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Protective effect of BDNF against beta-amyloid induced neurotoxicity *in vitro* and *in vivo* in rats

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ABSTRACT

We examined the potential protective effect of BDNF against β-amyloid-induced neurotoxicity *in vitro* and *in vivo* in rats. In neuronal cultures, BDNF had specific and dose–response protective effects on neuronal toxicity induced by $A\beta_{1-42}$ and $A\beta_{25-35}$. It completely reversed the toxic action induced by $A\beta_{1-42}$ and partially that induced by $A\beta_{25-35}$. These effects involved TrkB receptor activation since they were inhibited by K252a. Catalytic BDNF receptors (TrkB.FL) were localized *in vitro* in cortical neurons (mRNA and protein). In *in vivo* experiments, $A\beta_{25-35}$ was administered into the indusium griseum or the third ventricle and several parameters were measured 7 days later to evaluate potential $A\beta_{25-35}$/BDNF interactions, i.e. local measurement of BDNF release, number of hippocampal hilar cells expressing SRIH mRNA and assessment of the corpus callosum damage (morphological examination, pyknotic nuclei counting and axon labeling with anti-MBP antibody). We conclude that BDNF possesses neuroprotective properties against toxic effects of Aβ peptides.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by mild cognitive impairments at onset and deficits in multiple cortical functions in later stages. In the dementia stages, numerous senile plaques and neurofibrillary tangles accompanied by neuronal loss are observed. The senile plaques are essentially composed of amyloid β-peptide (Aβ), a 40–42 amino acid peptide fragment of the β-amyloid precursor (APP) (Glenner and Wong, 1984), but also of $A\beta_{25-35}$ oligomers (Gruden et al., 2007; Kubo et al., 2002). Aβ accumulation can result in oxidative stress, inflammation, and neurotoxicity, all of which can initiate the pathogenic cascade, ultimately leading to apoptosis and deterioration of the neurotransmission systems (Yankner, 1996).

Recent findings have suggested that a decrease in brain-derived neurotrophic factor (BDNF) levels could be associated to the pathogenesis of AD. BDNF is an endogenous protein from the neurotrophin family involved in the structural and functional plasticity of the brain (McAllister et al., 1999; Poo, 2001). It protects neurons against different kinds of brain insult (Lindvall et al., 1994; Tapia-Arancibia et al., 2004) and also plays important roles in the neural development and maintenance of central and peripheral neurons (Lewin and Barde, 1996; Thoenen, 1995). In AD patients, it has been observed that the precursor form of BDNF and mature BDNF (Peng et al., 2005; Michalski and Fahnestock, 2003) or its mRNA (Holsinger et al., 2000; Phillips et al., 1991) are decreased in the parietal cortex and hippocampus even in pre-clinical stages of AD. BDNF serum concentrations also vary over the course of the disease and are correlated with the severity of dementia (Laske et al., 2007). Strikingly, Murer et al. (1999) demonstrated in AD brains that neurons containing neurofibrillary tangles, a hallmark of the disease, do not contain BDNF-immunoreactive material whereas most intensely BDNF-labeled neurons were devoid of tangles. Taken together, these findings support a role of BDNF in the etiology of AD and suggest a potential neuroprotective action of BDNF in AD treatment.

We examined the presence of BDNF receptors and the impact of BDNF administration on the toxic effects of Aβ peptides ($A\beta_{25-35}$ and $A\beta_{1-42}$) in primary cultures of cortical neurons. In parallel, we investigated whether our *in vitro* results could be in keeping with some *in vivo* Aβ/BDNF interactions supporting eventual protective effects in areas related to cognitive functions, i.e. gyrus dentate and corpus callosum. Adult rats were thus injected with aggregated $A\beta_{25-35}$ peptide alone or accompanied by BDNF administration in the indusium

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griseum (IG) or in the 3rd ventricle (icv). The former is a medial cortical region near the corpus callosum and classically felt to be a displaced hippocampal anlage (Wyss and Sripanidkulchai, 1983). Following the different treatments, we examined local measurement of BDNF release, number of somatostatin (SRIH) neurons in the dentate gyrus hilus and morphological damage of corpus callosum.

Materials and methods

Animals

Adult pregnant females or male Sprague–Dawley rats (Depré, St Doulchard, France) (230–250 g) were housed for at least 1 week before the experiments and kept under constant temperature (21 ± 1°C) and lighting (light on from 07:00 am to 07:00 pm) regimens. Food pellets and water were freely available throughout the experiment. Procedures involving animals and their care were conducted in conformity with French laws on laboratory animals that are in compliance with international laws and policies (EC Council Directive 86/609, OJ L 358,1,24 November 1987). The Animal Welfare Committee at the University of Montpellier II approved all protocols and particular efforts were made to minimize the number of animals used and potential pain and distress.

Materials

$A\beta_{1-42}$, $A\beta_{25-35}$ or scrambled peptides were purchased from Bachem (Weil am Rhein, Germany) or NeoMPS (Strasbourg, France). Neurobasal media, B-27 supplement and fetal bovine serum were from GIBCO Invitrogen (Cergy Pontoise France). BDNF was a generous gift from Regeneron Pharmaceutical (USA) and NGF was from Genentech, Inc. (USA). All other chemicals, unless specifically mentioned, were purchased from Sigma-Aldrich (St Quentin Fallavier, France).

Antibodies against BDNF (sc-546, lot E0704), TrkB.FL (sc-12, lot J111) and TrkB (TK−) (sc-119, lot I1004 epitope mapping at the C-terminus recommended for detection of truncated receptors) were from Santa Cruz Biotechnology (Santa Cruz, CA). MBP mouse monoclonal antibody against myelin basic protein was from Boehringer, Mannheim, Germany. The Alexa Fluor 488 secondary antibodies (TrkB, BDNF and MBP detection) were from Molecular Probes (Leiden, Netherlands).

Neuronal cultures

Cerebral cortical cultures greatly enriched in neurons were prepared from embryonic day 17 rat fetuses obtained from Sprague–Dawley rats, as previously described (Tapia-Arancibia and Astier, 1989) with minor modifications. Cells plated at 2.5×10^5 cells/cm^2 were cultured in poly-D-lysine coated 24-well plates and maintained in Neurobasal medium supplemented with B-27 components (Invitrogen, Life Technologies, Cergy Pontoise, France) that contains a great number of trophic and protective antioxidant compounds and prevents the proliferation of glial cells (Brewer et al., 1993). The potential proliferation of remaining non-neuronal cells was inhibited by treatment with 10 μM cytosine arabinoside for 48 h between days 3 and 5 after plating. Cultures were grown for 6 days before treatments, which were performed between 6–8 days *in vitro* (DIV).

RNA extraction and cDNA synthesis

Total RNA was extracted from cortical cultures using the High Pure RNA Isolation Kit (Roche Diagnostique, Meylan, France) according to the manufacturer's instructions. RNA concentration and purity were evaluated by spectrometry on the basis of optical density (OD) measurements at 260 and 280 nm. cDNA synthesis was performed as already described (Silhol et al., 2007).

Table 1
Primers and expected sizes of PCR products with each primer pair

Gene	Forward primer	Reverse primer	Size (bp)
trkB.FL	5′-gatcttcacctacggcaagc-3′	5′-tcgccaagttctgaaggagt-3′	200
trkB.T1	5′-tcataagatccccctggatg-3′	5′-tgcttctcagctgcctgac-3′	242
cyclophilin	5′-ataatggcactggtggcaag-3′	5′-catgccttctttcaccttcc-3′	199

Quantitative real-time PCR

Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The PCR reactions were performed in a final volume of 20 μl with 1 × LC-DNA Master SYBR Green I mix, 3 mM $MgCl_2$, 0.5 μM of each primer and 1/5 diluted RT mixture for trkB.FL and trkB.T1, and 1/10 diluted RT mixture for cyclophilin (and water as negative control) was added as PCR template. The amplification conditions were the same as already described (Silhol et al., 2007). The amounts of target genes were normalized against cDNA of the housekeeping gene cyclophilin in the corresponding samples. Primers were developed using the Primer 3 software package (Rozen and Skaletsky, 2000). Primer sequences specific to the genes examined and predicted product sizes are shown in Table 1.

