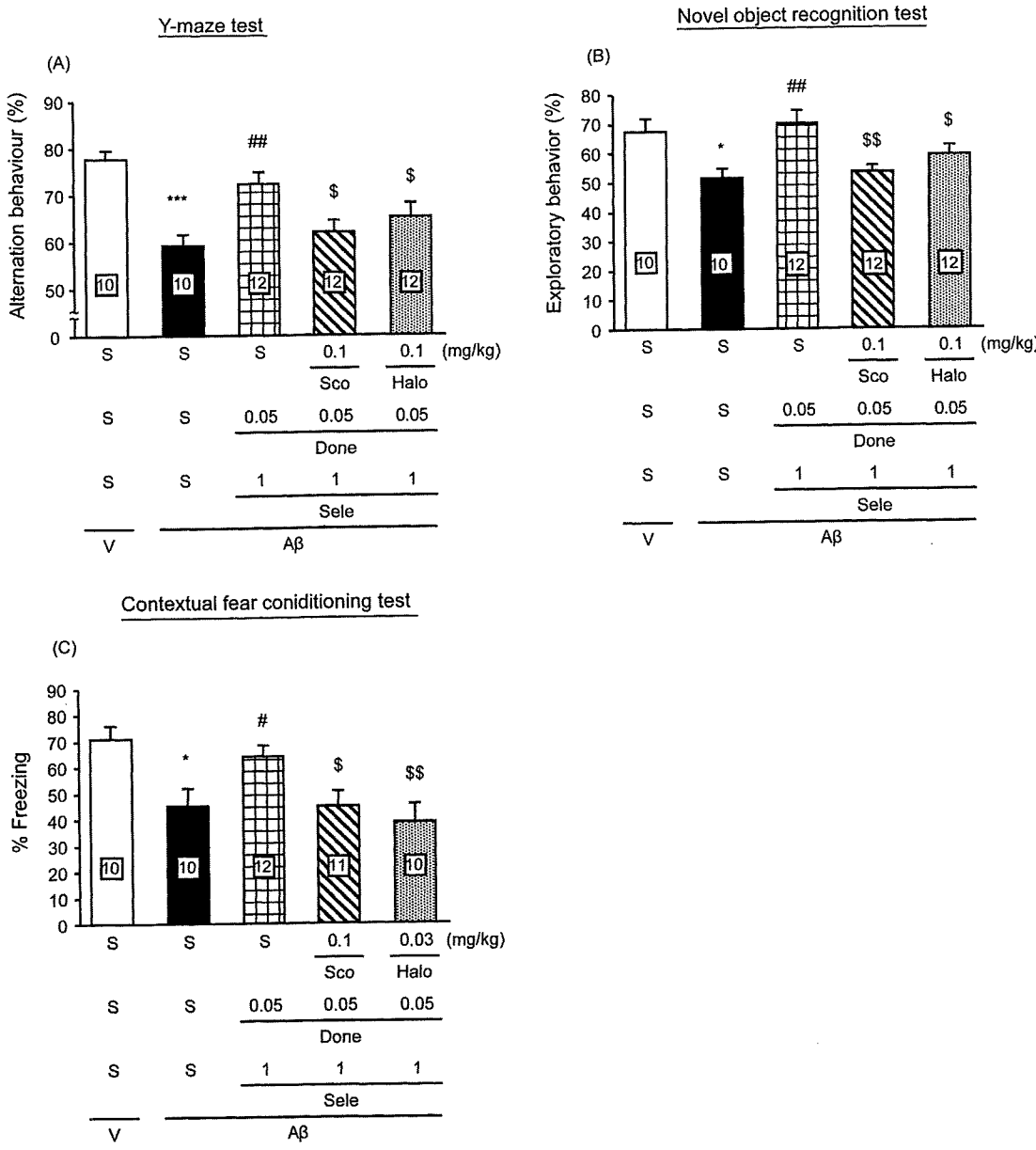

Fig. 4. Antagonistic effects of scopolamine and haloperidol against the synergistic cognition-improving effect of co-administration of selegiline and donepezil in $A\beta_{(25-35)}$-injected mice. Six, seven and nine days after i.c.v. injection of $A\beta_{(25-35)}$, the mice were subcutaneously administered 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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Review

Mouse model of relapse to the abuse of drugs: Procedural considerations and characterizations

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abstract

To identify genetic risk factors involved in relapse to the abuse of drugs in humans, it is essential for researchers to develop a reliable mouse model of relapse by extending well-established extinction-reinstatement procedures in rats. Because of technical difficulties such as the relatively short duration of catheter patency in mice, few reports are available on the characterization of extinction-reinstatement behavior in wild-type and genetically engineered mutant mice. In this review, efforts are made to describe practical considerations during the establishment of extinction-reinstatement procedure in mice, including drug-primed, cue-induced, and stress-triggered reinstatement of previously extinguished drug-seeking behavior. Next, attention will be given to some characteristics of extinction-reinstatement behavior in mice. The present review might provide a new impetus in the exploration of genetic risk factors involved in relapse to drug dependence/addiction in humans using extinction-reinstatement procedures in widely available mutant mice.

Contents

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