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RESEARCH PAPER

The anti-amnesic and neuroprotective effects of donepezil against amyloid $\beta_{25\text{-}35}$ peptide-induced toxicity in mice involve an interaction with the σ_1 receptor

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Background and purpose: The acetylcholinesterase inhibitor, donepezil, is also a high affinity σ_1 receptor agonist. We examined the involvement of σ_1 receptors in its anti-amnesic and neuroprotective properties against amyloid $\beta_{25\text{-}35}$ peptide-induced toxicity in mice.
Experimental approach: Mice were given an intracerebroventricular (i.c.v.) injection of A$\beta_{25\text{-}35}$ peptide (9 nmol) 7-9 days before being tested for spontaneous alternation and passive avoidance. Hippocampal lipid peroxidation was measured 7 days after A$\beta_{25\text{-}35}$ injection to evaluate oxidative stress. Donepezil, the σ_1 agonist PRE-084 or the cholinesterase (ChE) inhibitors tacrine, rivastigmine and galantamine were administered either 20 min before behavioural sessions to check their anti-amnesic effects, or 20 min before A$\beta_{25\text{-}35}$ injection, or 24 h after A$\beta_{25\text{-}35}$ injection and then once daily before behavioural sessions, to check their pre- and post-i.c.v. neuroprotective activity, respectively.
Key results: All the drugs tested were anti-amnesic, but only the effects of PRE-084 and donepezil were prevented by the σ_1 antagonist BD1047. Only PRE-084 and donepezil showed neuroprotection when administered pre i.c.v.; they blocked lipid peroxidation and learning deficits, effects inhibited by BD1047. Post i.c.v., PRE-084 and donepezil showed complete neuroprotection whereas the other ChE inhibitors showed partial effects. BD1047 blocked these effects of PRE-084, attenuated those of donepezil, but did not affect the partial effects of the other ChE inhibitors.
Conclusions and implications. The potent anti-amnesic and neuroprotective effects of donepezil against A$\beta_{25\text{-}35}$-induced toxicity involve both its cholinergic and σ_1 agonistic properties. This dual action may explain its sustained activity compared to other ChE inhibitors.
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Keywords: Donepezil; PRE-084; σ_1 receptor; cholinesterase inhibitor; amyloid β_{25-35} peptide; amnesia; neuroprotection; lipid peroxidation

Abbreviations: Aβ_{25-35}, amyloid β_{25-35} peptide; AChE, acetylcholinesterase; AD, Alzheimer's disease; APP, amyloid precursor protein; ChE, cholinesterase; CHP, cumene hydroperoxide; ER, endoplasmic reticulum; GFAP, glial fibrillary acidic protein; i.c.v., intracerebroventricularly; i.p., intraperitoneally; NMDA, *N*-methyl-D-aspartate; PLC, phospholipase C; PKC, protein kinase C

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of cognitive functions, leading to dementia and death. One of the main physiopathological features characterizing the disease is the presence of extracellular senile plaques, constituted mainly by accumulation of amyloid β (Aβ) proteins (Selkoe, 1991, 1994). Aβ proteins are generated from amyloid precursor protein (APP) by proteolytic cleavage and the main endogenous forms contain 40, 42 and 43 amino acids (Selkoe, 1991, 1994). Direct application of Aβ into primary neuronal cell cultures and other cell lines is highly toxic (Cotman and Anderson, 1995). Although the mechanism of the amyloid toxicity remains to be elucidated, its effects are dependent on the ability of the protein to aggregate into fibrillar amorphous structures (Pike *et al.*, 1993). Structure–activity studies using Aβ fragments revealed that the peptide bearing

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the 11 amino acids (25–35) retains the ability to self-aggregate and mediate the toxicity *in vitro* (Malouf, 1992; Mattson *et al*., 1992; Pike *et al*., 1995) and *in vivo* (Maurice *et al*., 1996). This toxicity involves oxidative stress induction by production of free radicals (Behl *et al*., 1992; Cafe *et al*., 1996; Pike *et al*., 1997), disruption of calcium homeostasis (Mattson *et al*., 1992, 1993), enhancement of excitotoxicity (Mattson *et al*., 1992) and apoptosis (Forloni *et al*., 1993). Indeed, the intracerebroventricular (i.c.v.) administration of $A\beta_{25–35}$ peptide into the rodent brain induced, within 1 or 2 weeks after administration, histological and biochemical changes, memory deficits (Flood *et al*., 1991; Kowall *et al*., 1991, 1992; Maurice *et al*., 1996) and oxidative stress (Stepanichev *et al*., 1998).

Cholinergic systems are very sensitive to amyloid toxicity and a well-characterized impairment of cholinergic neurons, within the nucleus basalis magnocellularis, or nucleus of Meynert in human, is involved in the rapid loss of learning and memory (Perry *et al*., 1978; Erme *et al*., 1992). Present strategies used to treat AD aim to improve or at least maintain central cholinergic functions, especially those involving cholinesterase (ChE) inhibitors. This allows a symptomatic alleviation of the cognitive deficits, but also putatively produces effective neuroprotection. Indeed, experimental evidence has shown that nicotinic receptor agonists are able to attenuate Aβ and glutamate toxicity in cultured neurons (Kihara *et al*., 1997; Zamani *et al*., 1997). Several ChE inhibitors, including tacrine, donepezil and galantamine, also attenuate $A\beta_{1–40}$- or $A\beta_{25–35}$-induced toxicity (Svensson and Nordberg, 1998; Arias *et al*., 2004; Kihara *et al*., 2004). This effect has been shown to involve an interaction with nicotinic receptors (Svensson and Nordberg, 1998) and more precisely the α7-nicotinic receptor subtype mediating activation of phosphatidylinositol 3-kinase (PI3K) (Kihara *et al*., 2004).

The different ChE inhibitors available at present exhibit different pharmacological profiles. In particular, whereas tacrine and rivastigmine inhibit acetylcholinesterase (AChE) and butyrylcholinesterase activities, donepezil is highly selective for AChE and galantamine acts as a weak ChE inhibitor (Ogura *et al*., 2000). Donepezil also interacts, within the same concentration range, with the σ_1 receptor (Kato *et al*., 1999), and this may contribute to its symptomatic and neuroprotective effects (Maurice *et al*., 2006; Meunier *et al*., 2006). The σ_1 receptor is an intracellular protein localized in the vicinity of the endoplasmic reticulum (ER). Its activation rapidly modulates the mobilization of inositol-1,2,4 trisphosphate receptor-gated calcium pools from the intracellular ER pools (Hayashi *et al*., 2000). This is of particular interest as Aβ toxicity has been shown to involve ER stress. Aβ proteins, and particularly $A\beta_{25–35}$ peptide, induced disturbances of the ER homeostasis and activation of stress-responsive genes, such as grp 78 or grp 94 (Yu *et al*., 1999; Ghribi *et al*., 2004). These genes are known to act as molecular chaperons regulating protein folding and translocation into the ER and protein secretion (Lee, 1992). Moreover, σ_1 receptor activation provokes its translocation, associated within lipid droplets to cholesterol and anchor proteins, from the ER towards plasma, mitochondria or nucleus membranes (Hayashi and Su, 2003). The σ_1 receptors may play a role in the compartmentalization and export of lipids to peripheries of cells (Hayashi and Su, 2003, 2005; Takebayashi *et al*., 2004). Lipid rafts have a role in a variety of cellular functions including vesicle transport, receptor clustering, internalization and coupling of receptors with the proteins involved in signal transduction (Simons and Ikonen, 1997). Activation of σ_1 receptors may, therefore, induce important effects on cell viability, differentiation and neuroprotection. Indeed, selective σ_1 receptor agonists are potent neuroprotective drugs, as observed in excitotoxicity models (for a review, see Maurice and Lockhart, 1997; Nakazawa *et al*., 1998) and recently against $A\beta_{25–35}$-induced toxicity in cortical neurons *in vitro* (Marrazzo *et al*., 2005).

In the present study, we examined the anti-amnesic and neuroprotective effects of donepezil, in comparison with the selective σ_1 receptor agonist PRE-084 and other ChE inhibitors, tacrine, rivastigmine and galantamine, against Aβ-induced toxicity *in vivo* in mice. Animals were administered aggregated $A\beta_{25–35}$ peptide, i.c.v., and the learning and memory impairments were checked after 1 week, using the spontaneous alternation and passive avoidance procedures. The level of lipid peroxidation, an index of oxidative stress, was measured in the hippocampus. Drugs were administered either 20 min before the behavioural procedures, that is, 1 week after $A\beta_{25–35}$, to examine the anti-amnesic effects; or 20 min before $A\beta_{25–35}$, that is, 1 week before the behavioural and biochemical measures, to examine the pre-i.c.v. protection; or 24 h after $A\beta_{25–35}$ and once-a-day for 1 week before the behavioural and biochemical measures, to examine the post-i.c.v. protection. In addition, the involvement of the σ_1 receptor in the pharmacological effects of the drugs was determined by pretreating the mice with the σ_1 receptor antagonist BD1047.

Methods

Animals

A total of 944 male Swiss mice, 1-month old and weighing 28–32 g, were used. They were purchased from the breeding centre of the Faculty of Pharmacy (Montpellier, France) and then kept in the animal facility building of the University of Montpellier II. Animals were housed in groups of 20 with access to food and water *ad libitum*, except during the experiments. They were kept in a temperature and humidity-controlled animal facility on a 12 h/12 h light/dark cycle (lights off at 1900 hours). Behavioral experiments were carried out between 0900 and 1400 hours, in a soundproof and air-regulated experimental room, to which mice were habituated to for at least 30 min. All animal procedures were conducted in strict adherence to the European Communities Council Directive of 24 November 1986 (86–609).

