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Interaction between β-amyloid protein and the α7 nicotinic acetylcholine receptor in cholinergic, gabaergic and calcium-binding proteins-containing neurons in the septum-diagonal band complex of the rat

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SUMMARY

The effects of intracerebral injection of the beta-amyloid protein (Aβ1-40) on the α7 subtype of nicotinic acetylcoline receptor protein (nAChR) in neurons of the septum-diagonal band (MS-nDBB) complex were studied in rats. Focal deposition of Aβ in the retrosplenial cortex resulted in a selective reduction in the number of α7nAChR-immunoreactive cells in different parts of the MS-nDBB complex, especially in the horizontal nucleus of the diagonal band of Broca (HDB). The analysis revealed a significant decrease of 37.27% in the number of α7nAChR-immunoreactive cells in the HDB ipsilateral to the Aβ-injected side as compared to the corresponding hemisphere of non-treated control animals, and a reduction of 31.55% was observed in the HDB ipsilateral to the Aβ-injected side as compared to the contralateral HDB, which corresponds to the control (PBS)-injected side. A significant reduction (up to 20%) of α7nAChR-containing neurons was also found in the medial septal nucleus when compared with the corresponding hemisphere in non-treated control animals. The results also revealed that α7nAChR-positive immunoreactivity is highly localized within cytoplasmic granules in cholinergic neurons and in a small subset of putative GABAergic cells of the MS-nDBB complex. In conclusion, these findings suggest an interaction of Aβ1-40 with the α7nAChR, which may contribute to impairments in cholinergic and GABAergic transmission in the MS-nDBB complex.

Keyworks: Alzheimer's disease – Posterior cingulate cortex – Basal forebrain – Cholinergic transmission – GABAergic transmission – Calcium binding protein

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Introduction

Alzheimer's disease (AD) is a neurological disorder that presently affects 20-30 million individuals around the world (Selkoe, 2005). The brains of AD patients manifest two characteristic lesions: extracellular amyloid and intracellular neurofibrillary tangles of hyperphosphorylated tau protein (Selkoe, 2003). The amyloid hypothesis states that the formation of amyloid peptides (Aβ) by neurons is the primary trigger of the pathogenesis of AD (Selkoe, 1991; Yankner and Mesulam, 1991; Walsh and Selkoe, 2004). However, although intensive efforts have been concentrated on factors affecting Aβ production, aggregation, and metabolism (Bigl et al., 2000; Vassar and Citron, 2000), it is still unclear how Aβ peptide causes toxicity. Recent *in vitro* studies suggest that the origin of the controversy may stem from the fact that Aβ peptide can polymerize spontaneously into fibrils, and many of the earlier *in vitro* studies did not first ensure whether the Aβ peptide used for treatment of cells was in fact monomeric, oligomeric or polymeric (Guan et al., 2001; Dahlgren et al., 2002; Dineley et al., 2002a; Bell et al., 2004). On the other hand, *in vitro* models of selected cell lines of neuronal or glial origin have important limitations (for review Harkany et al., 1999). Therefore, to resolve the limitations and difficulties of *in vitro* studies, in recent years several *in vivo* studies searched for evidence that both Aβ1-40 (Kowall et al., 1991; Giovannelli et al., 1995, 1998; Shin et al., 1997; Gonzalo-Ruiz and Sanz, 2002; Gonzalo-Ruiz et al., 2003, 2005, 2006; Reyes et al., 2004) and Aβ1-42 (Harkany et al., 2001) peptides are neurotoxic and that Aβ might contribute directly to the pathogenesis of AD.

Many studies have also shown that impaired cortical cholinergic neurotransmission contributes to the expression and processing of β-amyloid precursor protein (APP; Roβner et al., 1997; Leanza, 1998; Auld et al., 2002), and that cholinergic dysfunction may be related and may play an important role in the pathogenesis of AD (Whitehouse et al., 1981, 1982; Lehericy et al., 1993; Kar et al., 2004).