Neuronal treatments

Cultures were grown in the B27-supplemented Neurobasal serum-free medium. Since we observed that, in the presence of B27, amyloid peptides were not toxic for neurons up to high concentrations the B27 supplement was removed from the cultures during exposure to Aβ peptides between 6 and 8 DIV. Cells were washed for 1 h with Neurobasal medium and then incubated for 48 h in the presence or absence of Aβ peptides with or without BDNF. Control cells were incubated in Neurobasal medium alone.

Measurement of in vitro cell viability

Neuronal survival was determined at 8 DIV by trypan blue exclusion (Pike et al., 1993), which detects dead cells, or using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche), which detects active mitochondrial dehydrogenases of living cells to reduce MTT to a water-insoluble blue formazan product (Ivins et al., 1999). Since initial assays showed that the two methods gave comparable results, we continued our studies with the MTT method. In brief, cultures grown in 24-well plates were rinsed with 1 ml Locke's solution, then supplemented with MTT, and incubated for 2 h in a 5% CO_2 incubator at 37°C. The reaction product, solubilized in dimethylsulfoxide, was measured with an ELISA plate reader at 570 nm. The optical density of dimethyl sulfoxide was used as background and subtracted. MTT assays can quantify the survival promoting the effects of neurotrophins (Manthorpe et al., 1986); the viability of cortical neurons (Tong et al., 2004) or neuroblastoma cells (Olivieri et al., 2003) after Aβ treatment and cerebellar granule neuron apoptosis (Skaper et al., 1998). In each experiment, cell viability was determined from four wells for each condition and normalized to parallel controls with each well being treated as a single observation. Data were obtained from at least four separate cultures and expressed as means ± S.E.M. Statistical comparison was determined by an ANOVA test with Student's *t* test as the post hoc test.

Immunofluorescence labeling

Cells

On the day of the experiment, cells were fixed in 4% PFA in saline phosphate buffer (PBS). The antibodies used included rabbit IgG polyclonal antibodies against BDNF (1/50), TrkB.FL (recognizing the C-

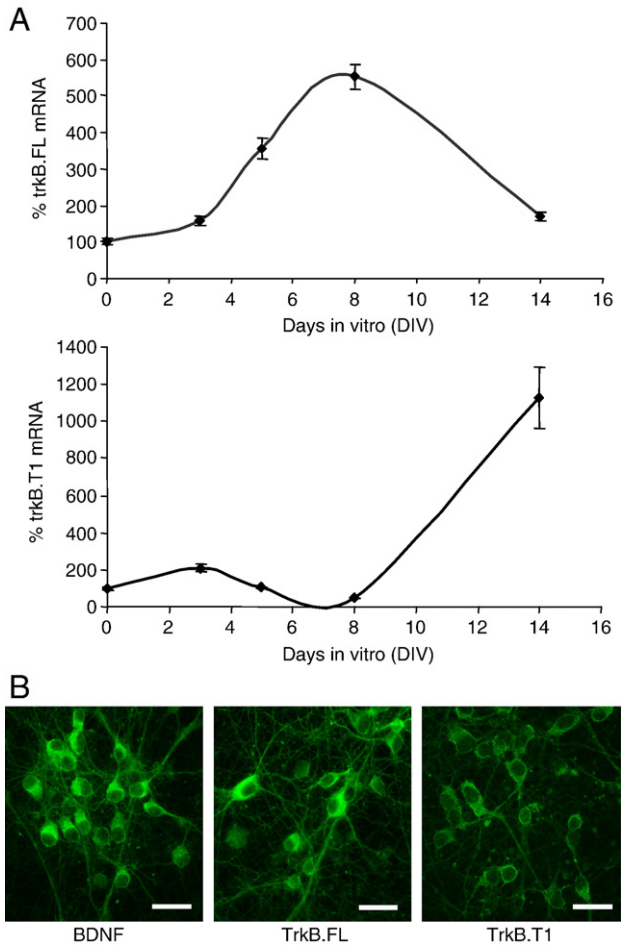

Fig. 1. Presence of BDNF and its receptors in primary cultures of rat cerebral cortical neurons. (A) Changes in trkB.FL and trkB.T1 mRNA expression as a function of days *in vitro* of cortical cultures determined by real-time PCR. A peak of trkB.FL mRNA levels was observed at 8 DIV, whereas trkB.T1 levels increased later, from 8 DIV. (B) Immunofluorescence detection of BDNF, TrkB.FL and TrkB.T1 proteins in cortical neurons at 8 DIV. At day 8, cells were fixed and immunostained with specific antibodies against BDNF, TrkB.FL and TrkB.T1 as indicated in Materials and methods. BDNF and TrkB.FL, the catalytic receptor, were strongly represented but the TrkB.T1 signal was weakly labeled. Scale bar = 50 μm.

terminal cytoplasmic domain of the receptor) (1/100) and TrkB.T1 (1/100). Cells were first incubated for 4 to 6 h in blocking buffer (PBS containing 0.1% Triton X-100 and 2% BSA) and then incubated overnight at 4°C in a humid chamber with primary BDNF and TrkB antibodies in the same buffer. After rinsing in PBS, they were incubated for 2 h at room temperature in a humid chamber with secondary antibody diluted in the blocking buffer: an anti-rabbit IgG conjugated with Alexa Fluor 488 (1/1000). After careful rinsing, cells were mounted in FluorSave reagent (VWR International, Strasbourg, France) and observed under a DMR fluorescent microscope (Leica, Rueil-Malmaison, France).

Slices

Adjacent sections (10 μm) obtained from brains of control and treated rats were incubated overnight at room temperature in a humid chamber with the primary antibody anti MBP (1/500) in PBS containing 0.4% Triton X-100 and 3% goat normal serum. After rinsing in PBS, they were incubated for 2 h at room temperature in a humid chamber with the secondary antibody, an anti-mouse IgG conjugated with Alexa Fluor 488 (1/1000) (Molecular Probes, Leiden, Netherlands) diluted in PBS containing 0.1% Triton X-100 and 1.5% goat normal serum. After careful rinsing, slices were mounted in FluorSave reagent (VWR international, Strasbourg, France) and observed under a DMR fluorescent microscope (Leica, Rueil-Malmaison, France).

In the two cases (cells and slices), controls of the immunostaining specificity consisted of (1) omitting the primary antibody and applying the secondary antibody alone and (2) exciting each fluorochrome by inappropriate wavelengths. This allowed us to confirm that the secondary antibody used did not induce artifactual fluorescent labeling and that there was no overlap in the emission spectra of the two fluorochromes.

Push-pull perfusion

After a 7 day laboratory acclimation period, rats were anesthetized with sodium pentobarbital (40 mg/kg) and a push-pull device (Phymep, Paris) consisting of a stainless steel cannula (0.5 mm outer diameter) fitted with a stylette was stereotaxically implanted into the IG according to Paxinos and Watson's (1997) coordinates (AP: 3.3 mm, L: 0.0 mm and DV: 2.8 mm). After surgery, the animals were caged separately, handled every day for 1 week and, after recovery of their preoperative body weight, subjected to push-pull perfusion or injections. Briefly, as previously reported (Arancibia, 1987; 2007), push-pull perfusion was performed on unanesthetized animals. Artificial cerebrospinal fluid was infused at a flow rate of 15 μl/min and samples (225 μl) were collected every 15 min for 2 h following intracerebral (ic) administration of $A\beta_{25-35}$ peptide or $A\beta_{25-35}$ peptide + BDNF. Perfusates were centrifuged and evaporated in a Speed-Vac concentrator (Savant Instruments, Hicksville, NY) and immediately stored at −20°C until BDNF ELISA immunoassay. As control groups, we used rats injected with either scramble $A\beta_{25-35}$ peptide or saline (sham-operated). As no differences were observed between these controls, they could be used indiscriminately.

