and induction of neuronal apoptotic death. Aβ proteins are also responsible for a generalized inflammatory response in brain structures associated with production of cytokines by activated astroglia and microglia (Frederickson, 1992) and exacerbated excitotoxic processes (Mattson *et al*, 1992).

Moreover, toxicity of Aβ has recently been shown to be highly dependent on the aggregation species (Chafekar *et al*, 2008). Aβ can exist in different assembly states and apart from the monomeric and mature fibrillar stages, different intermediate species have been identified, such as low molecular weight oligomers, larger globular oligomers, and protofibrils. This is true for $A\beta_{1-40}$ or $A\beta_{1-42/3}$ but also for $A\beta_{25-35}$ peptide. These different species greatly differ in their neurotoxic potential and molecular mechanism mediating the toxicity. For instance, impairment of long-term potentiation (Walsh *et al*, 2002) and ER stress (Chafekar *et al*, 2007) may be mediated by small oligomers, whereas the neuroinflammatory response may rather involve fibrillar Aβ (Eikelenboom *et al*, 2002). Preliminary observations of the laboratory showed that after *in vitro* aggregation, $A\beta_{25-35}$ peptide exist in these different species including small oligomers, amorphous oligomers, and fibrillar forms (S Marchal, L Givalois, T Maurice, unpublished work).

We described the nontransgenic model of AD induced in rodents by injection into the lateral ventricle of aggregated $A\beta_{25-35}$ peptide (Maurice *et al*, 1996; Delobette *et al*, 1997). The morphological and biochemical characterization of amyloid toxicity induced by $A\beta_{25-35}$ has been subsequently analyzed in details. $A\beta_{25-35}$ induces brain inflammation, oxidative stress, activation of proapoptotic caspases, impairment of long-term potentiation, cell loss in the hippocampus, and memory impairments (Stepanichev *et al*, 2004, 2006; Meunier *et al*, 2006). Recently, it was also observed that $A\beta_{25-35}$ injection activates the glycogen synthase kinase-3β, involved in cell survival regulation, T-phosphorylation and APP processing, suggesting that acute $A\beta_{25-35}$ injection results in production and seeding of endogenous $A\beta_{1-40/42}$ and T-phosphorylation (Klementiev *et al*, 2007). The model therefore appears as highly suitable to analyze the putative antiamnesic and neuroprotective activity of drugs with potential interest in AD, as recently used by several authors (Fang and Liu, 2006; Kuboyama *et al*, 2006; Meunier *et al*, 2006; Um *et al*, 2006; Alkam *et al*, 2007).

The σ_1 protein has only recently been identified as a chaperone protein located on membranes forming focal contacts between the ER and mitochondria (Hayashi and Su, 2007). In basal conditions, the σ_1 protein forms a complex with the other chaperone glucose-regulated protein 78 kDa (GRP78/BiP). Upon ER Ca^{2+} depletion or by ligand stimulation, the σ_1 protein dissociates from GRP78/BiP, leading to a prolonged Ca^{2+} signaling into mitochondria by IP_3 receptors (Hayashi and Su, 2007). Under intracellular Ca^{2+} signaling disruption and subsequent ER stress, the σ_1 protein translocates, to reach plasma membrane, recruiting Ca^{2+}-dependent intracellular cascades (Morin-Surun *et al*, 1999). On the plasma membrane, it contributes to form or modify the composition of lipid-rich microdomains, so-called lipid rafts (Hayashi and Su, 2001, 2003). Increasing or activating σ_1 proteins is expected to counteract ER stress response, whereas decreasing or inactivating them would enhance apoptosis (Hayashi and Su, 2007). Modifying σ_1 protein activation using selective activators/agonists therefore mediates a unique pharmacological action on Ca^{2+} homeostasis and signal transduction pathways, which has proven to allow an effective neuroprotection against several kinds of insults, including excitotoxicity, oxidative stress, and amyloid toxicity (for reviews, see Maurice *et al*, 2006; Monnet and Maurice, 2006). Indeed, preliminary experiments showed that, *in vitro*, the selective σ_1 activators PRE-084 and MR-22 attenuate the $A\beta_{25-35}$-induced expression of the proapoptotic protein Bax and neuronal death in rat cortical cultures (Marrazzo *et al*, 2005). We reported that, *in vivo*, PRE-084 prevents the $A\beta_{25-35}$-induced oxidative stress and learning impairments in mice (Meunier *et al*, 2006).

ANAVEX1-41 is a new aminotetrahydrofuran derivative (Vamvakides, 2002; Espallergues *et al*, 2007) acting as a σ_1 protein activator, with a high affinity (44 nM) and selectivity. The CEREP profile of the compound showed that it also presents nanomolar affinities (18–114 nM) for muscarinic receptors ($M_1 > M_3$, $M_4 > M_2$), some low micromolar affinity for sodium channel site 2, and negligible interaction with 60 other receptor and enzyme assays (data not shown). Its molecular profile is coherent with its antiamnesic and antidepressant effects (Espallergues *et al*, 2007). In this study, we analyzed its antiamnesic and neuroprotective potentials against $A\beta_{25-35}$-induced toxicity in mice. Learning deficits were measured using the spontaneous alternation test measuring spatial working memory and passive avoidance response measuring long-term contextual memory. The $A\beta_{25-35}$-induced toxicity was also analyzed at the morphological and biochemical levels. Finally, the involvement of the σ_1 protein or muscarinic receptors was examined using pretreatments with a selective antagonist, BD1047 or scopolamine, respectively.

MATERIALS AND METHODS

Animals

Male Swiss mice (Depré, St-Doulchard, France), aged 7 weeks and weighing 32 ± 2 g, were used in this study. Animals were housed in plastic cages in groups. They had free access to food and water, except during behavioral experiments, and they were kept in a regulated environment (23 ± 1°C, 40–60% humidity) under a 12 h light/dark cycle (light on at 0800 hours). Experiments were carried out between 0900 and 1700 hours, in an experimental room within the animal facility. Mice were habituated 30 min before each experiment. All animal procedures were conducted in strict adherence of European Union Directive of 24 November 1986 (86–609).

Drugs

Tetrahydro-*N*,*N*-dimethyl-5,5-diphenyl-3-furanmethanamine hydrochloride (ANAVEX1-41, formerly AE14) was synthesized in the laboratory (Anavex Life Sciences, Pallini, Greece). *N*-[2-(3,4-dichlorophenyl)ethyl]-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide (BD1047) was from Tocris Bioscience (Bristol, UK). All other materials, including scopolamine hydrobromide, xylenol orange, and cumene peroxide, were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Drugs used for *in vivo*

experiments were solubilized in physiological saline solution and administered intraperitoneally (i.p.) in a volume of 100 µl per 20 g body weight. The $A\beta_{25-35}$ peptide (Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met, $A\beta_{25-35}$) and scrambled $A\beta_{25-35}$ peptide (Ala-Lys-Ile-Gly-Asn-Ser-Ile-Gly-Leu-Met-Gly, Sc$A\beta$) were from NeoMPS (Strasbourg, France). They were dissolved in sterile bidistilled water at a concentration of 3 mg/ml and stored at −20°C until use. Before injection, peptides were aggregated by incubation at 3 mg/ml in sterile bidistilled water at 37°C for 4 days. They were administered intracerebroventricularly (i.c.v.) in a final volume of 3 µl per mouse, as previously described (Maurice *et al*, 1996, 1998).

Spontaneous Alternation Performances

Each mouse, naive to the apparatus, was placed at the end of one arm in a *Y*-maze (three arms, 40 cm long, 120° separate) and allowed to move freely through the maze during a single 8-min session. The series of arm entries, including possible returns into the same arm, was recorded visually. An alternation was defined as entries into all three arms on consecutive trials. The number of the total possible alternations was therefore the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations/total possible alternations) × 100. Animals performing less than eight arm entries in 8 min were discarded (ie, less than 5% of animals).

Step-Down Type Passive Avoidance Test

The apparatus consisted of a transparent acrylic cage (30 × 30 × 40 cm high) with a grid-floor, inserted in a soundproof outer box (35 × 35 × 90 cm high). A 15 W lamp lighted the cage during the experimental period. A wooden platform (4 × 4 × 4 cm) was fixed at the center of the grid-floor. Intermittent electric shocks (1 Hz, 500 ms, 40 V DC) were delivered to the grid-floor using an isolated pulse stimulator (Model 2100; AM Systems, Everett, WA, USA). The test consisted of two training sessions, at 90-min time interval, and a retention session, carried out 24 h after the first training. During training sessions, each mouse was placed on the platform. When it stepped down and placed its four paws on the grid-floor, shocks were delivered for 15 s. Step-down latency and the numbers of vocalizations and flinching reactions were measured. Shock sensitivity was evaluated by adding these two numbers. None of the treatments used in this study significantly affected the shock sensitivity. Animals that stepped down before 3 s has elapsed or that did not step down within 30 s were discarded (ie, less than 5% of the mice). Animals, which did not step down within 60 s during the second session, were considered as remembering the task and taken off, without receiving further electric shocks. The retention test was performed in a similar manner as training, except that the shocks were not applied to the grid-floor. Each mouse was again placed on the platform, and the latency was recorded, with an upper cutoff time of 300 s. Two parametric measures of retention were analyzed: the latency and the number of animals reaching the avoidance criterion, defined as correct if the latency measured during the retention session was greater than threefold the latency showed by the animal during the second training session and, at least, greater than 60 s.

Histology

Each mouse was anesthetized by intramuscular (i.m.) injection of ketamine, 80 mg/kg, and xylazine, 10 mg/kg, and quickly transcardially perfused with 50 ml of saline solution followed by 50 ml of paraformaldehyde 4%. Brains were removed and kept overnight in the fixative solution. They were cut in coronal sections (30 µm thickness) using a vibratome (Leica VT1000 S). Serial sections were selected to include the hippocampus formation and placed in gelatin-coated glass strip. Sections were stained with 0.2% cresyl violet reagent (Sigma-Aldrich), then dehydrated with graded ethanol, treated with toluene and mounted with DePeX medium (BDH Laboratories, Poole, UK). Examination of the CA1 area was performed using a light microscope (Dialux 22, Leitz), slices being digitalized through a CCD camera (Sony XC-77CE) with the NIH ImageJ software, to easily process CA1 measurement and pyramidal cells counts. Data were calculated as average of six slices and expressed as number of viable CA1 pyramidal cells per millimeter for each group.

Immunohistochemistry

Mice were anesthetized by i.m. injection of ketamine 10% and xylazine 2%, perfused transcardially with 50 ml of saline solution followed by 50 ml of paraformaldehyde 4%. Brains were removed and kept overnight in the fixative solution. Brain sections were cut in coronal sections (30 µm thickness) using a vibratome (Leica VT1000 S). Analysis of the glial response to neurodegeneration was carried out by immunolabeling sections, with mouse monoclonal antiglial fibrillary acidic protein (GFAP; Sigma-Aldrich; 1 : 1000).

Lipid Peroxidation Measures

Mice were killed by decapitation and brains were rapidly removed, weighed, and kept in liquid nitrogen until assayed. After thawing, brains were homogenized in cold methanol (1 : 10, w/v), centrifuged at 1000 *g* during 5 min and the supernatant collected. Homogenate was added to a solution containing $FeSO_4$ 1 mM, H_2SO_4 0.25 M, xylenol orange 1 mM, and incubated for 30 min at room temperature. Absorbance was measured at 580 nm ($A_{580}1$), and 10 µl of cumene hydroperoxide (CHP) 1 mM was added to the sample and incubated for 30 min at room temperature, to determine the maximal oxidation level. Absorbance was measured at 580 nm ($A_{580}2$). The level of lipid peroxidation was determined as CHP equivalents according to: CHP equiv. = $A_{580}1/A_{580}2 \times$ (CHP (nmol)) × dilution, and expressed as CHP equiv. per wet tissue weight.

