Antiamnesic and Neuroprotective Effects of the Aminotetrahydrofuran Derivative ANAVEX1-41 Against Amyloid β_{25-35}-Induced Toxicity in Mice

Vanessa Villard[1,2,3], Julie Espallergues[1,2,3], Emeline Keller[1,2,3], Tursun Alkam[4,5], Atsumi Nitta[4], Kiyofumi Yamada[4], Toshitaka Nabeshima[4,6], Alexandre Vamvakides[7] and Tangui Maurice*[1,2,3]

[1]INSERM U.710, Montpellier, France; [2]University of Montpellier 2, Montpellier, France; [3]EPHE, Paris, France; [4]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan; [5]Department of Basic Medicine, College of Traditional Uighur Medicine, Hotan, China; [6]Department of Chemical Pharmacology, Graduate School of Pharmaceutical Science, Meijo University, Nagoya, Japan; [7]Anavex Life Sciences, Pallini, Greece

The antiamnesic and neuroprotective activities of the new aminotetrahydrofuran derivative tetrahydro-N,N-dimethyl-5,5-diphenyl-3-furanmethanamine hydrochloride (ANAVEX1-41), a nonselective muscarinic receptor ligand and σ_1 protein activator, were examined in mice injected intracerebroventricularly with amyloid β_{25-35} ($A\beta_{25-35}$) peptide (9 nmol). $A\beta_{25-35}$ impaired significantly spontaneous alternation performance, a spatial working memory, and passive avoidance response. When ANAVEX1-41 (1–1000 µg/kg i.p.) was administered 7 days after $A\beta_{25-35}$, ie, 20 min before the behavioral tests, it significantly reversed the $A\beta_{25-35}$-induced deficits, the most active doses being in the 3–100 µg/kg range. When the compound was preadministered 20 min before $A\beta_{25-35}$, ie, 7 days before the tests, it prevented the learning impairments at 30–100 µg/kg. Morphological analysis of corticolimbic structures showed that $A\beta_{25-35}$ induced a significant cell loss in the CA1 pyramidal cell layer of the hippocampus that was prevented by ANAVEX1-41 (100 µg/kg). Increased number of glial fibrillary acidic protein immunopositive cells in the retrosplenial cortex or throughout the hippocampus revealed an $A\beta_{25-35}$-induced inflammation that was prevented by ANAVEX1-41. The drug also prevented the parameters of $A\beta_{25-35}$-induced oxidative stress measured in hippocampus extracts, ie, the increases in lipid peroxidation and protein nitration. ANAVEX1-41, however, failed to prevent $A\beta_{25-35}$-induced caspase-9 expression. The compound also blocked the $A\beta_{25-35}$-induced caspase-3 expression, a marker of apoptosis. Both the muscarinic antagonist scopolamine and the σ_1 protein inactivator BD1047 prevented the beneficial effects of ANAVEX1-41 (30 or 100 µg/kg) against $A\beta_{25-35}$-induced learning impairments, suggesting that muscarinic and σ_1 targets are involved in the drug effect. A synergic effect could indeed account for the very low active doses measured *in vivo*. These data outline the therapeutic potential of ANAVEX1-41 as a neuroprotective agent in Alzheimer's disease.
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INTRODUCTION

Alzheimer disease (AD) is an irreversible, progressive and degenerative disorder damaging the higher structures of the brain (Selkoe, 1989, 2004). It is actually incurable, as the available treatments, acetylcholinesterase inhibitors or a *N*-methyl-D-aspartate receptor antagonist with neuroprotective potential, memantine, are mainly symptomatic. The pathological cleavage of amyloid precursor protein (APP) is

responsible for the accumulation of amyloid-β ($A\beta$) proteins, aggregating into fibrillar oligomers and generating amyloid deposits that, in turn, form the senile plaques (Selkoe, 1989, 2004). Oligomers of $A\beta$ peptides are considered as the main factor mediating the devastating neurotoxicity observed in AD. $A\beta$ peptides vary in length from 40 to 43 amino acids, $A\beta_{1-42}$ occurring more frequently and forms fibrillar aggregates far more readily than $A\beta_{1-40}$ or $A\beta_{1-43}$ (Selkoe, 1989). Minor fragments were also identified including the highly toxic $A\beta_{25-35}$ peptide (Kubo *et al*, 2002; Gruden *et al*, 2007). The $A\beta$-mediated toxicity follows a very complex pattern. $A\beta$ oligomers form Ca^{2+} permeable pores on plasma membranes and interact with intracellular organelles regulating calcium homeostasis, the endoplasmic reticulum (ER) and mitochondria (Abramov *et al*, 2004), provoking a massive oxidative stress

*Correspondence: Dr T Maurice, INSERM U.710, EPHE, University of Montpellier II, c.c. 105, place Eugène Bataillon, 34095 Montpellier cedex 5, France, Tel: +33 4 67 14 36 23, Fax: +33 4 67 14 33 86, E-mail: Tangui.Maurice@univ-montp2.fr