Experimental series

Initially, the amnesic effects of aggregated $A\beta_{25–35}$ peptide, administered i.c.v., were checked. Animals were administered increasing doses of $A\beta_{25–35}$ or scrambled $A\beta_{25–35}$ (i.c.v.) and learning and memory impairments were examined after 7 days. Then, as depicted in Figure 1, the anti-amnesic effects

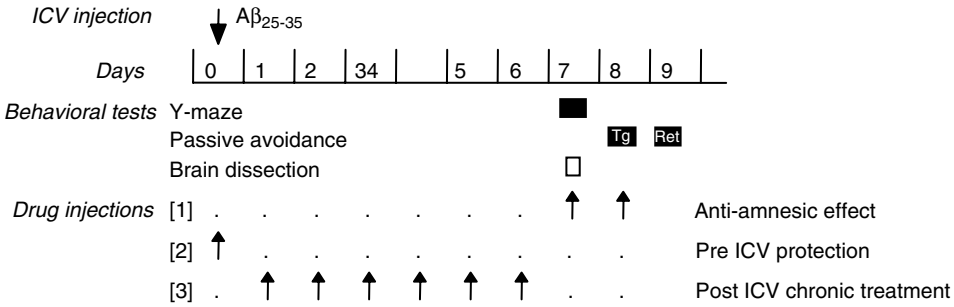

Figure 1 Experimental procedures. Animals were administered i.c.v. with amyloid Aβ_{25-35} peptide and examined for learning abilities after 7–9 days. Some animals were killed on day 7 for lipid peroxidation measurement in the hippocampus. Three drug administration schedules were used: (1) the anti-amnesic effects were tested by injecting drugs 20 min before behavioural testing, that is, 7–8 days after Aβ_{25-35} peptide; (2) the pre-i.c.v. neuroprotection was tested by injecting the drugs 20 min before Aβ_{25-35}-peptide, that is, 7–8 days before behavioural testing; (3) the post-i.c.v. neuroprotection was tested by injecting the drugs 24 h after Aβ_{25-35} peptide and once a day for 6–7 days, with the last injection at least 20 h before behavioural testing.

of the AChE inhibitors (donepezil, tacrine, rivastigmine, galantamine) or σ_1 receptor agonist (PRE-084) were examined by pre-test injections, 7–8 days after Aβ_{25-35} administration. The neuroprotective effects of each compound were examined using two protocols: (i) injecting the compound 20 min before Aβ_{25-35} administration, animals being tested after 7–9 days (pre-i.c.v. protection) or (ii) injections given 1 h after Aβ_{25-35} administration and repeatedly once a day for 6–7 days, animals being tested on day 7–9 (post-i.c.v. protection).

Spontaneous alternation performances

The spatial working memory was examined by measuring the spontaneous alternation behaviour of the mice in the Y-maze (Maurice *et al*., 1994, 1996, 1998). The maze was made of grey polyvinylchloride. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top and converged at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The series of arm entries, including possible returns into the same arm, were checked using an Apple IIe computer. An alternation was defined as entries into all three arms on consecutive occasions. The number of maximum alternations was, therefore, the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations/maximum alternations) $\times$ 100.

Step-through type passive avoidance response

The contextual long-term memory of the animals was assessed using the step-through passive avoidance procedure (Maurice *et al*., 2006; Meunier *et al*., 2006). The apparatus consisted of an illuminated compartment with white polyvinylchloride walls (15 $\times$ 20 $\times$ 15 cm high), a darkened compartment with black polyvinylchloride walls (15 $\times$ 20 $\times$ 15 cm high) and a grid floor. A guillotine door separated each compartment. A 60 W lamp positioned 40 cm above the apparatus lit the white compartment during the experimental period. Scrambled foot shocks (0.3 mA for 3 s) were delivered to the grid floor using a shock generator scrambler (Lafayette Instruments, Lafayette, MA, USA). The guillotine door was initially closed during the training session. Each mouse was placed into the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment and placed all its paws on the grid floor, the door was gently closed and the scrambled foot shock was delivered for 3 s. The step-through latency, that is, the latency spent to enter the dark compartment, and the number of vocalizations was recorded. The number of vocalizations did not differ between the groups, indicating that shock sensitivity was unaffected by the i.c.v. or i.p. treatments (data not shown). The retention test was carried out 24 h after training. Each mouse was placed again into the white compartment. After 5 s, the door was raised. The step-through latency was recorded up to 300 s. Animals entered the darkened compartment or were gently pushed into it and the escape latency, that is, the time spent to return into the white compartment, was also measured up to 300 s. The two parameters were measured although they do not rely on similar mechanisms. The step-through latency involves contextual reinforced stimuli and is a direct measure of passive avoidance behaviour. The escape latency relies on supplementary sensory information, the contact with the grid floor that *per se* activates specific retrieval pathways, but includes conflicting information: the absence of an electric shock in this compartment during the retention session. This parameter is more reliably measured in active avoidance paradigms and may, in our case, lead to less-sensitive differences between the groups.

Lipid peroxidation measures (modified ferrous oxidation-xylenol orange (FOX) assay)

The quantification of lipid peroxidation in tissue extracts is based on Fe(III)xylenol orange complex formation according to Hermes-Lima *et al*. (1995). Mice were killed by decapitation and brains were rapidly removed, weighed and kept in liquid nitrogen until assayed. After being thawed, homogenates were homogenized in cold methanol (1/5 w/v), centrifuged at 1000 *g* for 5 min and the supernatant was placed in an eppendorf tube. The reaction volume was determined in preliminary experiments. Increasing homo-

genate volumes (2–100 μl) prepared from control Swiss animals were sequentially added to $FeSO_4$ 1 mM, H_2SO_4 0.25 M, xylenol orange 1 mM and incubated overnight in a dark chamber at room temperature. Absorbance was measured at 580 nm, and the reaction volume was determined for an absorbance value of 0.7. Then, the reaction volume of each homogenate was added to $FeSO_4$ 1 mM, H_2SO_4 0.25 M, xylenol orange 1 mM and incubated for 30 min at room temperature. After the absorbance had been read at 580 nm ($A_{580}1$), 5 μl of cumene hydroperoxide (CHP) 1 mM was added to the sample and it was incubated for 30 min at room temperature, to determine the maximal oxidation level. The absorbance was measured at 580 nm ($A_{580}2$). The level of lipid peroxidation was determined as CHP equivalents according to: $CHPE = A_{580}1/A_{580}2 \times (CHP\ (nmol))$ and expressed as CHP equivalents per wet weight of tissue.

Drugs

Donepezil hydrochloride was obtained from Eisai Co. Ltd (Tokyo, Japan). 2-(4-Morpholino)ethyl 1-phenylcyclohexane-1-carboxylate (PRE-084) was provided by Dr Tsung-Ping Su (IRP, NIDA, NIH, Baltimore, MD, USA) and *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine (BD1047) by Dr Wayne D Bowen (Brown University, Providence, RI, USA). Rivastigmine tartrate was from Novartis (Basel, Switzerland). 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride (tacrine) and galantamine hydrobromide and other chemical reagents, including xylenol orange and cumene hydroperoxide, were from Sigma-Aldrich (St-Quentin-Fallavier, France). Doses refer to the salt form. The range of doses of the drug used were selected on the basis of those used in previous studies examining the anti-amnesic or neuroprotective effects of donepezil, PRE-084 or AChE inhibitors in pharmacological, hypoxic or $A\beta_{25-35}$ models of amnesia (Maurice *et al*., 1998, 2006; Meunier *et al*., 2006). Compounds were injected intraperitoneally (i.p.) in a volume of 100 μl per 20 g of body weight. For antagonism studies, the σ_1 receptor antagonist was administered before each drug and control animals received only one injection of vehicle (saline solution), as in numerous previous studies no differences in behavioural responses were observed after one or two injections of saline (i.p., data not shown). The amyloid β_{25-35} peptide ($A\beta_{25-35}$, SC489C) and scrambled $A\beta_{25-35}$ peptide (SC492) were from NeoMPS (Strasbourg, France). They were dissolved in sterile bidistilled water at a concentration of 3 mg ml^{-1} and stored at −20°C until use. Before being injected, peptides were aggregated by incubation at 3 mg ml^{-1} in sterile bidistilled water at 37°C for 4 days. They were administered intracerebroventricularly (i.c.v.), according to the method of Haley and McCormick (1957), in a final volume of 3 μl per mouse, as previously described (Maurice *et al*., 1996, 1998).

Statistical analyses

Y-maze test data and lipid peroxidation measures were expressed as mean value ± s.e.m. and analysed using Dunnett's or Newman–Keuls' multiple comparisons test after a one-way analysis of variance (ANOVA, *F*-values). Passive avoidance latencies did not show a normal distribution, as a cutoff time was set. They were thus expressed as median value and interquartile range and analysed using the Kruskal–Wallis non-parametric ANOVA (*H*-values), group comparisons being made with Dunn's non-parametric multiple comparisons test. The level of statistical significance was $P<0.05$.