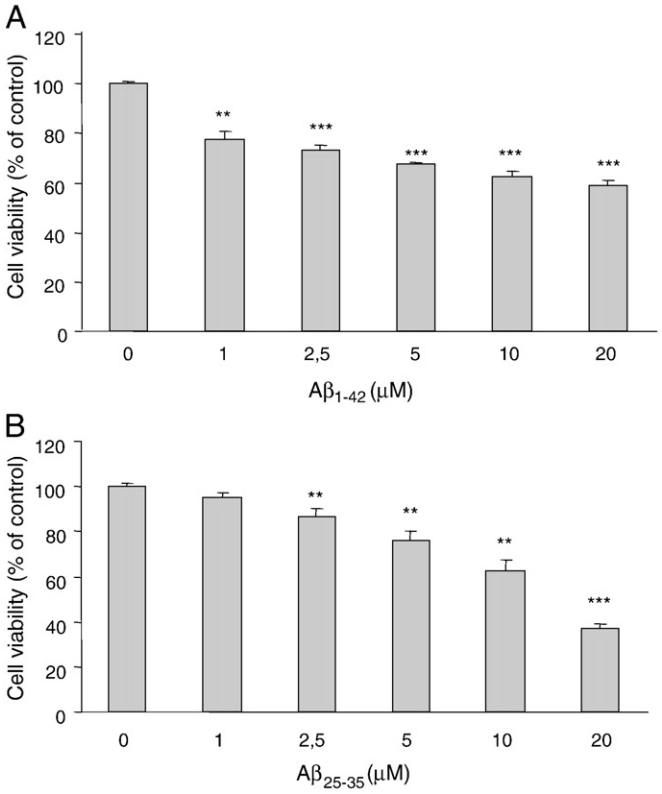

Fig. 2. Dose-dependent neurotoxicity of $A\beta_{1-42}$ and $A\beta_{25-35}$ peptides in rat cortical neurons. Increasing concentrations of $A\beta_{1-42}$ (A) and $A\beta_{25-35}$ (B) were added to cells in neurobasal medium on day 6 of culture and the toxicity was estimated at day 8 by the MTT assay. The experimental data were from four different cultures with $n=4$ dishes/culture at each concentration. Values represent mean ± SEM. $^{**}p<0.01$, $^{***}p<0.001$ compared with non-treated groups.

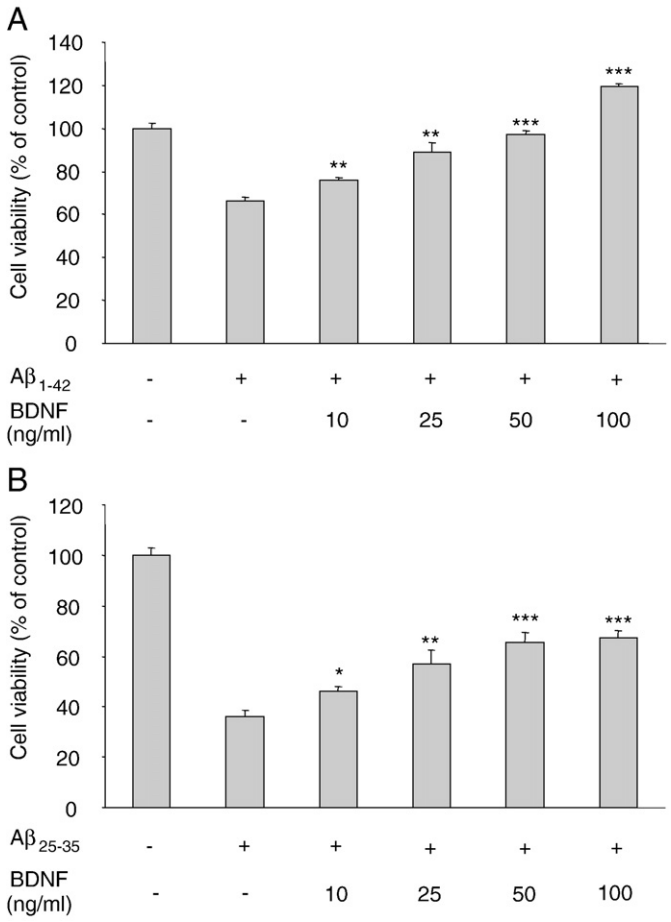

Fig. 3. Neuroprotective effect of BDNF on rat cortical neurons exposed to $A\beta_{1-42}$ and $A\beta_{25-35}$ for 2 days. 20 μM $A\beta_{1-42}$ (A) and 20 μM $A\beta_{25-35}$ (B) were added to cells in the presence of increasing concentrations of BDNF in neurobasal medium on day 6 of culture and toxicity was estimated at day 8 by the MTT assay. The experimental data were from three different cultures with $n=4$ dishes/culture at each BDNF concentration. Values represent mean ± SEM. $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared with groups without BDNF.

All push-pull experiments were performed in conscious rats at the same time of the day to avoid variations due to possible circadian BDNF release. Samples were collected for 2 h and at the end of the experiments animals were killed and their brains removed and fixed for histological examination or *in situ* hybridization. Rats with an unsuitable cannula location, or presenting a glial cells reaction around the scar, were excluded from the study.

BDNF release

BDNF release was measured with a conventional two site enzyme-immunoassay system (ELISA) assay, as previously reported (Arancibia et al., 2007), according to the manufacturer's protocol (Promega, Charbonnières, France). Briefly, each evaporated sample was reconstituted with 100 μl H_20 and used to determine BDNF release. The assay sensitivity was 15 pg/ml and the cross reactivity with other related neurotrophic factors was less than 3%. The BDNF concentration was expressed as picograms per fraction. The intra- and inter-assay coefficients of variation were 3% and 8%, respectively. Non-detectable values were considered, by convention, as being equal to the assay detection limit.

Toxicity in vivo studies

Aggregates of $A\beta_{25-35}$ or scrambled peptide were prepared by incubating the peptides at 3 mg/ml concentration in sterile water for 4 days at 37°C. Formation of aggregates was confirmed visually by phase-contrast microscope inspection (200X). Subsequently, 9 nmol/3 μl of aggregated $A\beta_{25-35}$ was administered to unanesthetized rats through the push segment of the push-pull cannula (1 μl/min). Sham-operated rats received the same volume of vehicle. Histological observations were performed 7 days after $A\beta_{25-35}$ administration. When the neuroprotective effect of BDNF was examined, it was administered (6 μg/6 μl) twice: 1 day before and 1 h before $A\beta_{25-35}$ administration and the studies were always performed 7 days later. At the 7th day, animals were killed by decapitation. A scheme showing the experimental design is illustrated in Fig. 6.

Histological examination

After *in vivo* experiments, rats were killed and their brains quickly frozen in liquid nitrogen. The frozen brains were mounted on a cryostat (Leica, Rueil-Malmaison, France) and serially cut into 10 μm coronal sections. The mounted slides were stained with Harris' hematoxylin solution for 3 min, dehydrated in alcohol and mounted in Entellan (Merck, Darmstadt, Germany). This method is used to assess the cytoarchitecture as well as degenerative changes in neurons in areas of neurodegeneration according well established criteria (Farber 1982; Garcia et al., 1995; Giovannelli et al., 1998; Stepanichev et al., 2004). Briefly, undamaged neurons were recognized as cells with round-shaped blue nuclei and clear perinuclear cytoplasm. Damaged neurons appeared as cells with altered nuclei (pyknosis, karyorrhexis and karyolysis) and cytoplasm with loss of hematoxylin affinity.