Western Blotting

For determination of protein nitration levels, mice were decapitated 5 days after $A\beta$ peptide injection. The hippocampus were removed on ice-cold glass plate and stored at −80°C. The hippocampus tissues were homo-

genized in ice-cold 20 mM Tris–HCl extraction buffer, pH 7.6, containing 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 1 mM sodium vanadate, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 1 mg/ml pepstatin, 1 mg/ml aprotinin, and 1 mg/ml leupeptin. Equal amounts of protein, 40 µg per lane, were resolved by a 10% SDS–polyacrylamide gel electrophoresis, and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were incubated in 3% skimmed milk in a washing buffer, Tris-buffered saline containing 0.05% (v/v) Tween 20, for 2 h at room temperature. Then, membranes were incubated at 4°C overnight with an antinitrotyrosine mouse clone1A6 (Upstate Cell Signaling, Lake Placid, USA; 1:1000) or with goat anti β-actin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100). After a wash, membranes were incubated with horseradish peroxidase-conjugated antimouse IgG (Sigma-Aldrich; 1:2000). Peroxidase activity was revealed by using enhanced chemiluminescence (ECL) reagent. Then, intensity of peroxidase activity was semiquantified using the ImageJ software. Results were corrected with the corresponding β-actin level and expressed as percentage of control group data.

For determination of GFAP, caspase-3 or caspase-9 expression, mice were decapitated 7 days after Aβ peptide injection. The hippocampi were removed on ice-cold glass plate and stored at −80°C. The hippocampus tissues were homogenized in ice-cold extraction buffer containing SDS 2% and proteases inhibitors (Roche). Equal amounts of protein, 40 µg per lane, were resolved by a 12% SDS–polyacrylamide gel electrophoresis, and then transferred electrophoretically to a nitrocellulose blot membrane (Schleicher Schuell 0.45 µm). The membranes were then blocked during 30 min at room temperature with 5% skim milk in Tris-buffered saline 20 mM (pH 7.6) containing 0.1% Tween 20 (TBS-T). The membranes were incubated at 4°C overnight with a mouse monoclonal anti-GFAP antibody (Sigma-Aldrich; 1:2000), or rabbit anticaspase-3 or rabbit anticaspase-9 antibody (Cell Signaling Technology; 1:1000 each), rinsed for 30 min in TBS-T and then incubated for 2 h with a goat antimouse or antirabbit secondary antibody (Sigma-Aldrich; 1:2000 each). Peroxidase activity was revealed by using ECL reagent. Then, intensity of peroxidase activity was semiquantified using the ImageJ software. Results were normalized to control values (anti β-tubulin; Sigma-Aldrich; 1:5000).

Statistical Analyses

Biochemical and behavioral data were expressed as mean ± SEM, except step-down latencies expressed as median and interquartile range. They were analyzed using one-way ANOVA (F-values), followed by the Dunnett's *post hoc* multiple comparison test. Passive avoidance latencies were analyzed a Kruskal–Wallis nonparametric ANOVA (H-values), as upper cutoff times were set, followed by the Dunn's multiple comparison test. The level of statistical significance was $p<0.05$.

RESULTS

Antiamnesic Effects of ANAVEX1-41 Against Aβ_{25-35}-Induced Learning Impairments

In the first series of experiments, the antiamnesic effects of ANAVEX1-41 was examined in mice centrally injected 7 days before with scrambled Aβ (ScAβ) or Aβ_{25-35} peptide. The spatial working memory was first examined in the *Y*-maze test, animals receiving ANAVEX1-41 20 min before the session. As shown in Figure 1a, the central administration of ScAβ peptide or the subsequent i.p. treatment with ANAVEX1-41, in the 1–1000 µg/kg dose range, failed to change the spontaneous alternation performance that was in the 65–70% range ($F<1$). The treatments also did not affect the total number of arm entries ($F<1$; Figure 1b). When mice were treated with Aβ_{25-35}, the alternation performance decreased highly significantly to 53% and the ANAVEX1-41 treatment reversed the deficit ($F_{(8,145)}=4.41$, $p<0.0001$; Figure 1c). The compound showed a significant effect at the dose of 3 µg/kg and the improvement remained significant up to the highest dose tested. The most effective dose appeared to be 10 µg/kg. Neither the Aβ_{25-35}, nor the ANAVEX1-41 treatments affected the number of arm entries ($F_{(8,145)}=1.62$, $p>0.05$; Figure 1d).

The long-term contextual memory was evaluated using the step-down type passive avoidance procedure. Animals were tested 8 days after the central administration of ScAβ or Aβ_{25-35} peptide and ANAVEX1-41 compound was administered 20 min before the first training session. The retention test was performed on day 9 after the peptide administration. As shown in Figure 2a and b, the ScAβ peptide or the subsequent treatment with ANAVEX1-41 in the 1–1000 µg/kg dose range, failed to affect the latency ($H=2.98$, $p>0.05$; Figure 2a) or percentage of animal-to-criterion that were in the 60–80% range (Figure 2b). In particular, the compound failed to show memory enhancing effect, as compared with V-treated animals. However, it must be noted that in procedures adapted to the measure of memory enhancing effects, the intensity of footshocks is lower than used in the present experiment. The central injection of Aβ_{25-35} peptide led to highly significant decreases in latency ($H=27.72$, $p<0.001$; Figure 2c) and percentage of animals-to-criterion (Figure 2d). The ANAVEX1-41 treatment resulted in a bell shaped but highly significant reversion of the deficits. Both parameters revealed an active dose range of 1–100 µg/kg.

Neuroprotective Effects of ANAVEX1-41 Against the Aβ_{25-35}-Induced Learning Deficits

The neuroprotective effects of ANAVEX1-41 were first analyzed on the appearance of Aβ_{25-35}-induced learning deficits. The drug was administered in the same, 1–1000 µg/kg i.p., dose range and only once, 20 min before the i.c.v. administration of the peptide. We previously reported that such procedure is highly effective for mixed cholinergic/σ_1 compounds (Meunier *et al*, 2006). The pretreatment with ANAVEX1-41 resulted, 7 days after in a bell shaped but significant prevention of the Aβ_{25-35}-induced spontaneous alternation impairments ($F_{(8,145)}=3.40$, $p<0.01$; Figure 3a). The active doses of compound were in the 10–100 µg/kg

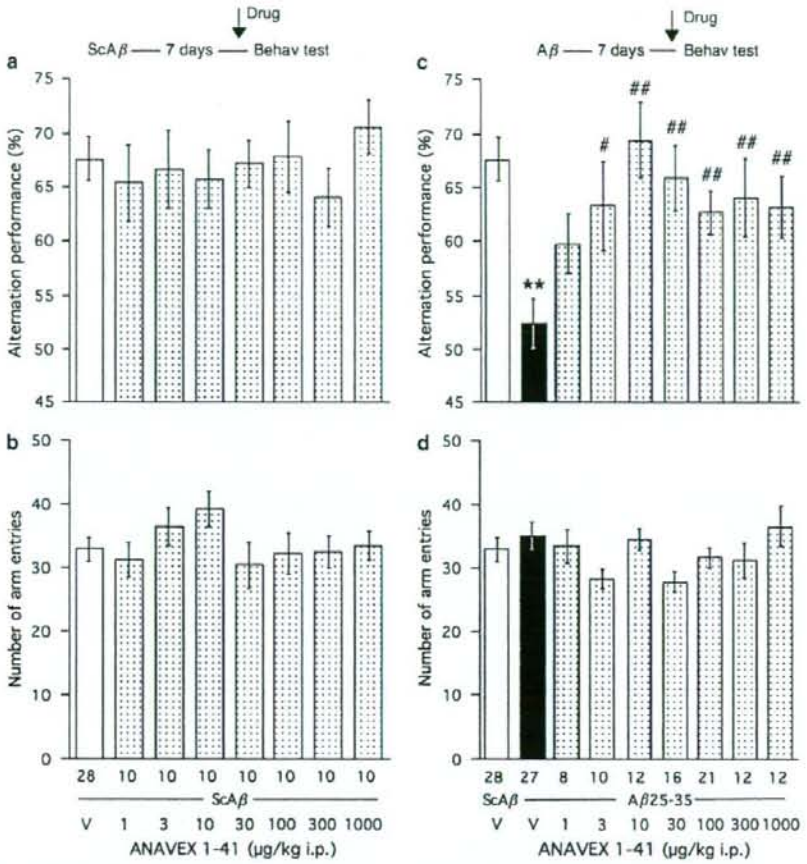

Figure 1 Antiamnesic effect of ANAVEX1-41 on $A\beta_{25-35}$-induced spontaneous alternation deficits in mice: alternation performances (a, c) and total numbers of arm entries (b, d). Mice were injected i.c.v. with ScAβ or $A\beta_{25-35}$ peptide (9 nmol). After 7 days, they were administered i.p. with the saline vehicle solution (V) or ANAVEX1-41 (1–1000 µg/kg), 30 min before being examined for spontaneous alternation in the Y-maze (see insert). The number of animals per group is indicated below the columns in (b) and (d). $^{**}p<0.01$ *vs* (ScAβ + V)-treated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* ($A\beta_{25-35}$ + V)-treated group; Dunnett's test.

range. No effect was observed in terms of number of arm entries ($F_{(8,145)} = 1.64$, $p > 0.05$; Figure 3b). The ANAVEX1-41 pretreatment also resulted in a significant prevention of the passive avoidance deficits, both in terms of latencies ($H = 45.2$, $p < 0.0001$; Figure 3c) and number of animals-to-criterion (Figure 3d). In this procedure, however, the active dose range was 30–300 µg/kg.

Neuroprotective Effects of ANAVEX1-41 Against $A\beta_{25-35}$-Induced Toxicity

Morphological validation of the protective effect of ANAVEX1-41 was examined using the most active dose of compound, 100 µg/kg. The pyramidal cell layer of the hippocampus is highly sensitive to the amyloid toxicity observed after $A\beta_{25-35}$ peptide injection in mice. We analyzed the number of viable cells in CA1 hippocampus area using cresyl violet staining (Figure 4). The $A\beta_{25-35}$ injection induced a −24.6% decrease in the number of viable cells in $A\beta_{25-35}$-treated mice ($F_{(3,20)} = 7.68$, $p < 0.01$; Figure 4c and e) as compared with ScAβ-treated mice (Figure 4a and e). In the same mice, no significant effect was measured in the CA3 area: 192 ± 6 cell per field ($n = 6$) for the ScAβ group *vs* 187 ± 10 cell per field ($n = 6$, $p > 0.05$) for the $A\beta_{25-35}$ group. The ANAVEX1-41 treatment failed to affect the number of viable cells in the ScAβ-treated group (Figure 4b and e), but significantly attenuated the diminution observed after $A\beta_{25-35}$ treatment (Figure 4d and e).