Results

Amnesic effects of $A\beta_{25-35}$ peptide administration in mice

$A\beta_{25-35}$ peptide, administered i.c.v., provoked marked learning impairments in mice after 1 week, as shown in Figure 2. $A\beta_{25-35}$ peptide, 1, 3 or 9 nmol per mouse i.c.v. dose-dependently, diminished the spontaneous alternation performance in the Y-maze (Figure 2a), without affecting significantly the locomotor response (Figure 2b). In parallel,

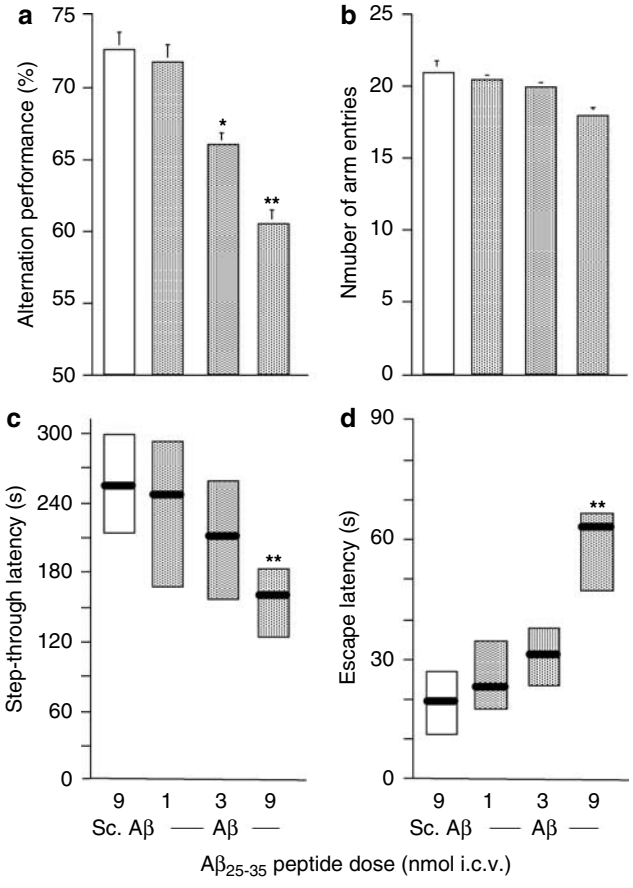

Figure 2 Dose–response effect of $A\beta_{25-35}$ peptide in mice. The $A\beta_{25-35}$ peptide (Aβ, 1, 3 or 9 nmol) or the scrambled $A\beta_{25-35}$ peptide (Sc.Aβ, 9 nmol) was administered i.c.v. The spontaneous alternation behaviour was examined on day 7: (**a**) spontaneous alternation percentage; (**b**) total number of arm entries. The step-through passive avoidance training was carried out on day 8 and retention examined on day 9: (**c**) step-through latency; (**d**) escape latency. The number of animals per group was $n=10$. One-way ANOVA: $F_{(3,36)}=7.24$, $P<0.001$ in (**a**), $F_{(3,36)}=2.06$, $P>0.05$ in (**b**). Kruskal–Wallis ANOVA: $H=9.96$, $P<0.05$ in (**c**), $H=12.91$, $P<0.01$ in (**d**). *$P<0.05$, **$P<0.01$ vs Sc.Aβ-treated group, Dunnett's test in (**a**), Dunn's test in (**c**, **d**).

passive avoidance deficits were observed, both the step-through latency (assessed using the entry into the dark compartment) and the escape latency (measured by the return in to the white compartment) showed dose-dependent diminutions as compared to scrambled Aβ_{25-35}-treated mice (Figure 2c and d).

Anti-amnesic effects of donepezil and other drugs against Aβ_{25-35} peptide-induced amnesia in mice

Donepezil, PRE-084 and the ChE inhibitors tacrine, rivastigmine and galantamine were injected (i.p.) 20 min before the Y-maze test session or 20 min before the passive avoidance training session, that is, 7 or 8 days, respectively,

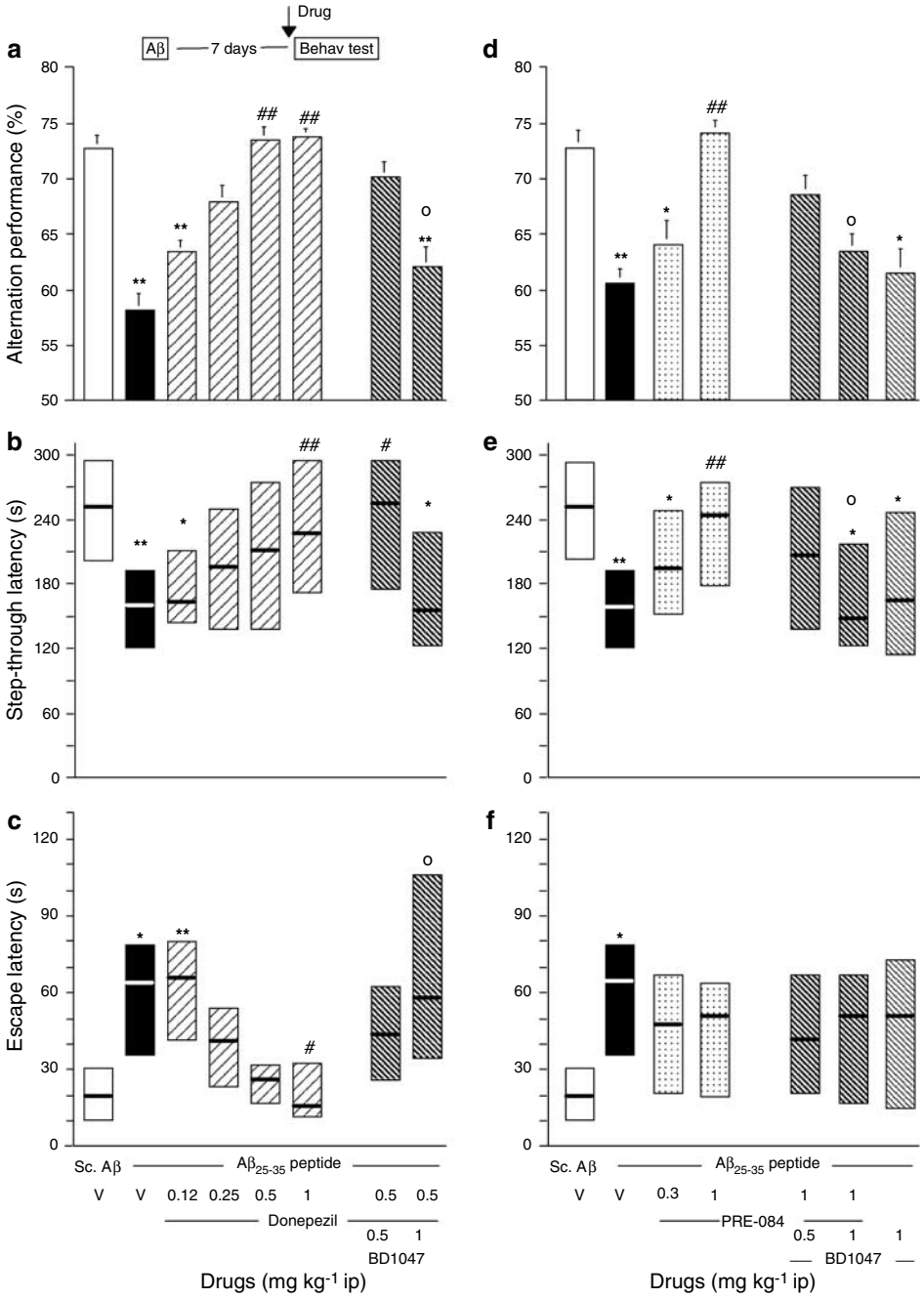

Figure 3 Beneficial effects of donepezil (**a–c**) and PRE-084 (**d–f**) against Aβ_{25-35} peptide-induced amnesia in mice. Mice were administered i.c.v. with Aβ_{25-35} peptide (9 nmol) or scrambled Aβ_{25-35} peptide (Sc.Aβ, 9 nmol). On day 7, animals were examined for spontaneous alternation performances (**a** and **d**). On day 8, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (**b** and **e**) and escape latency (**c** and **f**). Vehicle solution (saline, V), donepezil (0.12–1 mg kg^{-1}), PRE-084 (0.3–1 mg kg^{-1}) and/or BD1047 (0.5–1 mg kg^{-1}) were administered i.p. 20 min before the test (see insert). $n=10$ per group. $F_{(7,79)}=4.26$, $P<0.001$ in (**a**); $H=16.01$, $P<0.05$ in (**b**); $H=32.34$, $P<0.0001$ in (**c**); $F_{(6,63)}=4.15$, $P<0.01$ in (**d**); $H=15.87$, $P<0.05$ in (**e**); $H=13.08$, $P<0.05$ in (**f**). $^{*}P<0.05$, $^{**}P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs the V-treated Aβ_{23-35}-administered group; $^{\circ}P<0.05$ vs the donepezil (0.5 mg kg^{-1})- or PRE-084 (1 mg kg^{-1})-treated Aβ_{23-35}-administered group; Dunnett's test in (**a**, **d**), Dunn's test in (**b**, **c**, **e**, **f**).

after Aβ_{25-35} administration in mice. Donepezil was tested in the dose range 0.12–1 mg kg^{-1}. As shown in Figure 3a, it significantly reversed the alternation deficits at 0.5 and 1 mg kg^{-1}. The effect of donepezil at 0.5 mg kg^{-1} was blocked by pretreatment with BD1047 (Figure 3a). Donepezil also dose-dependently attenuated the Aβ_{25-35} peptide-induced passive avoidance deficits, both in terms of step-through latency (Figure 3b) and escape latency (Figure 3c). The effect of donepezil, 0.5 mg kg^{-1}, was attenuated, for the step-through latency parameter, or significantly antagonized, for the escape latency parameter, by pretreatment with the highest dose of BD1047 (Figure 3b and c).

PRE-084, the reference σ_1 receptor agonist, was administered at 0.3 and 1 mg kg^{-1}. At the highest dose tested, the compound reversed the Aβ_{25-35}-induced alternation deficits (Figure 3d). This effect of PRE-084 was blocked by the σ_1 receptor antagonist BD1047, which had no effect by itself (Figure 3d). In the passive avoidance test, the highest dose of PRE-084 significantly reversed the Aβ_{25-35}-induced decrease in step-through latency (Figure 3e) and nonsignificantly attenuated the Aβ_{25-35}-induced increase in escape latency (Figure 3f). Pretreatment with BD1047 significantly blocked the effects of PRE-084 on step-through latency (Figure 3e), but not its effects on escape latency (Figure 3f).

Tacrine, rivastigmine and galantamine were also tested. All three ChE inhibitors highly significantly reversed the Aβ_{25-35}-induced alternation deficits, at the highest doses tested, 1 mg kg^{-1}. Interestingly, the effects were unaffected by BD1047 pretreatment, even at the highest dose of this antagonist (Figure 4a). The passive avoidance procedure led to similar results, but with lower levels of significance. At 1 mg kg^{-1}, the three compounds attenuated the Aβ_{25-35}-induced decrease in step-through latency, but only the rivastigmine effect reached significance (Figure 4b). The effects were not affected by BD1047. Differences in terms of escape latency were very mild. As only the vehicle-treated Aβ_{25-35} group showed a significant increase of latency as compared with the vehicle-treated scrambled Aβ_{25-35} control group, it is probable that the drug treatments tended to attenuate the Aβ_{25-35}-induced deficits (Figure 4c).