Brains dissected from another independent group of animals were devoted to immunological assay with MBP antibody as above described.

In situ hybridization of SRIH

Somatostatin in *situ* hybridization was performed on frozen brains as previously described (Arancibia et al., 2001) with digoxigenin-labeled oligonucleotide probe. Briefly, a 45-mer oligonucleotide antisense probe for somatostatin-14 (5′ to 3′ sequence: CCAGAA-GAAAGTTCTTGCAGCCAGCTTTGCGTTCCCGGGGTGT) (Genosys, Cambridge, UK) was end-labeled with digoxigenin-11-dideoxyuridine-5-triphosphate. Negative controls were carried out by omitting the labeled probe from the hybridization buffer, or by incubating the sections with a 45-mer oligonucleotide sense probe based on the somatostatin-14 sequence.

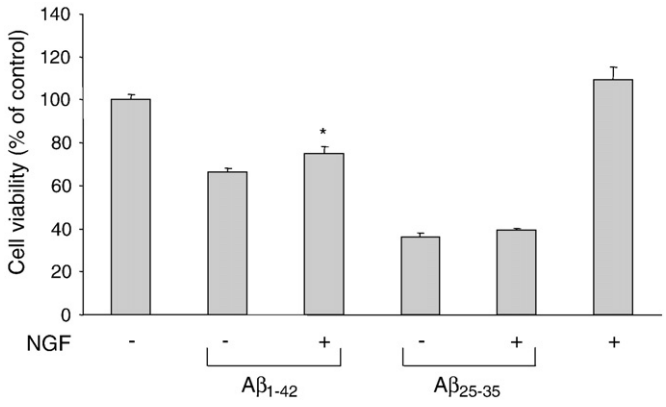

Fig. 4. Effect of NGF on rat cortical neurons exposed to $A\beta_{1-42}$ and $A\beta_{25-35}$ for 2 days. 20 μM $A\beta_{1-42}$ and 20 μM $A\beta_{25-35}$ were added to cells in the presence of 50 ng/ml NGF in neurobasal medium on day 6 of culture and toxicity was estimated at day 8 by the MTT assay. The experimental data were from two different cultures with $n=4$ dishes/culture for each experimental condition. Values represent mean ± SEM. $^*p<0.05$ compared with groups without NGF. NGF alone, only at this high concentration (50 ng/ml), increased cell survival by 10% and weakly protected cells, only against $A\beta_{1-42}$-induced toxicity.

Cellular counting and analysis

DIG-labeled cells and hematoxylin-stained cells (n = 5 rats/group; 8–10 sections per animal) were quantified using a Sony CCD XC-77 video camera with high resolution (570(H)3485 (V) TV lines) coupled to a Macintosh computer (Power PC G3) and NIH imageJ software (1.37v, W. Rasband, NIH, Bethesda, USA) (Arancibia et al., 2001; Givalois et al., 2004). For each animal, the cell number or the DIG-labeled cells were counted from 8 serial, non-adjacent 10 μm-thick coronal sections from the dorsal through the antero-posterior extent of the hippocampus between bregma −2.8 to −4.3 according the atlas of Paxinos and Watson. The count of stained sections was done by three different scientists unaware of the experimental conditions and independently from each other. Hilar neurons were considered according classical criteria (Lowenstein et al., 1992). Data were expressed as means ± S.E.M. Statistical comparisons of values were performed using a one-way analysis of variance (ANOVA; Statview 4.5) followed by a Fisher's PLSD test, as previously reported (Arancibia et al., 2001).

Results

In vitro data

Primary cultures of cortical neurons

We first investigated the developmental expression of mRNAs encoding TrkB.FL and TrkB.T1 receptors in cortical neurons in primary cultures by real-time PCR in three independent experiments and each with n = 4 wells (Fig. 1A). The results are presented as the ratio between trkB.FL or trkB.T1 and cyclophilin mRNAs and all of these parameters were determined in the same sample obtained from the same RT. We could thus compare the relative amounts of each mRNA at the same stage. We observed that trkB.FL mRNA levels progressively increased from 3 DIV to reach maximal levels at 8 DIV. In contrast, mRNA coding for the TrkB.T1 receptor was weakly expressed up to 8 DIV but its expression sharply increased at 14 DIV. Then, we used immunocytochemical staining to examine the presence of BDNF, TrkB.FL and TrkB.T1 proteins at 8 DIV since the toxicity and pharmacological experiments were performed at this stage. Fig. 1B shows that, at 8 DIV, neurons were strongly immunostained for BDNF and TrkB.FL but weakly stained for TrkB.T1.

Effect of increasing concentrations of $A\beta_{1-42}$ and $A\beta_{25-35}$ peptides on cell viability

As expected, 48 h exposure to $A\beta_{1-42}$ or to $A\beta_{25-35}$ induced a toxic dose-dependent effect on cortical neurons (Figs. 2A and B) with a maximal effect of around 40% and 60% at 20 μM, respectively. The scrambled peptides failed to affect cell survival compared with vehicle-treated control groups: 102 ± 4% at 20 μM $A\beta_{1-42}$ or 100 ± 4% at 20 μM $A\beta_{25-35}$ vs. 98 ± 3% control group. From these experiments, it was concluded that $A\beta_{25-35}$ was more toxic than $A\beta_{1-42}$ on *in vitro* cortical neurons.

Effect of increasing BDNF concentrations on $A\beta_{1-42}$ and $A\beta_{25-35}$-treated cells

We examined the ability of BDNF to protect neurons against beta amyloid peptide-induced toxicity. BDNF was able to significantly protect cortical neurons from both 20 μM $A\beta_{1-42}$ and $A\beta_{25-35}$-induced toxicity. This BDNF protection was dose-dependent and significant from 10 ng/ml and maximal at 50 ng/ml for $A\beta_{25-35}$-induced toxicity. Nevertheless, protection against $A\beta_{25-35}$-induced toxicity (Fig. 3B) was not complete (around 80%) whereas at 50 ng/ml BDNF completely reversed $A\beta_{1-42}$-induced toxicity (Fig. 3A). At 100 ng/ml, BDNF even surpassed the control group, probably by protecting cells from natural death. We also examined the effect of NGF (nerve growth factor) on $A\beta_{1-42}$- and $A\beta_{25-35}$-induced toxicity. In spite of the strong concentration used (50 ng/ml), this neurotrophin presented a very weak protective effect on 20 μM $A\beta_{1-42}$-induced toxicity and no effect on 20 μM $A\beta_{25-35}$-induced toxicity (Fig. 4).

Trk involvement in BDNF protective activity

To investigate the potential involvement of Trk receptors in the protective effects of BDNF described here, we treated cells with K252a, a Trk receptor inhibitor (Koizumi et al., 1988; Tanaka et al., 1997), 5 min before BDNF addition. K252a (100 nM) completely blocked the protective effects of BDNF on $A\beta_{1-42}$- and $A\beta_{25-35}$-induced toxicity (Figs. 5A, B). K252a added alone significantly ($^{a}p < 0.001$) decreased cell survival compared to the control group (40–50%), probably by blocking the protective effect of BDNF endogenously released by cultured cells.

In vivo data

Since our *in vitro* studies demonstrated that $A\beta_{25-35}$ was more toxic than $A\beta_{1-42}$, and that we previously showed that $A\beta_{25-35}$ retains the ability to self aggregate and mediate toxicity *in vivo* (Maurice et al., 1996; Meunier et al., 2006), we injected animals with this fragment either in the IG or in the 3rd ventricule.

