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ORIGINAL INVESTIGATION

Neuroprotection by the synthetic neurosteroid enantiomers *ent*-PREGS and *ent*-DHEAS against $A\beta_{25–35}$ peptide-induced toxicity in vitro and in vivo in mice

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Abstract
Rationale Pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulphate (DHEAS) are pro-amnesic, anti-amnesic and neuroprotective steroids in rodents. In Alzheimer's disease (AD) patient's brains, their low concentrations are correlated with high levels of Aβ and tau proteins. The unnatural enantiomer *ent*-PREGS enhanced memory in rodents. We investigated here whether *ent*-PREGS and *ent*-DHEAS could be neuroprotective in AD models.
Objective The effects of PREGS, *ent*-PREGS, DHEAS and *ent*-DHEAS against $A\beta_{25–35}$ peptide-induced toxicity were examined in vitro on B104 neuroblastoma cells and in vivo in mice.
Methods B104 cells pretreated with the steroids before $A\beta_{25–35}$ were analysed by flow cytometry measuring cell viability and death processes. Mice injected intracerebroventricularly with $A\beta_{25–35}$ and the steroids were analysed for their memory abilities. Additionally, lipid peroxidation levels in the hippocampus were measured.
Results *ent*-PREGS and PREGS significantly attenuated the $A\beta_{25–35}$-induced decrease in cell viability. Both steroids prevented the $A\beta_{25–35}$-induced increase in late apoptotic cells. PREGS further attenuated the ratio of necrotic cells. *ent*-DHEAS and DHEAS significantly reduced the $A\beta_{25–35}$-induced toxicity and prevented the cells from entering late apoptosis and necrosis. All steroids stimulated neurite outgrowth per se and prevented the $A\beta_{25–35}$-induced decrease. In vivo, *ent*-PREGS and *ent*-DHEAS significantly attenuated the $A\beta_{25–35}$-induced decrease in memory (spontaneous alternation and passive avoidance) and an increase in lipid peroxidation levels. In contrast to the natural steroids, both enantiomers prevented amnesia when injected 6 h before $A\beta_{25–35}$ in contrast to the natural steroids.
Conclusion The unnatural steroids *ent*-PREGS and *ent*-DHEAS are potent neuroprotective agents and could be effective therapeutical tools in AD.

Keywords Alzheimer's disease · Neurosteroid · Enantiomer · β-amyloid toxicity · Learning and memory · Oxidative stress · Pregnenolone sulphate · Dehydroepiandrosterone sulphate · Neuroprotection · Memory

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease in the elderly population (Selkoe 1997; Hardy and Gwinn-Hardy 1998). Amyloid-β (Aβ) species accumulating in the brain of AD patients as β-amyloid plaques are key actors in the pathogenesis (Blennow et al. 2006; Deshpande et al. 2006). The synthetic $A\beta_{25–35}$ fragment, the biologically active region of Aβ protein (Yankner et al. 1990; Pike et al. 1995), has been shown to disturb cellular integrity and function (for review, see (Kaminsky et al. 2010). It

induces neurodegeneration both in vitro and in vivo (Malouf 1992; Pike et al. 1995; Stepanichev et al. 2003; Meunier et al. 2006; Zussy et al. 2011). One of the earliest fundamental events associated with its toxicity is oxidative damage (Miranda et al. 2000; Butterfield et al. 2001). Aβ-associated free radical oxidative stress causes lipid peroxidation in brain cell membranes, resulting in cell death that underlies cognitive deficits (Mark et al. 1996; Sayre et al. 1997; Butterfield et al. 2002a, b). $A\beta_{25–35}$-induced neurotoxicity is also associated with other changes including perturbation of calcium homeostasis (Mattson et al. 1992; Harkany et al. 1999) and apoptosis (Forloni et al. 1996). $A\beta_{25–35}$ impairs memory formation after central administration in rodents (Maurice et al. 1998; Stepanichev et al. 2003; Holscher et al. 2007).

Preventing or protecting neuronal dysfunction and death has become an important component for alleviating memory impairments in AD. We have previously demonstrated that the concentrations of pregnenolone (PREGS) and dehydroepiandrosterone sulphate (DHEAS) are significantly reduced in the brain of AD patients as compared to non-demented controls and are correlated negatively with high Aβ levels and hyperphosphorylated tau proteins (Weill-Engerer et al. 2002). PREGS and DHEAS differentially regulate neuronal cell survival, in both in vitro and in vivo Aβ peptide-induced AD models. PREGS exacerbates the decrease in cell viability induced by $A\beta_{25–35}$ peptide in pheochromocytoma PC12 cell cultures (Akan et al. 2009). However, it shows neuroprotective action in mice centrally injected with the peptide (Yang et al. 2012). PREGS protects hippocampal neurogenesis in the APP/PS1 transgenic AD mouse model (Xu et al. 2012). In mouse cerebral cortex neuronal cultures, DHEAS selectively enhances dendrite growth (Compagnone and Mellon 1998). PREGS and DHEAS display both promnesiant and antiamnesiant activities in rodents (for review, see (Vallée et al. 2001a; Maurice et al. 2006). In particular, they dose dependently attenuated the memory deficits provoked by intracerebroventricular (i.c.v.) administration of $A\beta_{25–35}$ peptide in mice (Maurice et al. 1998) or memory deficits measured in APP/PS1 transgenic mice (Xu et al. 2012).

The synthetic enantiomers of PREGS (*ent*-PREGS) and of DHEAS (*ent*-DHEAS; Nilsson et al. 1998) have been used in pharmacological and electrophysiological studies as tools to provide insight into the enantioselectivity of steroid actions and the existence of chiral specific recognition sites on neurotransmitter receptor coupled channels such as γ-amino-butyric acid and *N*-methyl-D-aspartate (NMDA) receptors (Covey 2009; Covey et al. 2001). But some recent data showed a similar, if not higher, efficacy of steroid enantiomers over natural steroids in several pharmacological tests. PREGS potentiation of NMDA receptors is known to improve learning (Mathis et al. 1996; Akwa et al. 2001; Petit et al. 2011). Using a two-trial arm recognition task in a Y-maze, we have shown that *ent*-PREGS is more active than PREGS, the effective i.c.v. doses in rats and mice being roughly 10× lower than that of PREGS (Akwa et al. 2001). In addition, *ent*-PREGS acts independently of NMDA receptor activity (Akwa et al. 2001; Petit et al. 2011). By contrast, PREGS is an order of magnitude more effective than *ent*-PREGS in reversing scopolamine-induced amnesia in rats (Vallée et al. 2001b). To the best of our knowledge, the action of *ent*-DHEAS upon memory function has not been reported whereas DHEAS is established as a memory enhancer in rodents (Flood et al. 1988; Maurice et al. 1997; Markowski et al. 2001; Farr et al. 2004).

Neither *ent*-PREGS nor *ent*-DHEAS have been evaluated for their capacity as neuroprotective agents in rodent models of AD and related neurodegenerative processes. The aim of the present study was therefore to investigate the effects of synthetic *ent*-PREGS against $A\beta_{25–35}$-induced toxicity in B104 neuroblastoma cell cultures, as compared to that of natural PREGS, with a focus on cell survival and neurite outgrowth. In vivo, we examined the ability of *ent*-PREGS to attenuate the oxidative stress and learning impairments induced by i.c.v. administration of $A\beta_{25–35}$ peptide, using the spontaneous alternation and passive avoidance tests in mice. Behavioural studies were extended to *ent*-DHEAS, and the duration of actions of the enantiomers was compared to that of the natural steroids.

Experimental procedures

Preparation of steroids and Aβ peptides

PREGS was purchased from Steraloids (Newport, RI, USA). DHEAS was from Sigma-Aldrich (Saint Quentin-Fallavier, France) *ent*-PREGS and *ent*-DHEAS (ammonium salts) were chemically synthesised as previously reported (Nilsson et al. 1998). For experiments in B104 neuroblastoma cells, the concentrations of *ent*-PREGS and PREGS were obtained by successive dilutions of a 2 mg/ml stock solution in serum-free culture medium. Final concentration of ethanol in culture medium was less than 0.1 %. For studies in mice, *ent*-PREGS and *ent*-DHEAS were dissolved in sterile doubly distilled water. PREGS and DHEAS were solubilized in 5 % dimethylsulfoxide.

Lyophilized $A\beta_{25–35}$ used in B104 cell cultures was from Bachem (Weil am Rhein, France). $A\beta_{25–35}$ (5 mg) was initially dissolved in 0.5 ml of sterile deionized H_2O (10 μg/μl), vortexed and stored at −80 °C until use. Dilutions were further performed in free serum culture medium to obtain appropriate concentrations. For experiments in mice, lyophilized $A\beta_{25–35}$ peptide (SC489C) and scrambled $A\beta_{25–35}$ peptide (SC492) were from NeoMPS (Strasbourg, France). They were dissolved in sterile distilled water at a concentration of 3 mg/ml and stored at −20 °C until use. Before being injected, peptides were incubated at 1 mg/ml in sterile distilled water at 37 °C for 4 days as previously described (Maurice et al. 1996,

1998). $A\beta_{25–35}$ was used in its aggregated form in both in vitro and in vivo studies. Indeed, this truncated Aβ fragment unlike the full-length peptide rapidly forms fibrils and exhibits toxicity immediately upon its solubilisation in water (Yankner et al. 1990; Pike et al. 1995).

Cell culture

We used the B104 neuroblastoma cell line which originates in the rat central nervous system (Schubert et al. 1974). Cells were a gift from Dr A. Meiniel (INSERM U384, Faculty of Medicine, Clermont-Ferrand, France). They have the advantage over primary cortical neuronal cell cultures of a fast growth rate. They display numerous neuronal characteristics such as electrical membrane excitability (Schubert et al. 1986), expression of neurotransmitters/receptors (Hales and Tyndale 1994; Tyndale et al. 1994) and 14-3-2 neuron-specific protein (Schubert et al. 1974). These features make them an attractive model for the study of human neurological disease and for testing neurotoxicity of putative drugs. Cells were plated in poly-L-lysine-coated plates (6 or 24 wells) and grown in a controlled environment with a humidified atmosphere containing 5 % CO_2 at 37 °C, in complete culture medium containing RPMI 1640 medium supplemented with 10 % fetal calf serum, 5 % horse serum and a mixture of 1 % penicillin/L-glutamine/streptomycin (Gibco, Life Technologies, Saint-Aubin, France).

Experiments on B104 neuroblastoma cell cultures

Steroid effects on B104 cell viability: dose–response study Experiments were performed in order to determine whether *ent*-PREGS and *ent*-DHEAS were toxic to B104 cells as compared to PREGS and DHEAS, respectively. After initial 24-h plating with 8×10^4 cells/well in six-well plates, the complete culture medium was replaced by free serum medium containing variable concentrations of each steroid ranging from 0.25 to 20 μM or no steroid (control). Cell survival was evaluated 24 h later by flow cytometry.

$A\beta_{25–35}$ toxicity on B104 cells In order to determine the minimum concentration of $A\beta_{25–35}$ that reduced cell viability in steroid neuroprotection experiments, a preliminary dose–response study were carried out. After an initial 24-h cell plating, B104 cells was treated with 5, 10 and 20 μM of peptide in serum free medium for 24 h. The percentage of viable cells was determined by flow cytometry.

Steroid neuroprotection against $A\beta_{25–35}$ toxicity To test the potential neuroprotective effects of the steroids against Aβ peptide toxicity, B104 cells were seeded on poly-L-lysine-coated plates at 8×10^4 in six-well plates. After an initial 24-h cell plating, the complete culture medium was replaced with fresh medium containing increasing doses of steroids and incubated for 24 h. The culture medium was then replaced by serum-free medium containing the same above steroid treatments. $A\beta_{25–35}$ was then added 30 min after steroid treatments and cells were incubated for an additional 24 h. The percentages of viable, apoptotic and necrotic cells were calculated by flow cytometry.

Steroid effects on neurite outgrowth Low-density cultures of B104 neuroblastoma cells are suitable for the analysis of the enhanced neurite outgrowth (El Bitar et al. 1999). Three thousands cells in complete culture medium were seeded and allowed to attach for 24 h. They were incubated with *ent*-PREGS, PREGS, *ent*-DHEAS or DHEAS or no steroid (control) in the presence or absence of $A\beta_{25–35}$ in free serum culture medium for 3, 5 or 7 days. Cells were examined on each indicated day of culture under a Nikon Labophot 2 photonic microscope. Images were captured with an Infinity2 camera equipped with Infinity software. Tracing and quantification of the longest neurite per cell was made by using the NeuronJ software. Three randomly chosen fields per well were chosen in which the longest neurite per cell was examined in three wells having the same treatment. The experiment was repeated four times for *ent*-PREGS and three times for PREGS.