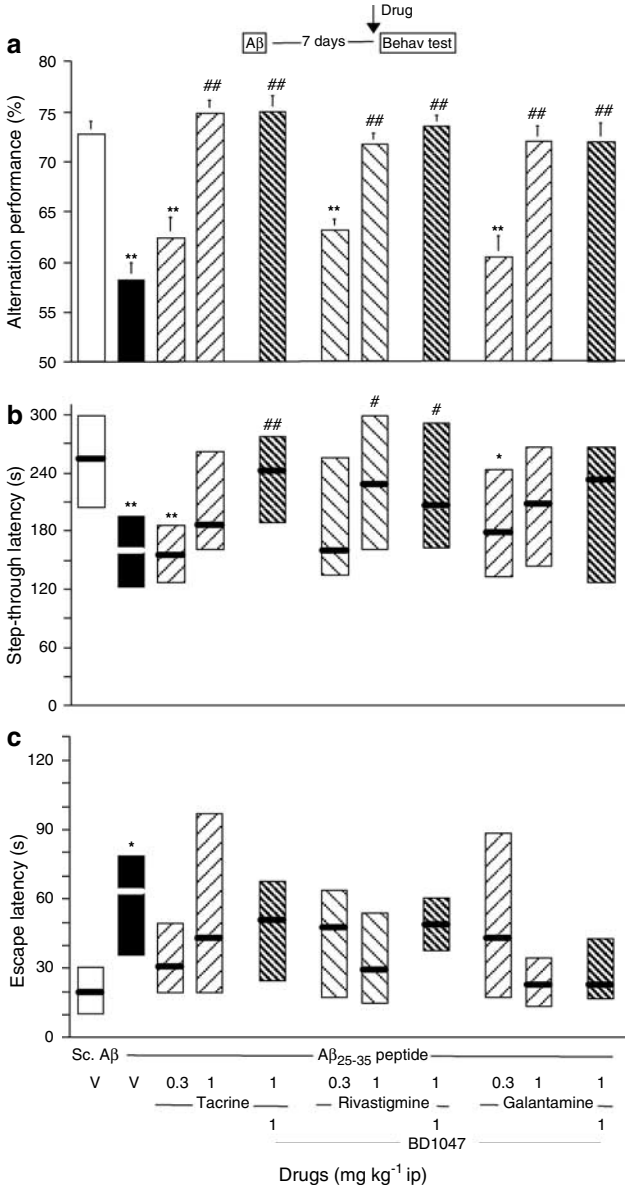

Figure 4 Beneficial effects of tacrine, rivastigmine and galantamine against Aβ_{25-35} peptide-induced amnesia in mice. Mice were administered i.c.v. with Aβ_{25-35} peptide (9 nmol) or scrambled Aβ_{25-35} peptide (Sc.Aβ, 9 nmol). After 7 days, animals were examined for spontaneous alternation performances (**a**). On day 8, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (**b**) and escape latency (**c**). Vehicle solution (saline, V), tacrine (0.3–1 mg kg^{-1}), rivastigmine (0.3–1 mg kg^{-1}), galantamine (0.3–1 mg kg^{-1}) and/or BD1047 (1 mg kg^{-1}) were administered i.p. 20 min before the test. $n=10$ per group. $F_{(10,99)}=4.38$, $P<0.0001$ in (**a**); $H=27.09$, $P<0.05$ in (**b**); $H=25.74$, $P<0.05$ in (**c**). *$P<0.05$, **$P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs the V-treated Aβ_{25-35}-administered group; Dunnett's test in (**a**), Dunn's test in (**b**, **c**).

Neuroprotective effects of donepezil and other drugs against Aβ_{25-35} peptide-induced toxicity after pre-i.c.v. administration

Compounds were injected i.p. 20 min before the peptide and the behavioural observations were initiated 7 days (Y-maze test session) or 8 days (passive avoidance training) later. The lipid-peroxidized products were measured in mice that did not experience the behavioural tests, 7 days after Aβ_{25-35} administration. Donepezil, when tested at 0.12–1 mg kg^{-1} i.p., significantly reversed the alternation deficits at 0.5 and 1 mg kg^{-1} (Figure 5a). The effect of donepezil at 0.5 mg kg^{-1} was completely blocked by pretreatment with BD1047 (Figure 5a). Donepezil also dose-dependently attenuated the Aβ_{25-35} peptide-induced passive avoidance deficits, both in terms of step-through latency (Figure 5b) and escape latency (Figure 5c). The effect of donepezil, 0.5 mg kg^{-1}, was unaffected, for the step-through latency parameter, but significantly blocked, for the escape latency parameter, by pretreatment with the highest dose of BD1047 (Figure 5b and c).

PRE-084 was again administered at 0.3 and 1 mg kg^{-1}. At the highest dose tested, it prevented the Aβ_{25-35}-induced alternation deficits (Figure 5d). This effect of PRE-084 was

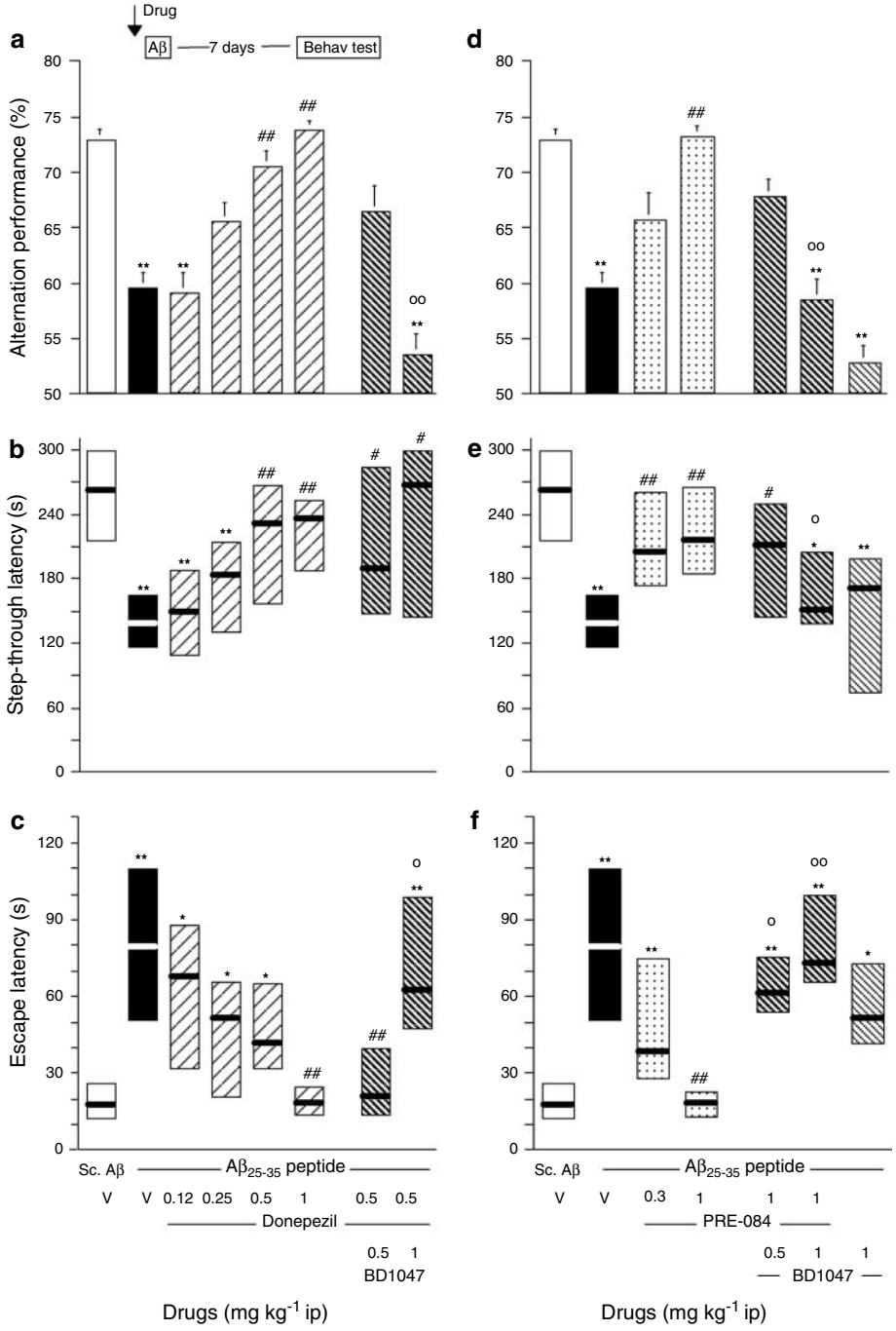

Figure 5 Pre i.c.v. neuroprotective effects of donepezil (**a**–**c**) and PRE-084 (**d**–**f**) in Aβ_{25-35} peptide-injected mice. Mice were administered, i.p., either vehicle solution (saline, V), donepezil (0.12–1 mg kg^{-1}), PRE-084 (0.3–3 mg kg^{-1}) and/or BD1047 (0.5–3 mg kg^{-1}) 20 min before being administered i.c.v. with Aβ_{25-35}-peptide (9 nmol) (see insert). Control animals received scrambled Aβ_{25-35} peptide (Sc.Aβ, 9 nmol). After 7 days, animals were examined for spontaneous alternation performances (**a** and **d**). On day 8 after peptide injection, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (**b** and **e**) and escape latency (**c** and **f**). $n=10$–12 per group. $F_{(7,77)}=5.93$, $P<0.0001$ in (**a**); $H=27.60$, $P<0.001$ in (**b**); $H=31.13$, $P<0.0001$ in (**c**); $F_{(6,67)}=6.28$, $P<0.0001$ in (**d**); $H=22.89$, $P<0.001$ in (**e**); $H=31.31$, $P<0.0001$ in (**f**). $^{*}P<0.05$, $^{**}P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs the V-treated Aβ_{25-35}-administered group; $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$ vs the donepezil (0.5 mg kg^{-1})- or PRE-084 (1 mg kg^{-1})-treated Aβ_{25-35}-administered group; Dunnett's test in (**a**, **d**), Dunn's test in (**b**, **c**, **e**, **f**).

dose-dependently blocked by BD1047, the compound being devoid of effect by itself (Figure 5d). In the passive avoidance test, PRE-084 significantly reversed the Aβ_{25-35}-induced decrease in step-through latency (Figure 5e) and increase in escape latency (Figure 5f). Pretreatment with BD1047 significantly blocked the PRE-084 effects on both parameters (Figure 5e and f).