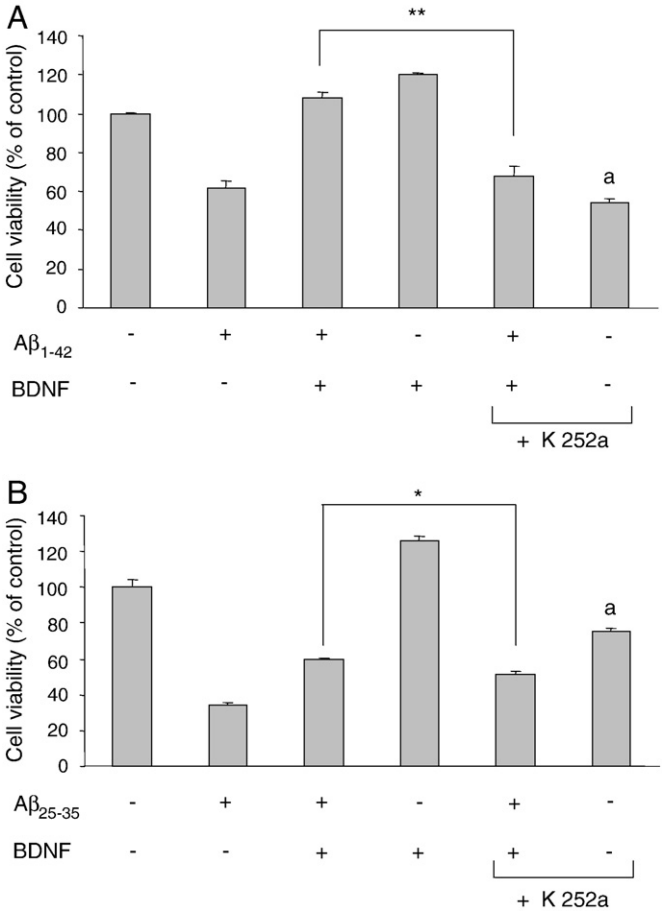

Fig. 5. Trk involvement in BDNF protection against toxicity induced by $A\beta_{1-42}$ and $A\beta_{25-35}$ peptides in rat cortical neurons. 100 nM K252a was added 5 min before 50 ng/ml BDNF and 20 μM $A\beta_{1-42}$ (A) and $A\beta_{25-35}$ (B) peptide addition to rat cortical neurons. Substances were added to cells in neurobasal medium on day 6 of culture and toxicity was estimated at day 8 by the MTT assay. The experimental data were from two different cultures with n=4 dishes/culture for each experimental condition. Values represent mean ± SEM. $^{**}p<0.01$ compared with the group treated with 50 ng/ml BDNF and 20 μM $A\beta_{1-42}$ but without K252a, $^{*}p<0.05$ compared with the group treated with 50 ng/ml BDNF and 20 μM $A\beta_{25-35}$ without K252a. $^{a}p<0.01$ compared to the control group.

Effect of $A\beta_{25-35}$ on in vivo BDNF release measured by push-pull perfusion of IG

Seven days after chirurgical cannula implantation, BDNF release from IG was estimated using push-pull perfusion. Under control conditions, BDNF release exhibited basal values ranging between 7 ± 4 pg/15 min, with peaks of 40 ± 5 pg/15 min throughout 120 min of perfusion. At the end of this procedure, animals were injected with $A\beta_{25-35}$ (group 1) or BDNF (group 2) and allowed to rest for 7 days before the next perfusion. Animals of group 2 received, 24 h after the 1st BDNF injection, a 2nd administration of BDNF + $A\beta_{25-35}$, and a new push-pull perfusion on the same animal was performed on day 14. Animals of group 1 ($A\beta_{25-35}$ alone without BDNF) were also perfused 7 days after the first perfusion. A scheme of the experimental design is shown in Fig. 6A. In group 1, $A\beta_{25-35}$ administered rats, the values of BDNF release estimated in the perfusate by ELISA assay did not yield differences as compared to values obtained for control rats (representative experiments are shown in Fig. 6B). In contrast, animals of group 2 yielded BDNF release values that were significantly higher than the control values (first perfusion of the same animal, 7 days previously). These higher values concerned the basal release (281 ± 26%), peaks (205 ± 11%) or total release (247 ± 22%). Quantitative data are shown in Fig. 6C.

Secondarily, we used the icv route of Aβ administration to compare the results of BDNF release obtained through IG administration with those obtained from other medial regions (3rd ventricle) close to areas examined in this study. We succeeded in showing, and for the first time, detectable basal BDNF levels in the third ventricle in rats, which, similarly to BDNF release arising from IG, was not affected by Aβ treatment. BDNF release was 11. 7 ± 2.2 pg/15 min ($A\beta_{25-35}$ group) vs. 11.9 ± 1.8 pg/15 min (control group) ($n = 5$).

Number of dentate gyrus hilar cells expressing SRIH mRNA in histological sections from hippocampus

A significant ($p < 0.02$) decrease in the number of cells expressing SRIH mRNA was observed in the dorsal hilar region of Aβ-treated animals (Fig. 7B) as compared to sham-operated animals (Fig. 7A) [135 ± 13 cells ($A\beta_{25-35}$ group) vs. 170 ± 7 cells (control group)]. In contrast, when both BDNF and $A\beta_{25-35}$ were co-administered, the harmful effect of $A\beta_{25-35}$ alone was completely reversed (Fig. 7C) and even surpassed the control values (219 ± 8 cells), as shown in the