The extent of brain inflammation after $A\beta_{25-35}$ and subsequent ANAVEX1-41 treatment was analyzed by measuring reactive astrocytes using GFAP immunohistolabeling (Figure 5). As the i.c.v. injection is expected to provoke by itself a massive glial reaction, ScAβ-treated groups were compared with animals receiving only the i.p. treatment with vehicle solution (Figure 5a, g, m and s) or ANAVEX1-41 (100 µg/kg; Figure 5b, h, n and t). Several

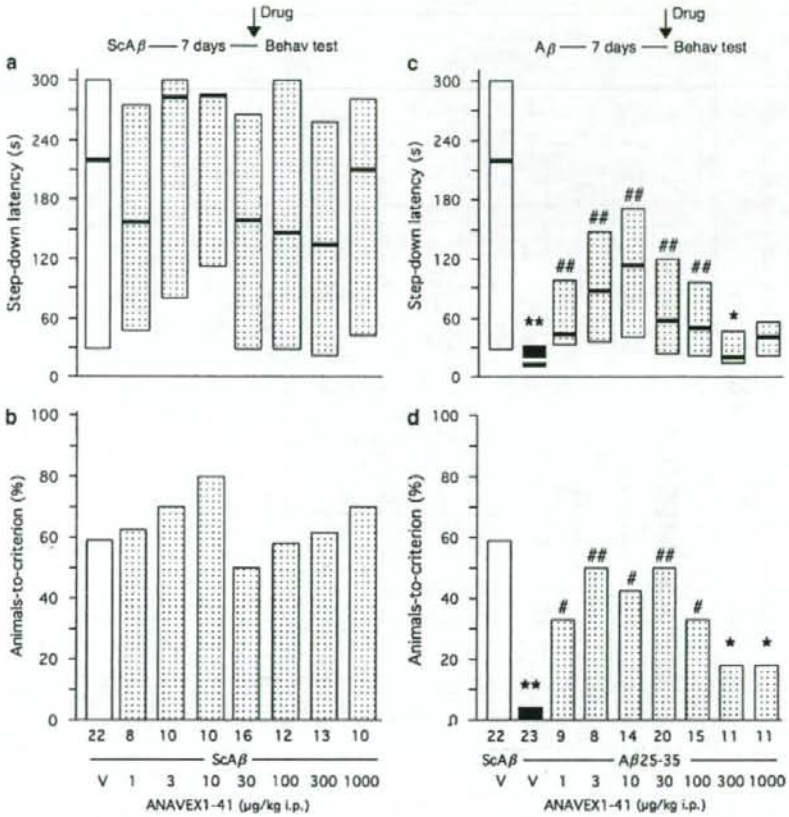

Figure 2 Effect of ANAVEX1-41 on $A\beta_{25-35}$-induced passive avoidance deficits in mice: step-down latency (a, c) and percentage of animals-to-criterion (b, d). Mice were injected i.c.v. with ScAβ or $A\beta_{25-35}$ peptide (9 nmol). After 7 days, they were administered i.p. with saline vehicle solution (V) or ANAVEX1-41 (1–1000 µg/kg), 30 min before the first training session (see insert). The number of animals is indicated below the columns in (b) and (d). $^{*}p<0.05$, $^{**}p<0.01$ *vs* (ScAβ + V)-treated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* ($A\beta_{25-35}$ + V)-treated group; Dunn's test in (a) and (c), χ^2-test in (b) and (d).

brain structures were analyzed and Figure 5 presents typical pictures in the retrosplenial (Figure 5a–f) and parietal (Figure 5g–l) cortices, where astrocytic clusters could be observed, and in the CA1 (Figure 5m–r) and CA3 (Figure 5s–x) areas of the hippocampus. In vehicle-treated animals, disseminated clusters containing few astrocytes were observed in the cortical areas (Figure 5a and g). The pattern of labeling was unchanged after ANAVEX1-41 i.p. injection and/or ScAβ i.c.v. injection (Figure 5b–d and h–j). $A\beta_{25-35}$ injection, however, provoked after 7 days a marked increase in the number of labeled astrocytes and in their branching, resulting in densification of astrocytic clusters. This was observed in the retrosplenial cortex (Figure 5e), but not in the parietal area (Figure 5k). The ANAVEX1-41 treatment resulted in a blockade of $A\beta_{25-35}$-induced increase of GFAP labeling (Figure 5f). In the hippocampus, astrocytes were regularly disseminated throughout the oriens and stratum radiatum layers surrounding the pyramidal cell layers (indicated by asterisks), at both the CA1 and CA3 levels (Figure 5m and s). These patterns were unchanged after ANAVEX1-41 i.p. injection and/or ScAβ i.c.v. injection (Figure 5n–p and t–v). The $A\beta_{25-35}$ injection, however, provoked a massive densification of astrocytic labeling both in CA1 (Figure 5q) and CA3 (Figure 5w). The ANAVEX1-41 treatment resulted in an attenuation of the $A\beta_{25-35}$-induced increase of GFAP labeling (Figure 5r and x).

Quantification of the increase in GFAP expression was performed in the hippocampus by western blotting. As shown in Figure 6, the ScAβ i.c.v. treatment or/and the ANAVEX1-41 i.p. treatment were without effect on GFAP expression. The $A\beta_{25-35}$ treatment significantly increased GFAP expression and this increase was blocked by ANAVEX1-41 ($F_{(5,49)} = 5.59$, $p<0.001$; Figure 6). These data strengthened the qualitative immunohistochemical observations.

Several biochemical parameters of amyloid toxicity were also analyzed in the hippocampus extracts to validate the neuroprotective activity of ANAVEX1-41. First, amyloid peptides, and particularly $A\beta_{25-35}$, induce a massive oxidative stress in forebrain structures. We therefore analyzed in the levels of lipid peroxidation (Figure 7a) and protein nitration (Figure 7b) and induction of caspase-9 expression, a marker of mitochondrial damage (Figure 7c).

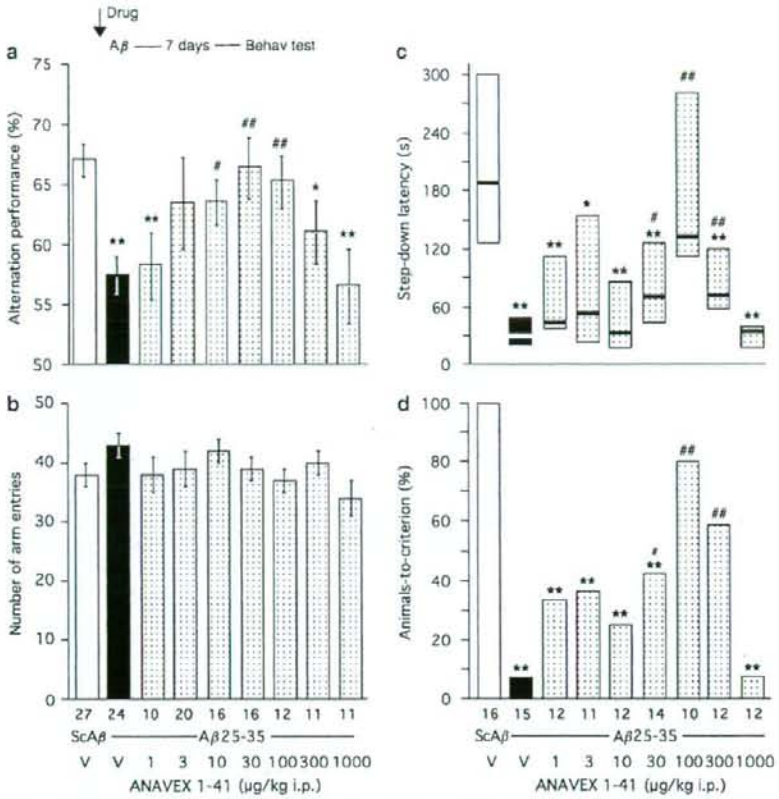

Figure 3 Neuroprotective effect of ANAVEX1-41 on $A\beta_{25-35}$-induced learning deficits in mice: alternation performance (a) and number of arm entries (b) in the *Y*-maze test; step-down latency (c) and percentage of animals-to-criterion (d) in the passive avoidance test. Mice were administered i.p. with vehicle solution (saline, V) or ANAVEX1-41 (1–1000 µg/kg) 20 min before being injected i.c.v. with ScAβ or $A\beta_{25-35}$ peptide (9 nmol). After 7 days, they were examined for spontaneous alternation or trained in the passive avoidance test (see insert). The number of animals per group is indicated below the columns in (b) and (d). $^{*}p<0.05$, $^{**}p<0.01$ *vs* (ScAβ + V)-treated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* ($A\beta_{25-35}$ + V)-treated group; Dunnett's test in (a), Dunn's test in (c), χ^2-test in (d).

Second, amyloid toxicity results in cell death through caspase-dependent apoptosis pathways. We therefore measured the induction of caspase-3 expression (Figure 7d).

$A\beta_{25-35}$ induced a +117% increase in the level of peroxidized lipids that could be measured in the hippocampus ($F_{(6,82)}=8.07$, $p<0.0001$; Figure 7a). ANAVEX1-41, tested in the 10–1000 µg/kg i.p. dose range, highly significantly, but in a U-shaped manner, prevented the $A\beta_{25-35}$-induced increase in lipid peroxidation. The protective effect was measured at 30 and 100 µg/kg (Figure 7a). The western blot analysis of protein nitration revealed only a single band for nitrated proteins at the size of 70 kDa (Figure 7b, see Supplementary Figure 1 for the whole blot). $A\beta_{25-35}$ induced a +30% increase in nitrotyrosine immunoreactivity ($F_{(3,23)}=8.99$, $p<0.001$; Figure 7b). The pretreatment with ANAVEX1-41, 100 µg/kg i.p., tended to decrease the level of nitrotyrosine immunoreactivity in ScAβ-treated mice (−19%, $p>0.05$) but highly significantly prevented the $A\beta_{25-35}$-induced increase (Figure 7b). The western blot analysis of caspase-9 expression revealed only a single band at the size of 49 kDa that corresponded to procaspase-9 (Figure 7c). $A\beta_{25-35}$ induced a +38% increase in caspase-9 expression ($F_{(3,51)}=4.13$, $p<0.05$; Figure 7c). The pretreatment with ANAVEX1-41, 100 µg/kg i.p., failed to affect caspase-9 expression in ScAβ- or $A\beta_{25-35}$-treated animals (Figure 7c). The western blot analysis of caspase-3 expression revealed only a single band at the size of 35 kDa that corresponded to the cleaved form of caspase-3 (Figure 7d). $A\beta_{25-35}$ induced a +32% increase in caspase-3 induction ($F_{(3,34)}=4.31$, $p<0.05$; Figure 7d). The pretreatment with ANAVEX1-41, 100 µg/kg i.p., significantly prevented the $A\beta_{25-35}$-induced increase (Figure 7d). However, the treatment also resulted in a significant increase in the level of caspase-3 induction in ScAβ-treated mice (+20%, $p<0.05$; Figure 7d).