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 Aβ_{1-40} or A$\beta_{1-42/3}$ but also for Aβ_{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, Aβ_{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 Aβ_{25-35} peptide (Maurice et al, 1996; Delobette et al, 1997). The morphological and biochemical characterization of amyloid toxicity induced by Aβ_{25-35} has been subsequently analyzed in details. Aβ_{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 Aβ_{25-35} injection activates the glycogen synthase kinase-3β, involved in cell survival regulation, T-phosphorylation and APP processing, suggesting that acute Aβ_{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 Aβ_{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 Aβ_{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 Aβ_{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 Aβ_{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 Aβ$_{25-35}$ peptide (Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met, Aβ$_{25-35}$) and scrambled Aβ$_{25-35}$ peptide (Ala-Lys-Ile-Gly-Asn-Ser-Ile-Gly-Leu-Met-Gly, ScAβ) 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$_2$SO$_4$ 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 × (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 Aβ 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
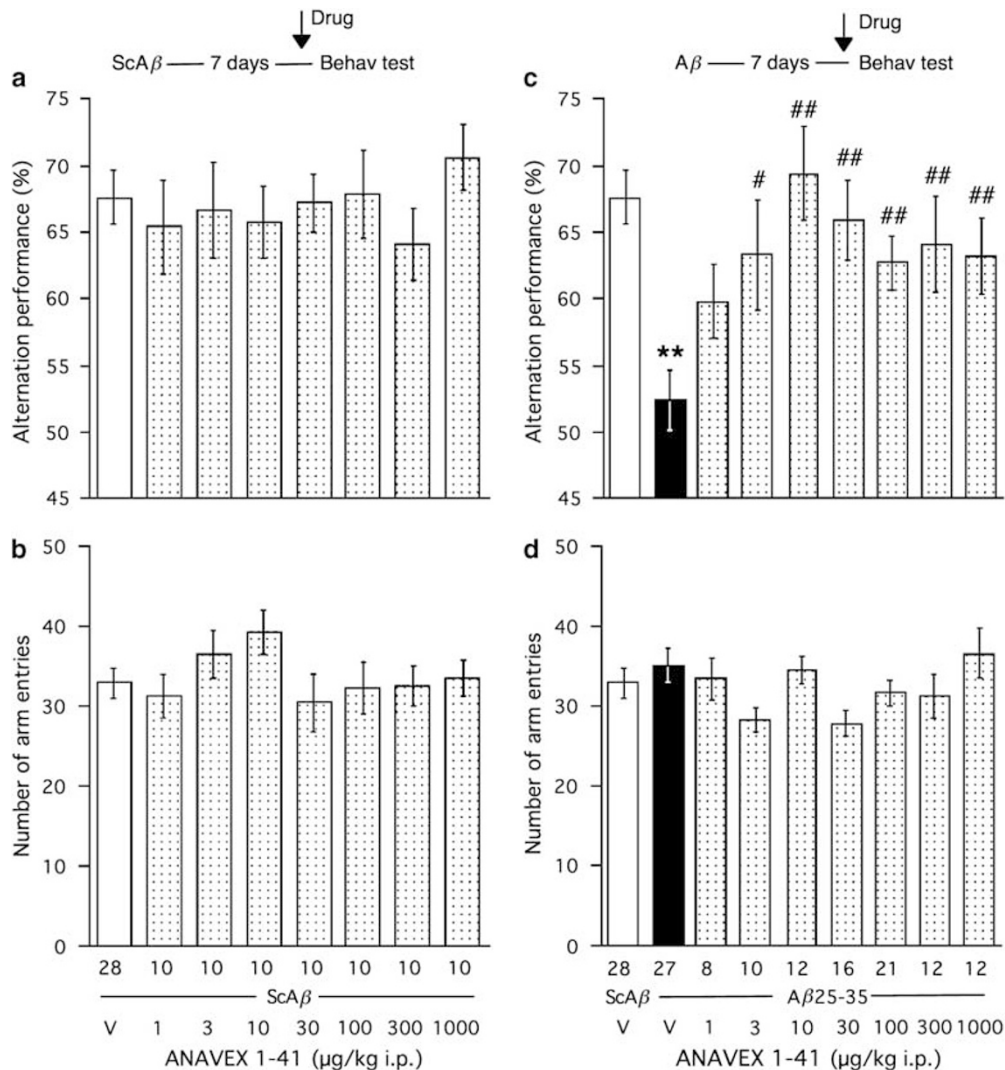
Figure I Antiamnesic effect of ANAVEX1-41 on Aβ$_{25-35}$-induced spontaneous alternation deficits in mice: alternation performances (a, c) and total numbers of arm entries (b, d). Mice were injected i.c.v. with ScAβ or Aβ$_{25-35}$ peptide (9 nmol). After 7 days, they were administered i.p. with the 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 (Sc.Aβ + V)-treated group; $^{#}p < 0.05$, $^{##}p < 0.01$ vs (Aβ$_{25-35}$ + 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 Aβ$_{25-35}$-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 Aβ$_{25-35}$ peptide injection in mice. We analyzed the number of viable cells in CA1 hippocampus area using cresyl violet staining (Figure 4). The Aβ$_{25-35}$

injection induced a −24.6% decrease in the number of viable cells in Aβ$_{25-35}$-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 Aβ$_{25-35}$ 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 Aβ$_{25-35}$ treatment (Figure 4d and e).