Flow cytometric assay

Flow cytometry was utilised to detect the percentage of intact, apoptotic and necrotic cells. Cells were washed with PBS, harvested by trypsinisation, centrifuged at 400*g* for 5 min and assayed with the Alexa-Fluor488®/annexin-V dead cell apoptosis kit (Invitrogen, Life Technologies) according to instructions of the manufacturer. Staining was detected on a FACSCalibur flow cytometer (Becton-Dickinson, Heidelberg, Germany). At least 10,000 cells per treatment condition were analysed on CellQuest Pro software (Becton-Dickinson). Cells in early apoptosis were annexin-V/Alexa-Fluor488® positive and PI negative. Late apoptotic cells were annexin-V positive/PI. Necrotic cells were only stained by PI. Living cells show little or no fluorescence.

Animals

Male Swiss mice aged 8 to 9 weeks old and weighing 32–35 g, were used (Depré, Saint-Doulchard, France). They were group housed in the animal facility building of the University of Montpellier 2, with free access to food and water, except during experiments. They were kept in a temperature- and humidity-controlled animal facility on a 12/12 h light/dark cycle (lights off at 7:00 pm). Behavioural experiments were carried out between 9:00 am and 4:00 pm in a sound-attenuated and air-regulated room to which mice were habituated for at least 30 min. All animal procedures were conducted in strict adherence to the European Union Directive of September 22, 2010 (2010/63/UE).

Peptides and steroid injections

Aβ peptides (9 nmol), steroids (0.05, 0.2, 0.5 and 2 nmol) or vehicle (V) were simultaneously administered i.c.v. in mice, under isoflurane 2.5 % anaesthesia, through a 28-gauge stainless-steel needle, 3 mm long. An injection volume of 3 μl was delivered gradually within 30 s and the needle left in place for an additional 30 s before being removed, as previously described (Maurice et al. 1996, 1998). Mice (n=10–12 per group) were examined for memory alteration 1 week after treatments, by an experimenter blind to the treatments. In the experiment depicted in Fig. 14, under isoflurane anaesthesia, mice were implanted with a polyethylene cannula, 0.75 mm inner diameter and 6 mm length (Phymep, Paris, France), fixed using acrylic cement. The tip of the cannula was placed onto the right ventricle, with stereotaxic coordinates from the Bregma being, in millimeter, A −0.5, L −1, V 2.5. Injections began 36 h after surgery. Steroids were injected at −12 h, −6 h and simultaneously before $A\beta_{25–35}$ peptide.

Experimental protocol

On day 7 after injections, mice were examined for spatial working memory performances using the spontaneous alternation test in the Y-maze. Non-spatial long-term memory was then evaluated using the step-through passive avoidance test, with training and retention sessions carried out on days 8 and 9, respectively. At the end of behavioural experiments, mice were euthanized by decapitation and their hippocampi were collected to measure the levels of lipid peroxidation.

Spontaneous alternation performances in the Y-maze

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

Step-through type passive avoidance response

The non-spatial long-term memory was assessed using the step-through passive avoidance procedure (Maurice et al. 1998; Meunier et al. 2006). The apparatus consisted of an illuminated compartment with white polyvinylchloride walls and a darkened compartment with black polyvinylchloride walls (each 15 cm×20 cm and 15 cm high) and a grid floor. A guillotine door separated each compartment. The white compartment was illuminated during the experimental period by a 60 W lamp positioned 40 cm it. Scrambled footshocks (0.3 mA for 3 s) were delivered to the grid floor using a shock generator scrambler (Lafayette Instruments, Lafayette, MA, USA). The guillotine door was initially closed during the training session. Each mouse was placed into the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment and placed all its paws on the grid floor, the door was gently closed and the scrambled foot shock was delivered for 3 s. The step-through latency (i.e., latency spent to enter the dark compartment) and shock sensitivity were measured. The latter was estimated as: 0, no reaction; 1, flinching reactions; and 2, fliching and vocalisation reactions. None of the treatment used in this study affected the shock sensitivity of the mice (data not shown). After 24 h (on day 9 after peptide injection), each mouse was placed again into the white compartment. After 5 s, the door was raised. The step-through latency was recorded up to 300 s.

Lipid peroxidation measures

The quantification of lipid peroxidation in tissue extracts is based on $Fe^{(III)}$/xylenol orange complex formation according to Hermes-Lima et al. (1995) and as previously reported (Meunier et al. 2006; Villard et al. 2009). Brain were weighed and kept in liquid nitrogen until assayed. After being thawed, brains were homogenised in cold methanol (1/5 w/v), centrifuged at 1,000g for 5 min and the supernatant collected. The 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 in a dark chamber at room temperature. Absorbance was measured at 580 nm ($A_{580}1$) and 10 μl of cumene hydroperoxide (CHP) 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 equivalents= $A_{580}1/A_{580}2\times$(CHP (nmol))×dilution, and expressed as CHP equivalents per wet tissue weight.

Statistical analyses

Data were expressed as mean±SEM. Statistical analyses were performed using InVivoStat® or Prism® software. For the experiments on neuroprotection in B104 cells, data were analysed using a one-way ANOVA (F values) according to treatment or a two-way ANOVA with treatment and concentration as independent factors, followed by planned comparisons on the predicted means to compare the levels of the

selected effect. Multiple means were compared using Fisher's protected least significant difference (PLSD) test. For neurotrophicity experiments in B104 cells, the lengths of the longest neurite were calculated by using NeuronJ® software and analysed using a two-way ANOVA with day and treatment as independent factors. For mice experiments, measures of spontaneous alternation and lipid peroxidation were analysed using one-way ANOVA, followed by the Dunnett's post hoc multiple comparison test. Passive avoidance latencies did not show a normal distribution as upper cutoff times were set. They were thus expressed as median value and interquartile range and were analysed using a Kruskal–Wallis non-parametric ANOVA (*H* values), group comparisons being made with Dunn's non-parametric multiple comparisons tests. The level of statistical significance was $p<0.05$. For reading clarity, all statistical values are detailed in the figure legends.

Results

Dose–response effects of *ent*-PREGS and PREGS on B104 neuroblastoma cell viability

ent-PREGS was incubated at 0.25, 0.5, 2, 5, 10, 20 μM with parallel controls (without steroid), in low density (3×10^3 cells) cultured B104 cells for 24 h (Fig. 1a). The percentage of viable cells was measured by flow cytometry and we observed that *ent*-PREGS did not affect the B104 cells viability in the 0.25–20 μM concentration range as compared to controls (Fig. 1a). Seventy-five to 88 % of viable cells were observed in *ent*-PREGS treated cell cultures as in controls. Under the same conditions, PREGS also did not affect cell viability in the same concentration range, as compared to controls (Fig. 1b).

Dose–response of $A\beta_{25–35}$ peptide on B104 neuroblastoma cell viability

Treatment of the B104 cells for 24 h with $A\beta_{25–35}$ peptide elicited a dose-dependent reduction in viability, as compared to control cells (Fig. 2). Significant decreases in the percentage of viable cells were already observed with the lowest dose of $A\beta_{25–35}$.

Neuroprotective effect of *ent*-PREGS or PREGS against $A\beta_{25–35}$-induced decrease in B104 neuroblastoma cell viability

The neuroprotective effect of *ent*-PREGS was analysed on the reduction of cell viability provoked by $A\beta_{25–35}$. *ent*-PREGS was added at variable concentrations to cell cultures alone or prior to the peptide. As shown in Fig. 3a, the enantiomer failed to affect cell viability in the 0.25–10 μM concentration range. Exposure to $A\beta_{25–35}$ peptide induced a significant decrease of B104 cell viability, as compared with untreated control cells

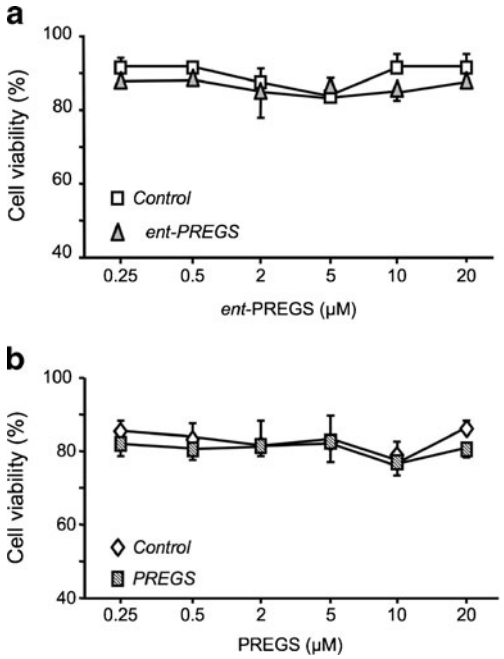

Fig. 1 Dose–response of *ent*-PREGS and PREGS on B104 cell viability. The percentage of viable cells was quantified by flow cytometry. Two-way ANOVA: $F_{(1,40)}=2.16$, $p>0.05$ for treatment, $F_{(5,40)}=0.74$, $p>0.05$ for concentration, $F_{(5,40)}=0.12$, $p>0.05$ for the treatment×concentration interaction in **a**; $F_{(1,20)}=2.64$, $p>0.05$ for treatment, $F_{(5,20)}=0.66$, $p>0.05$ for concentration, $F_{(5,20)}=0.50$, $p>0.05$ for the interaction in **b**

($p<0.001$; Fig. 3a). Pre-treatment with *ent*-PREGS attenuated the $A\beta_{25–35}$-induced decrease in cell viability in a dose-dependent manner, with significant effects at 5 and 10 μM ($p<0.01$; Fig. 3a).

The effect of PREGS pretreatment on the decrease of cell viability induced by $A\beta_{25–35}$ was determined. As shown in Fig. 3b, PREGS in the 0.25–5 μM concentration range failed to affect cell viability alone. Treatment with $A\beta_{25–35}$-induced a significant decrease of B104 cell viability as compared to untreated control cells ($p<0.001$; Fig. 3b). This decrease was significantly attenuated by PREGS at all concentrations

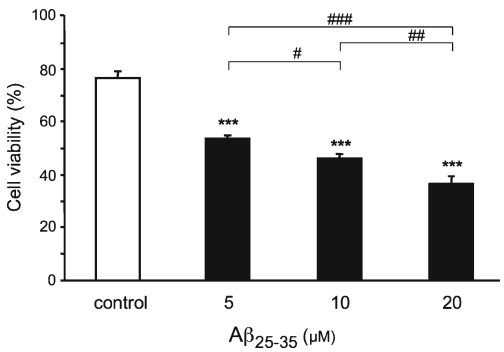

Fig. 2 Effects of $A\beta_{25–35}$ peptide on B104 cell viability. Cells were treated with $A\beta_{25–35}$ peptide (5, 10 and 20 μM) or without peptide (control) for 24 h. The percentage of viable cells was determined by flow cytometric analysis. One-way ANOVA: $F_{(3,8)}=79.6$, $p<0.001$. ***$p<0.001$ *vs* control cells, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ among the indicated groups, Fisher's PLSD test

tested. Particularly, PREGS at the lowest (0.25 μM) and highest concentration tested (5 μM) led to complete prevention of $A\beta_{25–35}$ toxicity, since group data were not statistically different from control cell data.

ent-PREGS prevents $A\beta_{25–35}$ peptide-induced late apoptotic death in B104 neuroblastoma cells

We analysed the *ent*-PREGS effect on each type of cell death induced by $A\beta_{25–35}$. Both necrosis (Behl et al. 1994) and apoptosis (Loo et al. 1993) have been linked to $A\beta_{25–35}$-induced toxicity. The use of annexin-V as the Alexa-fluor conjugate in combination with propidium iodide allows one to distinguish between early and late apoptotic cells, and secondary necrotic cells, by flow cytometry as illustrated in a typical experiment shown in Fig. 4a. We first calculated the percentage of total dead cells by summing that of apoptotic and necrotic cells following the treatments of *ent*-PREGS (at different concentrations) in the presence or absence of $A\beta_{25–35}$, and in untreated controls cells (Fig. 4b). $A\beta_{25–35}$ significantly increased the percentage of total dead cells by threefold ($p<0.001$). *ent*-PREGS prevented in a concentration-dependent manner $A\beta_{25–35}$-induced death and this effect was significant at the concentrations of 5 and 10 μM ($p<0.001$ Fig. 4b). The percentage of cells in early apoptotic phase was not significantly different whatever the treatment (Fig. 4c). However, there was a highly significant effect of treatment during late apoptosis (Fig. 4d). The percentage of cells in late apoptosis was highly significantly increased by the $A\beta_{25–35}$ treatment ($p<0.001$). The *ent*-PREGS co-treatment prevented this increase in a concentration-dependent manner, significantly at 5 and 10 μM ($p<0.01$; Fig. 4d). $A\beta_{25–35}$ significantly enhanced the percentage of necrotic cells. Interestingly, *ent*-PREGS (0.25–10 μM) failed to prevent this $A\beta_{25–35}$ effect (Fig. 4e).