Tacrine, rivastigmine and galantamine, when injected before the Aβ_{25-35} peptide, failed to affect the resulting alternation deficits (Figure 6a). In the passive avoidance

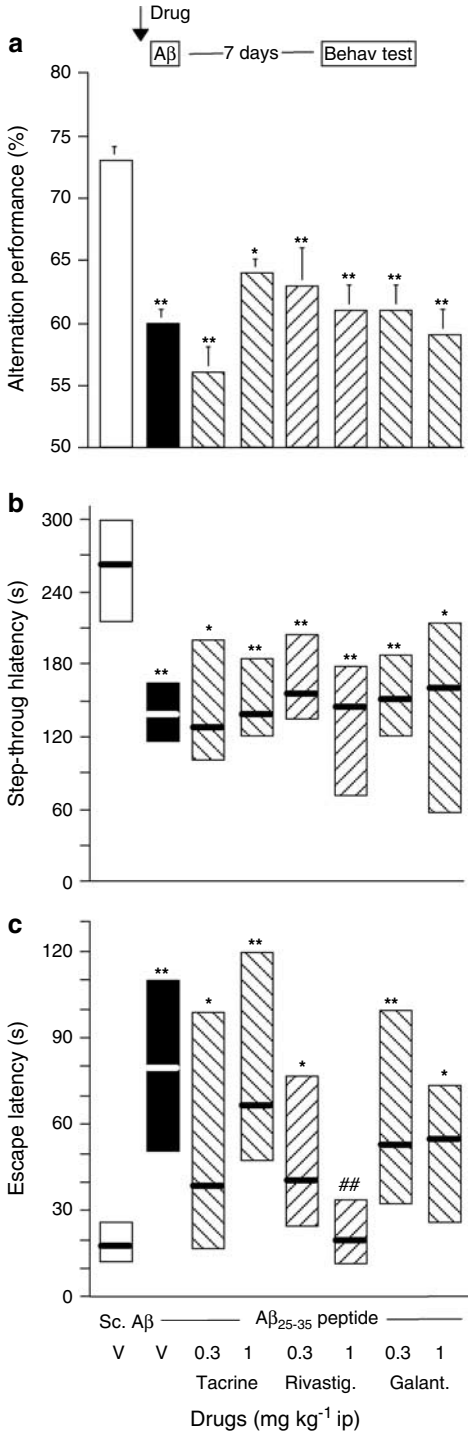

Figure 6 Pre-i.c.v. neuroprotective effects of tacrine, rivastigmine and galantamine in Aβ_{25-35} peptide-injected mice. Mice were administered, i.p., either vehicle solution (saline, V), tacrine (0.3–1 mg kg^{-1}), rivastigmine (0.3–1 mg kg^{-1}) or galantamine (0.3–1 mg kg^{-1}) 20 min before being administered i.c.v. with Aβ_{25-35} peptide (9 nmol) (see insert). Control animals received scrambled Aβ_{25-35} peptide (Sc.Aβ, 9 nmol). After 7 days, animals were examined for spontaneous alternation performances (**a**). On day 8 after peptide injection, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (**b**) and escape latency (**c**). $n=10$–12 per group. $F_{(7,76)}=2.94$, $P<0.05$ in (**a**); $H=16.63$, $P<0.05$ in (**b**); $H=28.71$, $P<0.001$ in (**c**). *$P<0.05$, **$P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#\#}P<0.01$ vs the V-treated Aβ_{25-35}-administered group; Dunnett's test in (**a**), Dunn's test in (**b**, **c**).

procedure, analysis of the step-through latency led to similar results (Figure 6b). Rivastigmine, but not tacrine or galantamine, allowed a significant amelioration of the escape latency, at the highest dose tested (Figure 6c). Nevertheless, the ChE inhibitors were overall poorly active.

The neuroprotective effects of the drugs were also tested on the oxidative stress response induced by Aβ_{25-35} peptide (Figure 7). Aβ_{25-35} peptide augmented the levels of lipid-peroxidized products in the mouse hippocampus 7 days after injection (+83%). Pre-administration of donepezil (0.5 mg kg^{-1}, i.c.v.) or PRE-084 (1 mg kg^{-1}, i.c.v.), at their behaviourally active doses, resulted in a complete blockade of the lipid peroxidation augmentation (Figure 7a). Pre-administration of BD1047 significantly blocked the donepezil and PRE-084 effects, the σ_1 receptor antagonist being without effect by itself (Figure 7a). Tacrine, rivastigmine or galantamine, tested at 1 mg kg^{-1}, failed to show any effect (Figure 7b).

Neuroprotective effects of donepezil and other drugs against Aβ_{25-35} peptide-induced toxicity post-i.c.v. administration

We finally examined the neuroprotective effects of the compounds injected 24 h after the peptide and once a day for 6 days (before the Y-maze test session) or 7 days (before the passive avoidance training). The lipid-peroxidized products were measured in mice that did not experience the behavioural tests, 7 days after Aβ_{25-35} administration. Donepezil, when tested at 0.12–1 mg kg^{-1} i.p., significantly reversed the alternation deficits at 0.5 and 1 mg kg^{-1} (Figure 8a). The effect of donepezil at 0.5 mg kg^{-1} was partially, but significantly attenuated by pretreatment with BD1047 (Figure 8a). Donepezil also dose-dependently attenuated the Aβ_{25-35} peptide-induced decrease in step-through latency (Figure 8b) and increase in escape latency (Figure 8c) in the passive avoidance procedure. The effects of donepezil, 0.5 mg kg^{-1}, on both parameters, were blocked by pretreatment with the highest dose of BD1047 (Figure 8b and c).

PRE-084 dose-dependently prevented the Aβ_{25-35}-induced alternation deficits (Figure 8d) and passive avoidance deficits (Figure 8e and f). These effects of PRE-084 were dose-dependently blocked by BD1047, the compound being devoid of effect by itself (Figure 8d–f).

Tacrine, rivastigmine and galantamine were also effective in blocking the Aβ_{25-35}-induced toxicity when administered post i.c.v. Tacrine and galantamine, but not rivastigmine, attenuated the Aβ_{25-35}-induced spontaneous alternation deficits (Figure 9a). The effects were unaffected by BD1047 (Figure 9a). Tacrine, rivastigmine and galantamine ameliorated the passive avoidance deficits induced by Aβ_{25-35}, both in terms of step-through latency (Figure 9b) or escape latency (Figure 9c). Pretreatment with BD1047 failed to affect the effects of these drugs significantly. Interestingly, with this administration procedure, galantamine was more potent at 0.3 mg kg^{-1} than at 1 mg kg^{-1} (Figure 9a–c).

The neuroprotective effects of the drugs post i.c.v. administration were also tested on the oxidative stress response induced by Aβ_{25-35} peptide injection (Figure 10). Donepezil (0.5 mg kg^{-1}) or PRE-084 (1 mg kg^{-1}), at their behaviourally active doses, significantly attenuated the

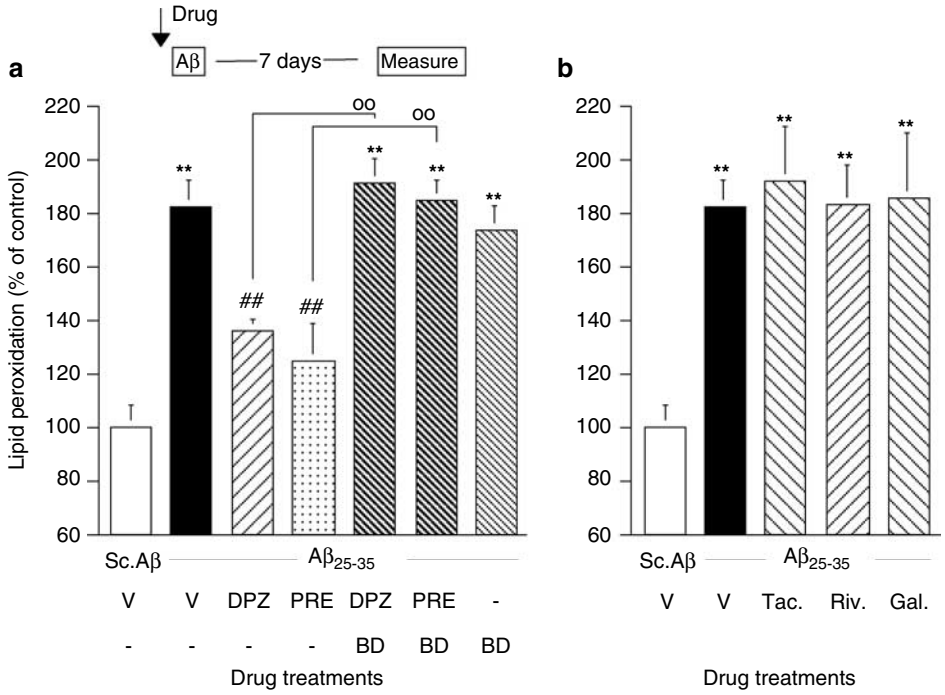

Figure 7 Pre-i.c.v. neuroprotective effects of the compounds, assessed using measures of the lipid peroxidation levels in the hippocampus of Aβ_{25-35} peptide-injected mice. Mice were administered, i.p., either vehicle solution (saline, V), donepezil (0.5 mg kg^{-1}), PRE-084 (1 mg kg^{-1}) and/or BD1047 (1 mg kg^{-1}) (**a**); or tacrine (1 mg kg^{-1}), rivastigmine (1 mg kg^{-1}), galantamine (1 mg kg^{-1}) and/or BD1047 (1 mg kg^{-1}) (**b**) 20 min before being administered i.c.v. with Aβ_{25-35} peptide (9 nmol) (see insert). Lipid peroxidation levels were measured on day 7. $n=6$–8 per group. $F_{(6,39)}=16.21$, $P<0.0001$ in (**a**); $F_{(4,29)}=7.54$, $P<0.001$ in (**b**). $^{**}P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#\#}P<0.01$ vs the V-treated Aβ_{25-35}-administered group, oo $P<0.01$ vs the donepezil (0.5 mg kg^{-1})- or PRE-084 (1 mg kg^{-1})-treated Aβ_{25-35}-administered group; Newman–Keuls' test.