The extent of brain inflammation after Aβ$_{25-35}$ 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
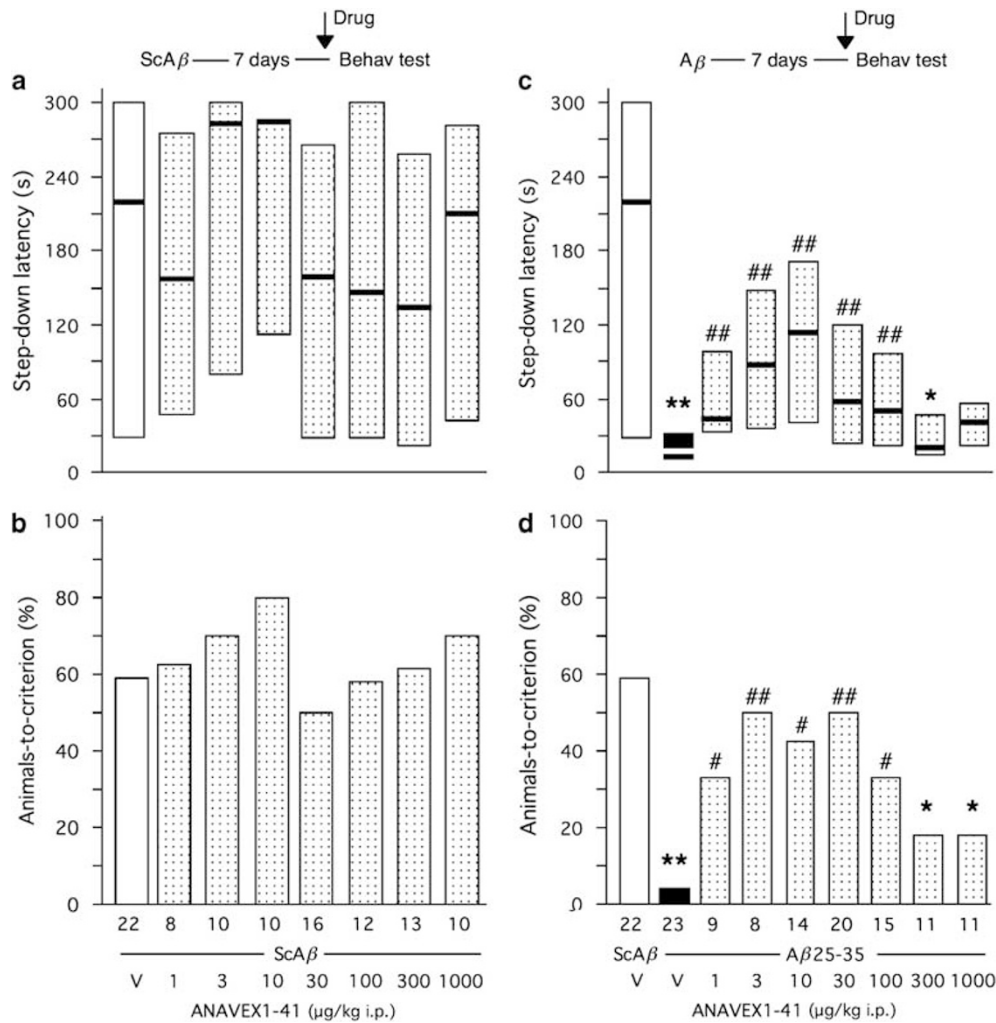
Figure 2 Effect of ANAVEX1-41 on $A\beta_{25-35}$-induced passive avoidance deficits in mice: step-down latency (a, c) and percentage of animals-to-criterion (b, d). Mice were injected i.c.v. with $ScA\beta$ or $A\beta_{25-35}$ peptide (9 nmol). After 7 days, they were administered i.p. with saline vehicle 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 ($ScA\beta + V$)-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 $ScA\beta$ 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 $ScA\beta$