PREGS prevents $A\beta_{25–35}$ peptide-induced late apoptotic and necrotic death in B104 neuroblastoma cells

We tested the PREGS effects under the same conditions as used for *ent*-PREGS. Figure 5a illustrates a typical experiment showing the percentage of cells in early or late apoptosis, and necrotic cells as determined by flow cytometry in untreated control B104 cell cultures and after treatment by $A\beta_{25–35}$ without or with PREGS. $A\beta_{25–35}$ significantly increased the percentage of total dead cells comparing to control ($p<0.001$; Fig. 5b). PREGS dose dependently prevented the death

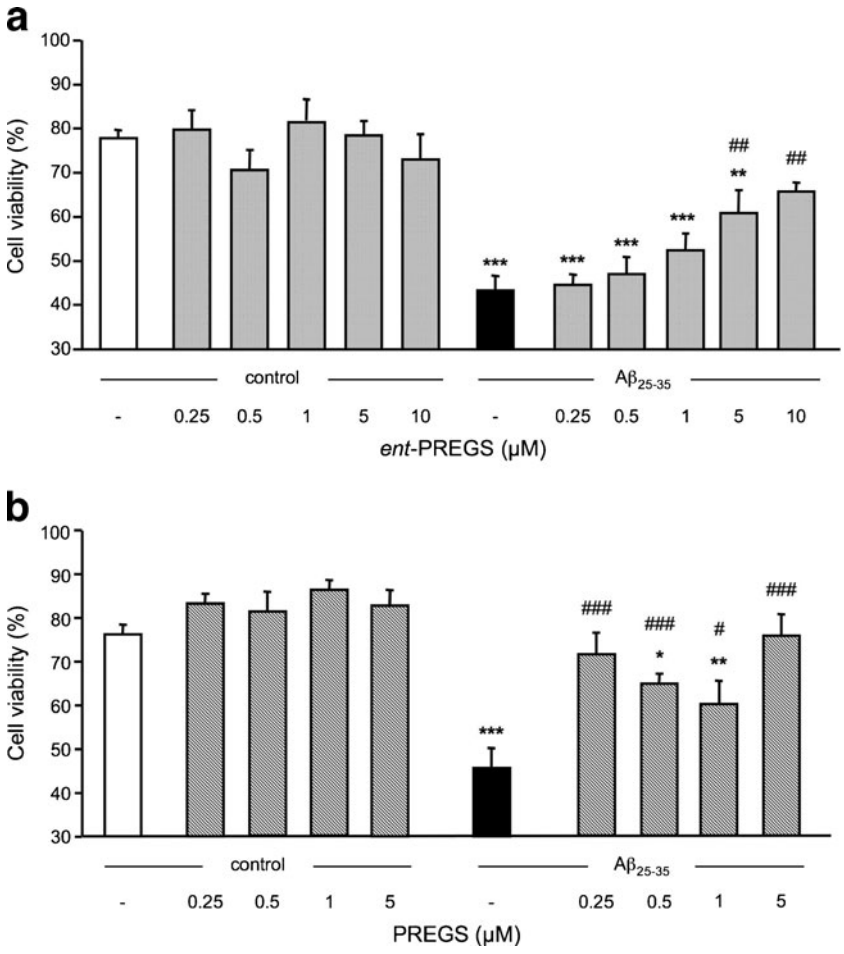

Fig. 3 Prevention of $A\beta_{25–35}$-induced decrease in B104 cell viability by *ent*-PREGS (**a**) or PREGS (**b**). Cells were pre-treated with increasing concentrations of *ent*-PREGS or PREGS for 30 min, followed or not by exposure to $A\beta_{25–35}$ (5 μM) for 24 h. Cell viability was determined by flow cytometry analysis. One-way ANOVA: $F_{(11,37)}=9.96$, $p<0.001$ in **a**; $F_{(9,33)}=12.0$, $p<0.001$ in **b**. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. $A\beta_{25–35}$-treated cells, Fisher's PLSD test

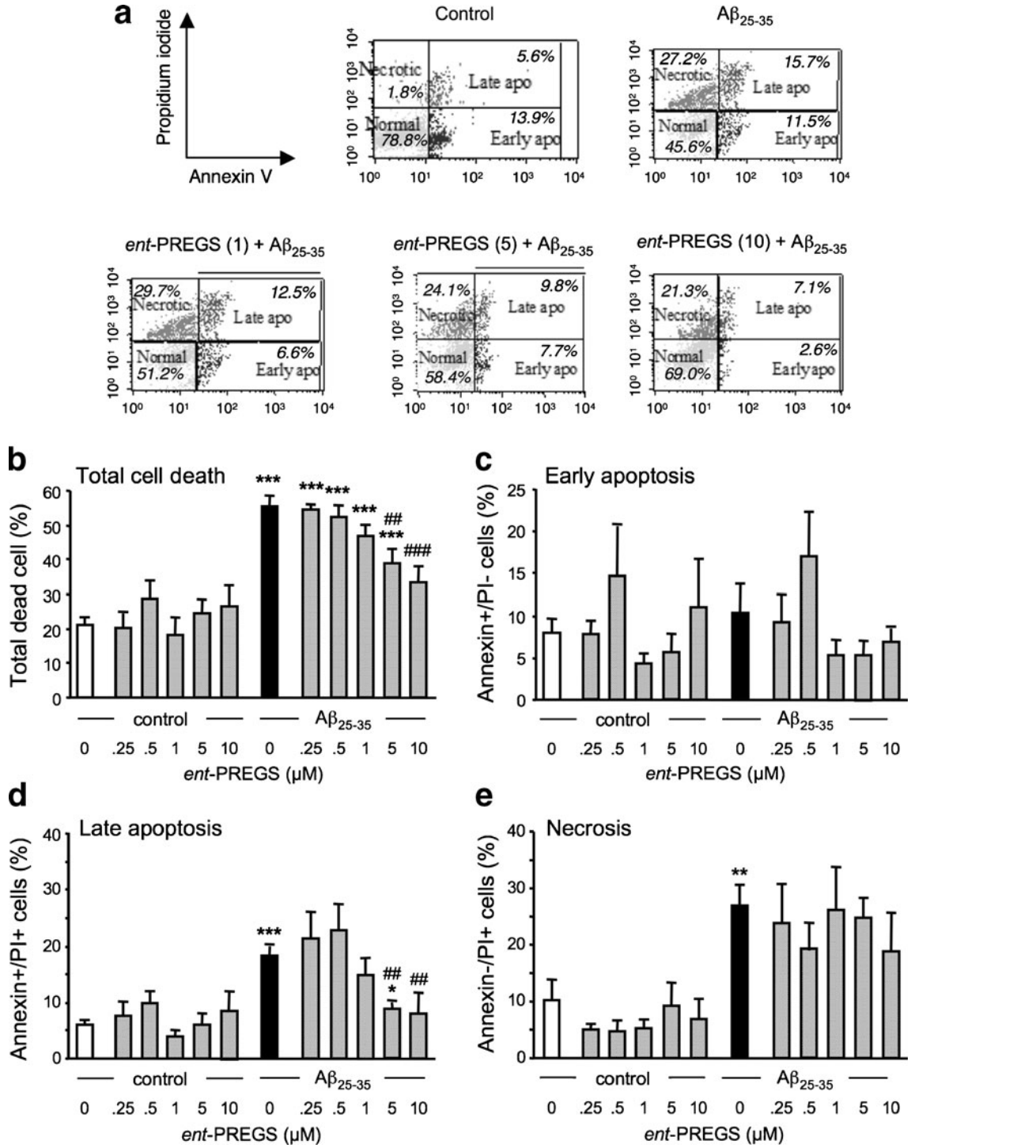

Fig. 4 Cytometric analysis of the prevention by *ent*-PREGS (0.25–10 μM) of $A\beta_{25-35}$-induced B104 cell death: **a** representative annexin-V-Alexa 488/propidium iodide (PI) double stainings of B104 cells treated with $A\beta_{25-35}$ peptide±*ent*-PREGS (1, 5, 10 μM). The percentages of cells analysed by flow cytometry in each quadrant under each condition are indicated: *lower left* Alexa $488^-/PI^-$, normal intact cells; *lower right* Alexa $488^+/PI^-$, early apoptototic cells; *upper left* Alexa $488^-/PI^+$, necrotic cells; and *upper right* Alexa $488^+/PI^+$, late apoptotic cells. *Graphs* show the quantification of the percentages of dead cells (**b**), early apoptotic cells (**c**), late apoptotic cells (**d**), and necrotic cells (**e**). One-way ANOVA: $F_{(11,37)}=14.9$, $p<0.001$ in **b**; $F_{(11,37)}=0.90$, $p>0.05$ in **c**; $F_{(11,37)}=6.39$, $p<0.001$ in **d**; $F_{(11,37)}=2.77$, $p<0.01$ in **e**. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control cells; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. $A\beta_{25-35}$-treated cells; Fisher's PLSD test

induced by $A\beta_{25-35}$ and this effect was significant for all concentrations tested, in the 0.25–5 μM concentration range. However, the percentage of dead cells in cultured cells treated with both $A\beta_{25-35}$ peptide and PREGS remained significantly higher than in control cells, for 0.5 and 1 μM (Fig. 5b). The PREGS treatment alone had no effect as compared with

control cultures. No significant effect of the treatments on cells in early apoptotic phase was measured (Fig. 5c). However, a highly significant effect of the treatments was measured on cells during late apopoptis (Fig. 5d). The percentage of cells in late apoptosis was significantly increased by $A\beta_{25–35}$. It was significantly lowered by PREGS at all concentrations tested, to the level of control cultures. The percentage of necrotic cells was significantly increased by $A\beta_{25–35}$ (Fig. 5e). Pre-treatment with PREGS attenuated the peptide effect, significantly at 0.25 and 5.0 μM (Fig. 5e).

Neuroprotective effect of *ent*-DHEAS or DHEAS against $A\beta_{25–35}$-induced decrease in B104 neuroblastoma cell viability

The neuroprotective effect of *ent*-DHEAS was analysed for the reduction of cell viability induced by $A\beta_{25–35}$. *ent*-DHEAS was added at variable concentrations to cell cultures alone or prior to the peptide. As shown in Fig. 6a, the enantiomer did not affect cell viability in the 0.25–10 μM concentration range. Exposure to $A\beta_{25–35}$ induced a significant decrease of B104 cell viability, as compared with untreated control cells ($p<0.001$; Fig. 6a). Pre-treatment with *ent*-DHEAS attenuated the $A\beta_{25–35}$-induced decrease in cell viability with significant effects at all concentrations tested ($p<0.001$; Fig. 6a).

The effect of DHEAS pretreatment on the decrease of cell viability induced by $A\beta_{25–35}$ was determined. As shown in Fig. 6b, DHEAS in the 0.25–5 μM concentration range failed to affect cell viability. Treatment with $A\beta_{25–35}$ induced a significant decrease of B104 cell viability as compared to untreated control cells ($p<0.001$; Fig. 6b). This decrease was significantly attenuated by DHEAS at all concentrations tested ($p<0.001$, Fig.6b).

ent-DHEAS prevents $A\beta_{25–35}$-induced apoptotic and necrotic death in B104 neuroblastoma cells

The percentage of early and late apoptotic cells, as well as secondary necrotic cells was analysed by flow cytometry in untreated control B104 cell cultures and after treatment by $A\beta_{25–35}$ without or with *ent*-DHEAS, as illustrated in a typical experiment (Fig. 7a). $A\beta_{25–35}$ significantly increased the percentage of total dead cells by threefold ($p<0.001$). *ent*-DHEAS prevented in a concentration-dependent manner $A\beta_{25–35}$-induced death and this effect was significant at the 0.25–10 μM concentration range ($p<0.001$, Fig. 7b). The significant increase in the percentage of cells in early apoptotic phase after $A\beta_{25–35}$ treatment ($p<0.01$) was strongly and significantly reduced by *ent*-DHEAS whatever the concentration ($p<0.001$, Fig. 7c). The percentage of cells in late apoptosis was also highly significantly increased by the $A\beta_{25–35}$ treatment ($p<0.001$). The *ent*-DHEAS co-treatment significantly prevented this increase in a concentration-dependent manner from 0.25 to 10 μM ($p<0.001$; Fig. 7d). $A\beta_{25–35}$ significantly enhanced the percentage of necrotic cells ($p<0.05$). This effect was prevented by *ent*-DHEAS only at the highest concentrations of 5 μM ($p<0.05$) and 10 μM ($p<0.01$; Fig. 7e).