Involvement of (i) Muscarinic receptors and (ii) σ_1 Protein in the Neuroprotective Effect of ANAVEX1-41

The compound is equally active, with binding affinities in the 18–114 nM range, on muscarinic M_1–M_4 receptors and the σ_1 protein (Espallergues *et al*, 2007). To determine

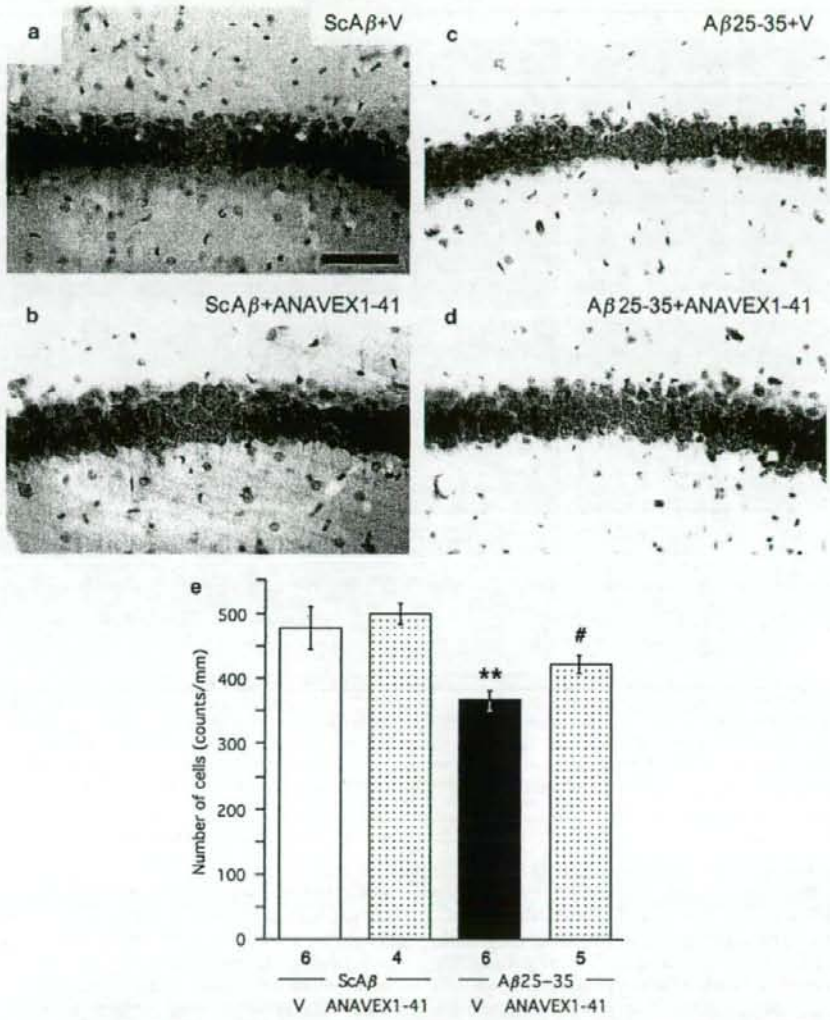

Figure 4 Neuroprotective effect of ANAVEX1-41 on $A\beta_{25-35}$-induced toxicity in mice: pyramidal cell loss in the CA1 area of the hippocampal pyramidal cell layer, 7 days after $A\beta_{25-35}$ injection. (a–d) Representative microphotographs of coronal sections of cresyl violet stained hippocampal CA1 subfield. (e) Averaged levels of viable cells. Mice were administered i.p. with saline vehicle solution (V) or ANAVEX1-41 (100 μg/kg), 20 min before being administered i.c.v. with $A\beta_{25-35}$ peptide (9 nmol). Scale bar shown in (a) = 100 μm. At least six slices were counted per mice and the number of mice used per group is indicated below the columns in (e). $^{**}p<0.01$ *vs* (ScAβ + V)-treated group; $^{\#}p<0.05$ *vs* ($A\beta_{25-35}$ + V)-treated group; Dunnett's test.

whether both pharmacological targets are involved in the protective effects of the compound, we coadministered: (i) the muscarinic receptor antagonist scopolamine (0.5 mg/kg) or (ii) the σ_1 protein inactivator BD1047 (1 mg/kg) with the active doses of ANAVEX1-41 (30, 100 μg/kg). The learning abilities were analyzed after 7 days using the *Y*-maze and passive avoidance procedures. As shown in Figure 8a, the muscarinic receptor antagonist attenuated the ANAVEX1-41 effect, nonsignificantly against the 30 μg/kg dose of ANAVEX1-41 and significantly against 100 μg/kg ($F_{(6,119)} = 5.14$, $p = 0.0001$). The BD1047 treatment led to a similar effect (Figure 8b). BD1047 attenuated the ANAVEX1-41 effect, nonsignificantly against the 30 μg/kg dose of ANAVEX1-41 and significantly against 100 μg/kg ($F_{(6,114)} = 4.55$, $p<0.001$; Figure 8b). In the passive avoidance test, scopolamine pretreatment also fully prevented the ANAVEX1-41 (100 μg/kg) effect, but not the ANAVEX1-41 (30 μg/kg) effect, similarly for latency ($H = 30.6$, $p<0.0001$; Figure 9a) and animals-to-criterion (Figure 9b). However, different results were obtained in the contextual memory procedure with BD1047. The σ_1 protein inactivator significantly blocked the beneficial effect of 30 μg/kg

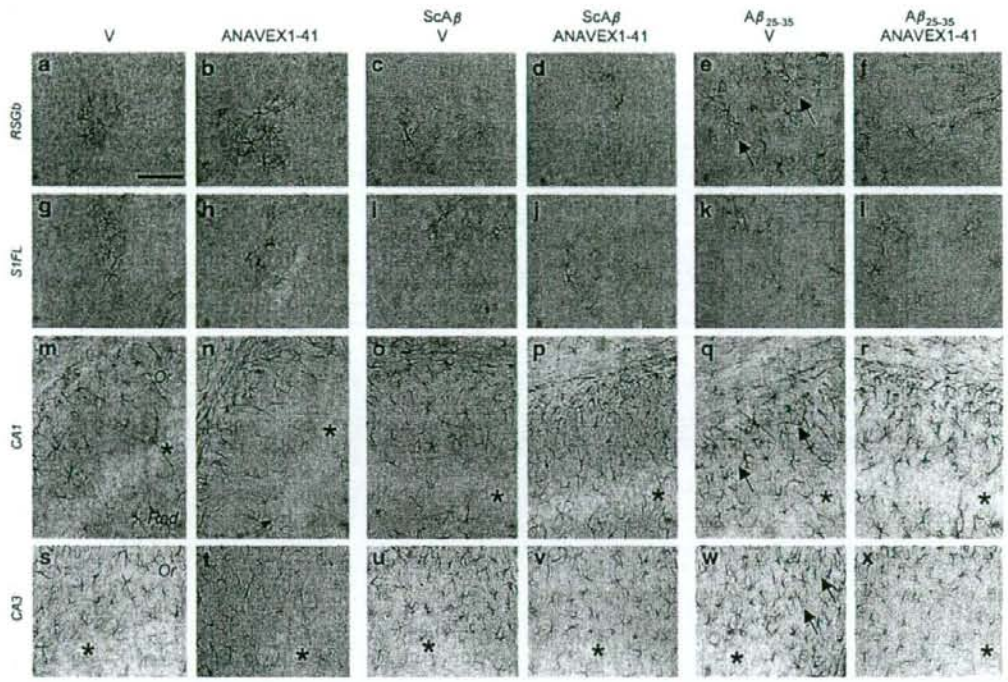

Figure 5 Morphological analysis of astrocytic reaction using GFAP immunolabeling in $A\beta_{25-35}$-treated mice. Animals were treated i.p. with saline vehicle solution (V) or ANAVEX1-41 (100 μg/kg) and received no i.c.v. treatment (two left columns), ScAβ (9 nmol; two central columns) or $A\beta_{25-35}$ (9 nmol; two right columns) and were killed after 7 days for immunohistological analysis. Coronal 30 μm thick sections were stained with anti-GFAP antibody and several brain areas were visually analyzed. Representative microphotographs are shown in two cortical areas, the retrospenial granular basal cortex (RSGb; a–f) and S1 cortex forelimb region (S1FL; g–l), and two hippocampal formation areas, the CA1 (m–r) and CA3 (s–x). The pyramidal cell layers are indicated by asterisks. Arrows outlined densifications of astrocyte labeling. Abbreviations: Or, oriens layer; Rad, stratum radiatum. At least three slices per mice and four mice per conditions were analyzed. Scale bar in (a) = 300 μm.

ANAVEX1-41, both in terms of step-down latency ($H = 39.7$, $p < 0.0001$; Figure 9c) and percentage of animals-to-criterion. The compound only nonsignificantly attenuated the ANAVEX1-41 (100 μg/kg) effect, particularly in terms of percentage of animals-to-criterion (Figure 9d), suggesting that protection through activation of σ_1 protein is differentially effective on short-term and long-term memory mechanisms.

DISCUSSION

The first data in this study showed that ANAVEX1-41 attenuated the learning deficits observed 1 week after the central injection of $A\beta_{25-35}$ peptide in mice. In the brain of rats or mice, $A\beta_{25-35}$ peptide induces, after acute injection or chronic infusion, biochemical changes, morphological alterations, and behavioral impairments reminiscent of AD physiopathology. In particular, $A\beta_{25-35}$-treated rodents showed spontaneous alternation, passive avoidance, and water-maze learning deficits (Maurice *et al*, 1996; Delobette *et al*, 1997) clearly related to alterations in cholinergic and glutamatergic corticolimbic systems (Maurice *et al*, 1996; Olariu *et al*, 2001). ANAVEX1-41, administered before the behavioral procedures, reversed the $A\beta_{25-35}$-induced deficits with a very low active dose range because the maximum antiamnesic effect was measured at 10 μg/kg for both the short-term and long-term memory tests. This observation confirms that ANAVEX1-41 is a very potent antiamnesic drug. The compound acts as a σ_1 protein activator, with a K_i value of 44 nM (Espallergues *et al*, 2007). Such pharmacological action is known to mediate antiamnesic effects, particularly against $A\beta_{25-35}$-induced learning impairments. Numerous σ_1 protein activators including (+)-SKF-10 047, (+)-pentazocine, SA4503, or PRE-084 attenuated $A\beta_{25-35}$-induced learning impairments (Maurice *et al*, 1998; Meunier *et al*, 2006). Indeed, activation of the σ_1 protein rapidly results in amplification of Ca^{2+} mobilization from intracellular stores, facilitating Ca^{2+}-dependent intracellular pathways and activation of intracellular kinases (Morin-Surun *et al*, 1999; Hayashi and Su, 2001; Dong *et al*, 2005). In turn, σ_1 protein activators increase hippocampus glutamatergic transmission by facilitating glutamate release, activation of glutamate receptors and long-term potentiation (Monnet *et al*, 1992; Dong *et al*, 2005). They may also directly facilitate cholinergic neurotransmission by inducing acetylcholine release in the

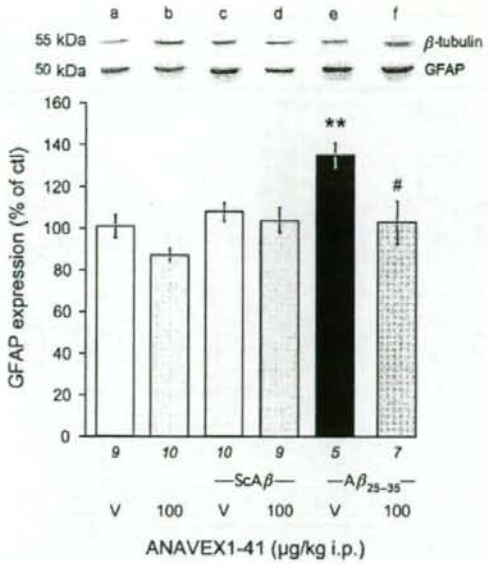

Figure 6 Effect of ANAVEX1-41 on GFAP expression measured by western blot in the hippocampus of $A\beta_{25-35}$-treated mice. Animals were treated i.p. with saline vehicle solution (V) or ANAVEX1-41 (100 μg/kg) and received no i.c.v. treatment (two left columns), $ScA\beta$ (9 nmol; two central columns) or $A\beta_{25-35}$ (9 nmol; two right columns) and were killed after 7 days for western blot analysis. GFAP 50 kDa variations were compared with untreated mice and normalized with β-tubulin expression levels. Typical micrographs are shown in the upper panel. The number of animals per group is indicated below each column. The number of animals per group is indicated below the columns. Lanes on the blots: *a*, V; *b*, ANAVEX1-41; *c*, $ScA\beta$ + V; *d*, $ScA\beta$ + ANAVEX1-41; *e*, $A\beta_{25-35}$ + V; *f*, $A\beta_{25-35}$ + ANAVEX1-41. $^{**}p<0.01$ *vs* the V-treated group; $^{\#}p<0.05$ *vs* the ($A\beta_{25-35}$ + V)-treated group; Dunnett's test.

hippocampus and cortex (Matsuno *et al*, 1995; Horan *et al*, 2002).