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 $ScA\beta$ 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).
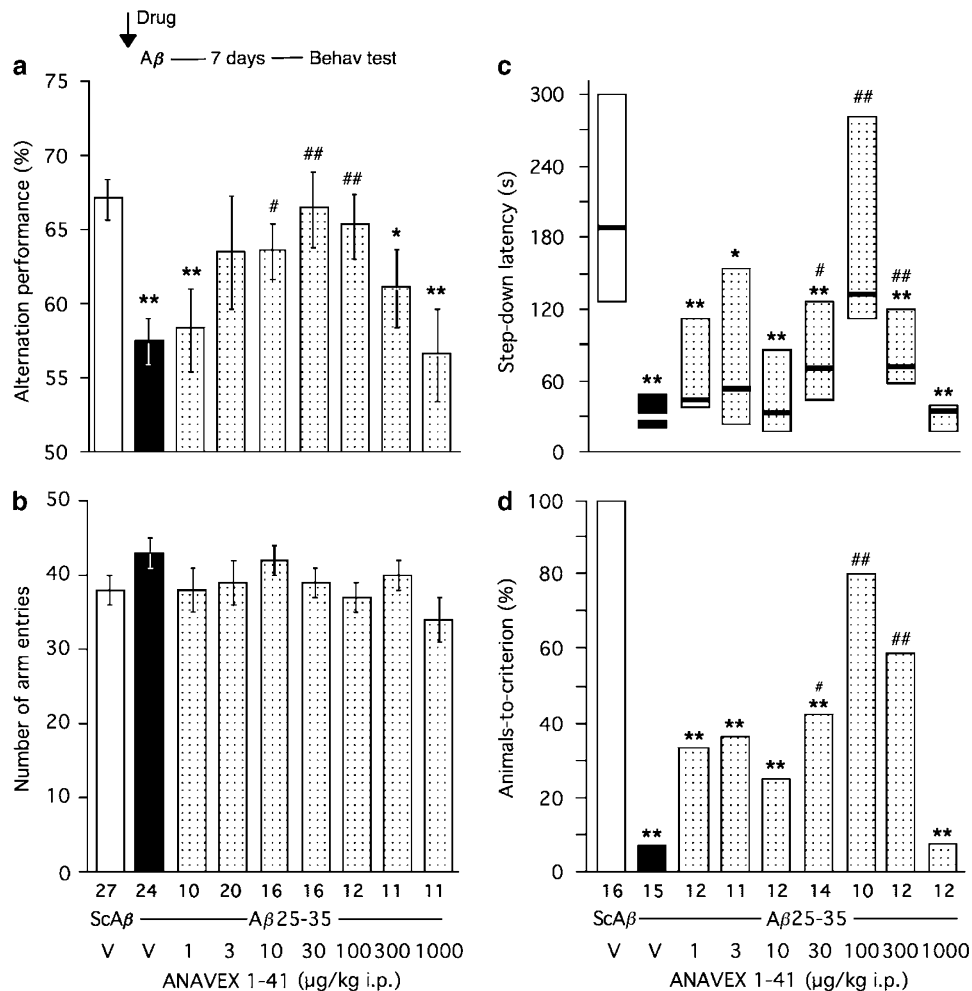
Figure 3 Neuroprotective effect of ANAVEX1-41 on $A\beta_{25-35}$-induced learning deficits in mice: alternation performance (a) and number of arm entries (b) in the Y-maze test; step-down latency (c) and percentage of animals-to-criterion (d) in the passive avoidance test. Mice were administered i.p. with vehicle solution (saline, V) or ANAVEX1-41 ($1-1000\,\mu g/kg$) 20 min before being injected i.c.v. with $ScA\beta$ 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 ($ScA\beta+V$)-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 $10-1000\,\mu g/kg$ 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 $100\,\mu g/kg$ (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, $100\,\mu g/kg$ i.p., tended to decrease the level of nitrotyrosine immunoreactivity in $ScA\beta$-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, $100\,\mu g/kg$ i.p., failed to affect caspase-9 expression in $ScA\beta$- 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, $100\,\mu g/kg$ 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 $ScA\beta$-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
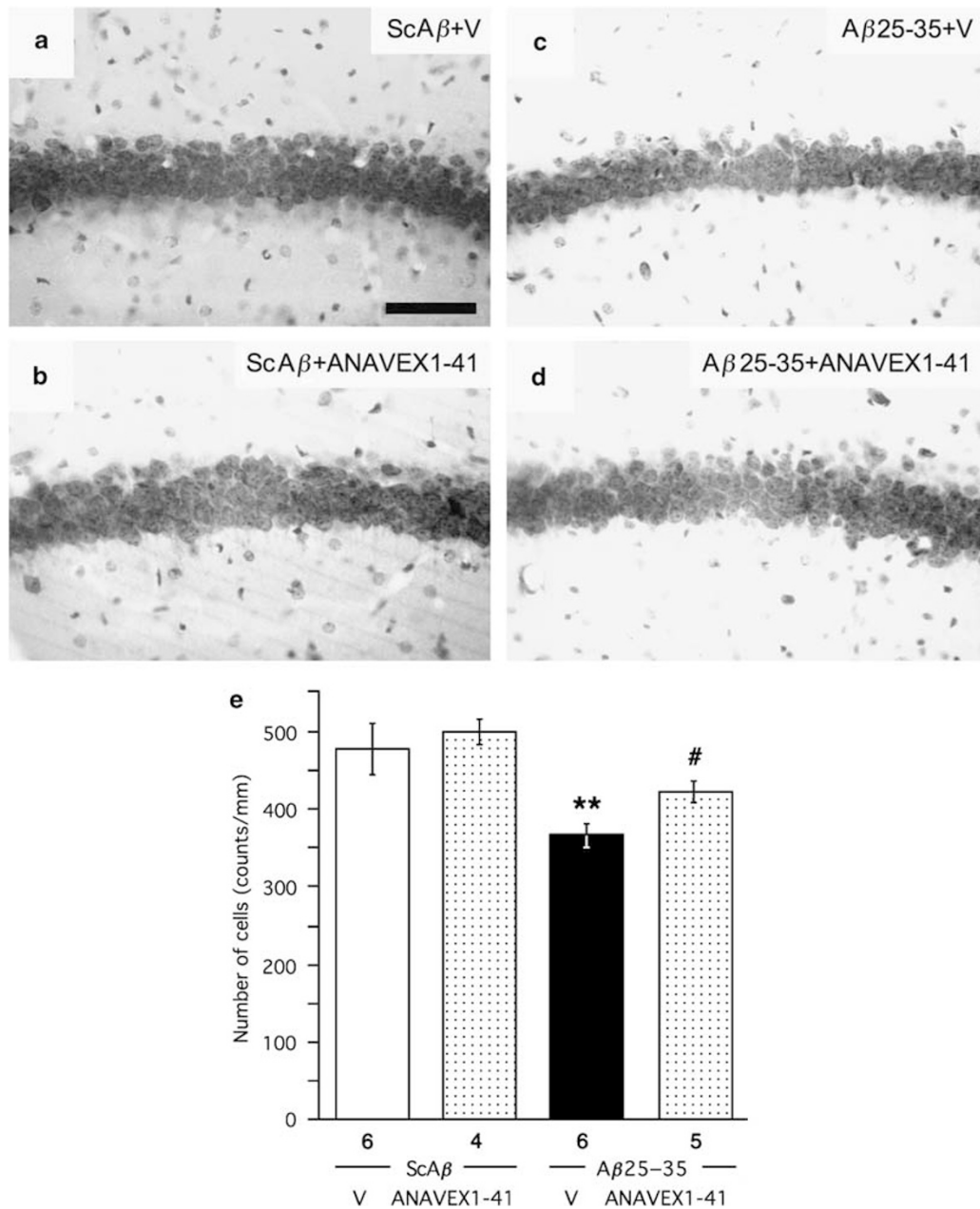
Figure 4 Neuroprotective effect of ANAVEX1-41 on $A\beta_{25-35}$-induced toxicity in mice: pyramidal cell loss in the CA1 area of the hippocampal pyramidal cell layer, 7 days after $A\beta_{25-35}$ injection. (a–d) Representative microphotographs of coronal sections of cresyl violet stained hippocampal 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