Moreover, losses of nicotinic acetylcholine receptors (nAChRs), including the α7 subtype, have been observed in several regions of the AD brain (Lee et al., 2000; Wevers et al., 2000; Court et al., 2001; Nordberg, 2001), and this deficit has been associated with the cognitive dysfunction observed in AD patients (Nordberg, 1994; Whitehouse and Kalaria, 1995). Recent investigations have provided strong evidence that the Aβ peptide interacts with nAChRs (Guan et al., 2001; Liu et al., 2001; Nagele et al., 2002; Lee and Wang, 2003; Wang et al., 2003), suggesting that these receptors would be important targets for the pathological effects of Aβ in connection with AD. In addition, behavioural studies have shown that nicotinic mechanisms contribute to attention, learning, and memory (Levin et al.,1999; Van Kampen et al., 2004; Young et al., 2004). Consistent with these findings are recent studies demonstrating improved learning rates and attention in AD patients following treatments that specifically enhance the function of nAChRs (Kem, 2000; Levin and Rezvani, 2000; Sabbagh et al., 2002).

Finally, since cortical cholinergic dysfunction has been correlated with the cognitive impairments involved in this disease (for review, see Fibiger, 1991) and since the cerebral cortex, including the retrosplenial cortex, is one of the brain areas intimately involved in cognitive processes (Gabriel et al., 1983; Sutherland and Hoesing, 1993), over recent years the retrosplenial cortex has received particular attention (Gonzalo-Ruiz and Morte, 2000; Gonzalo-Ruiz and Sanz, 2002, Gonzalo-Ruiz et al., 2003). Nevertheless, it is still unclear how cholinergic deficits and Aβ formation and deposition might be related to one another. Thus, because α7 nAChR is thought to be a key molecule involved in AD, the purpose of the present study was to determine whether local extracellular deposition of Aβ peptide might affect the amount of the α7nAChR protein in the medial septum-diagonal band (MS-nDBB) complex, an important area associated with the cholinergic dysfunction observed in AD (Whitehouse et al., 1981, 1982; Lehericy et al., 1993). In addition, since a previous study carried out by us (Gonzalo-Ruiz and Sanz, 2002) had revealed that intracerebrally injected Aβ induces a significant reduction in cholinergic and non-cholinergic neurons in the MS-nDBB complex, here special emphasis was placed on the discovery of any interaction between Aβ and the α7 nAChR protein in cholinergic and non-cholinergic neurons of the MS-nDBB complex.

Materials and methods

Experimental animals and anaesthesia

Female Wistar albino rats (n= 9; 250 to 300 g; 9 to 12 months old) were used. They were kept under standard laboratory conditions (20ºC ambient temperature, 12 h light/dark cycle, tap water and regular rat chow *ad libitum*). The animals were anaesthetised with Nembutal (45mg/kg, injected intraperitoneally) for the surgical procedure (injection of Aβ1-40 peptide or vehicle solution). Prior to perfusion with fixative, the animals were reanaesthetized in the same manner but with up to double the dose used for the surgical procedure. In addition, another group of non-treated rats (n = 4, control animals) was also included in this study. In each case, the animals were housed and handled according to national legislation and the guidelines approved by the Animal Care Committee of the University of Valladolid, which comply with or are even more stringent than the EEC Directive 86/609.

Injection of Aβ1-40 peptide or vehicle solution

Anaesthetised animals were placed in a stereotaxic frame. A hole was made in the parietal bone with a dental drill and the dura was opened with a fine hypodermic needle. A synthetic peptide corresponding to the first 40 amino acids of Aβ protein (Aβ1-40) (BACHEM) was dissolved in 0.01 phosphate-buffered saline (PBS) at a concentration of 2 μg/μl and incubated at 37ºC for one week before use, as previously described (Gonzalo-Ruiz and Sanz, 2002). A single unilateral microinjection of Aβ1-40 (2 μg in 1 μl of PBS) was performed into the left retrosplenial cortex using stereotaxic coordinates derived from the atlas of Paxinos and Watson (1986). All microinjections were made using a 10 μl Hamilton syringe, with 26-gauge stainless steel needle, lowered slowly into place. The needle was left in place for 3-5 min before the injection was started, and then the fragments were injected slowly at a rate of 0.1 μl/min. The needle was left in place for an additional 3-5 min before being slowly withdrawn. As a control, in the same operating session and using a different microsyringe, a single microinjection of identical volume of vehicle solution (1 μl of PBS) at a rate of 0.1 μl/min was made into the corresponding regions of the right retrosplenial cortex using the same stereotactic coordinates as for the Aβ injection in the retrosplenial cortex. After the injection, the scalp was sutured and the animals were allowed to recover from the anaesthetic.