DHEAS prevents $A\beta_{25–35}$ peptide-induced late apoptotic and necrotic death in B104 neuroblastoma cells

Figure 8a illustrates a typical experiment showing the percentage of cells in early or late apoptosis, and necrotic cells determined by flow cytometry in untreated control B104 cell cultures and after treatment by $A\beta_{25–35}$ without or with DHEAS. $A\beta_{25–35}$ significantly increased the percentage of total dead cells compared to control ($p<0.001$, Fig. 8b). DHEAS dose dependently prevented the death induced by $A\beta_{25–35}$ and this effect was significant for all concentrations tested in the 0.25–5 μM concentration-range. The percentage of cells in the early apoptotic phase was not significantly modified whatever the treatment. A highly significant effect of the treatments was measured on late apoptopic cells (Fig. 8d). The percentage of cells in late apoptosis was significantly increased by $A\beta_{25–35}$ ($p<0.001$). It was significantly lowered by DHEAS at all concentrations tested ($p<0.001$). The percentage of necrotic cells was significantly increased by $A\beta_{25–35}$ ($p<0.05$, Fig. 8e). Pre-treatment with DHEAS attenuated the peptide effect significantly at 1 and 5.0 μM (Fig. 5e).

Neurotrophic effects of *ent*-PREGS and PREGS in B104 neuroblastoma cells

The effects of *ent*-PREGS and PREGS on B104 cell morphology was investigated by analysing neurite outgrowth. Cells were plated at low density and incubated in the absence (control) or presence of *ent*-PREGS or PREGS for 3, 5 and 7 days. Figure 9a shows representative phase-contrast photomicrographs of cells. Over time, exposure to *ent*-PREGS or PREGS lead to striking differences in neurite length in steroid-treated cells as compared to controls. The length of the longest neurite per cell was significantly increased by *ent*-PREGS and by PREGS as compared to control at days 3, 5 and 7 (Fig. 9b). The *ent*-PREGS treatment increased neurite length over time. Significant differences were observed between days 3 and 7, days 5 and 7, and days 3 and 5 (Fig. 9b). The PREGS treatment increased the length of the longest neurite significantly only between days 5 and 7 (Fig. 9b).

Neurotrophic effects of *ent*-DHEAS and DHEAS in B104 neuroblastoma cells

The effects of *ent*-DHEAS and DHEAS on B104 neurite outgrowth were determined. Cells plated at low density were incubated in the absence (control) or presence of *ent*-DHEAS

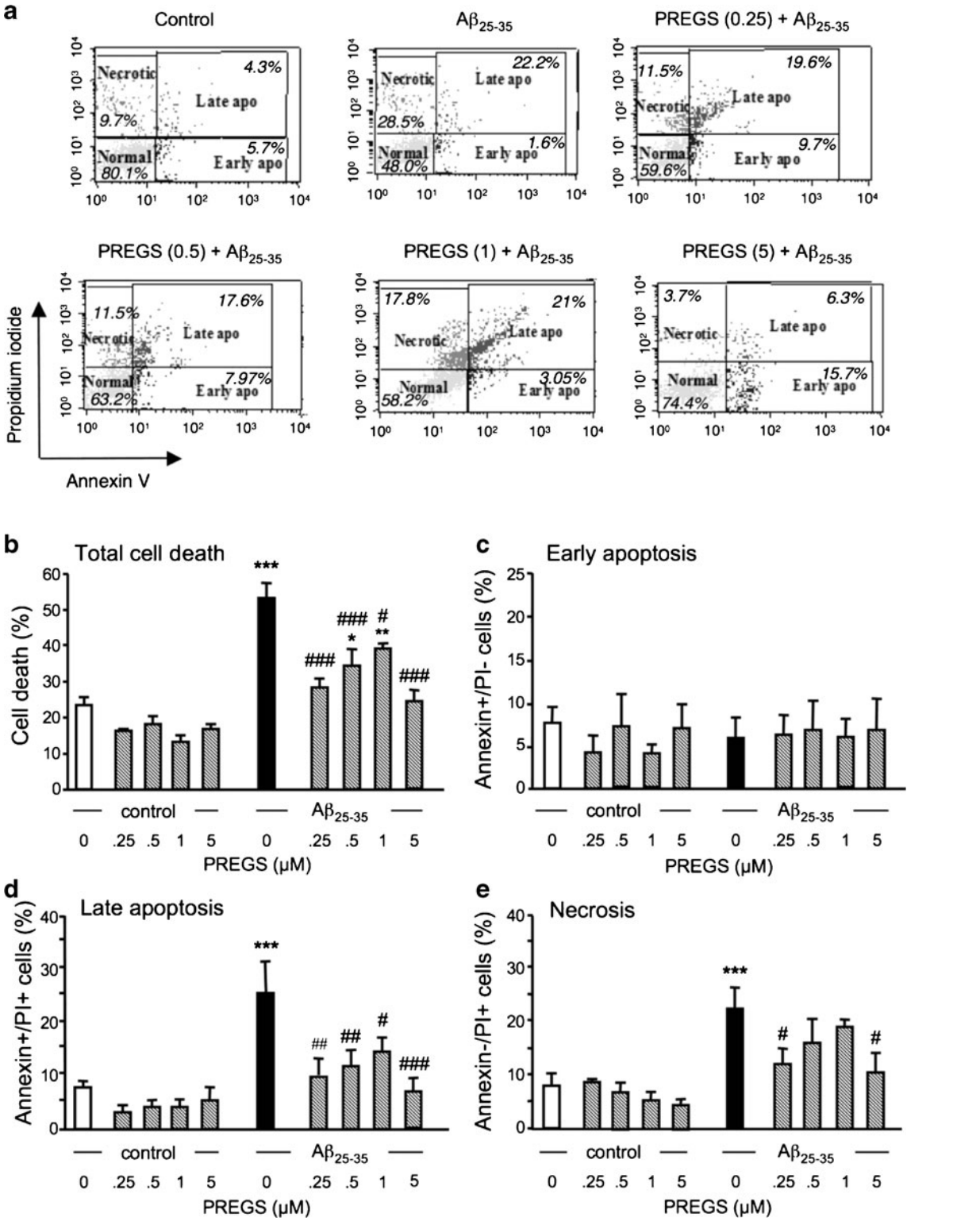

Fig. 5 Cytometric analysis of the prevention by PREGS (0.25–5 μM) of $A\beta_{25-35}$-induced B104 cell death: **a** representative annexin-V-Alexa 488/propidium iodide (PI) double staining of B104 cells treated with $A\beta_{25-35}$ peptide±PREGS. The percentages of cells analysed by flow cytometry in each quadrant under each condition are indicated: *lower left* Alexa $488^-/PI^-$, normal intact cells; *lower right* Alexa $488^+/PI^-$, apoptototic cells; *upper left* Alexa $488^-/PI^+$, necrotic cells; and *upper right*, Alexa $488^+/PI^+$, late apoptotic cells. *Graphs* show the quantifications of the percentages of dead cells (**b**), early apoptotic cells (**c**), late apoptotic cells (**d**), and necrotic cells (**e**). One-way ANOVA: $F_{(9,33)}=12.0$, $p<0.001$ in **b**; $F_{(9,33)}=0.15$, $p>0.05$ in **c**; $F_{(9,33)}=4.62$, $p<0.001$ in **d**; $F_{(9,33)}=4.32$, $p<0.001$ in **e**. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. $A\beta_{25-35}$-treated cells; Fisher's PLSD test

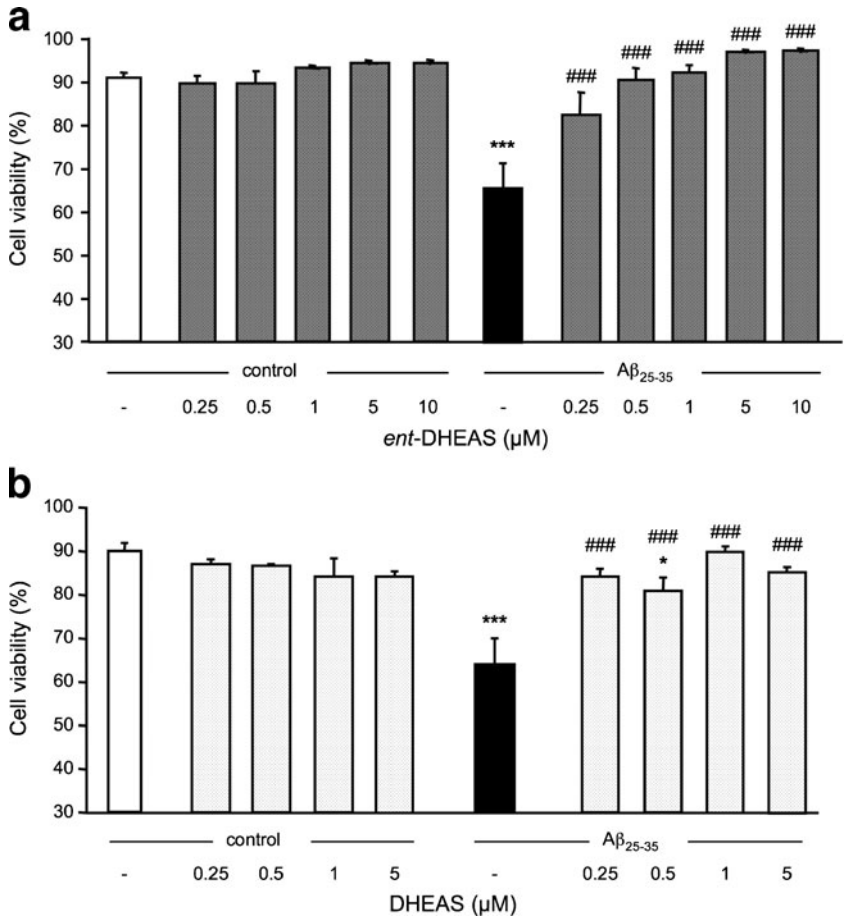

Fig. 6 Prevention of $A\beta_{25–35}$-induced decrease in B104 cell viability by *ent*-DHEAS (**a**) or DHEAS (**b**). Cells were pre-treated with increasing concentrations of *ent*-DHEAS or DHEAS for 30 min, followed or not by exposure to $A\beta_{25–35}$ (20 μM) for 24 h. Cell viability was determined by flow cytometry analysis. One-way ANOVA: $F_{(11,27)}=6.75$, $p<0.001$ in **a**; $F_{(9,23)}=6.36$, $p<0.001$ in **b**; ***$p<0.001$ vs. control cells; ###$p<0.001$ vs. $A\beta_{25–35}$-treated cells, Fisher's PLSD test

or DHEAS for 3, 5 and 7 days. Representative phase-contrast photomicrographs of cells are shown in Fig. 10a. The length of the longest neurite per cell was significantly increased by *ent*-DHEAS as compared to control, at days 3, 5 and 7 (Fig. 10b). It was also significantly increased by DHEAS as compared to control at days 3, 5 and 7 (Fig. 10b). The *ent*-DHEAS treatment slightly, but not significantly increased neurite length over time. In contrast, significant differences were observed with DHEAS treatment between days 3 and 7 and, days 5 and 7 (Fig. 10b).

ent-PREGS and PREGS prevents $A\beta_{25–35}$ peptide-induced decrease in neurite outgrowth in B104 neuroblastoma cells

Cells were untreated or treated with $A\beta_{25–35}$ alone or together with *ent*-PREGS or PREGS for 3, 5 and 7 days. Exposure to $A\beta_{25–35}$ peptide significantly decreased the length of the longest neurite at each day tested, as compared with untreated control cells (Fig. 11a) Pretreatment with *ent*-PREGS significantly attenuated the $A\beta_{25–35}$-induced decrease in neurite length at days 3, 5 and 7 (Fig. 11a). Pretreatment with PREGS also significantly diminished the $A\beta_{25–35}$-induced decrease in neurite length at days 5 and 7 but not at day 3 (Fig. 11a).

ent-DHEAS and DHEAS prevents $A\beta_{25–35}$ peptide-induced decrease in neurite outgrowth in B104 neuroblastoma cells

Cells were untreated or treated with $A\beta_{25–35}$ alone or together with *ent*-DHEAS or DHEAS for 3, 5 and 7 days. Treatment with $A\beta_{25–35}$ peptide significantly decreased the length of the longest neurite at each day tested, as compared with untreated control cells (Fig. 11b). Pretreatment with *ent*-DHEAS significantly attenuated the $A\beta_{25–35}$-induced decrease in neurite length at days 3, 5 and 7 (Fig. 11b). Pretreatment with DHEAS also significantly diminished the $A\beta_{25–35}$-induced decrease in neurite length at day 3, 5 and 7 (Fig. 11b).