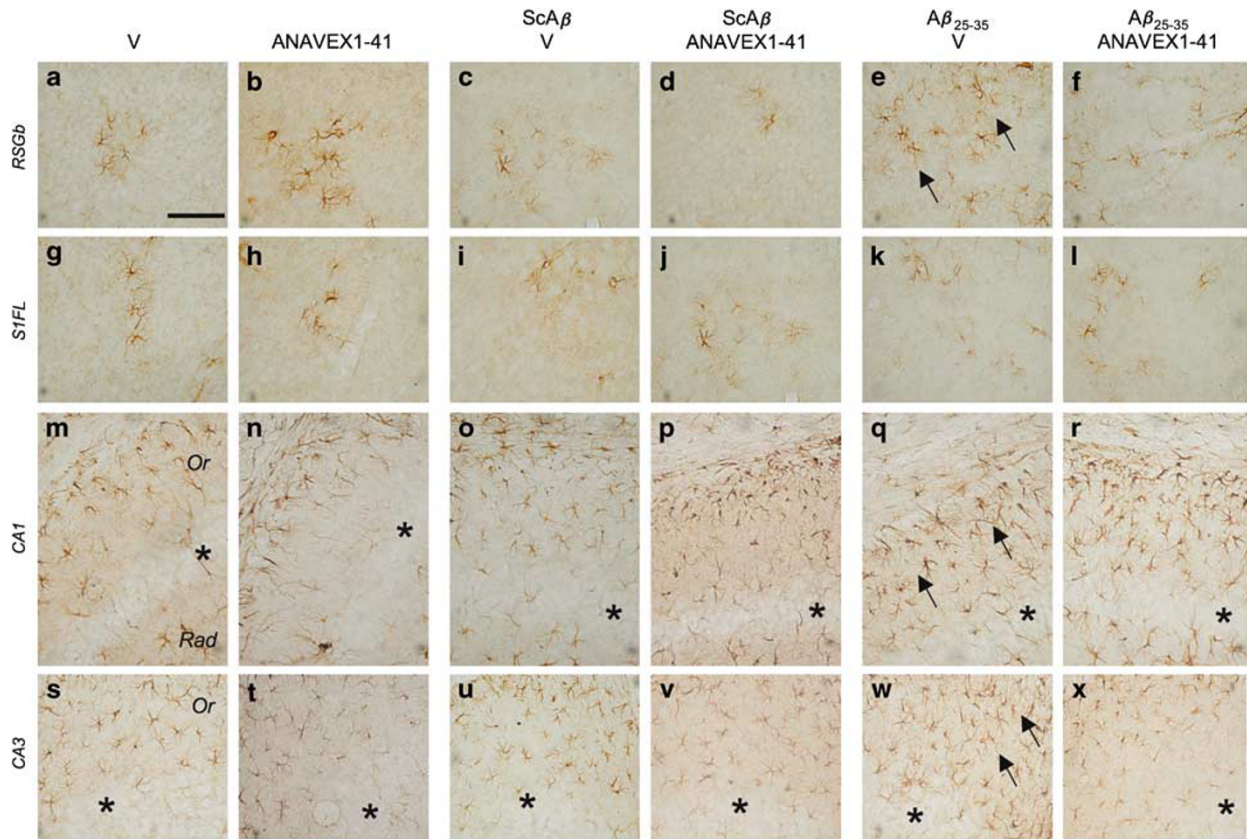**Figure 5** Morphological analysis of astrocytic reaction using GFAP immunolabeling in Aβ₂₅₋₃₅-treated mice. Animals were treated i.p. with saline vehicle solution (V) or ANAVEX1-41 (100 μg/kg) and received no i.c.v. treatment (two left columns), ScAβ (9 nmol; two central columns) or Aβ₂₅₋₃₅ (9 nmol; 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 Aβ₂₅₋₃₅ peptide in mice. In the brain of rats or mice, Aβ₂₅₋₃₅ peptide induces, after acute injection or chronic infusion, biochemical changes, morphological alterations, and behavioral impairments reminiscent of AD physiopathology. In particular, Aβ₂₅₋₃₅-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 Aβ₂₅₋₃₅-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 Aβ₂₅₋₃₅-induced learning impairments. Numerous σ_1 protein