Fixation

Following short post-injection survival periods (four days – two weeks), the rats were reanaesthetized and the brain tissue fixed by intracardiac perfusion of 60 ml of 0.9% saline at 20ºC containing heparin (1.000 IU) to flush blood from the vascular system, followed by ca 350-400 ml of fixative solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB) at pH 7.2 (also perfused at 20ºC) for 30 min to perform the histochemical and immunohistochemical procedures (see below). The survival time after Aβ injection was selected on the basis of our previous and concurrent studies with the same peptide (Gonzalo-Ruiz and Sanz, 2002; Gonzalo-Ruiz et al., 2003, 2005, 2006).

Tissue preparation, histochemical and immunohistochemical procedures

Immediately after perfusion, the brain was removed, trimmed, sectioned in the coronal plane at 40μm using a freezing microtome, and collected serially as seven series of adjacent sections. All sections were stored under similar conditions at 4ºC (between 1 and 10 days) before being processed for histochemical and immunocytochemical studies. One series of sections, which contained the entire brain, was mounted on gelatinized slides and stained with 0.1% cresyl violet to identify the injection site and the area of Aβ toxicity. A second series of sections, which contained the full antero-posterior extent of the MS-nDBB complex, was immunoreacted for single antigen localization of α7 subtype of nAChRs (method 1). A third series of sections incorporating the full antero-posterior extent of the MS-nDBB complex was processed for sequential double-immunohistochemical localization of choline acetyltransferase (ChAT) and α7 nAChR, parvalbumin (PARV) and α7 nAChR, or GABA and α7 nAChR, respectively (method 2). A further two series of sections were subjected to immunocytochemistry to localize M1 and M2 subtypes of muscarinic acetylcholine receptors [as described elsewhere (Gonzalo-Ruiz et al., 2004b)].

In addition to the single- and double-labelled material derived from these animals, further complementary sections from several other Aβ-injected animals (n=4), immunore-

acted for single antigens (e.g., against human Aβ peptide; method 1) or for the sequential double-immunolabelling of calbindin (Calb) and α7 nAChR (method 2), were also available for study.

Method 1: Single-labelling immunohistochemistry

Immunostaining was performed on free-floating sections. Sections processed for Aβ-immunohistochemistry and for the immunohistochemical localization of α7 nAChR (using a rabbit polyclonal antibody against α7 nAChR) were first immersed for 1 h in 10% normal goat serum (NGS) in 0.01 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 0.1 M lysine, whereas the sections processed for immunohistochemical localization of α7 nAChR using a goat polyclonal antibody were immersed for 1 h in 10% normal rabbit serum (NRS) in PBS containing 0.3% Triton X-100 and 0.1 M lysine. After rinsing in PBS, endogenous peroxidase activity was blocked with 1% hydrogen peroxide (H_2O_2) in PBS for 30 min, after which the sections were rinsed in PBS again. Sections processed for immunohistochemical localization of α7 nAChR were then incubated in a solution containing either rabbit polyclonal antibody against α7 nAChR (1:200 dilution; Santa Cruz Biotech, California, USA) or goat polyclonal antibody against α7 nAChR (1:200 dilution; Santa Cruz Biotech, California, USA), while those for Aβ(1-40) were incubated in a solution containing a mouse monoclonal antibody against human Aβ (CLONE: 6E10; 1:1000 dilution; Signet Pathol. Systems, Inc., Dedham, MA) for 18-24 h at 4ºC. After incubation in the primary antibody, sections were washed either in 1% NGS (for Aβ and α7 nAChR using rabbit polyclonal antibody) or in 1% NRS (for α7 nAChR using goat polyclonal antibody) and then incubated in biotinylated goat anti-rabbit IgG (Vector, 1:200 in PBS with 1% NGS) for α7 nAChR (using rabbit polyclonal antibody), in biotinylated goat anti-mouse IgG (Vector, 1:200 in PBS with 1% NGS) for Aβ immunohistochemistry, or in biotinylated rabbit anti-goat IgG (Vector, 1:200 in PBS with 1% NRS) for the immunohistochemical localization of α7 nAChR using goat polyclonal antibody. Sections were then washed in PBS and immersed in avidin-biotin-HRP complex (Vector Laboratories Burlingame, CA; 1:100 dilution) for 60 min. The immunoreaction product was visualized using 0.005% diaminobenzidine (DAB) and 0.01% H_2O_2 in PB (this chromogen produces a diffuse brown reaction product). After immunostaining, sections were rinsed in several changes of PB, mounted on gelatine-coated microscope slides, air-dried, dehydrated in ascending concentrations of ethanol, cleared with xylene, coverslipped with Permount, and examined and photographed under bright-field illumination.