Protective effects of *ent*-PREGS against $A\beta_{25–35}$-induced memory deficits

In order to analyze in vivo the steroid enantiomer effects, *ent*-PREGS, or vehicle, was co-administered i.c.v. with $A\beta_{25–35}$ in mice at day 0 and the learning performances of the mice were analysed after 1 week. Mice were first tested for their spontaneous alternation performance in the Y-maze, a spatial working memory test. $A\beta_{25–35}$ treatment resulted in a significant decrease in alternation

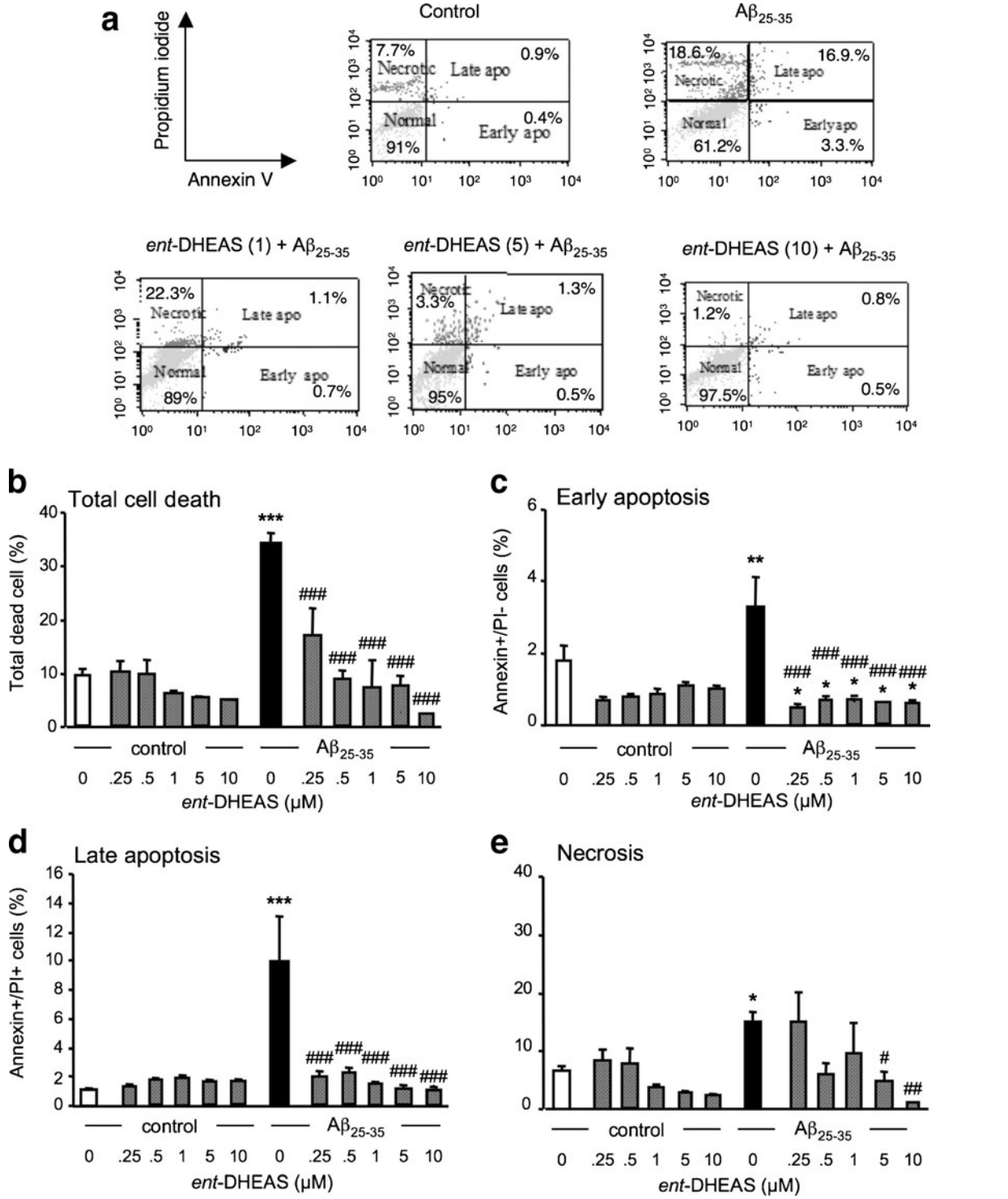

Fig. 7 Cytometric analysis of the prevention by *ent*-DHEAS (0.25–10 μM) of $A\beta_{25-35}$-induced B104 cell death: **a** representative annexin-V-Alexa 488/propidium iodide (PI) double stainings of B104 cells treated with $A\beta_{25-35}$ peptide±*ent*-DHEAS (1, 5, 10 μM) followed by $A\beta_{25-35}$ peptide. The percentages of cells analysed by flow cytometry in each quadrant under each condition are indicated: *lower left* Alexa $488^-/PI^-$, normal intact cells; *lower right* Alexa $488^+/PI^-$, early apoptotic cells; *upper left* Alexa $488^-/PI^+$, necrotic cells; and *upper right* Alexa $488^+/PI^+$, late apoptotic cells. *Graphs* show the quantification of the percentages of dead cells (**b**), early apoptotic cells (**c**), late apoptotic cells (**d**), and necrotic cells (**e**). One-way ANOVA: $F_{(11,27)}=6.75$, $p<0.001$ in **b**; $F_{(11,27)}=4.07$, $p<0.001$ in **c**; $F_{(11,27)}=5.49$, $p<0.001$ in **d**; $F_{(11,27)}=2.27$, $p<0.04$ in **e**. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. $A\beta_{25-35}$-treated cells; Fisher's PLSD test

performance as compared to the (ScAβ+V)-treated group (Fig. 12a). The pre-treatment with *ent*-PREGS led to a dose-dependent attenuation of $A\beta_{25-35}$-induced deficits, with significant effects at doses higher than 0.2 nmol.

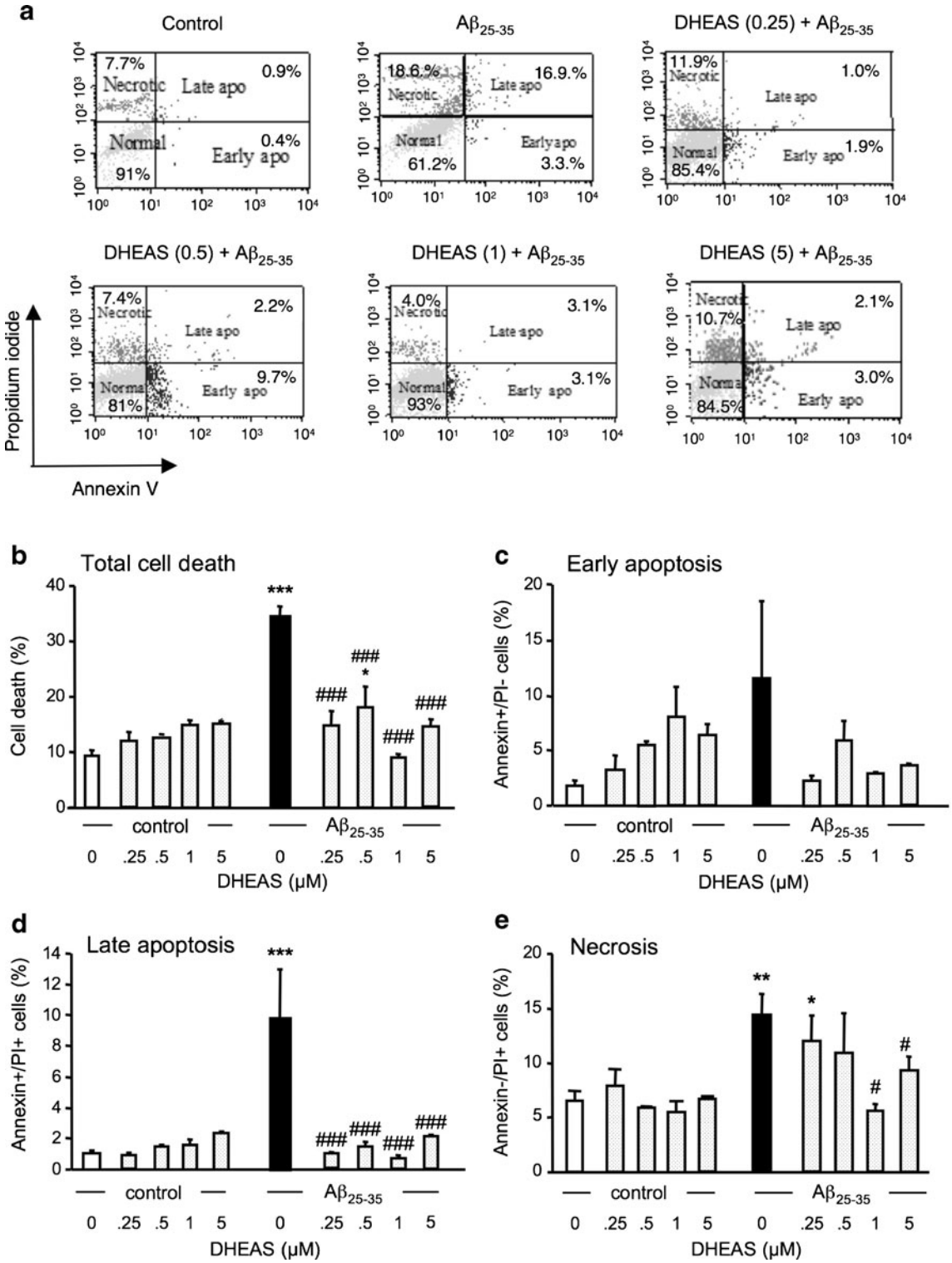

Fig. 8 Cytometric analysis of the prevention by DHEAS (0.25–5 μM) of $A\beta_{25–35}$-induced B104 cell death: **a** representative annexin-V-Alexa 488/propidium iodide (PI) double staining of B104 cells treated with $A\beta_{25–35}$ peptide alone and cells pre-treated with DHEAS followed by $A\beta_{25–35}$ peptide. The percentages of cells analysed by flow cytometry in each quadrant under each condition are indicated: *lower left* Alexa $488^-/PI^-$, normal intact cells; *lower right* Alexa $488^+/PI^-$, early apoptototic cells; *upper left* Alexa $488^-/PI^+$, necrotic cells; and *upper right*, Alexa $488^+/PI^+$, late apoptotic cells. *Graphs* show the quantifications of the percentages of dead cells (**b**), early apoptotic cells (**c**), late apoptotic cells (**d**), and necrotic cells (**e**). One-way ANOVA: $F_{(9,23)}=6.36$, $p<0.001$ in **b**; $F_{(9,23)}=1.28$, $p>0.05$ in **c**; $F_{(9,23)}=4.01$, $p<0.01$ in **d**; $F_{(9,23)}=2.62$, $p<0.05$ in **e**. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. control cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. $A\beta_{25–35}$-treated cells; Fisher's PLSD test