activators including (+)-SKF-10 047, (+)-pentazocine, SA4503, or PRE-084 attenuated Aβ₂₅₋₃₅-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
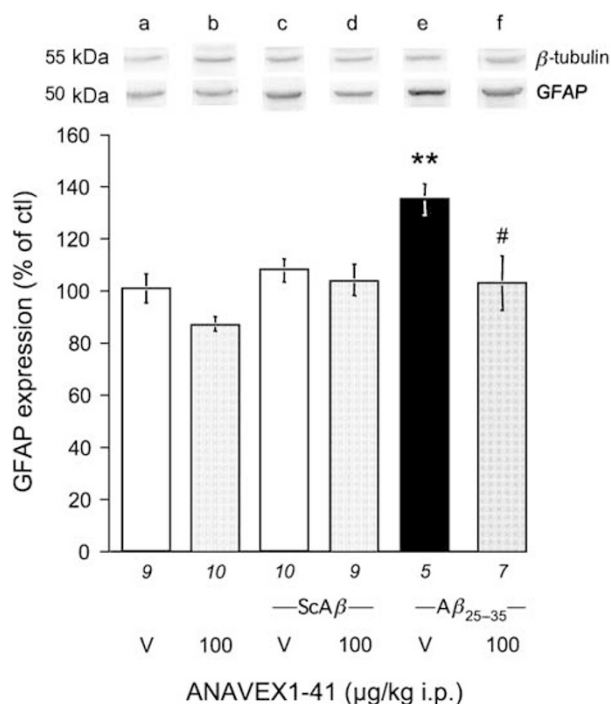
Figure 6 Effect of ANAVEX1-41 on GFAP expression measured by western blot in the hippocampus of $A\beta_{25-35}$-treated mice. Animals were treated i.p. with saline vehicle solution (V) or ANAVEX1-41 (100 µg/kg) and received no i.c.v. treatment (two left columns), ScAβ (9 nmol; two central columns) or $A\beta_{25-35}$ (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, ScAβ + V; d, ScAβ + 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
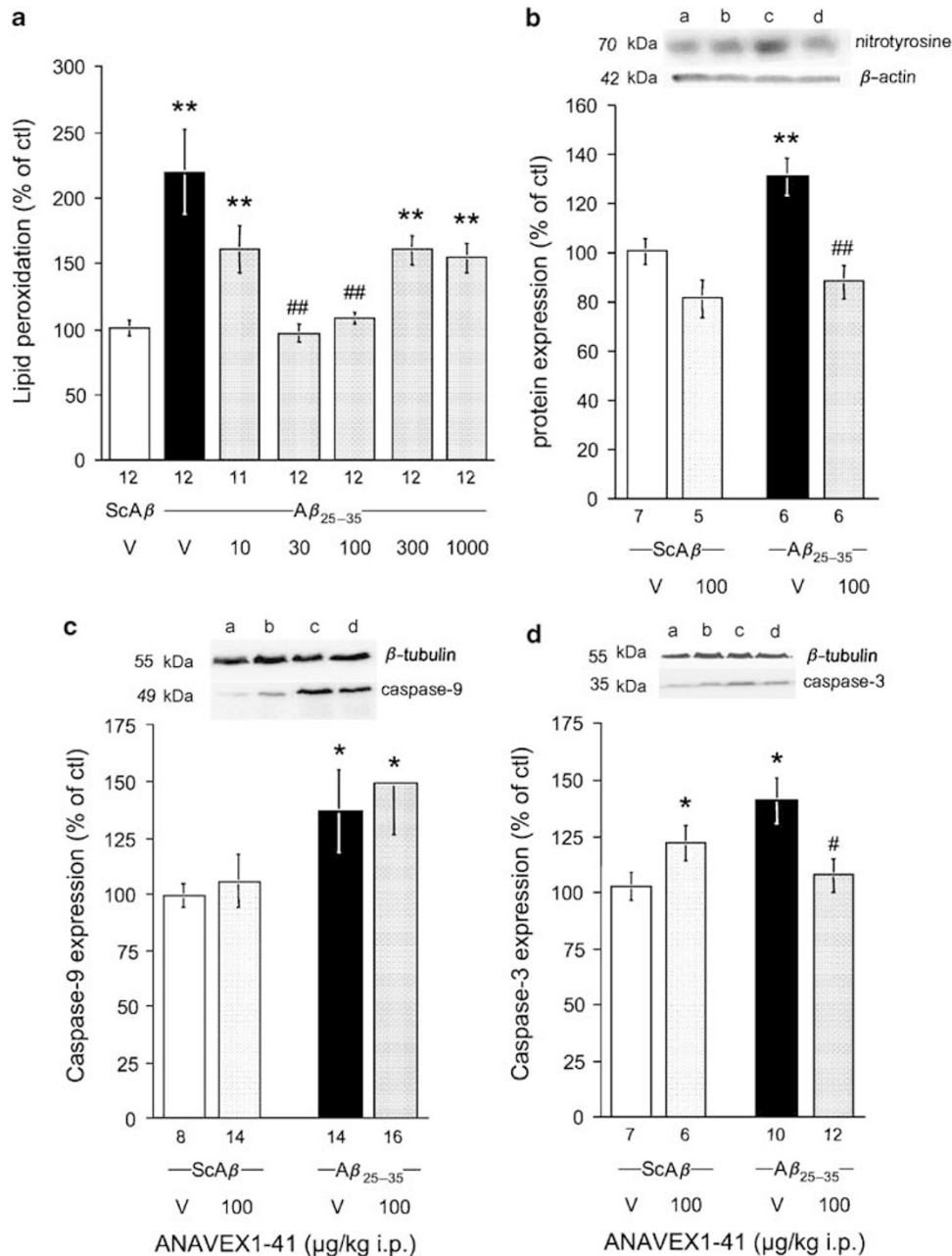
Figure 7 Neuroprotective effects of ANAVEX1-41 measured using biochemical markers in the hippocampus in $A\beta_{25-35}$ peptide-injected mice: (a) lipid