As a control, some sections from each series were incubated as described above but in the absence of primary antibody or after replacing the primary antibody with the respective normal serum (e.g., rabbit, goat or mouse). There was a complete absence of Aβ-immunoreactivity and of α7nAChR-immunoreactive neurons or neuropil in such control sections.

Method 2: Double-labelling immunohistochemistry (ChAT/α7nAChR, PARV/α7nAChR, GABA/α7nAChR, Calb/α7nAChR)

Sections processed for ChAT were first immersed for 1 h in 10% normal rabbit serum (NRS), whereas the sections processed for PARV, Calb or GABA were immersed for 1 h in 10% NGS in PBS containing 0.3% Triton X-100 and 0.1 M lysine. After rinsing in PBS, endogenous peroxidase activity was blocked with 1% H_2O_2 in PBS for 30 min and then rinsed in PBS again. Sections processed for the immunohistochemical localization of ChAT were then incubated in a solution containing a rat monoclonal antibody (1:4 dilution; Boehringer-Mannheim, Germany). Those for PARV and Calb were incubated in a solution containing a mouse monoclonal antibody against PARV (1:1000 dilution; Swant, Switzerland) or against Calb (1:1000 dilution; Swant, Switzerland) respectively, while those processed for GABA were incubated in a solution containing a guinea-pig polyclonal antibody against GABA (1:500 dilution, Eugene Tech) for 18-24 h at 4ºC. After incubation in the primary antibody, sections were washed either in 1% NRS (for ChAT) or in 1% NGS (for PARV, Calb or GABA) and then incubated in biotinylated rabbit anti-rat IgG (Vector, Burlingame, CA, USA; 1:100 in PBS with 1% NRS) for ChAT immunohistochemistry; in biotinylated goat anti-mouse IgG (Vector, 1:200 in PBS with 1% NGS) for PARV and Calb immunohistochemistry, or in biotinylated goat anti-guinea-pig IgG (Vector; 1:200 in PBS with 1% NGS) for the immunohistochemical localization of GABA. Sections were

then washed in PBS and immersed in avidin-biotin-HRP complex (Vector Laboratories Burlingame, CA; 1:100 dilution) for 60 min. The immunoreaction product was visualized using 0.005% DAB and 0.01% H_2O_2 in PB. Sections were rinsed through several changes of PBS over a period of 60 min, and were then incubated with another primary antibody, against the second antigen, using a rabbit polyclonal antibody against α7 nAChR (1:200 dilution, Santa Cruz Biotech) for 18-24 h at 4ºC. After incubation, sections were washed in PBS and then processed for α7 nAChR immunohistochemistry, as described above. The second antigen was then visualized with BDHC (Levey et al., 1986). This chromogen produced a granular blue reaction product. After immunostaining, all sections were rinsed through several changes of PB, mounted on gelatinized microscope slides, air-dried, dehydrated, covered with Permount, and examined and photographed under bright-field illumination.

As a control, some sections from each series were processed for the two-colour co-localization procedure, as described above, but in the absence of one or other primary antiserum, or without either antisera, or after replacing the primary antibody with the respective normal serum (e.g. rat, mouse or rabbit). Control sections were processed through the secondary antiserum, DAB, and BDHC steps, exactly as the other sections. These control procedures established that non-specific single- or double-labelling of neuronal somata or processes did not occur under the conditions employed in our study.