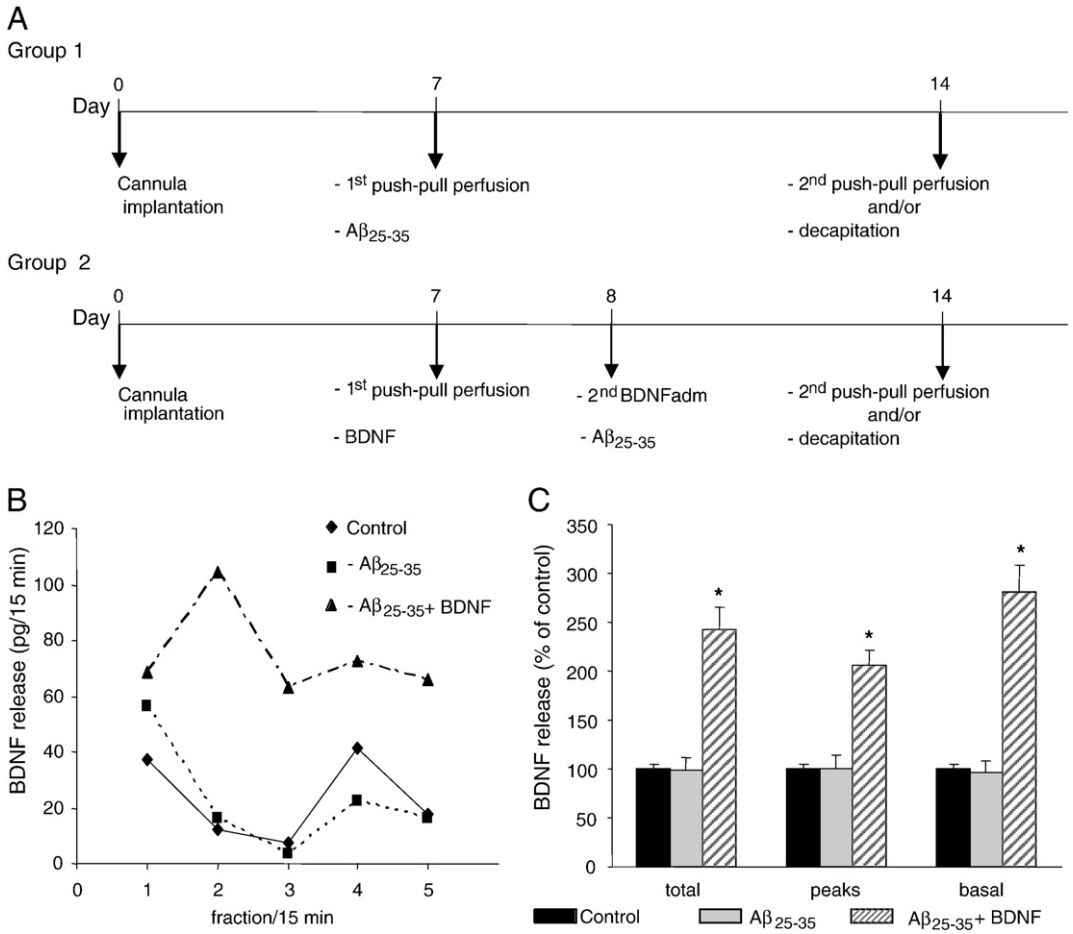

Fig. 6. *In vivo* BDNF release from IG in push-pull cannulated, unanesthetized, free-moving rats 7 days after stereotaxic surgery. (A) Experimental *in vivo* design. All animals underwent surgery to implant the ic or icv push-pull cannula on day 0. The first group underwent a first push-pull perfusion at the 7th day, at the end of which the $A\beta_{25-35}$ or scramble peptide or saline solution was administered (group 1). When BDNF release was analyzed, a 2nd push-pull perfusion was performed 7 days later, on day 14. Then the animals were decapitated and their brain was removed and frozen for morphological studies. Otherwise, the animals were killed 7 days after $A\beta_{25-35}$ administration. In the second paradigm (group 2), animals underwent a first push-pull perfusion at the 7th day, at the end of which BDNF was injected. The next day the animals received a second dose of BDNF followed by $A\beta_{25-35}$ administration. On day 14, a 2nd push-pull perfusion was performed or, if BDNF release was not determined, the animals were decapitated and their brain was removed and frozen for morphological studies. (B) Individual representations. Three different experimental conditions are illustrated: sham-control rat (♦), $A\beta_{25-35}$ injected rat (■) and $A\beta_{25-35}$+BDNF (▲) injected rats. Note that peak-like secretion was observed in the three experimental conditions. (C) Statistical representation including all animals studied. BDNF secretion was calculated as total, peaks and basal amount of BDNF. Note that administration of $A\beta_{25-35}$ alone had no effect on BDNF release, but administration of $A\beta_{25-35}$+BDNF significantly modified the three parameters.

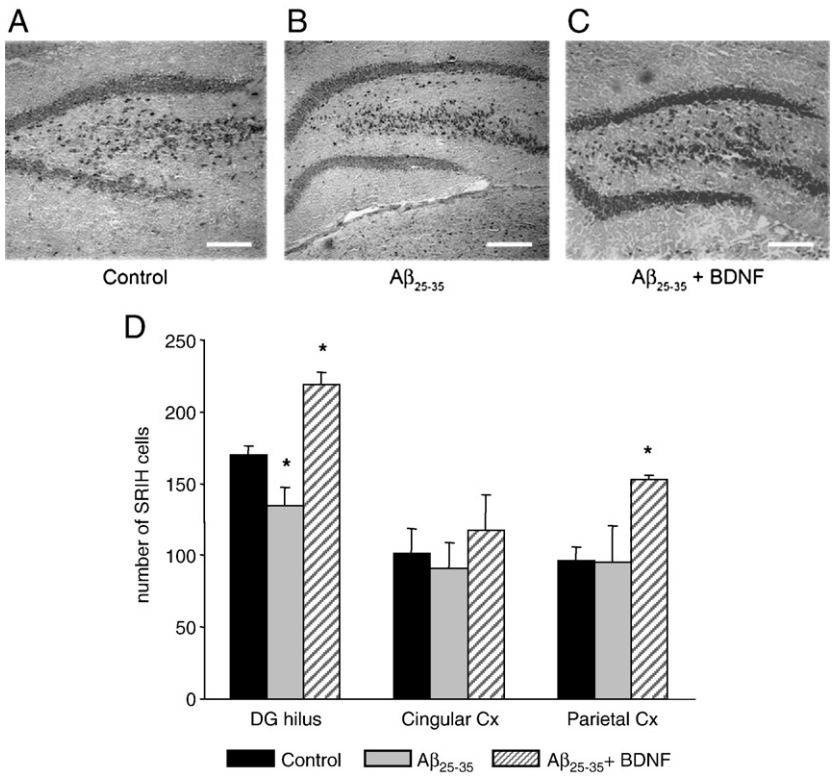

Fig. 7. Representative histological sections of rat hippocampus from differently treated animals analyzed by in situ hybridization to detect cells expressing SRIH mRNA in the dentate gyrus hilus. Control histological section corresponding to a sham-operated rat (A), to an $A\beta_{25-35}$ injected rat (B) and to an $A\beta_{25-35}$ + BDNF injected rat (C). (D) Histogram illustrating the previous conditions using the number of cells expressing SRIH mRNA as the quantification index. In addition to the hilus, two other regions were also analyzed and represented, i.e. the cingular cortex and the parietal cortex.

statistical representation (Fig. 7D) (n = 5 rats/group). The analysis of hematoxylin-stained sections adjacent to those processed for *in situ* hybridization experiments allowed us to express data as percentage of somatostatin cells vs. total cells found in the dorsal hilus: 32.5 ± 2.4% (control group), 23.8 ± 3.2% ($A\beta_{25-35}$ group) and 47.5 ± 3.7% ($A\beta_{25-35}$ + BDNF group). Examination of cingular and parietal cortex revealed that Aβ treatment had no toxic effect on the number of SRIH neurons but that BDNF administration induced a significant positive effect on parietal SRIH neurons (Fig. 7D).

Effect of $A\beta_{25-35}$ administration on histological integrity of corpus callosum and on immunostaining labeling

Fig. 8 shows the corpus callosum morphology in serial micrographs after $A\beta_{25-35}$ administration or when co-administered with BDNF according to the protocol specified in Fig. 6. The axonal histological lesion was characterized by a white matter rarefaction and a clear loss of tissue integrity laterally limited by the lateral ventricles. For example at −1.8 AP bregma, the lesion was laterally extended up to 1.9 mm whereas at bregma −3.3, the lesion attained laterally 3.7 mm. This injury pattern was observed throughout the corpus callosum as demonstrated in our serial study in slices included between bregma +0.2 and −3.8. Damaged cells with pyknotic nuclei were revealed by the hematoxylin staining method. Histological observations and counting of damaged cells (Table 2) showed that BDNF co-treatment significantly prevented the $A\beta_{25-35}$ harmful effects.

Table 2
Counting of pyknotic nuclei in the corpus callosum

Control	$A\beta_{25-35}$	BDNF + $A\beta_{25-35}$
98 ± 11	312 ± 22**	75 ± 5

$p<0.01$ vs. control.

Fig. 9 shows the corpus callosum morphology at higher magnification in controls, $A\beta_{25-35}$, and $A\beta_{25-35}$ + BDNF-treated animals. In control animals, MBP immunostaining of corpus callosum appeared uniform and the staining showed the typical railroad structure. When animals received $A\beta_{25-35}$ administration the MBP pattern revealed a clear lesion in the corpus callosum consisting in myelin fragmentation which was appreciably attenuated in BDNF-treated animals. Myelin damage appeared as a toxic sign whose improvement by BDNF treatment, although evident, was less striking than other histological features observed here. This fact is probably in keeping with the latency required to rescue axons.