ANAVEX1-41, however, also acts as a muscarinic ligand. We have previously reported that the compound shows K_i values in the low nanomolar range for muscarinic receptors subtypes (18–114 nM), with a profile by ascending order of potency: $M_1 > M_3 > M_4 > M_2$ (Espallergues *et al*, 2007). All subtypes of muscarinic receptors are expressed in the hippocampus and cortex (Levey *et al*, 1995) and post-synaptic M_1 and autoreceptor M_2 subtypes have been shown to be crucially involved in learning and memory processes (Ghelardini *et al*, 1999; but see also Quirion *et al*, 1995; Miyakawa *et al*, 2001; Seeger *et al*, 2004). Nonselective muscarinic antagonists, such as scopolamine and atropine, impair performance in various learning and memory tasks in rodents, including eight-arm radial maze learning (Eckerman *et al*, 1980), contextual fear conditioning (Anagnostaras *et al*, 1995), water-maze learning (Sutherland *et al*, 1982), or passive avoidance (Espallergues *et al*, 2007).

The combined activity of ANAVEX1-41 at σ_1 protein and muscarinic receptors is expected to lead to synergistic effect on memory. Indeed, activation of the σ_1 protein and M_2 autoreceptors antagonism by ANAVEX1-41 (Vamvakides, 2002; Espallergues *et al*, 2007) may facilitate Ca^{2+}-dependent acetylcholine release from presynaptic terminals in the hippocampus and cortex, as shown with other compounds (Quirion *et al*, 1995; Matsuno *et al*, 1995; Horan *et al*, 2002). As previously discussed (Espallergues *et al*, 2007), it is obvious that, at the very low pharmacologically active doses (10–100 μg/kg) measured for ANAVEX1-41, the compound acts both as σ_1 activator and muscarinic receptor ligand and provokes complex concomitant effects on neurotransmission that will affect: (i) acetylcholine release, by presynaptic σ_1 protein-mediated and M_2 autoreceptor-mediated effects; (ii) phospholipase C activation induced by muscarinic receptor activation but amplified by σ_1 protein-mediated activity; and (iii) IP_3 formation and activation of ER IP_3 receptors, again amplified by the σ_1 protein activation. Noteworthy, the active dose shown by ANAVEX1-41 is unrelated to the drug *in vitro* affinities for either σ_1 protein or muscarinic receptor subtypes. For comparison, PRE-084, a selective σ_1 activator with a similar affinity of 44 nM (Su *et al*, 1991), is antiamnesic at 0.5–1 mg/kg against $A\beta_{25-35}$-induced learning impairments (Meunier *et al*, 2006). One of the most promising muscarinic compound, AF102B, inhibiting ^{3}H-quinuclidinyl benzilate binding with K_i values in the 1–5 nM concentration range (Fisher *et al*, 1991), is active at 1–5 mg/kg against the learning deficits induced in rats by bilateral i.c.v. injection of the cholinotoxin ethylcholine aziridinium ion (AF64A; Nakahara *et al*, 1989). ANAVEX1-41, with a similar affinity for σ_1 protein as PRE-084 and even lower affinities for muscarinic subtypes as AF102B, showed an *in vivo* activity at 10 μg/kg, ie, almost 100 times lower than the cited drugs. These data must be tempered after considering the protein binding and brain/plasma ratio in humans, but suggests strong synergic effects between the σ_1 and muscarinic targets. The precise mechanism of action remains to be analyzed more adequately using *in vitro* preparations, but it clearly relies on facilitated Ca^{2+} mobilization and activation of Ca^{2+}-dependent intracellular signaling induced by muscarinic receptor and σ_1 protein during learning-induced neuronal activation.

The second part of the study analyzed the neuroprotective potential of ANAVEX1-41 in $A\beta_{25-35}$-treated mice. For this purpose, the compound was administered at the same time as $A\beta_{25-35}$, ie, 7 days before the behavioral, morphological or biochemical analyses, a procedure known to allow the observation of neuroprotective effects for mixed cholinergic and σ_1 drugs (Meunier *et al*, 2006). The compound induced a bell shaped but significant prevention of $A\beta_{25-35}$-induced learning deficits, with an active dose about 100 μg/kg. At the morphological level, $A\beta_{25-35}$ induced a limited but significant cell loss in the CA1 pyramidal cell layer of the hippocampus (Stepanichev *et al*, 2004) and a marked inflammation in corticolimbic structures that could be visualized by analyzing the GFAP immunolabeling in reactive astrocytes (Stepanichev *et al*, 2003; Klementiev *et al*, 2007). Interestingly, although a significant cell loss could be measured in particularly vulnerable areas, like CA1 in mice, GFAP immunolabeling increased in a more diffuse manner, in structures associated with the amyloid deposits, as observed in the retrospenial granular basal cortex and oriens layer of the hippocampus. ANAVEX1-41, tested at 100 μg/kg, significantly attenuated the $A\beta_{25-35}$-induced cell loss in CA1 and increase in GFAP expression, as shown by

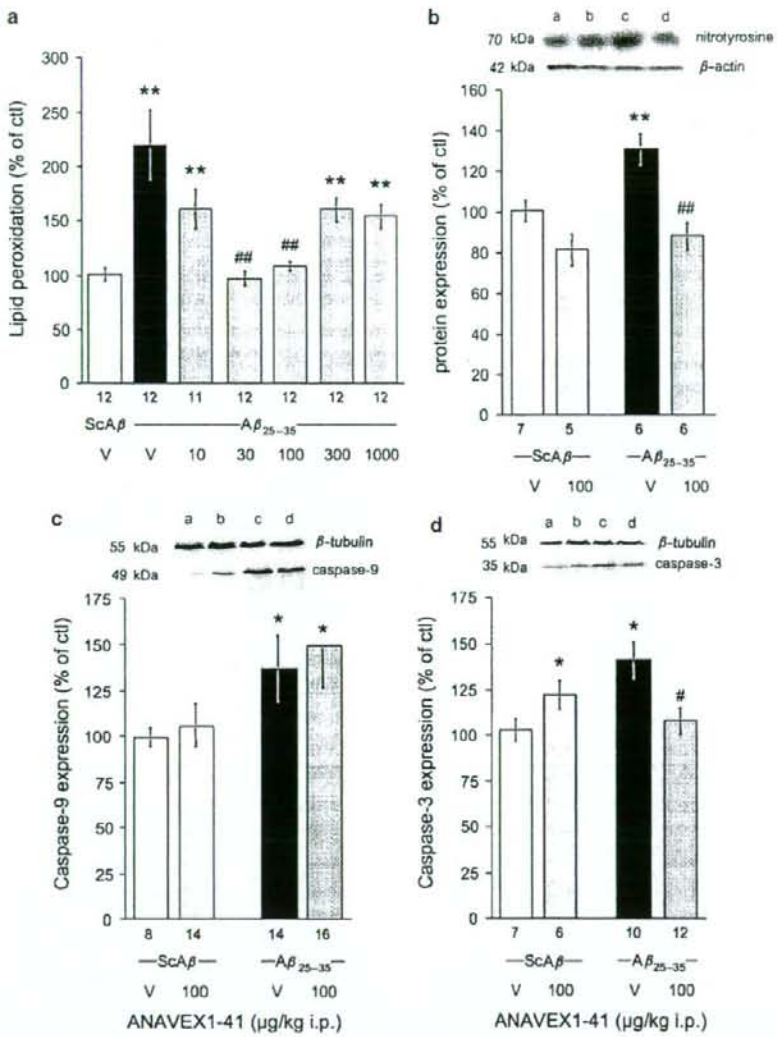

Figure 7 Neuroprotective effects of ANAVEX1-41 measured using biochemical markers in the hippocampus in $A\beta_{25-35}$ peptide-injected mice: (a) lipid peroxidation levels, (b) protein nitration levels; (c) caspase-9 expression; (d) caspase-3 expression. Mice were administered i.p. with saline vehicle solution (V) or ANAVEX1-41, 10–1000 µg/kg in (a) or 100 µg/kg in (b) and (c), 20 min before the i.c.v. injection of ScAβ or $A\beta_{25-35}$ peptide (9 nmol). Lipid peroxidation levels and caspases induction were measured on day 7 and protein nitration on day 5. The number of animals per group is indicated below the columns. Lanes on the blots: *a*, ScAβ + V; *b*, ScAβ + ANAVEX1-41; *c*, $A\beta_{25-35}$ + V; *d*, $A\beta_{25-35}$ + ANAVEX1-41. $^{*}p<0.05$, $^{**}p<0.01$ *vs* the (ScAβ + V)-treated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* the ($A\beta_{25-35}$ + V)-treated group; Dunnett's test.

western blot. It appeared then that ANAVEX1-41 is able to counteract the morphological damages induced by amyloid toxicity in sensitive structures.

The neuroprotective effect of the compound was also tested using selected biochemical markers. First, Aβ induces a strong oxidative stress, as observed in cell culture models (Behl *et al*, 1994) or in the hippocampus and cortex of rodents centrally injected with the peptides (Meunier *et al*, 2006). We therefore analyzed the level of lipid peroxidation in the hippocampus, 7 days after $A\beta_{25-35}$. Peroxynitrite anion, $ONOO^-$, is formed from nitric oxide and superoxide anion during oxidative stress and is responsible for a widespread biological damage in the AD brains (Smith *et al*, 1997). $A\beta_{25-35}$-induced formation of $ONOO^-$ could be indirectly indicated by the level of nitrated proteins, 5 days after peptide injection (Alkam *et al*, 2007). Moreover, Aβ-induced oxidative stress is because of production of reactive oxygen species by the mitochondria, by premature electron leakage to oxygen through the respiratory electron transport chain, and dysfunction of enzymes responsible for

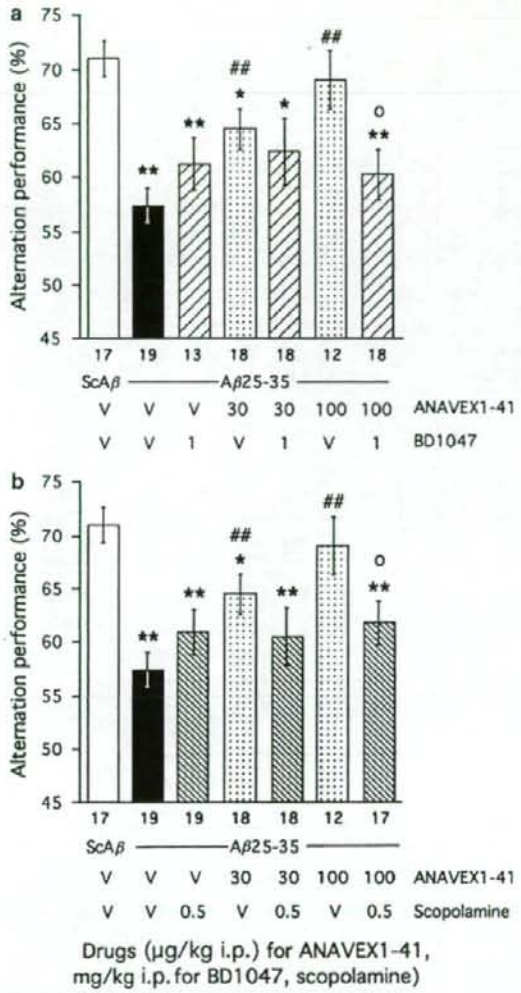

Figure 8 Effect of the preadministration of the muscarinic antagonist scopolamine (b) or the σ_1 receptor antagonist BD1047 (a) on the ANAVEX1-41 protective effect against the Aβ_{25-35}-induced alternation deficits in mice. Mice were administered i.p. with saline vehicle solution (V), scopolamine (0.5 mg/kg), BD1047 (1 mg/kg) and/or ANAVEX1-41 (30, 100 µg/kg), 20 min before ScAβ or Aβ_{25-35} (9 nmol). After 7 days, they were examined for spontaneous alternation in the Y-maze. The number of animals per group is indicated below the columns. $^{*}p<0.05$, $^{**}p<0.01$ *vs* (ScAβ + V)-treated group; $^{\#\#}p<0.01$ *vs* (Aβ_{25-35} + V)-treated group; $^{o}p<0.05$ *vs* (Aβ_{25-35} + ANAVEX1-41)-treated group; Dunnett's test.