Qualitative analysis

Sections stained with cresyl violet were used to identify each injection site, whereas sections stained immunocytochemically with antibodies against Aβ1-40 peptide were used to show Aβ1-40 deposition. In each series of sections stained immunocytochemically with antibodies against either α7 nAChR or Aβ, or double-labelled for either ChAT/α7 nAChR, PARV/α7 nAChR, GABA/α7 nAChR, or Calb/α7 nAChR all the sections throughout the MS-nDBB complex (each separated by approximately 280 μm) were examined systematically. The specificity of the immunoreaction was checked by comparing sections stained either with single antiserum (e.g., α7nAChR, or Aβ) or double-labelled (e.g., ChAT/α7 nAChR, PARV/α7nAChR, GABA/α7nAChR, or Calb/α7nAChR) and control material, respectively. Structures immunostained by antibodies but not seen in the control slides, were considered to be specifically immunolabelled.

Quantitative analysis of α7 nAChR-positive neurons in the MS-nDBB complex

Complete series of the rostro-caudal extent of the nuclei of interest, such as the medial septal nucleus (MS) and the nuclei of diagonal band of Broca (nDBB), especially the horizontal nucleus of the nDBB (HDB), were taken for quantitative analysis of α7 nAChR-like-immunoreactive neurons (Aβ-injected rats, n=8; uninjected control animals, n=4). Immunocytochemically-processed material was viewed under bright-field illumination with an Olympus microscope (BX50) interfaced with a colour video camera (Hamamatsu) and a NeuroLucida digitizing morphometry system (MicroBrightField, Inc., Colchester). Live colour images of the immunohistological material were displayed using a 10x objective on a high-resolution video monitor at a final magnification of approximately 100x (10x oculars and 10x objectives). The boundaries of the MS and HDB nuclei were traced and digitized for quantitative analysis from a series of sections specifically immunolabelled with antibody against α7nAChR.

Within those sections previously digitized from a complete series of the rostro-caudal extent of the MS and HDB nuclei, cell counting was performed with the aid of a computerized image analysis system using a NeuroLucida morphometry system. Profiles of immunostained cell bodies were analysed in the left and right side of the MS, as well as separately in both the left and right HDB, i.e., ipsilateral and contralateral to the injection of Aβ into the retrosplenial cortex. All α7 nAChR-immunoreactive cell bodies were plotted, indicating their localization within each section, and drawn onto the computer atlas templates for each nucleus and for each animal. The outlines of soma of the cell bodies were also drawn onto the same computer atlas templates for morphometric measurements of each cell type. The same slides were analyzed single blind by two different investigators to ensure that results were independent of individual bias.

The total number of α7 nAChR-immunoreactive neurons within each nucleus

of interest was calculated by multiplying the number of cells counted in a series of sections (made up to 5 -7 sections per animal) by seven series of sections that had been collected for a given animal. The total number of α7 nAChR-immunoreactive cells in the Aβ-injected side was compared with that in the contralateral side of the same sections and with that seen at the corresponding hemisphere in non-treated control animals. In addition, the effect of Aβ peptide on the number of α7 nAChR-positive cells in the MS-nDBB complex was evaluated by detecting the difference between the number of α7 nAChR-positive cells in the PBS-injected side and those counted in the Aβ-injected side in each nucleus of interest.

Quantitative analysis of double-antigen localization (ChAT/α7nAChR, PARV/α7nAChR, GABA/α7nAChR, Calb/α7nAchR) in the MS-nDBB complex

Sections throughout the MS and HDB nuclei processed for sequential double-immunolabelling of ChAT/α7nAChR, PARV/α7nAChR, GABA/α7nAChR or Calb/α7nAChR were taken for the quantitative analysis of single- and double-immunoreactive neurons, using a NeuroLucida morphometric system, as described above. Profiles of single- and double-immunostained cell bodies were counted in small selected areas of the left and right side of the MS, as