Discussion

Our findings show that BDNF has neuronal protective effects against Aβ peptide toxicity *in vivo* and *in vitro*. The mechanisms by which amyloid peptides are neurotoxic are not yet understood and attempts to find protective molecules are exciting and promising. As BDNF is able to protect neurons against cellular damage (Knüsel et al., 1992; Lindvall et al., 1994), we hypothesized that it would be protective against Aβ aggression. Given that in this paradigm cortical neurons express TrkB receptors (mRNA and protein) and the signal-transducing BDNF-specific receptor (Tapia-Arancibia et al., personal observations), we assessed the protective effect of BDNF against toxicity induced by Aβ peptides on neuronal survival *in vitro*. Consistent with previous observations (Geci et al., 2007; Pike et al., 1995; Yao et al., 2005), our data showed a dose-dependent toxic effect of amyloid peptides in cortical neurons. Our comparative *in vitro* study, performed under similar experimental conditions, indicated that $A\beta_{25-35}$ was more toxic for cellular survival than $A\beta_{1-42}$.

Interestingly, we found that BDNF had a protective dose–response effect on neuronal toxicity induced by the two Aβ peptides but with some differences. Actually, this protection was more pronounced on toxicity

induced by $A\beta_{1-42}$ than that induced by $A\beta_{25-35}$ since it completely reversed its toxic action on neuronal survival, whereas it partially reversed the toxicity induced by $A\beta_{25-35}$. BDNF-mediated protection involved TrkB receptor activation since the effect was completely inhibited by K252a, a potent tyrosine kinase inhibitor (Koizumi et al., 1988). NGF, another neurotrophin of the same family, had a very weak effect in rescuing cells from amyloid peptide-induced death, which indicated a specific action of BDNF at similar concentrations.

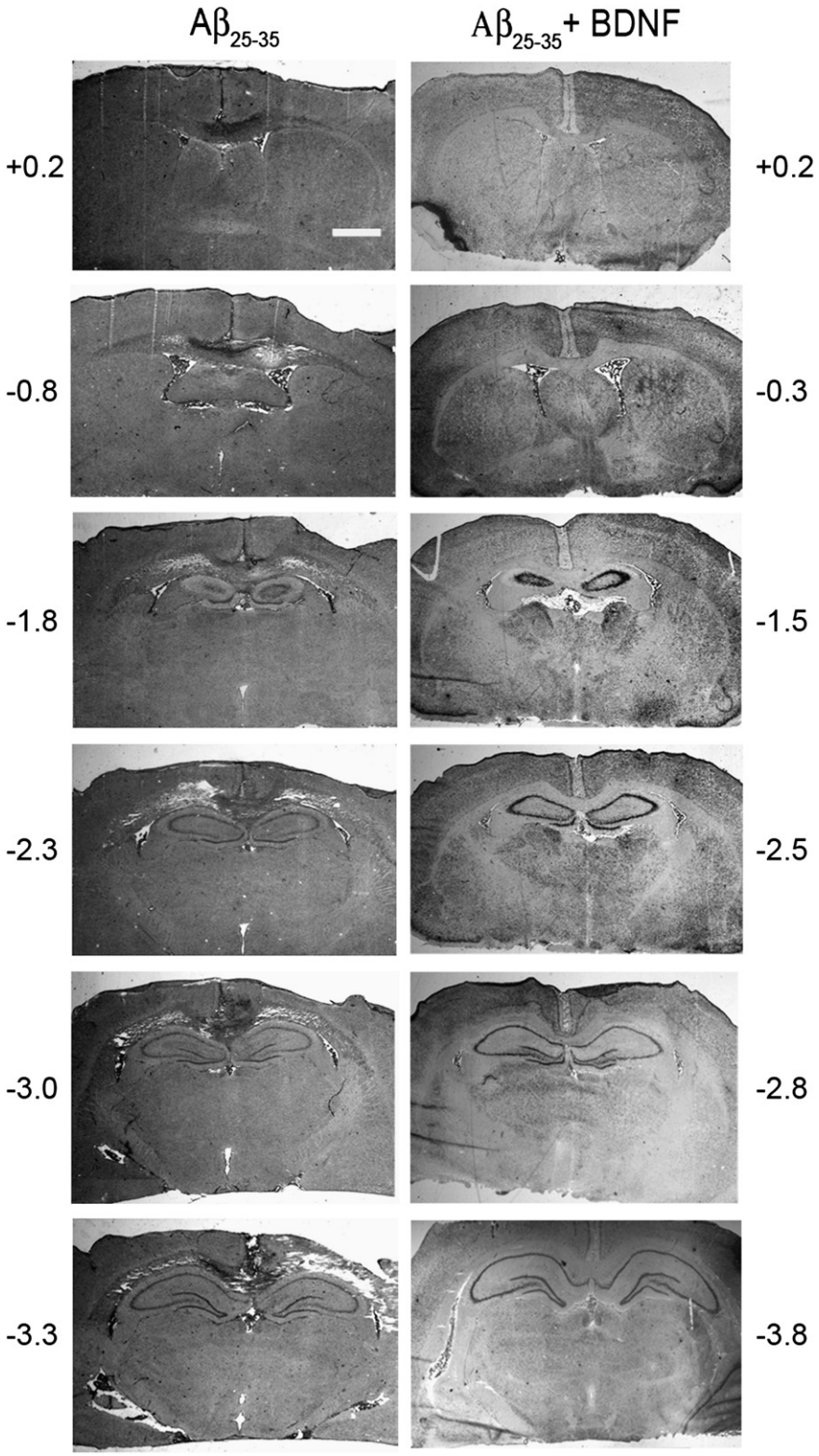

Fig. 8. Representative micrographs of histological features of the corpus callosum stereologically ranging between bregma +0.2 and −3.8, in an $A\beta_{25-35}$ injected rat and an $A\beta_{25-35}$+BDNF injected rat. Note that in these hematoxylin-stained slices, the corpus callosum exhibits a clear loss of tissue integrity and cellular damage in the $A\beta_{25-35}$ injected rat. Scale bar=25 μm.