limiting the superoxide production, such as NAPDH-dependent oxidase, NADH-dependent diaphorase, and superoxide dismutase (Kim *et al*, 2003). Several markers could be used to selectively assess the appearance of mitochondrial damage, such as release of cytochrome *c* into the cytosol or, as we analyzed, induction of caspase-9. Finally, we also analyzed the induction of caspase-3, known to be a key mediator of Aβ-mediated apoptosis. Results showed that ANAVEX1-41 blocked the Aβ_{25-35}-induced increase in lipid peroxidation, at 30 and 100 µg/kg, in the hippocampus. The compound also blocked the increase in protein nitration. This antioxidant effect, however, may not primarily involve the mitochondria because Aβ_{25-35}-induced increase in caspase-9 was not attenuated by ANAVEX1-41. Noteworthy, the σ_1 protein is expressed at the surface of the mitochondria and at focal contacts between the ER and mitochondria (Hayashi and Su, 2007). We have previously observed that the σ_1 protein activator PRE-084 blocks the Aβ_{25-35}-induced increase in lipid peroxidation (Meunier *et al*, 2006), suggesting that activation of the σ_1 protein results in an antioxidant effect mediated at the mitochondrial level. Our biochemical data suggest that ANAVEX1-41 also induces a strong antioxidant effect that may, however, not primarily involve a protection of mitochondrial integrity through σ_1 protein activation. Otherwise, oxidative stress has been shown to impair M_1 and M_2 muscarinic receptor signaling activity, through increased phosphorylation and sequestration (Mou *et al*, 2006), an effect that may impede the pharmacological action of ANAVEX1-41 at muscarinic receptors. A precise mechanistic study has therefore to be carried out to identify the mechanism of the antioxidant action of ANAVEX1-41. The compound is nevertheless protective against the resulting apoptosis, as it blocked the induction of caspase-3. This observation could be considered as one of the cellular correlates of the protecting effect of ANAVEX1-41, already described at the morphological and behavioral levels.

The mechanism of the neuroprotective activity of ANAVEX1-41 is likely to involve, as detailed above regarding its antiamnesic action, a complex interaction between its muscarinic and σ_1 targets. We observed that scopolamine or BD1047 could significantly inhibit the protective effect of ANAVEX1-41, at least in terms of learning deficits. A synergistic σ_1/muscarinic mechanism could also be evoked to account for the neuroprotective efficacy of ANAVEX1-41, in particular, through the phospholipase C involvement and regulation of intracellular Ca^{2+} homeostasis.

In summary, we reported that ANAVEX1-41, a new mixed muscarinic receptor ligand and σ_1 protein activator, is a very active antiamnesic and neuroprotective drug against Aβ_{25-35} peptide-induced amnesia and toxicity in the mouse. Its similar efficacy at muscarinic and σ_1 targets suggest a unique, concomitant action, most probably at the presynaptic and intraneuronal levels, on neurotransmitter release, activation of membrane receptors and intracellular transduction systems.

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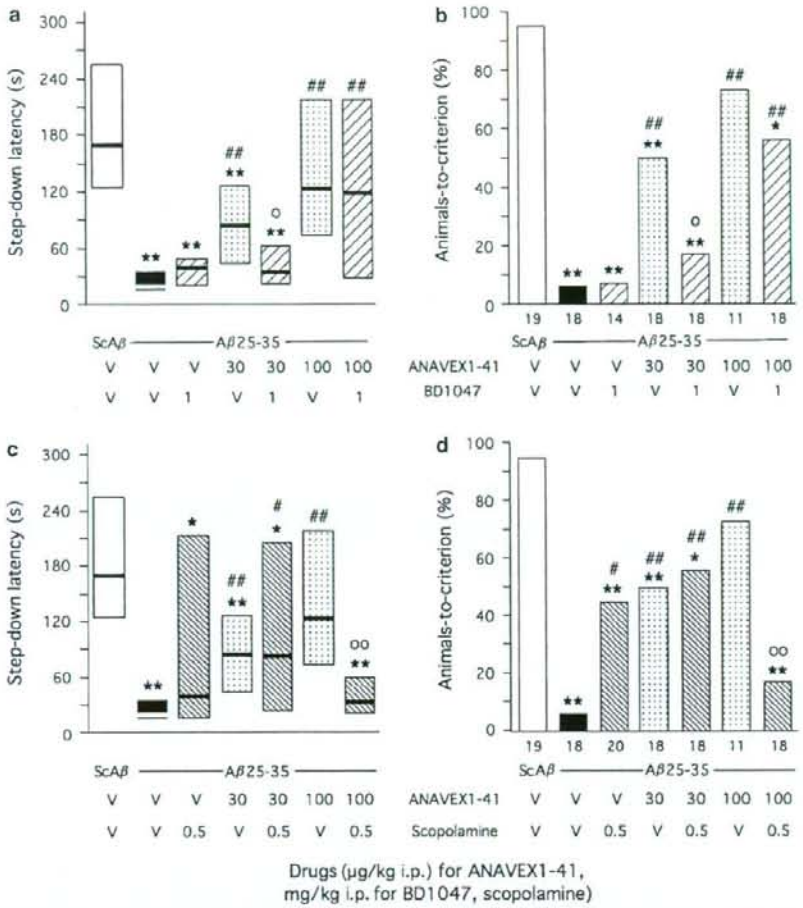

Figure 9 Effect of the preadministration of scopolamine (c, d) or BD1047 (a, b) on the ANAVEX1-41 effect against the $A\beta_{25-35}$-induced passive avoidance deficits in mice: step-down latency (a, c) and percentage of animals-to-criterion (b, d). Mice were administered i.p. with saline vehicle solution (V), scopolamine (0.5 mg/kg), BD1047 (1 mg/kg), and/or ANAVEX1-41 (30, 100 µg/kg), 20 min before ScAβ or $A\beta_{25-35}$ (9 nmol). They were trained on day 7 and retention was performed on day 8. The number of animals is indicated below the columns. $^{*}p<0.05$, $^{**}p<0.01$ *vs* (ScAβ + V)-treated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* ($A\beta_{25-35}$ + V)-treated group; $^{\circ}p<0.05$, $^{\circ\circ}p<0.01$ *vs* ($A\beta_{25-35}$ + ANAVEX1-41)-treated group; Dunn's test in (a) and (c), χ^2-test in (b) and (d).

DISCLOSURE/CONFLICT OF INTEREST

T Maurice is a member of the scientific advisory board of Anavex Life Sciences. Other authors declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Research report

Restraining tumor necrosis factor-alpha by thalidomide prevents the Amyloid beta-induced impairment of recognition memory in mice

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Abstract

No effective remedy has currently been realized to prevent the cognitive impairments of Alzheimer's disease (AD). The interruption of the toxic pathways of amyloid beta peptide (Aβ) still remains promising for the treatment. The involvement of tumor necrosis factor-alpha (TNF-α) in the toxicity of $A\beta_{1-40}$ in recent reports provide a fresh target for the interruption. In the current study, we evaluated the feasibility of a strategy that target TNF-α to prevent the impairment of memory induced by Aβ. The *i.c.v.*-injection of $A\beta_{25-35}$ increased the hippocampal mRNA expression of both TNF-α and inducible nitric oxide synthase (iNOS), of which the former was stronger. The knock-out of TNF-α (TNF-α (−/−)) in mouse prevented the increase of iNOS mRNA induced by $A\beta_{25-35}$. Not only the inhibition of iNOS activity but also TNF-α (−/−) prevented the nitration of proteins in the hippocampus and the impairment of recognition memory in mice induced by $A\beta_{25-35}$. Daily treatment with thalidomide (20 mg/kg), a preferential degrader of TNF-α mRNA, or *i.c.v.*-injection of an anti-TNF-α antibody (10 ηg/mouse) prevented the nitration of proteins in the hippocampus and the impairment of recognition memory induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ in mice. These results suggested the practicability of targeting TNF-α as a preventive strategy against Aβ-mediated cognitive impairments.

Keywords: Amyloid beta ($A\beta_{25-35}$); Tumor necrosis factor-alpha; Protein nitration; Recognition memory; Thalidomide

1. Introduction

Treating the cognitive impairments of Alzheimer's disease (AD) is a complex practice due to its multifactoral pathology that including the increase of toxic amyloid beta peptide (Aβ), neuroinflammation, oxidative damage, and neurodegeneration in critical brain regions involved in memory and cognition [18,42,49,53,63,66]. Although, there have been many recent advances in the understandings of the pathological process of AD, therapeutic approaches still remain limited to targeting the toxic pathways of Aβ, the assumed central player in the pathogenesis of the disease [15,19,30,38,39,41,50].

The neurotoxicity of Aβ has recently been implicated by the involvement of tumor necrosis factor-alpha (TNF-α), a multifunctional cytokine that triggers a wide range of cellular responses [35]. TNF-α is found upregulated in AD patients [8,17,43,56], and involved in $A\beta_{1-40}$-induced inhibition of long-term potentiation in hippocampal slices and the impairment of spatial memory in mice [36,65]. $A\beta_{1-40}$ induces toxicity by a mechanism of TNF-α dependent overexpression of inducible nitric oxide synthase (iNOS) [2,13,69]. In general, TNF-α is regarded as an inducer of the expression of iNOS [11,23,51]. The induced iNOS produces high amount of NO [25,59] which interacts rapidly with superoxide to form prox-

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ynitrite [6,46]. Proxynitrite mediates the extensive nitration of proteins in the brain of AD and evidently correlated with the increased level of cerebral Aβ and the severity of cognitive impairment [27,28,54,57]. The impairments of memory induced by Aβ in mice are prevented by the genetic deletion of TNF-α receptor or iNOS as well as by scavenging of peroxynitrite [3,36,40]. The implications of the involvement of TNF-α in iNOS-driven peroxynitrite and memory failure prompt a fresh strategy of targeting the synthesis of TNF-α for the prevention of Aβ-induced cognitive impairment. In this study, we have reconfirmed the critical involvement of TNF-α in the neurotoxicity of $A\beta_{25-35}$, the most toxic Aβ fragment that detected in the brain of AD patient [29,44,68], by utilizing TNF-α (−/−) mouse and evaluated the usefulness of targeting TNF-α by using thalidomide to prevent the Aβ-induced impairment of memory in mice.

2. Material and methods

2.1. Animals

Male C57BL/6 (wild type, WT) mice, male TNF-α knock out (TNF-α (−/−)) mice [55], and male ICR mice (Nihon SLC Co., Shizuoka, Japan), were used. The animals were housed in a controlled environment (23 ± 1 °C, 50 ± 5% humidity) and allowed food and water ad lib. The room lights were kept on between 8:00 .a.m and 8:00 p.m. All experiments were performed in accordance with the guidelines for animal experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in "Principles of Laboratory Animal Care".