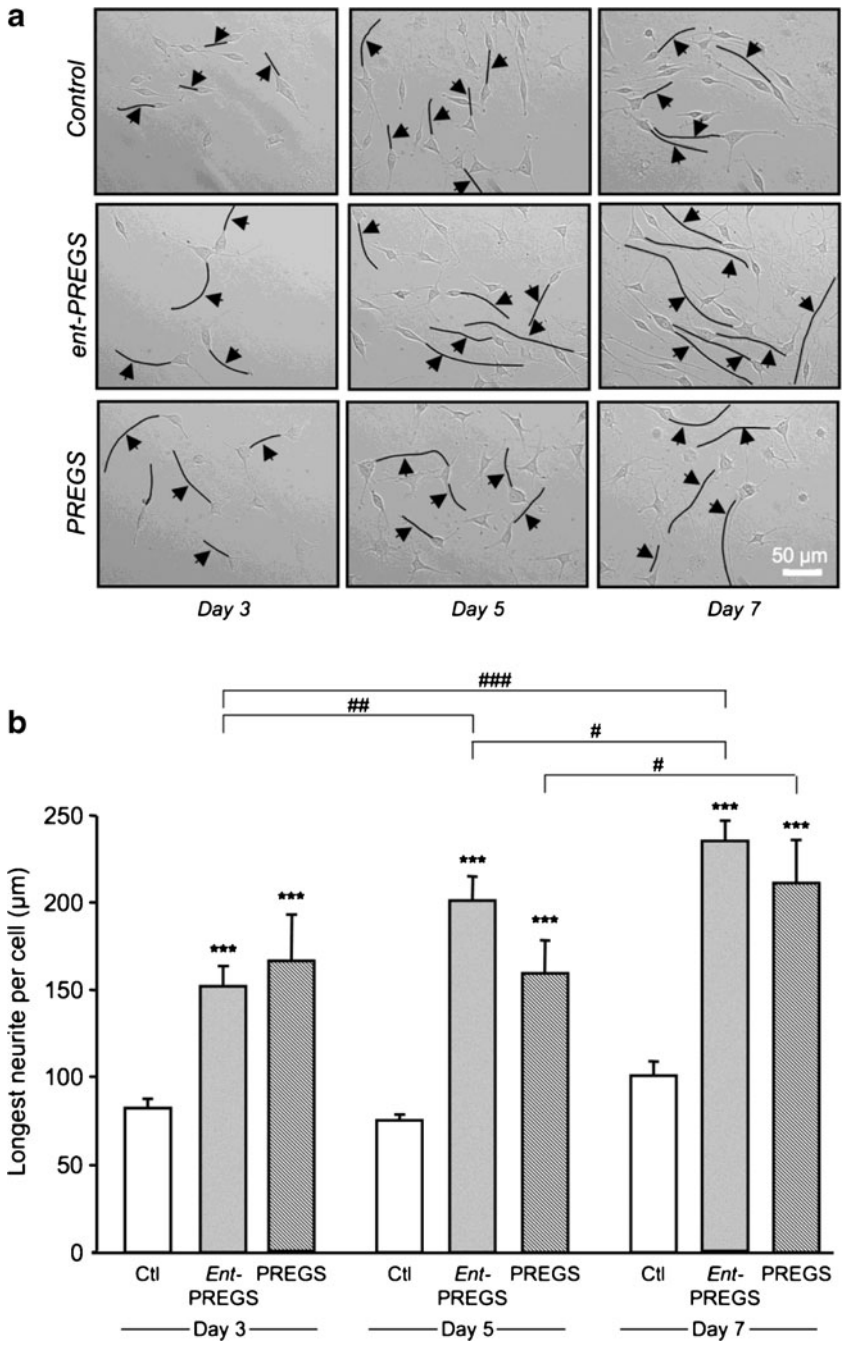

Fig. 9 Effects of *ent*-PREGS and PREGS on neurite outgrowth in B104 cultured cells: **a** representative photomicrographs of control and steroid-treated cells at days 3, 5 or 7. The longest neurite per cell was marked by a *black line* and indicated by an *arrow* (magnification ×10). *Scale bar* 50 µm. **b** Histograms of the length of the longest neurite per cell corresponding to the pool of three to four independent experiments. An average of 30–40 longest neurites was counted per treatment group and per day. Two-way ANOVA: $F_{(2,317)}=79.7$, $p<0.001$ for the steroid treatment; $F_{(2,317)}=11.7$, $p<0.001$ for the day; $F_{(4,317)}=2.02$, $p>0.05$ for the treatment×day interaction. ***$p<0.001$ vs. control (ctl); $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ for pairwise comparisons of steroid treatment between days; Fisher's PLSD test

Mice performances were then examined using the step-through passive avoidance test (Fig. 12b). The administration of $A\beta_{25–35}$ peptide significantly reduced the latency in comparison with the controls. The $A\beta_{25–35}$ peptide-induced impairment was attenuated by *ent*-PREGS, significantly at 0.5 nmol of *ent*-PREGS only (Fig. 12b). Of note, the performances of the groups treated with the two highest doses (0.5 and 2 nmol) of *ent*-PREGS in both tests were similar to that of the (ScAβ+V)-treated control group, showing a complete blockade of the $A\beta_{25–35}$ peptide-induced deficits.

Protective effects of *ent*-DHEAS against the $A\beta_{25–35}$-induced memory deficits

We extended the in vivo studies by testing *ent*-DHEAS. *ent*-DHEAS was also co-administered i.c.v. in the 0.05–2 nmol dose range with $A\beta_{25–35}$ peptide and behavioural performances were analysed 1 week later. $A\beta_{25–35}$ resulted in a significant decrease in alternation performance as compared to (ScAβ+V)-treated groups (Fig. 13a). *ent*-DHEAS significantly attenuated $A\beta_{25–35}$ peptide-induced deficits, with significant effects at the two highest doses tested. In the passive

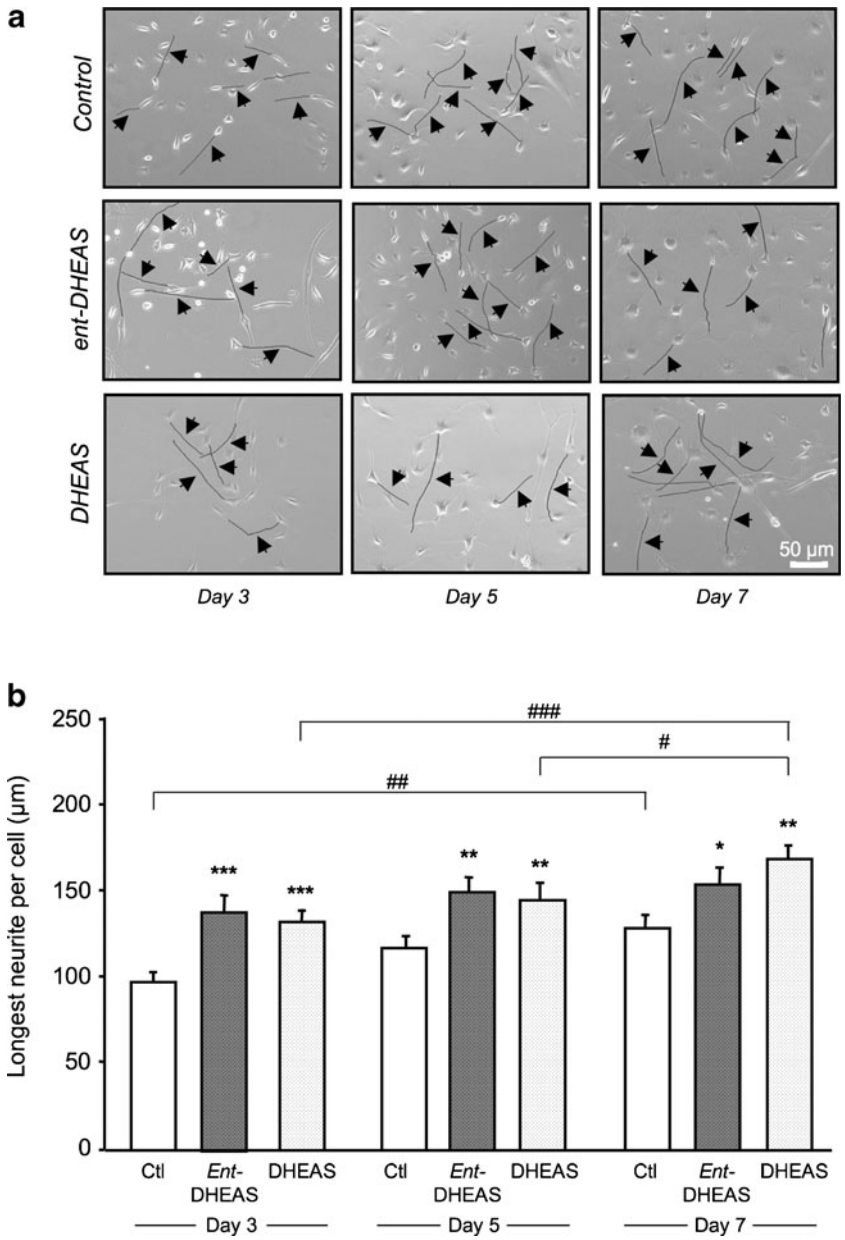

Fig. 10 Effects of *ent*-DHEAS and DHEAS on neurite outgrowth in B104 cultured cells: **a** representative photomicrographs of control and steroid-treated cells at days 3, 5 or 7. The longest neurite per cell was marked by a *black line* and indicated by an *arrow* (magnification ×10). *Scale bar* 50 µm. **b** Histograms of the length of the longest neurite per cell corresponding to the pool of three independent experiments. An average of 30–35 longest neurites was counted per treatment group and per day. Two-way ANOVA: $F_{(2,290)}$=16,3, p<0.001 for the steroid treatment; $F_{(2,290)}$=8,19, p<0.001 for the day; $F_{(4,290)}$=0.64, p>0.05 for the treatment×day interaction. *p<0.05, **p<0.01, ***p<0.001 vs. control (ctl); $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 for pairwise comparisons of steroid treatment between days; Fisher's PLSD test

avoidance test, $A\beta_{25–35}$ decreased significantly the step-through latency as compared to (ScAβ+Veh)-treated mice (Fig. 13b). *ent*-DHEAS pre-treatment (0.5 and 2 nmol) significantly prevented the diminution in latency induced by $A\beta_{25–35}$ (p<0.05; Fig. 13b). At the highest dose tested, the latency was not significantly different from the control (ScAβ+V)-treated group data, showing a complete prevention of $A\beta_{25–35}$-induced deficits.

Long-term effects of *ent*-PREGS and *ent*-DHEAS pre-injected 6 or 12 h before the peptide

Since we considered that the steroid enantiomers are unlikely to be substrates for enzymes involved in steroid biosynthesis in the brain, thereby causing them to remain unchanged longer than natural steroids, we pre-injected the enantiomers at their most active dose (0.5 nmol) at different time-points before the $A\beta_{25–35}$ peptide and checked the resulting protection in terms of behavioural deficits after 7 days (spontaneous alternation) or 8–9 days (passive avoidance). Results are summarised in Fig. 14. $A\beta_{25–35}$ peptide-induced alternation deficits are prevented when *ent*-PREGS or *ent*-DHEAS is injected 6 h but not 12 h before the peptide on day 0 (Fig. 14a, b). Under these administration schedules, neither PREGS nor DHEAS appeared active. Similarly, in the passive avoidance, a significant prevention of $A\beta_{25–35}$-induced decrease in step-through latency was observed when *ent*-PREGS or *ent*-DHEAS is injected 6 h before the peptide (Fig. 14a), but not 12 h before (Fig. 14d).

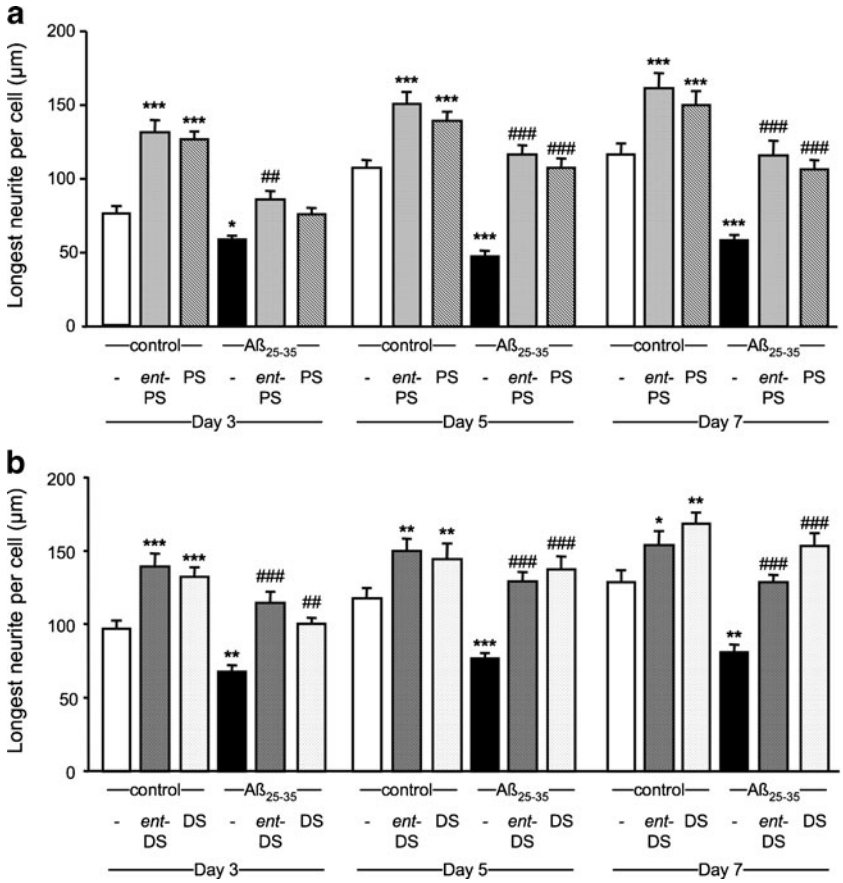

Fig. 11 Neuroprotective effects of steroids against $A\beta_{25–35}$-induced decrease on neurite outgrowth in B104 cultured cells. Histograms of the length of the longest neurite per cell correspond to the pool of three to four independent experiments. An average of 30–40 longest neurites was counted per treatment group and per day. **a** Effects of *ent*-PREGS (*ent*-PS, 5 μM) and PREGS (PS, 5 μM) alone or in presence of $A\beta_{25–35}$ (20 μM). Two-way ANOVA: $F_{(5,664)}=79.5$, $p<0.001$ for the steroid treatment; $F_{(2,664)}=26.1$, $p<0.001$ for the day; $F_{(10,664)}=2.1$, $p>0.05$ for the treatment×day interaction. **b** Effects of *ent*-DHEAS (*ent*-DS, 5 μM) and DHEAS (DS, 5 μM) alone or in the presence of $A\beta_{25–35}$ (20 μM). Two-way ANOVA: $F_{(5,585)}=40.3$, $p<0.001$ for the steroid treatment; $F_{(2,585)}=20.8$, $p<0.001$ for the day; $F_{(10,664)}=1{,}4$, $p>0.05$ for the treatment×day interaction. *$p<0.05$, ***$p<0.001$ vs. control; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ vs. $A\beta_{25–35}$-treated cells. Fisher's PLSD test