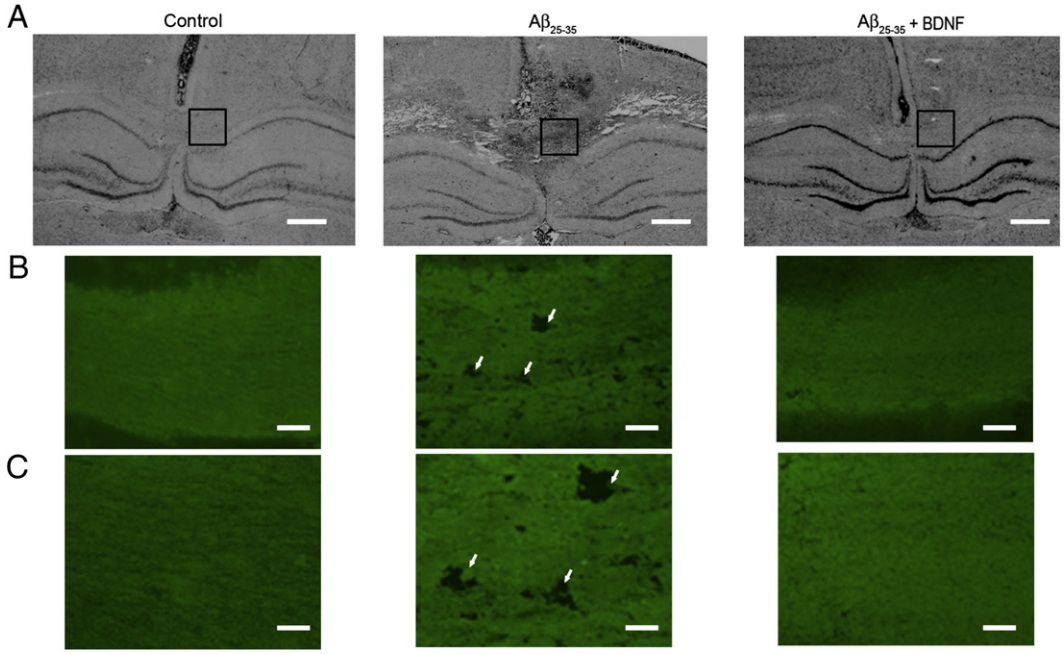

Fig. 9. Representative histological micrographs of brain rats subjected to different experimental treatments. (A) Corpus callosum morphology under control conditions (left); degeneration after $A\beta_{25-35}$ administration (middle); protection by BDNF co-administrated with $A\beta_{25-35}$ (right) (60X magnification). Panels B and C show areas indicated in the inserts in (A) from histological sections stained with antibody anti-MBP (myelin basic protein) at 200X and 400X magnifications, respectively. Arrows show corpus callosum damage after $A\beta_{25-35}$ administration. Right micrographs show protection by previous BDNF administration. Scale bars = 150 μm (A); 250 μm (B) and 500 μm (C).

In view of these results, we used the experimental paradigm of intracerebral injection of $A\beta_{25-35}$ peptide in the *in vivo* studies. The amyloid β-derived peptide $A\beta_{25-35}$ contains hydrophobic transmembrane residues 25–35 (GSNKGAIIGLM) of the Aβ protein and aggregates as insoluble fibrils (Yankner et al., 1990) that retain the toxic effect of larger Aβ peptides (Pike et al., 1993). We have already reported that the $A\beta_{25-35}$ peptide retains the ability to self-aggregate and induce *in vivo* toxicity (Maurice et al., 1996; Meunier et al., 2006). In addition, it has been described that soluble D-ser$^{26}A\beta_{1-40}$, possibly produced during aging, is released from plaques and converted by proteolysis to toxic D-ser$^{26}A\beta_{25-35}$ which enhances excitotoxicity in AD (Kubo et al., 2002). As a route of $A\beta_{25-35}$ administration, we essentially chose an *in situ* injection into the IG, a single median region, thus avoiding the bilateral injection protocol used by others (Stepanichev et al., 2004). The IG, although it has been reported to be refractory to some kind of neuronal aggressions, surrounds some critical regions engaged in the AD onset, i.e. cingular cortex, corpus callosum and hippocampus, and represents a phylogenic old olfacto-recipient outpost of the hippocampus (Adamek et al., 1984).

After $A\beta_{25-35}$ administration, we observed no modification in BDNF release from IG measured in the push-pull perfusate, which probably indicates that there are no changes in endogenous BDNF content at this $A\beta_{25-35}$ concentration. In contrast, previous exogenous administration of BDNF strongly increased BDNF release according to *in vitro* data showing that BDNF increases BDNF release (Canossa et al., 1997). The expression of trkB.FL mRNA in cingular cortex surrounding the IG region as well as in the hilar region of the dentate gyrus (Arancibia et al. personal observations; Merlio et al., 1992; Yan et al., 1997) further supports the physiological significance of our findings concerning BDNF release (IG) and BDNF protective action (hilus and corpus callosum).

We also examined the number of hippocampal hilar cells expressing SRIH mRNA, which represents a valuable parameter to investigate the toxic effect of $A\beta_{25-35}$ (Aguado-Llera et al., 2007). This particular class of interneurons was chosen because of their sensitivity to excitotoxicity and vulnerability to a variety of insults and neurological diseases including Alzheimer disease (Chan-Palay, 1987; Lowenstein et al., 1992; Tallen, 2007; Ylinen et al., 1991). It has been hypothesized that vulnerability of hilar SRIH interneurons, which is different from that of interneurons from the rest of the hippocampus, is due to their lack of Ca^{2+} binding proteins (Tallent, 2007). The striatal-enriched protein tyrosine phosphatase (STEP), a key regulator of ERK/MAPK signaling (Choi et al., 2007), would be involved in this excitotoxic event. The significant reduction in the number of SRIH hilar cells after the Aβ administration reported here is in keeping with recent *in vitro* data (Geci et al., 2007). Although we did not inject Aβ directly into the hippocampus, Aβ might exert their effects in distant targets of the application site as it has been reported (Sigurdsson et al., 1997; Stepanichev et al., 2000), inducing for example a neural disconnection between some neuronal structures (Ahmed et al., 1995; Kunzle, 2004). Disconnection of DG from the entorhinal cortex, its major input, is observed in early stages of AD (Ohm, 2007). The hilar interneurons (also innervated by fibers arising from entorhinal cortex) which control the DG granule cell activity (Amaral et al., 2007) would be affected in this process. The reduction in SRIH interneurons reported here was completely prevented by previous BDNF administration, which could have easily diffused (Yan et al., 1994; Anderson et al., 1995) from the IG or transported from the 3rd ventricle to the dentate gyrus (Mufson et al., 1996). Interestingly, SRIH has also been described as a neurotrophic factor (Schwartz et al., 1998; Blake et al., 2004) presumably involved in the AD etiology (Dournaud et al., 1994; Vecsei and Klivenyi, 1995), notably in its early onset (Ramos et al., 2006). Moreover, it has been reported that somatostatin is involved in the catabolism of Aβ through neprilysin activation (Saito et al., 2005), a rate-limiting enzyme for Aβ degradation (Hama and Saido, 2005). BDNF-induced SRIH increase could result in neprilysin-mediated Aβ degradation thus contributing to the neuroprotective effect.

Corpus callosum damage was other of the parameters examined in our *in vivo* experiments after $A\beta_{25-35}$ administration alone or combined with BDNF administration. Its atrophy or rarefaction has been shown to be a reliable and sensitive *in vivo* marker of cortical neuronal loss associated with cognitive impairment in AD (Hampel et al., 2002; Teipel et al., 1998, 2003) and correlated with dementia

severity in these patients (Wiltshire et al., 2005; Yamauchi et al., 2000). Our data showed that Aβ administration causes nuclei cell pyknosis and corpus callosum disruption, indicating fragmentation of the axonal cytoarchitecture. Previous treatment with BDNF notably attenuated these $A\beta_{25-35}$-induced damage. Our data using anti-MBP antibody are in keeping with our histological results.

The neuronal injuries reported here could be explained by an oxidative damage of $A\beta_{25-35}$ (Stepanichev et al., 2004) considered as a fundamental pathogenic mechanism of Alzheimer's disease (Perry et al., 2004). BDNF could act as an antioxidative factor since it is known that it increased the level of activity of some antioxidant enzymes (Mattson et al., 1995). Aβ/BDNF interaction could also be explained by Aβ interference with signaling pathways used by BDNF to exert its protective effects, i.e. on BDNF-induced Arc (activity-regulated cytoskeleton-associated gene) protein expression (Echeverria et al., 2007; Wang et al., 2006), CREB phosphorylation (Tong et al., 2004) or its nuclear translocation (Arvanitis et al., 2007). Arc synthesis controls local actin synthesis, synaptic plasticity and cognitive functions (Wang et al., 2006). Whatever the case, β-amyloid peptides may engender a dysfunctional encoding state in neurons, leading to neurodegeneration (Tong et al., 2004), and BDNF signaling might be compromised early in the course of AD (Murer et al., 1999).

Overall, both the *in vitro* and *in vivo* results presented here validated our hypothesis that exogenous administration of BDNF exerts neuroprotective actions against toxic effects of Aβ peptides in regions related to cognitive functions providing new insight for future therapeutic approaches.

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