2.2. Treatment and experimental design

$A\beta_{25-35}$ and $A\beta_{35-25}$ (Bachem, Bubendorf, Switzerland) were dissolved in sterile double-distilled water in a concentration of 1 mg/ml and stored at −20 °C before use and incubated for aggregation at 37 °C for 4 days before the injection. $A\beta_{1-40}$ and $A\beta_{40-1}$ (Bachem, Bubendorf, Switzerland) were dissolved in 35% acetonitrile/0.1% trifluoroacetic acid to a stock concentration of 1 mg/ml and stored at −20 °C before use. Peptides including $A\beta_{25-35}$ and $A\beta_{35-25}$ were *i.c.v.*- injected at a volume of 3 μl. Peptides including $A\beta_{40-1}$ and $A\beta_{1-40}$ were *i.c.v.*-injected at a volume of 5 μl. $A\beta_{35-25}$ and $A\beta_{40-1}$ were injected as the control. All peptides were injected as described previously [3,34]. Aminoguanidine (Wako, Saitama, Japan) was dissolved in saline and *i.p.*-injected at the dose of 100 mg/kg/day as described previously [36,59]. Thalidomide (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and *p.o.*-administrated at the dose of 20 mg/kg/day that was selected according to the previous literature on its pharmacodynamic study in mice [12]. Anti-TNF-α antibody (α-TNF-α) (R&D Systems, Minneapolis, MN) was *i.c.v.*-injected at the dose of 10 ηg that followed by the injection of Aβ peptides with 15 min interval as described previously [36]. The schedule of administration of peptides and drugs as well as biochemical and behavioral investigations are shown in Fig. 1.

2.3. Real-time reverse transcription-polymerase chain reaction

Mice were decapitated at the indicated time-points after the *i.c.v.*-injection of $A\beta_{25-35}$ (day 0). The hippocampi were removed on ice-cold glass plate and stored at −80 °C. The hippocampal tissue was homogenized and total RNA was extracted using an RNeasy total RNA isolation kit, following the supplier's protocol (Qiagen, Valencia, CA). cDNA was synthesized by using a Superscript™ reverse transcriptase kit (Invitrogen, Carlsbad, CA). The primer sequences were given below: for iNOS (Gene Bank access: NM_010927), forward primer: 5′-GGGCAGCCTGTGAGACCTT-3′; reverse primer: 5′-GCATTGGAAGTGAAGCGTTTC-3′; TaqMan probe: TGTCCGAAGCAAACATCACATTCAGATCC; For TNF-α (Gene Bank access: NM_023517), forward primer: 5′-CTTTCGGTTGCTCTTTGGTTGAG-3′; reverse primer: 5′-GCAGCTCTGTCTGTTGGATCAG-3′; TaqMan probe: TGCGACAGCACAAGTCACAGCCCC; for the brain-derived neurotrophic factor (BDNF) (Gene Bank access: BC034862), forward primer: 5′-GCAAACATGTCTATGAGGGTTCG-3′; reverse primer: 5′-ACTCGCTAATACTGTCACACACG-3′; TaqMan probe: ACTCCGACCCTGCCCGCCGT; for glial cell-derived neurotrophic factor (GDNF) (Gene Bank access: NM_010275), forward primer: 5′- GAAGAGAGAGGAATCGGCAGG-3′; reverse primer: 5′-TGGCCTCTGCGACCTTTC-3′; TaqMan probe: AGCTGCCAGCCCAGAGAATTCCAGAG; For all, the experimental amplification protocol consisted of a first round at 95 °C for 3 min and then 30 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 60 s, and extension at 72 °C for 1 min, with a final extension reaction carried out at 72 °C for 10 min. PCR was carried out on Bio-Rad iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The signal was detected according to the manufacturer's instructions. The expression levels were calculated as described previously [62].

2.4. Western blotting

Animals were decapitated on day 7 after the injection of Aβ peptides. The hippocampi were removed on ice-cold glass plate and stored at −80 °C. The hippocampal tissues were homogenized as described previously [3]. Briefly, the hippocampal tissues were homogenized in ice-cold extraction buffer (150 μl of 20 mM Tris–HCl buffer (pH 7.6) containing 150 mM NaCl, 2 mM EDTA · 2Na, 50 mM sodium fluoride, 1 mM sodium vanadate, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mg/ml pepstatin, 1 mg/ml aprotinin, and 1 mg/ml leupeptin). Equal amounts of protein, 20 μg/lane, were resolved by a 10% SDS-polyacrylamide gel electrophoresis, and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). Membranes were incubated in 3% skim milk in a washing buffer (Tris-buffered saline containing 0.05% (v/v) Tween-20) for 2 h at room temperature. Then the membranes were incubated at 4 °C overnight with a diluted (1:1000) anti-nitrotyrosine mouse clone1A6 (Upstate cell signaling, Lake Placid, USA) or with goat anti-actin primary antibody (1:100) (Santa Cruz Biotechnology Inc., Santa Crutz, CA). After a wash, membranes were incubated with horseradish peroxidase-labeled anti-mouse IgG (1:2000) (Kirkegaard & Perry Laboratories, Baltimore, MD) or with donkey anti-goat IgG secondary antibody (1:2000) (Santa Cruz Biotechnology Inc., Santa Crutz, CA). Immunoreactive complexes on the membrane were detected using Western blotting detection reagents (Amersham Biosciences Inc., Piscataway, NJ) according to the manufacturer's instructions, and exposed to X-ray film. The intensity of each protein band on the film, analyzed with the Atto Densitograph 4.1 system (Atto, Tokyo, Japan), and was corrected with the corresponding β-actin level. The results were expressed as the percentage of that of the control.

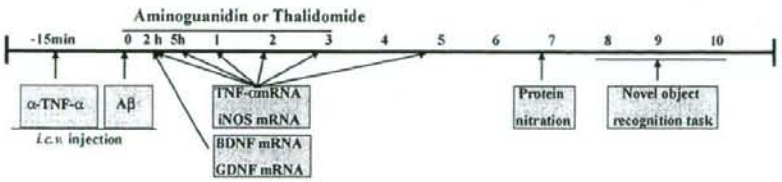

Fig. 1. The experimental schedule.

2.5. Novel object recognition task

This task, based on the spontaneous tendency of rodents to explore a novel object more often than a familiar one [16], was performed during Day 8–Day 10 after the *i.c.v.* injection of Aβ peptides as described previously [3]. A plastic chamber (35 × 35 × 35 cm) was used in low light condition during the light phase of the light/dark cycle. The general procedure consisted of three different phases: a habituation phase, an acquisition phase, and a retention phase. On the first day (habituation phase), mice were individually subjected to a single familiarization session of 10 min, during which they were introduced in the empty arena, in order to become familiar with the apparatus. On the second day (acquisition phase) animals were subjected to a single 10 min session, during which floor-fixed two objects (A and B) were placed in a symmetric position from the centre of the arena, 15 cm from each and 8 cm from the nearest wall. The two objects, made of the same wooden material with the similar color and smell, were different in shape but identical in size. Mice were allowed to explore the objects in the open field. A preference index for each mouse was expressed as a ratio of the amount of time spent exploring object A (TA × 100)/(TA + TB), where TA and TB are the time spent on exploring object A and object B, respectively. On the third day (retention phase), mice were allowed to explore the open field in the presence of two objects: the familiar object A and a novel object C in different shape but in similar color and size (A and C). A recognition index, calculated for each mouse, was expressed as the ratio (TC × 100)/(TA + TC), where TA and TC are the time spent during retention phase on object A and object C, respectively. The time spent exploring the object (nose pointing toward the object at a distance ≤1 cm) was recorded by hand.

2.6. Statistical analyses

The results are expressed as the mean ± S.E. Statistical significance was determined with one-way ANOVA followed by the Bonferroni multiple comparisons test. $p < 0.05$ was taken as a significant level of difference.

3. Results

3.1. TNF-α was involved in $A\beta_{25-35}$-induced impairment of recognition memory

The expression of TNF-α and iNOS mRNA in the hippocampus of mice was investigated at different time points after the *i.c.v.*-injection of $A\beta_{25-35}$. At 2 h time point, $A\beta_{25-35}$ increased the mRNA expressions of TNF-α and iNOS, the expression of the former was stronger than that of the latter (Fig. 2A). $A\beta_{25-35}$ did not increase the expression of iNOS mRNA in TNF-α (−/−) mouse at the two-hour time point (Fig. 2B).

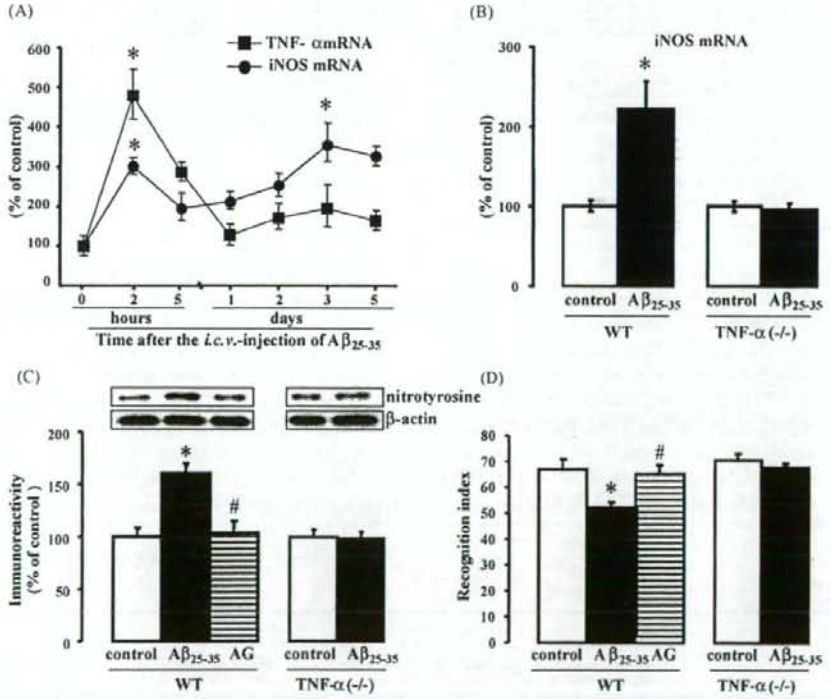

Fig. 2. TNF-α is required for the iNOS-mediated nitration of proteins and impairment of memory induced by $A\beta_{25-35}$. At different time points after the *i.c.v.*-injection of $A\beta_{25-35}$ (day 0) in mice, the mRNA expression levels of TNF-α and iNOS in the hippocampus were investigated by using real-time RT-PCR. A: $A\beta_{25-35}$ induced a sharp increase of TNF-α and iNOS mRNA in two hours after injection, the expression of the former was stronger than that of the latter. Data were presented as the mean ± S.E. ($n = 4$). * $p < 0.05$ vs. control. B: TNF-α (−/−) prevented the increase of iNOS mRNA which was induced by $A\beta_{25-35}$ in WT mice. Data were presented as the mean ± S.E. ($n = 4$). * $p < 0.05$ vs. control. C–D: Either the selective inhibition of iNOS activity by AG or TNF-α (−/−) prevented the $A\beta_{25-35}$-induced nitration of proteins in the hippocampus (day 7) and the impairment of novel object recognition memory (day 8–10). Data were presented as the mean ± S.E., ($n = 4$ for C, $n = 10$ for D), * $p < 0.05$ vs. control, # $p < 0.05$ vs. $A\beta_{25-35}$; TNF-α: tumor necrosis factor-alpha; iNOS: inducible nitric oxide synthase; WT: wild type; TNF-α (−/−): TNF-α knock out; AG: aminoguanidine; $A\beta_{25-35}$: Amyloid beta peptide (25–35).