peroxidation levels, (b) protein nitration levels; (c) caspase-9 expression; (d) caspase-3 expression. Mice were administered i.p. with saline vehicle 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
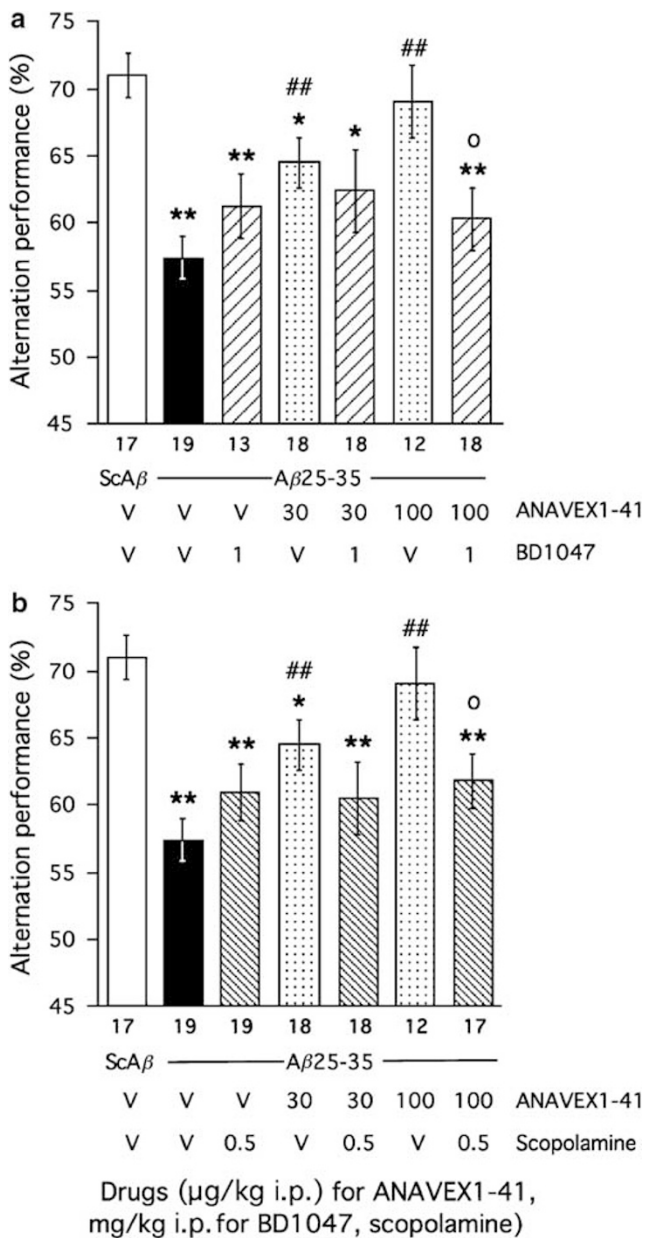
Figure 8 Effect of the preadministration of the muscarinic antagonist scopolamine (b) or the σ_1 receptor antagonist BD1047 (a) on the ANAVEX1-41 protective effect against the $A\beta_{25-35}$-induced alternation deficits in mice. Mice were administered i.p. with saline vehicle solution (V), scopolamine (0.5 mg/kg), BD1047 (1 mg/kg) and/or ANAVEX1-41 (30, 100 µg/kg), 20 min before ScAβ or $A\beta_{25-35}$ (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 ($A\beta_{25-35}$ + V)-treated group; °$p < 0.05$ vs ($A\beta_{25-35}$ + ANAVEX1-41)-treated group; Dunnett's test.