Aβ_{25-35} peptide-induced lipid peroxidation (−80 and −70%, respectively, Figure 10a). Preadministration of BD1047 decreased this effect of donepezil (40% reduction), but not significantly, whereas the effect of PRE-084 was completely and significantly blocked. The σ_1 receptor antagonist was devoid of effect by itself (Figure 10a). Tacrine, rivastigmine and galatamine, tested at 1 mg kg^{-1}, also showed some efficacy in preventing the increase in lipid-peroxidized products formation and this was significant for rivastigmine and galantamine (Figure 10b). The BD1047 pretreatment did not affect these drug effects (Figure 10b).

Discussion

The Aβ protein, the major component of neuritic plaques found in AD, has been implicated as a potential contributor to the disease's progressive neuropathology. After *in vitro* exposure to aggregates of synthetic Aβ peptide, the neurites of rat-cultured hippocampal neurons adopt a dystrophic appearance. The morphological changes in the neurites include beading, fragmentation, terminal swelling and tortuous growth patterns. The degenerative changes are similar to those observed in neurites associated with neuritic plaques, suggesting that Aβ may induce the neuritic abnormalities of AD neuropathology (Pike *et al*., 1991). The truncated Aβ_{25-35} fragment includes extracellular and transmembrane residues that have been reported to represent an active region of Aβ (Yankner *et al*., 1990). Structure–activity studies revealed that peptides containing the highly hydrophobic (29–35) region formed stable aggregations (Pike *et al*., 1993). Numerous *in vitro* studies have provided evidence that Aβ_{25-35} induces neuronal death by necrosis or apoptosis (Behl *et al*., 1994; Ivins *et al*., 1999), resulting from exposure to the peptide. Moreover, a correlation between the ability of Aβ peptide fragments to self-aggregate and their neurotoxicity was observed in long-term neuronal cultures, consistent with the hypothesis that Aβ protein aggregation contributes to neurodegeneration in AD (Pike *et al*., 1993).

Two nontransgenic rodent models of AD have been studied in the past 10 years to analyse the molecular, morphological and behavioural consequences of amyloid toxicity *in vivo*, namely the infusion of either A$\beta_{1-40/42}$ protein or Aβ_{25-35} peptide. A rapid review of the literature revealed the parallels between these two strategies that relied on the use of the endogenous amyloid protein or a synthetic neurotoxic peptide. After 14 days of an i.c.v. infusion of Aβ_{1-40} protein, immunohistochemical accumulation of the protein was observed throughout the hippocampus and cerebral cortex (Nitta *et al*., 1994). The immunolabelling of Aβ_{25-35}, after i.c.v. injection, has not yet been reported. However, Congo red-stained deposits have been observed throughout the hippocampal formation and cortex (Maurice *et al*., 1996), with a similar morphological aspect as that observed for the Aβ_{1-40} protein (Giovannelli *et al*., 1998). Indeed, Kowall *et al*. (1992) initially reported that both Aβ_{1-40} and Aβ_{25-35} injected into the rat cortex produced localized necrosis at the injection site surrounded by a zone

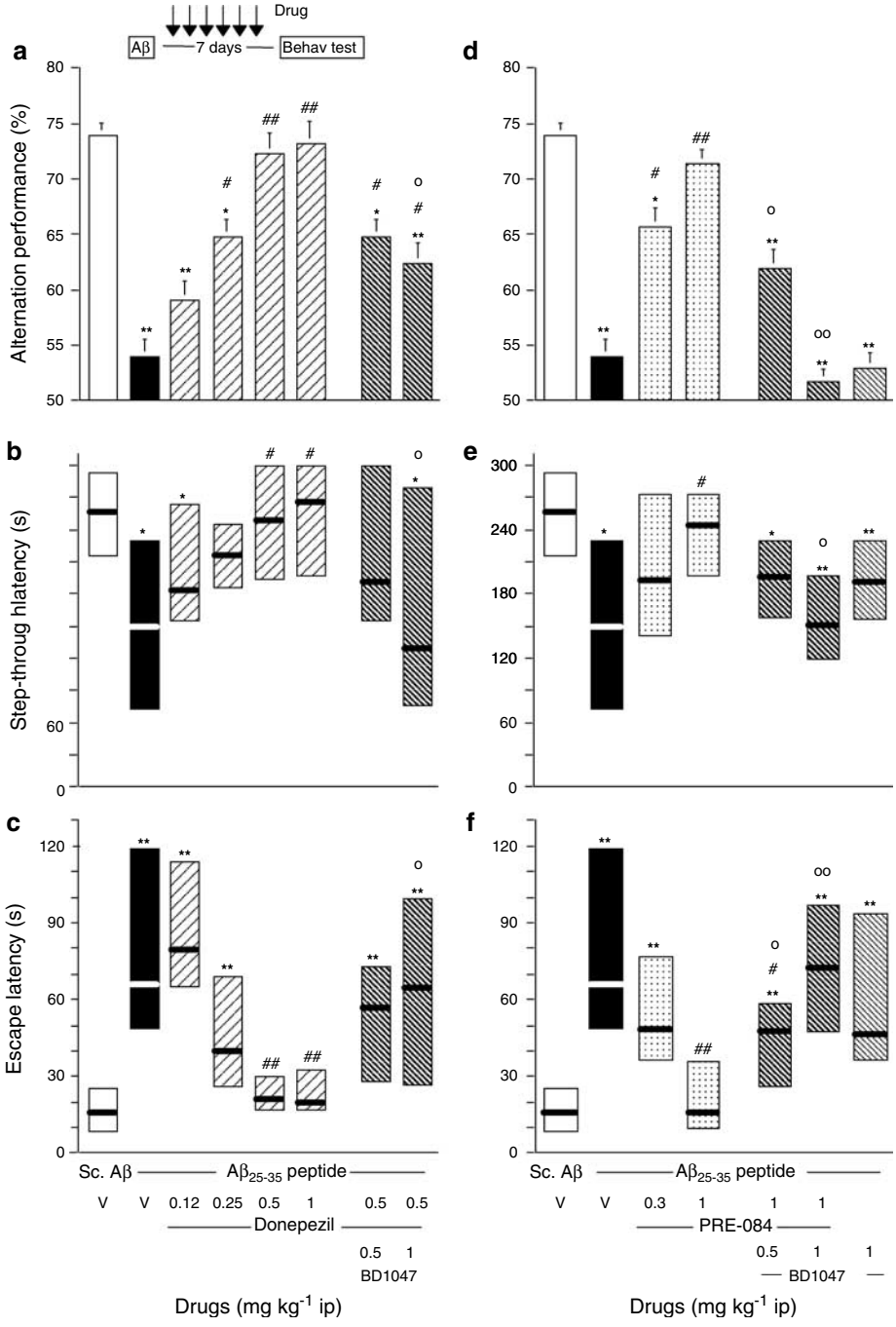

Figure 8 Post i.c.v. neuroprotective effects of donepezil (**a–c**) and PRE-084 (**d–f**) in Aβ_{25-35} peptide-injected mice. Mice were administered, i.p., either vehicle solution (saline, V), donepezil (0.12–1 mg kg^{-1}), PRE-084 (0.3–1 mg kg^{-1}) and/or BD1047 (0.5–1 mg kg^{-1}) 24 h after the peptide injection and once a day for 6 days (see insert). On day 7, animals were examined for spontaneous alternation performances (**a**, **d**). On day 8, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (**b**, **e**) and escape latency (**c**, **f**). $n=10$–12 per group. $F_{(7,83)}=5.61$, $P<0.0001$ in (**a**); $H=15.63$, $P<0.05$ in (**b**); $H=38.79$, $P<0.0001$ in (**c**); $F_{(6,71)}=11.20$, $P<0.0001$ in (**d**); $H=16.74$, $P<0.05$ in (**e**); $H=33.59$, $P<0.0001$ in (**f**). $^{*}P<0.05$, $^{**}P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs the V-treated Aβ_{25-35}-administered group; $^{o}P<0.05$, $^{oo}P<0.01$ vs the donepezil (0.5 mg kg^{-1})- or PRE-084 (1 mg kg^{-1})-treated Aβ_{25-35}-administered group; Dunnett's test in (**a**, **d**), Dunn's test in (**b**, **c**, **e**, **f**).

of neuronal loss and gliosis. Morphological damages in the CA1–2 and dendate gyrus areas of the hippocampus, together with increased GFAP immunoreactivity, were observed 2 weeks after cessation of an i.c.v. infusion of Aβ_{1-40} (Nitta *et al.*, 1997). Aβ_{1-42}, administered i.c.v., increased the immunoreactivities of glial fibrillary acidic protein (GFAP), the astrocyte marker, and interleukin-1β in the hippocampus (Cho *et al.*, 2005). Aβ_{25-35} also causes reactive gliosis in the ipsilateral hemisphere, as demonstrated by upregulation of GFAP expression and the presence