Effects of *ent*-PREGS and *ent*-DHEAS on the levels of hippocampal lipid peroxidation

To validate that *ent*-PREGS and *ent*-DHEAS protected against $A\beta_{25–35}$ peptide toxicity, we analysed the levels of lipid peroxidation in the hippocampus 7 days after i.c.v. injection of the peptide (Fig. 15). $A\beta_{25–35}$ induced a significant 31–38 % increase in the level of peroxidized lipid, as compared with the control (ScAβ+V)-treatment data (Fig. 15a, b; black columns). *ent*-PREGS significantly prevented this increase at the dose of 2 nmol ($p<0.05$; Fig. 15a). *ent*-DHEAS also significantly prevented this increase at the doses of 0.5 ($p<0.01$) and 2 nmol ($p<0.05$; Fig. 15b).

Discussion

In the present study, we provide the first evidence that synthetic enantiomers of steroids efficiently protect against Aβ peptide toxicity in vitro and in vivo. In vitro, both PREGS and *ent*-PREGS did not affect the viability of B104 neuroblastoma cells at concentrations up to 20 μM, indicating that they are not detrimental to the cells, nor do they induce their proliferation, under the conditions used. It is clear that the effect of PREGS on cell viability depends on its concentration and the cell type. In rat primary hippocampal cell cultures, PREGS was also devoid of any effect on cell viability, even at 100 μM (Weaver et al. 1998). In rat PC12 cell cultures, however, the cell viability profile under PREGS treatment was bell shaped with a maximal response at 1 μM (Akan et al. 2009). How *ent*-PREGS affects the viability of other cell types remains to be determined.

Administration of $A\beta_{25–35}$ peptide decreased the viability of B104 neuroblastoma cells in a dose-dependent manner. A powerful and significant toxicity was observed in the 5–20 μM range, with the highest concentration showing a relatively high reduction (about 30 %) of cell survival, showing toxicity in this concentration range, as previously described in

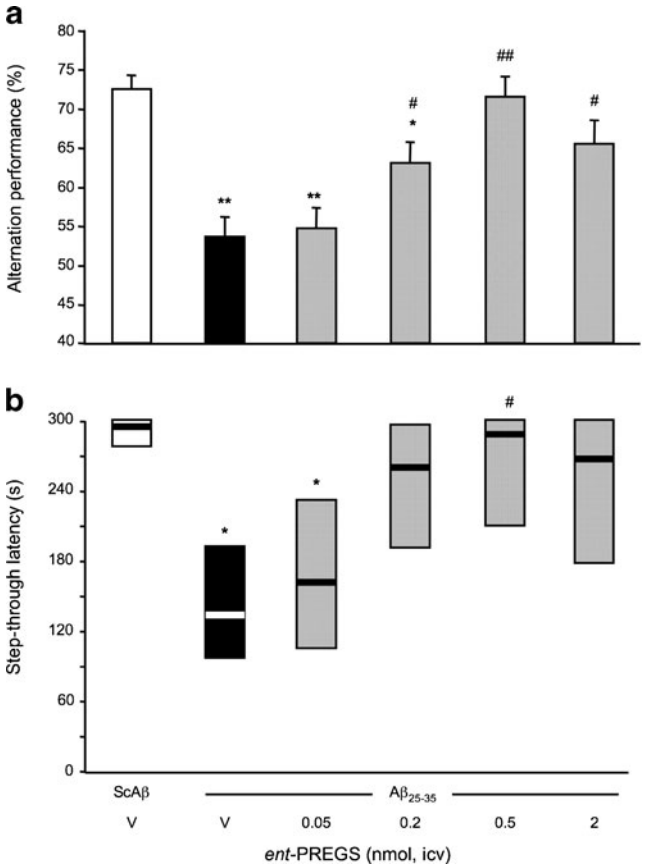

Fig. 12 Protective effects of *ent*-PREGS against the $A\beta_{25–35}$-induced memory deficits in mice: **a** spontaneous alternation performances and **b** step-through passive avoidance. Mice were administered i.c.v. with distilled water (V) or *ent*-PREGS (0.05–2 nmol) simultaneously with $A\beta_{25–35}$ peptide (9 nmol). The i.c.v. injection of ScAβ (9 nmol) was used as control. Spontaneous alternation performances in the Y-maze were measured on day 7. Passive avoidance training was carried out on day 8 and retention on day 9. $F_{(5,51)}=7.92$, $p<0.0001$, $n=6–10$ per group in **a**; $H=11.4$, $p<0.05$, $n=6–10$ in **b**. $^{*}p<0.05$, $^{**}p<0.01$ vs. the (ScAβ+V)-treated group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. the ($A\beta_{25–35}$+V)-treated group; Dunnett's test in **a**; Dunn's test in **b**

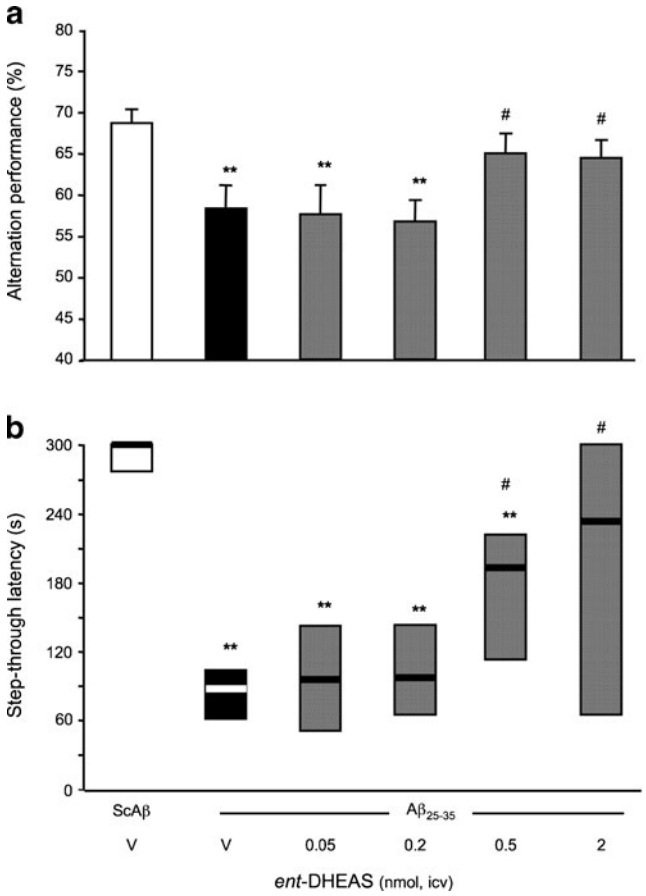

Fig. 13 Protective effects of *ent*-DHEAS against the $A\beta_{25–35}$ peptide-induced memory deficits in mice: **a** spontaneous alternation performances and **b** step-through passive avoidance. Mice were administered i.c.v. with distilled water (V) or *ent*-DHEAS (0.05–2 nmol) simultaneously with $A\beta_{25–35}$ peptide (9 nmol). The i.c.v. injection of ScAβ (9 nmol) was used as control. Spontaneous alternation performances in the Y-maze were measured on day 7. Passive avoidance training was carried out on day 8 and retention on day 9. $F_{(5,94)}=3.86$, $p<0.01$, $n=10$ in **a**; $H=15.3$, $p<0.01$, $n=12$ in **b**. $^{**}p<0.01$ vs. the (ScAβ+V)-treated group; $^{\#}p<0.05$ vs. the ($A\beta_{25–35}$+V)-treated group; Dunnett's test in **a**, Dunn's test in **b**

other cell lines including mouse hippocampal HT-22 cells (Gursoy et al. 2001) or SKN-SH human neuroblastoma cells (Gridley et al. 1997).

The neuroprotective effects of steroids were compared in terms of effectiveness (which refers to the ability of the steroid to produce a beneficial effect), efficacy (which refers to the maximum response achievable) and potency (which refers to the amount required to produce an effect of given intensity). Both *ent*-PREGS and PREGS were effective as they significantly and dose-dependently prevented the decreased of cell viability induced by $A\beta_{25–35}$. Steroid application for 24 h prior to the peptide resulted in different profiles of cytoprotection. The magnitude of protection by PREGS was higher than that of *ent*-PREGS at the same concentrations, 0.25–5 μM, indicating that the natural steroid may be more efficient than its synthetic analogue in counteracting the toxic effect of the peptide. In addition, PREGS was more potent than its enantiomer as the minimally active concentration of PREGS was 0.25 μM as compared to 5 μM for *ent*-PREGS. Complete protection of Aβ-induced toxicity was observed at 5 μM for PREGS and 10 μM for *ent*-PREGS. These results revealed that *ent*-PREGS and PREGS have different pharmacological activities in terms of intensity and active dose. Whether or not this enantioselectivity is indicative of different mechanisms of actions for the natural and enantiomeric steroids remains to be determined, but this possibility is intriguing. *ent*-DHEAS and DHEAS were also found to be neuroprotective by preventing the decrease of cell viability induced by $A\beta_{25–35}$. The actions of both steroids were similar and they were highly efficient and highly potent. At the lowest steroid concentration, a complete protection of Aβ-induced toxicity was reached. Since PREGS neuroprotection is