showed that ANAVEX1-41 blocked the $A\beta_{25-35}$-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 $A\beta_{25-35}$-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 $A\beta_{25-35}$-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 mechanism 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 $A\beta_{25-35}$ 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 pre-synaptic and intraneuronal levels, on neurotransmitter release, activation of membrane receptors and intracellular transduction systems.

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 $A\beta$-mediated apoptosis. Results
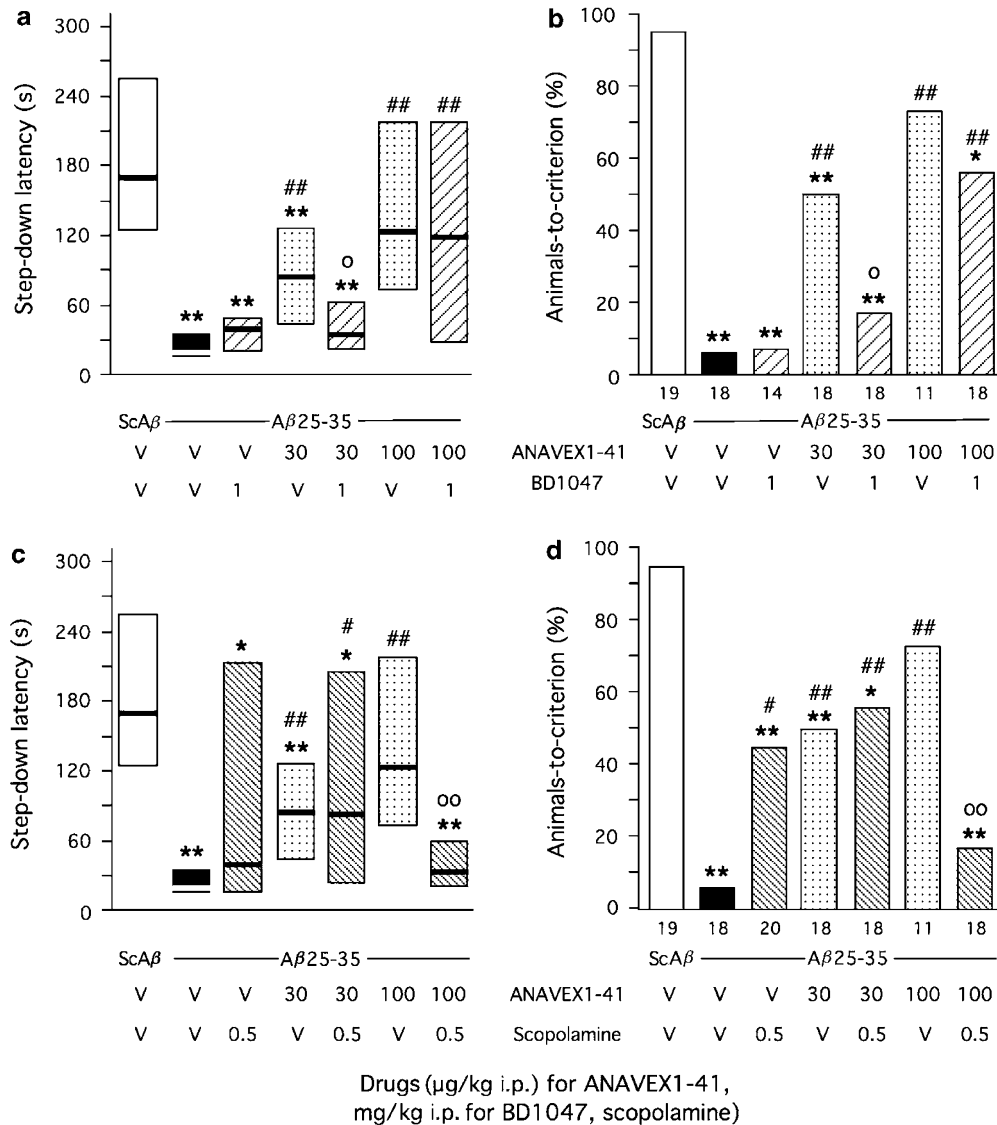
Figure 9 Effect of the preadministration of scopolamine (c, d) or BD1047 (a, b) on the ANAVEX1-41 effect against the Aβ$_{25-35}$-induced passive avoidance deficits in mice: step-down latency (a, c) and percentage of animals-to-criterion (b, d). Mice were administered i.p. with saline vehicle solution (V), scopolamine (0.5 mg/kg), BD1047 (1 mg/kg), and/or ANAVEX1-41 (30, 100 μg/kg), 20 min before ScAβ or Aβ$_{25-35}$ (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 (Sc.Aβ + V)-treated group; #$p < 0.05$, ##$p < 0.01$ vs (Aβ$_{25-35}$ + V)-treated group; °$p < 0.05$, °°$p < 0.01$ vs (Aβ$_{25-35}$ + ANAVEX1-41)-treated group; Dunn's test in (a) and (c), χ^2-test in (b) and (d).

DISCLOSURE/CONFLICT OF INTEREST

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)