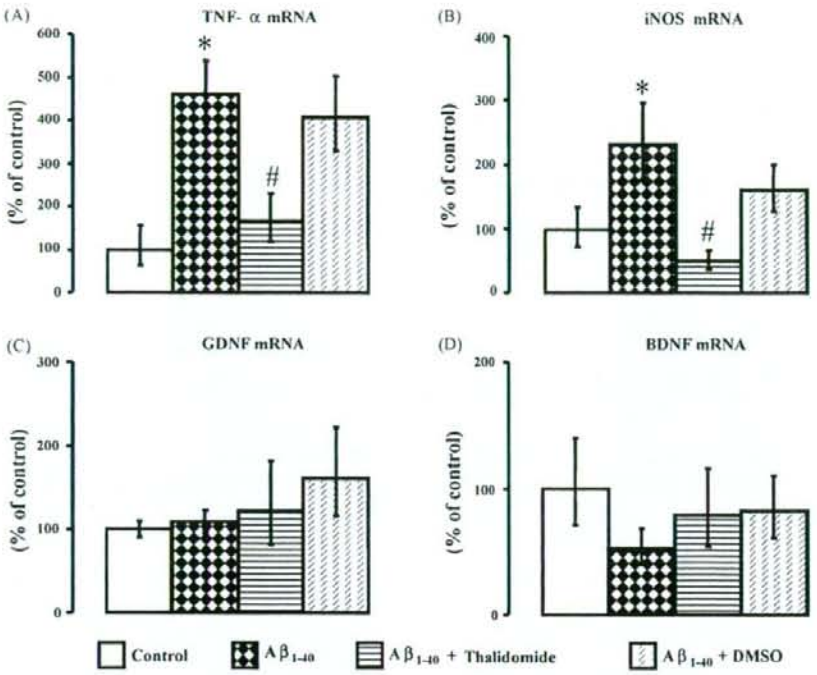

Fig. 3. Thalidomide suppressed the increase of TNF-α mRNA induced by $A\beta_{1-40}$. Thalidomide (20 mg/kg) was administrated *p.o.* one hour before the *i.c.v.*-injection of $A\beta_{1-40}$ in mice. A–D: Two hours after the injection of $A\beta_{1-40}$, the mRNA levels of TNF-α, iNOS, BDNF, and GDNF in the hippocampus were investigated by using real-time RT-PCR. Thalidomide suppressed the increase of TNF-α and iNOS mRNA induced by $A\beta_{1-40}$. The mRNA levels of BDNF and GDNF in the hippocampus were not changed either by $A\beta_{1-40}$ injection or thalidomide treatment. Data were presented as the mean ± S.E., $n = 4$, $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. $A\beta_{1-40}$; TNF-α: tumor necrosis factor-alpha; iNOS: inducible nitric oxide synthase; GDNF: glial cell-derived neurotrophic factor; BDNF: brain-derived neurotrophic factor; $A\beta_{1-40}$: Amyloid beta peptide (1–40). DMSO: dimethyl sulfoxide.

The selective inhibition of iNOS activity by aminoguanidine [14] or TNF-α (−/−) prevented $A\beta_{25-35}$ induced nitration of proteins in the hippocampus or the impairment of recognition memory in mice (Fig. 2C and D). The western blott analysis of protein nitration revealed only a single band for nitrated proteins at the size of 70 kDa. Through extensive studies with different anti-nitrotyrosine antibodies from different origin, we found that the detection of this single band is owing to the selectivity of the anti-nitrotyrosine mouse antibody [3]. These results, together with previous reports [2,13,36,65], confirmed that TNF-α is essential for the neurotoxicity of Aβ.

3.2. *Restraining TNF-α by thalidomide prevented Aβ-induced impairment of recognition memory*

The consistent involvement of TNF-α in the neurotoxicity of Aβ prompted us to examine an available candidate for anti-TNF-α remedy. Thalidomide, an immunomodulatory drug, has recently been suggested for the treatment of neurodegenerative disease as a selective inhibitor of TNF-α [21,60]. Thalidomide inhibits the synthesis of TNF-α through the degradation of mRNA [37]. We reexamined the selectivity of thalidomide-induced degradation of TNF-α mRNA. Thalidomide suppressed the increase of both TNF-α and iNOS mRNA induced by $A\beta_{1-40}$ (Fig. 3A and B). The decrease of iNOS mRNA might be due to the decrease of TNF-α [32]. The mRNA expressions of brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF), however, were not affected either by $A\beta_{1-40}$ injection or thalidomide treatment (Fig. 3C and D). Either the daily treatment with thalidomide or the *i.c.v.*-injection of an anti-TNF-α antibody prevented the nitration of proteins in the hippocampus and the impairment of recognition memory induced by $A\beta_{25-35}$ or $A\beta_{1-40}$ (Fig. 4A and B). The preventive effects of the anti-TNF-α antibody was consistent with previous reports [36,65], and supported the validity of targeting TNF-α by thalidomide to prevent the neurotoxicity of Aβ.

4. Discussion

Providing effective medication or practical strategy for the treatment of the cognitive deficits in AD can have a dramatic effect on the quality of life of a patient.

As a strategy to prevent the cognitive decline in AD, anti-oxidants are well recommended based on the association of the increased cerebral oxidative damage with the progress of the disease [7,22,45]. The oxidative damage in the brain of AD is mostly ascribed to the increased level of Aβ that induces the peroxynitrite-mediated nitration of proteins and the resultant

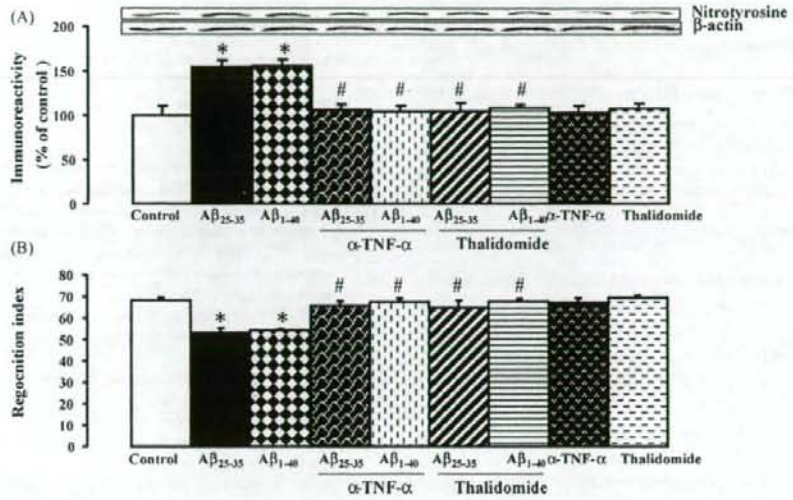

Fig. 4. Thalidomide prevented the nitration of proteins and the impairment of memory induced by Aβ. A–B: Thalidomide (20 mg/kg) was administrated *p.o.* daily until day 3 after the *i.c.v.*-injection of $A\beta_{25-35}$ or $A\beta_{1-40}$ (Day 0). An anti-TNF-α antibody (α-TNF-α) (10 ηg/mouse) was *i.c.v.*-injected 15 min prior to the injection of $A\beta_{25-35}$ or $A\beta_{1-40}$. Either the daily treatment with thalidomide or the *i.c.v.*-injection of α-TNF-α antibody prevented the nitration of proteins in the hippocampus (day 7) and the impairment of novel object recognition memory (day 8–10) induced by $A\beta_{25-35}$ or $A\beta_{1-40}$. Data were presented as the mean ± S.E., ($n = 4$ for E, $n = 10$ for F), $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. $A\beta_{25-35}$ or $A\beta_{1-40}$; α-TNF-α: anti-TNF-α antibody; $A\beta_{35-25}$: Amyloid beta peptide (35–25). $A\beta_{1-40}$: Amyloid beta peptide (1–40).

irreversible loss of function [9,10,46,53,54,67]. It is therefore thus suggested that preventing the formation of peroxynitrite should be the early strategy for interrupting the cognitive failure in AD. Peroxynitrite is the product of the inter-action of nitric oxide (NO) and superoxide [6,46]. Although the half-life of NO is extremely short, peroxynitrite could be formed at a rate more than three times faster than the scavenging of superoxide by superoxide dismutase, implying criticality of the over-produced NO [5]. The overproduction of NO by Aβ is ascribed to the overpexpression of iNOS [1,23,25,40,47,59,64]. The deletion of iNOS or inhibition of the activity prevents peroxynitrite-mediated damage or the impairment of memory induced by $A\beta_{1-40}$ [25,40,58]. Observation of iNOS overexpression in the brains of AD patients [33,61], supports an anti-iNOS strategy [40,59]. However, the involvement of iNOS in the important physiology of life such as sexuality and sleep in the recent reports makes the strategy less favorable [20,26]. Therefore, restraining the overproduction of iNOS without affecting its normal expression and function is desired. The involvement of the TNF-α in iNOS-mediated neurotoxicity of $A\beta_{1-40}$ [2,13,36,65], points out a better strategy of targeting TNF-α to prevent the iNOS-driven-peroxynitrite-mediated impairment of memory in the neurotoxicity of Aβ.

In the present study, we examined the feasibility of a strategy of targeting TNF-α for the prevention of Aβ-induced impairment of memory. To confirm the involvement of TNF-α in the Aβ-induced impairment of recognition memory, $A\beta_{25-35}$, the most toxic Aβ species detected in AD brain, and TNF-α (−/−) mouse were utilized. $A\beta_{25-35}$ did not induce the impairment of recognition memory in TNF-α (−/−) mouse. The serious involvement of TNF-α was consistent with the previous reports [2,13,36,65], and boosted the prospect of an anti-TNF-α strategy to prevent the neurotoxicity of Aβ. Considering the current availability for practice, thalidomide was selected due to its preferential degradative effect on the mRNA of TNF-α [32,37]. Thalidomide preferentially suppressed the increase of TNF-α mRNA and prevented the Aβ-induced impairment of recognition memory. An anti-TNF-α antibody also provided prevention against the impairment of recognition memory induced by Aβ, validating the usefulness of the strategy of targeting TNF-α.

A variety of inhibitory agents of the synthesis or the activity of TNF-α is widely investigated and currently used as remedy in practice [4,35]. However, the penetrating ability of the blood–brain-barrier (BBB) of these agents is one of the factors to challenge their application for the ailments in the central nervous system. With the ability of penetrating of BBB, thalidomide is recently emerged as good candidate for the treatment for neurodegenerative diseases due to its potent inhibitory property for the synthesis of TNF-α [21,24,60]. The drug has recently been reintroduced for treating leprosy, although once was withdrawn from the general market because of its catastrophic adverse effect of teratogenesis [31]. The treatment with thalidomide in AD may have very limited chance, in the elderly, of causing teratogenesis in the progeny. Since thalidomide has a neglectable weak inhibitory effect for NOS including iNOS [52], the attenuation of iNOS-related pathology could most likely be contributed by its inhibition of the synthesis of TNF-α [32]. These reports qualify thalidomide as an ideal agent for reducing the iNOS/peroxynitrite related pathology via restraining the increase of TNF-α without evidently harming the physiological function of iNOS. The neuroprotective effects of thalidomide

in an animal model of inflamed brain pathology of AD in a recent study also recommend and support the use of the drug [48].

In conclusion, the usefulness of restraining TNF-α by thalidomide to prevent Aβ-induced impairment of memory in mice would provide a practical fresh strategy for the management of cognitive deficits in AD.

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