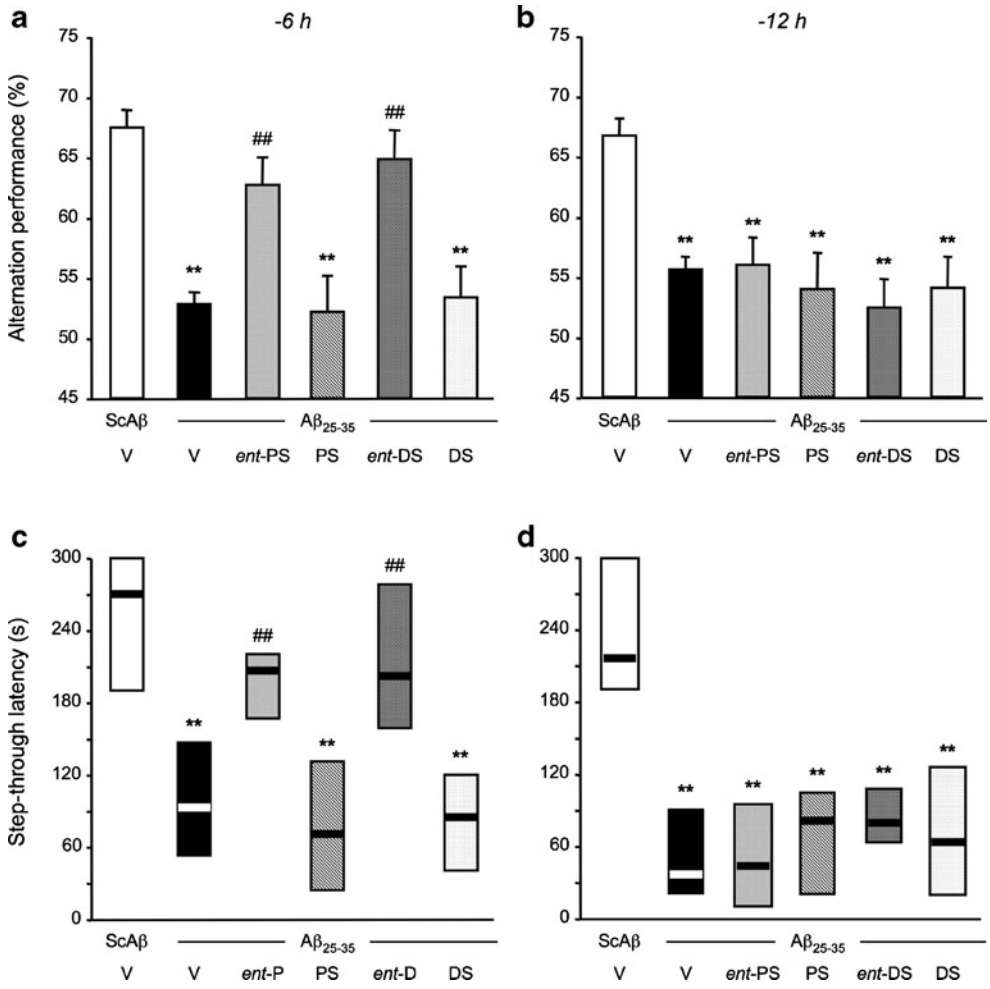

Fig. 14 Comparison of the protective efficacy of *ent*-PREGS, *ent*-DHEAS and their respective natural stereoisomers pre-injected 6 h (**a**, **c**) or 12 h (**b**, **d**) prior to $A\beta_{25–35}$ peptide in mice: **a**, **b** spontaneous alternation performances on day 7; **c**, **d** passive avoidance response on days 8 and 9. Mice were cannulated and administered i.c.v. with distilled water (V) or ent-PREGS (*ent*-PS), PREGS, *ent*-DHEAS (*ent*-DS) or DHEAS (each at 0.5 nmol) either 6 or 12 h before the $A\beta_{25–35}$ peptide (9 nmol). The i.c.v. injection of ScAβ (9 nmol) was used as control. $F_{(5,69)}=10.0$, $p<0.0001$, $n=11–12$ per group in **a**; $F_{(5,71)}=5.92$, $p<0.0001$, $n=12$ per group in **b**; $H=27.9$, $p<0.0001$, $n=11–12$ in **c**; $H=24.3$, $p<0.001$, $n=12$ in **d**. $^{**}p<0.01$ vs. the (ScAβ + V)-treated group; $^{\#\#}p<0.01$ vs. the ($A\beta_{25–35}$+V)-treated group; Dunnett's test in **a** and **b**; Dunn's test in **c** and **d**

enantioselective and DHEAS was not enantioselective, this finding may also imply differences in the mechanism of action for PREGS and DHEAS.

It is established that cell death induced by amyloid peptides, including $A\beta_{25–35}$, involves apoptotic processes (Forloni et al. 1996; Ekinci et al. 2000) and necrosis (Behl 1997), differ according to the cell type. In the present work, the combination of annexin-V and PI in flow cytometric analyses allowed us to distinguish between living, necrotic and apoptotic cells in the early or late phase. Exposure of B104 cells to $A\beta_{25–25}$ for 24 h led to significant increases of the percentage of cells in late—not early—apoptosis and necrosis. The increased cell death in late apoptotic phase may be the result of serum withdrawal before steroid and peptide applications. Solovyan et al. (1998) have shown evidence of late phase apoptosis occurring in NB2a neuroblastoma cells after serum deprivation. *ent*-PREGS and PREGS exert protective effects against Aβ toxicity by both preventing late-phase apoptotic toxicity, but with different potencies (*ent*-PREGS action was significant at the high 5–10 μM concentration while PREGS was already efficient at the lowest 0.25 μM concentration).

One major event of late apoptosis is the potentiation of DNA damages in the cell nucleus. One can then speculate that $A\beta_{25–35}$-dependent DNA damages may be reduced under steroid treatment. The fact that PREGS also inhibit Aβ-induced necrotic cell death might be one explanation for its higher potency and efficacy in decreasing cell viability as compared to *ent*-PREGS. Both steroids had no effect on early-phase apoptosis, suggesting that they may not intervene in the initiation of the apoptotic cascades that involves cell membrane and organelle damage without nuclear alterations. With regards to *ent*-DHEAS and DHEAS, they both prevented Aβ toxicity by reducing late apoptotic and necrotic cells with similar potencies and efficacy.

In addition to their protective properties, *ent*-PREGS and PREGS highly promoted neurite process growth over time, with the same efficacy. *ent*-DHEAS and DHEAS also enhanced neurite outgrowth with similar efficacy. Since these trophic effects were not enantioselective, we do not have any evidence that they are the result of steroids directly binding to a receptor. This raises the possibility that the trophic effects might be indirect (e.g., receptors responding differently due to changes in the surrounding membrane caused by the steroids).

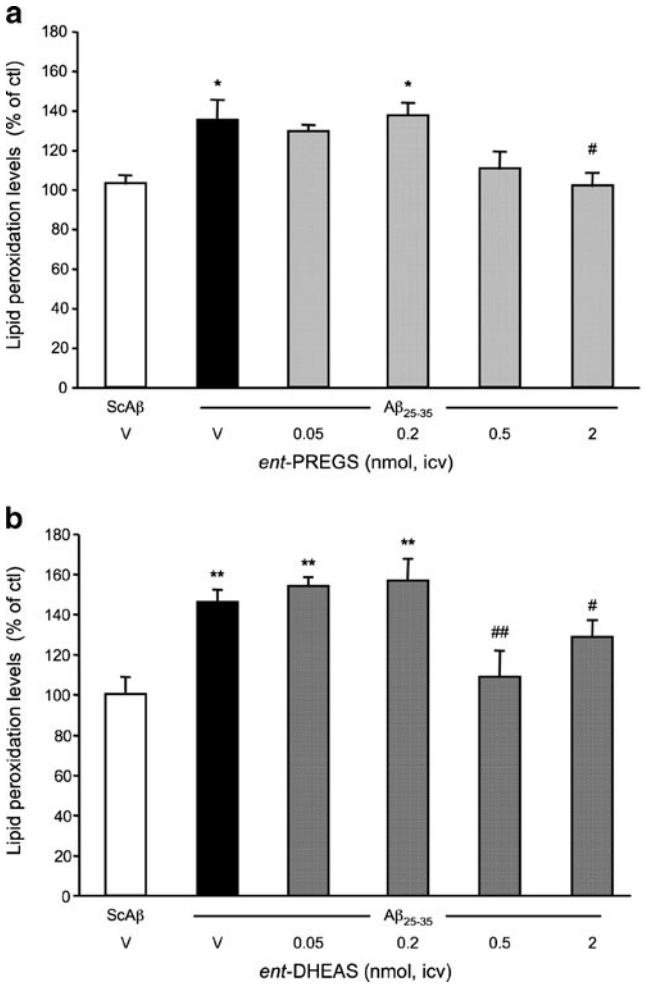

Fig. 15 Neuroprotective effects of *ent*-PREGS and *ent*-DHEAS against the $A\beta_{25–35}$ peptide-induced oxidative stress in the hippocampus of mice: measure of lipid peroxidation levels. Mice were administered i.c.v. with distilled water (V) or *ent*-PREGS (0.05–2 nmol) in **a** or *ent*-DHEAS (0.05–2 nmol) in **b**, simultaneously with $A\beta_{25–35}$ peptide (9 nmol). The i.c.v. injection of ScAβ (9 nmol) was used as control. Lipid peroxidation levels were measured on day 9. $F_{(5,30)}=4.69$, $p<0.01$, $n=6$ per group in **a**; $F_{(5,69)}=7.54$, $p<0.0001$, $n=10–11$ per group in **b**. $^{*}p<0.05$, $^{**}p<0.01$ vs. the (ScAβ+V)-treated group; $^{\#\#}p<0.01$ vs. the ($A\beta_{25–35}$+V)-treated group; Dunnett's test

We then analysed the neuroprotective potential of both *ent*-PREGS and *ent*-DHEAS in vivo in $A\beta_{25–35}$-teated mice. *ent*-Steroids were administered at the same time as $A\beta_{25–35}$, i.e., 1 week before the behavioural and biochemical analyses. $A\beta_{25–35}$ induces delayed deficits in spontaneous alternation and passive avoidance 1 week after injection with an active i.c.v. dose of 9 nmol, an effect consistent with our earlier reports (Maurice et al. 1998; Villard et al. 2009). *ent*-PREGS induced a bell shaped but significant prevention of Aβ-induced amnesia. The dose of 0.5 nmol i.c.v. was the most effective on both alternation and avoidance responses. Interestingly, equal potency was observed for the promnesiant effect of *ent*-PREGS in mice tested in the two-trial arm recognition task (Akwa et al. 2001). Coadministration of *ent*-DHEAS with Aβ also prevented Aβ-induced memory deficits, with similar potency as *ent*-PREGS. In contrast to *ent*-PREGS, the behavioural deficits were reversed with increasing doses of *ent*-DHEAS, the most active being the highest dose tested (2 nmol). We can thus conclude that both steroid enantiomers protect from the deficits of the short- and long-term memory induced by $A\beta_{25–35}$, with *ent*-PREGS being more potent than *ent*-DHEAS. Using the same behavioural paradigms, we previously showed that systemic administration of PREGS or DHEAS can attenuate with the same potency (20 mg/kg) $A\beta_{25–35}$-induced memory deficits 1 week after $A\beta_{25–35}$ i.c.v. injection (Maurice et al. 1998). After daily chronic systemic injections for 7 days of PREGS (20 mg/kg), an improvement of $A\beta_{25–35}$-impaired spatial memory can be observed in the Morris water maze test (Yang et al. 2012).

Results from pre-administration studies clearly suggested that *ent*-PREGS and *ent*-DHEAS maintain a longer duration of action that their natural counterparts, since their effect could be detectable in a pretreatment timeframe of at least 6 h. Because of their opposite absolute configuration, *ent*-PREGS and *ent*-DHEAS are not expected to be substrates for the biosynthetic enzyme that convert PREGS and DHEAS to other steroids. A high production of pregnenolone (PREG) and DHEA (identified by gas chromatography–mass spectrometry) could be observed in the rat brain, 5 min after i.c.v injections of PREGS or DHEAS, respectively, while *ent*-PREGS and *ent*-DHEAS were not converted into the respective unsulphated *ent*-steroids (Akwa, unpublished data). These experiments suggested that both steroid enantiomers were not substrates of the brain 3β-hydroxysteroid sulfatase, and were probably less susceptible to further metabolism for this reason. PREGS however can form PREG in the brain in rodents (Zwain and Yen 1999; Compagnone and Mellon 2000), which can protect against $A\beta_{25–35}$-induced cell death with the same efficacy (at low concentration of 0.5 μM), for example in mouse hippocampal (HT-22) (Gursoy et al. 2001) and PC12 (Gursoy et al. 2001) cell lines.

The neuroprotective effects of *ent*-PREGS and *ent*-DHEAS were also tested on lipid peroxidation which is an important and early biochemical mechanism of the oxidative stress in AD- and Aβ-induced neurotoxicity (Butterfield et al. 2001, 2002b). Evidence is presented here for the antioxidant effects of *ent*-PREGS and *ent*-DHEAS as pre-treatment of both enantiomers strongly prevented $A\beta_{25–35}$-induced lipid peroxidation in the hippocampus. This is consistent with the neuroprotective effect of the synthetic enantiomer of 17β-E_2 against H_2O_2 toxicity in human neuroblastoma SH-N-SY cells (Wang et al. 2006).

In conclusion, we report the first demonstration of active and powerful neuroprotection by the synthetic enantiomers of PREGS and DHEAS against $A\beta_{25–35}$ peptide-induced cell death, amnesia or oxidative stress. The higher efficacy and longer duration of action of *ent*-PREGS and *ent*-DHEAS in

improving memory deficits, as compared to their natural counterparts, may provide better therapeutic benefits in early stages of AD.

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Conflict of interest JM and VV are now employees of Amylgen (Montpellier). TM is the scientific director of Amylgen and scientific board adviser of Anavex Life Sciences (Hoboken, NJ, USA). DFC holds equity in Sage Therapeutics Inc. The companies were not involved, scientifically or financially, in the present experiments. The authors declare that they have no other conflict of interest.

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