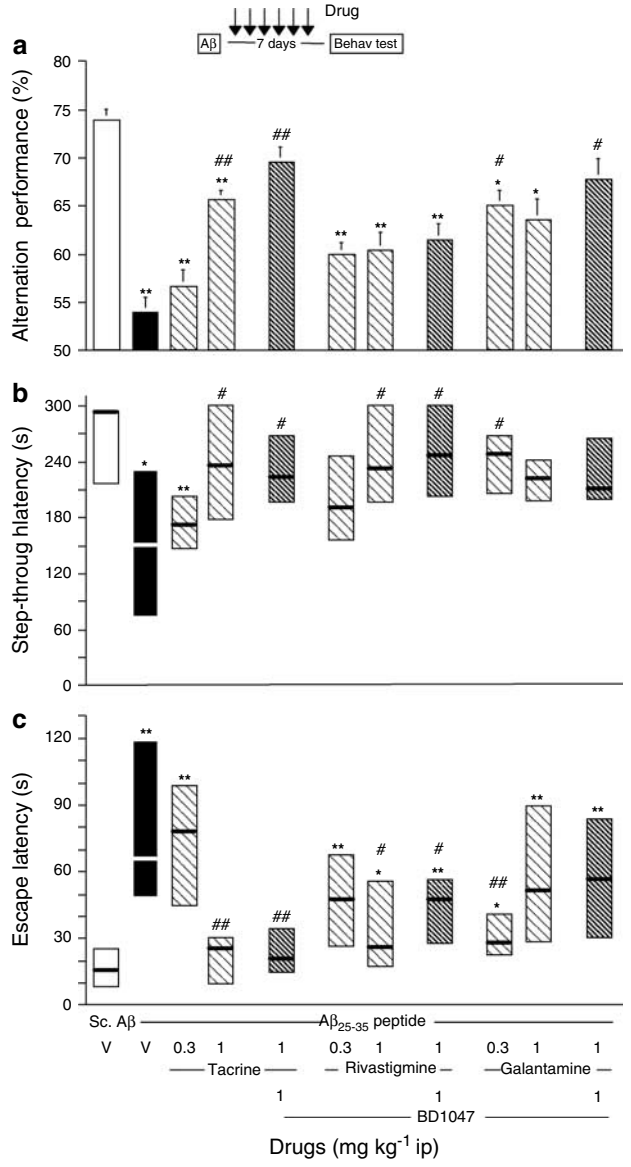

Figure 9 Post-i.c.v. neuroprotective effects of tacrine, rivastigmine and galantamine in Aβ_{25-35} peptide-injected mice. Mice were administered, i.p., either vehicle solution (saline, V), tacrine (0.3–1 mg kg^{-1}), rivastigmine (0.3–1 mg kg^{-1}), galantamine (0.3–1 mg kg^{-1}) and/or BD1047 (1 mg kg^{-1}) 24 h after the peptide injection and once a day for 6 days (see insert). On day 7, animals were examined for spontaneous alternation performances (**a**). On day 8, animals were trained for passive avoidance task and retention was examined on day 9, in terms of step-through latency (**b**) and escape latency (**c**). $n=10$–12 per group. $F_{(10,103)}=3.82$, $P<0.001$ in (**a**); $H=19.23$, $P<0.05$ in (**b**); $H=41.65$, $P<0.0001$ in (**c**). $^{*}P<0.05$, $^{**}P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs the V-treated Aβ_{25-35}-administered group; Dunnett's test in (**a**), Dunn's test in (**b**, **c**).

of hypertrophic astrocytes in the hippocampus (Stepanichev *et al*., 2003). After caspase-3 activity had been induced in the hippocampus and cortex of rats (Stepanichev *et al*., 2003), an injection of Aβ_{25-35} produced a moderate but significant reduction in the number of neurons in the CA1 or CA3 hippocampal areas (Stepanichev *et al*., 2003, 2005, 2006; Mamiya *et al*., 2004; unpublished results).

Oxidative stress contributes to the Aβ_{1-42}-induced toxicity *in vivo*, as shown by induction of cytosolic Cu,Zn-superoxide dismutase (SOD) and mitochondrial Mn-SOD in the hippocampus and cortex. Production of malondialdehyde (lipid peroxidation) and protein carbonyl (protein oxidation) remains elevated 10 days after Aβ_{1-42} injection (Jhoo *et al*., 2004). Reduction of SOD immunoreactivity was also clearly evidenced after Aβ_{1-40} fusion (Kim *et al*., 2003). Chronic Aβ_{1-40} infusion caused a robust peroxynitrite formation and subsequent tyrosine nitration of proteins, particularly synaptophysin, in the hippocampus (Tran *et al*., 2003). Similarly, Aβ_{25-35} induces significant oxidative stress, measured within 1 week after injection, as an increase in lipid peroxidation and superoxide generation (Mamiya *et al*., 2004; Stepanichev *et al*., 2004; this study).

In both models, the amyloid toxicity directly affects neuronal physiology. Cholinergic and glutamatergic systems appear to be the most sensitive ones. The impact of Aβ peptides on cholinergic systems was studied mainly by biochemical techniques. Choline acetyltransferase activity, nicotine-induced acetylcholine release and nicotine- and high K^{+}-induced dopamine release were significantly decreased in the frontal cortex and hippocampus of Aβ_{1-40}-infused rats (Nitta *et al*., 1994; Itoh *et al*., 1996). The effects of Aβ_{25-35} were examined after its chronic infusion and it was shown to decrease nicotine-evoked acetylcholine release from the frontal cortex/hippocampus of rats and reduce protein kinase C (PKC) activation, measured as a decrease in [^{3}H]phorbol dibutyrate binding (Olariu *et al*., 2001). The impact of Aβ peptides on glutamatergic systems was examined by use of an electrophysiological approach. The amplitude of field excitatory postsynaptic potentials recorded in the CA1 region of awake rats was reduced 24 h after the injection of Aβ_{1-40} and this effect was prevented by treatment with *N*-methyl-D-aspartate (NMDA) receptor antagonists, suggesting that Aβ_{1-40} produced a delayed reduction in the function of glutamatergic synapses, probably as a result of an initial overactivation of the NMDA receptor-mediated component of transmission (Cullen *et al*., 1996). Itoh *et al*. (1999) confirmed that long-term potentiation induced by tetanic stimulations in CA1 pyramidal cells, which was readily observed in the vehicle control rats, was also impaired in the Aβ_{1-40}-infused rats. Similarly, i.c.v. administration of aggregated Aβ_{25-35} was followed 1 month later by significant changes in the dynamics of long-term potentiation in the hippocampus *in vivo*, expressed as powerful and stable increases in the amplitude of evoked potentials (Trubetskaya *et al*., 2003).

These Aβ-induced toxicity and functional deficits are responsible for the delayed learning and memory deficits observed. Continuous i.c.v. infusion of Aβ_{1-40} induced memory impairments in the water-maze task and passive avoidance test when compared with control Aβ_{40-1} infused rats (Nitta *et al*., 1994, 1997). Single bilateral i.c.v. injection of Aβ_{25-35} in male Wistar rats induced, after 1month, learning impairments in the radial-arm maze, which appeared to be more marked for the working memory component than for reference memory in the water-maze test or passive avoidance test (Stepanichev *et al*., 2003, 2005, 2006). However, another study has clearly demonstrated

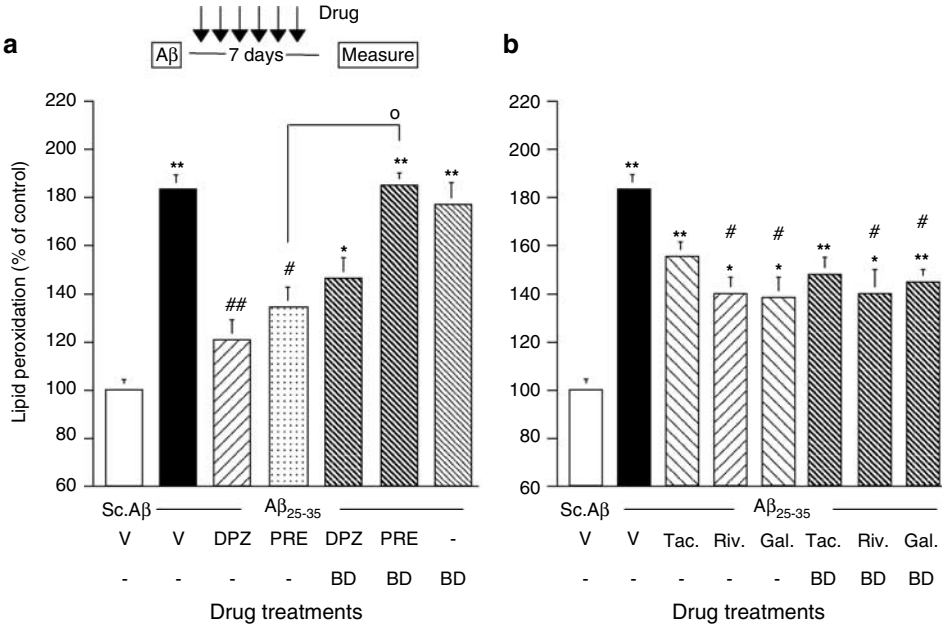

Figure 10 Post-i.c.v. neuroprotective effects of the compounds, assessed using measures of the lipid peroxidation levels in the hippocampus of Aβ_{25-35} peptide-injected mice. Mice were administered, i.p., either vehicle solution (saline, V), donepezil (0.5 mg kg^{-1}), PRE-084 (1 mg kg^{-1}) and/or BD1047 (1 mg kg^{-1}) (**a**); or tacrine (1 mg kg^{-1}), rivastigmine (1 mg kg^{-1}), galantamine (1 mg kg^{-1}) and/or BD1047 (1 mg kg^{-1}) (**b**) 24 h after the peptide injection and once a day for 6 days (see insert). Lipid peroxidation levels were measured on day 7. $n=6$–8 per group. $F_{(6,39)}=6.38$, $P<0.0001$ in (**a**); $F_{(7,44)}=3.77$, $P<0.01$ in (**b**). $^{*}P<0.05$, $^{**}P<0.01$ vs the V-treated Sc.Aβ-administered group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs the V-treated Aβ$_{25-35}$-administered group, $^{o}P<0.05$; Newman–Keuls' test.

reference memory impairment in the water-maze after an i.c.v. injection of Aβ_{25-35} (15 nmol) in Wistar rats (Delobette *et al*., 1997). Moreover, Aβ_{25-35}-induced deficits in passive avoidance response, spontaneous alternation and place learning in the water-maze have repeatedly been demonstrated in mice (Maurice *et al*., 1996, 1998; Mamiya *et al*., 2004; this study). Interestingly, the Aβ_{25-35}-induced deficits in rats were still observable 6 months after injection in a spontaneous alternation and social recognition test (Stepanichev *et al*., 2003).

In the present study, we therefore use the validated model of aggregated Aβ_{25-35} peptide injection in mice to examine the anti-amnesic and neuroprotective effects of donepezil, in comparison with other, more selective, ChE inhibitors and a reference σ_1 receptor agonist. Donepezil, with a 14.6 nM affinity for the σ_1 receptor and an IC_{50} of 21.5 nM for inhibition of acetylcholinesterase activity, has been shown to be equipotent for the two targets (Kato *et al*., 1999). Other cholinesterase inhibitors are more selective cholinomimetics and have only very low affinity for the σ_1 receptor. For instance, tacrine shows an affinity of 6 μM for the σ_1 receptor (Kato *et al*., 1999) as compared with an IC_{50} of 77 nM for the inhibition of acetylcholinesterase activity (Ogura *et al*., 2000). In parallel, PRE-084 is a poor muscarinic ligand, with an affinity for [^{3}H]quinuclidinyl benzilate-binding sites of about 14 μM (Su *et al*., 1991) and no reported affinity for nicotinic receptors or AChE. The purpose of this study was, firstly, to identify any neuroprotective effect of donepezil on an *in vivo* nontransgenic model of AD; secondly, to analyse whether the interaction of the compound with the σ_1 receptor is involved in its putative neuroprotective activity and thirdly, to demonstrate the neuroprotective potential of σ_1 receptor agonists against Aβ toxicity *in vivo*.

In the first part of the study, we observed that donepezil, the selective ChE inhibitors, tacrine, rivastigmine and galantamine, and the σ_1 receptor agonist PRE-084, all have potent anti-amnesic activity against the learning deficits induced by i.c.v. injection of aggregated Aβ_{25-35} peptide in mice. In animals treated with Aβ_{25-35} 1 week before, the acute pre-test injections of the compounds allowed recovery of spontaneous alternation or passive avoidance deficits. These results confirm previous similar observations. Firstly, donepezil, administered at 2.5 mg kg^{-1} *per os* in rats, alleviated the deficits of delayed-matching to position paradigm in rats infused bilaterally with Aβ_{1-40} peptide into the hippocampus (Yamada *et al*., 2005). The symptomatic effect of donepezil was compared to that of memantine and demonstrated a complete recovery of the Aβ-induced deficits. Tacrine, or direct injection of nicotine, have been shown to alleviate the deficits of spontaneous alternation, passive avoidance and place learning in a water-maze induced by Aβ_{25-35} in mice (Maurice *et al*., 1996). Moreover, the anti-amnesic effect of PRE-084 has also been described previously in the Aβ_{25-35} mouse model of AD (Maurice *et al*., 1998). Notably, the observation that pretreatment with BD1047 significantly blocked the anti-amnesic effect of donepezil showed that the drug did not behave as a pure ChE inhibitor, but that an interaction with the σ_1 receptor is involved in its behavioural action. We previously demonstrated that the anti-amnesic effect of donepezil against the learning deficits induced by blockade of the NMDA receptor in dizocilpine-treated mice (Maurice *et al*., 2006), or by

hypoxia in CO gas-exposed mice (Meunier *et al*., 2006), is also blocked by the σ_1 receptor antagonist. In all cases, BD1047 almost abolished the anti-amnesic effect of donepezil, suggesting that its effects on σ_1 receptors and cholinergic systems are not purely additive. Donepezil appears to have a unique pharmacological action when compared with other selective cholinesterase inhibitors. This implies that drugs acting nonselectively as cholinomimetics and σ_1 receptor agonists may present a very specific mode of action. Indeed, the physiological consequences of σ_1 receptor activation are intracellular regulation of Ca^{2+} mobilization and activation of phospholipase C (PLC) and PKC pathways (Morin-Surun *et al*., 1999; Hayashi *et al*., 2000), which will in turn affect the signal transduction downstream to acetylcholine receptor activation and result in a complete blockade by selective σ_1 receptor antagonists.

The second part of the study examined the neuroprotective efficacy of donepezil, ChE inhibitors and PRE-084 against $A\beta_{25-35}$ peptide-induced toxicity. The toxicity was evaluated at two levels: a biochemical index of the oxidative stress induced in the hippocampus, by measure of the lipid peroxidation products, and the resulting learning deficits as a direct behavioural consequence. The protection against the $A\beta_{25-35}$ peptide application was examined by administration of the drugs before the peptide, whereas the long-term protection against the delayed neurodegeneration induced by amyloid deposits was examined by administration of the drugs semichronically between the peptide injection and behavioural or biochemical measures. Both types of measure led to concordant results indicating that donepezil and PRE-084 are potent at exerting protection when administered pre-i.c.v., in a BD1047-sensitive manner, whereas other ChE inhibitors were without effect. When the drugs were injected semichronically, both σ_1 and cholinomimetics showed some neuroprotective efficacy.

Donepezil has been reported to protect against $A\beta_{1-40}$ or $A\beta_{25-35}$ toxicity in cell culture models. In particular, this compound, as well as tacrine, protected PC12 cells from $A\beta_{25-35}$ toxicity when applied 2 h before the peptide (Svensson and Nordberg, 1998). In rat septal neurons, donepezil, but not galantamine or tacrine, blocked the $A\beta_{1-40}$ toxicity when added 24 h before the peptide. Selective effects of donepezil were measured in terms of LDH release, and thioflavin-T fluorescence (Kimura *et al*., 2005). Particularly from this last, highly relevant study, a parallel can be drawn between *in vitro* and *in vivo* studies suggesting that donepezil pre-administered before $A\beta$ peptides induced a more effective neuroprotection than selective ChE inhibitors. Recently, the neuroprotective activities of PRE-084 and (−) MR-22, another selective σ_1 receptor agonist, were also described against $A\beta_{25-35}$ peptide-induced toxicity in rat cortical neurons (Marrazzo *et al*., 2005). Each compound was applied before $A\beta_{25-35}$ and significant enhancement of cell survival and diminutions of the expression of the proapoptotic protein Bax were measured. The neuroprotective activity of selective σ_1 receptor agonists, previously described in excitotoxic models *in vitro* and *in vivo* (for reviews, see Maurice and Lockhart, 1997; Maurice *et al*., 1999), is also effective against amyloid toxicity. Moreover, a complete reversion of $A\beta_{25-35}$-induced oxidative stress and learning deficits by PRE-084 and a complete blockade of donepezil's effects by BD1047 were observed when drugs were administered pre-i.c.v. This, together with the lack of efficacy of selective ChE inhibitors, strongly suggests that the σ_1 receptor is mainly involved in the neuroprotective effect of donepezil or, at least, that under these particular experimental conditions, the drug acts mainly as a σ_1 receptor agonist. In other words, donepezil, through its σ_1 receptor agonist property, is more efficient than other ChE inhibitors in blocking the toxic effect of newly synthetized β-amyloid peptides. Moreover, the pre-i.c.v. administration of donepezil before $A\beta_{25-35}$ offers suitable conditions to examine the involvement of σ_1 receptors in its effects.

When drugs were administered repeatedly after the $A\beta_{25-35}$ peptide injection, the donepezil effects were partly antagonized by BD1047. Also, tacrine, rivastigmine and galantamine induced moderate, partly significant effects on both the behavioural and biochemical measures. Therefore, both cholinomimetics and σ_1 receptor agonists exhibit effective neuroprotection in these experimental conditions, reflecting the neuroprotective ability against the amyloid deposits formed. Cholinomimetics, such as galantamine, have been shown to induce phosphorylation of Akt, through activation of PI3K mediated via activation of the α7 nicotinic receptor (Kihara *et al*., 2004). The mechanism of the σ_1 receptor-mediated neuroprotection is still elusive. As the primary effect of $A\beta_{25-35}$, after penetrating the neurons, is the induction of mitochondrial and ER stress and as σ_1 receptors are known to be located in resting conditions on the ER and mitochondrial membranes (Hayashi *et al*., 2000), the σ_1 receptor-mediated effect may result from an inhibition of the mitochondrial or ER stress. In particular, σ_1 drugs have been shown to regulate Ca^{2+} mobilization and activate PLC/PKC pathways, which may help to attenuate the cellular effects of mitochondrial or ER dysfunctions (Morin-Surun *et al*., 1999; Hayashi *et al*., 2000). The σ_1 receptor-mediated effects could also involve a blockade of the penetration of the peptide, putatively by long-term effects involving recomposition of intracellular compartments and membrane composition (Hayashi and Su, 2005). The precise mechanisms are currently being investigated.

Conclusions

The results from this study confirm that donepezil is able to alleviate the memory deficits induced by $A\beta_{25-35}$ peptide injection in mice and show, for the first time, that the drug is able to protect against the appearance of $A\beta_{25-35}$ peptide-induced toxicity, measured in terms of peroxidized lipid formation and resulting learning impairments. In particular, the compound was effective not only when it was repeatedly injected after the $A\beta_{25-35}$ peptide but also when it was administered before $A\beta_{25-35}$ and this, selectively through its σ_1 receptor agonist action. Therefore, the development of σ_1 receptor acting drugs, selective or not, may lead to original neuroprotective strategies for the treatment of β-amyloid-induced toxicity.

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Conflict of interest

The authors state no conflict of interest.

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