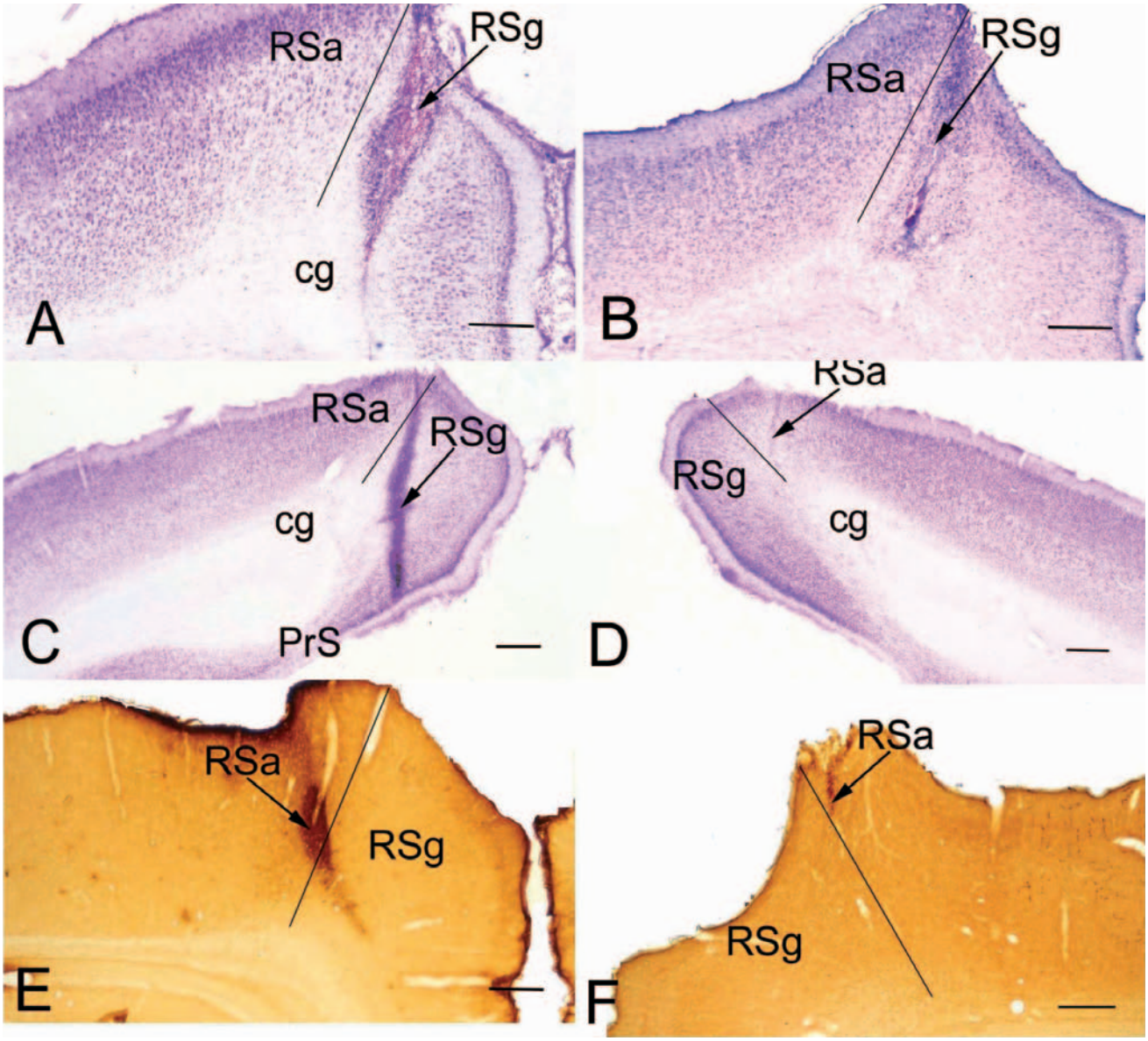

Figure 1. A-D: Low-magnification photomicrographs of coronal sections through the retrosplenial cortex from cresyl violet-stained sections showing representative injections of Aβ1-40 in the left RSg (A-C, arrows) and a representative injection of PBS centred on the right RSg (D, arrow). **(E, F):** Photomicrographs of coronal sections through the retrosplenial cortex from sections subjected to Aβ-immunohistochemistry (using an antibody against Aβ1-40) showing a dark staining at the level of a representative injection of Aβ into the left RSa (E, arrow), and a light background staining at the level of an PBS control injection into the right RSa (F, arrow). Scale bars: 250 μm (A-F).

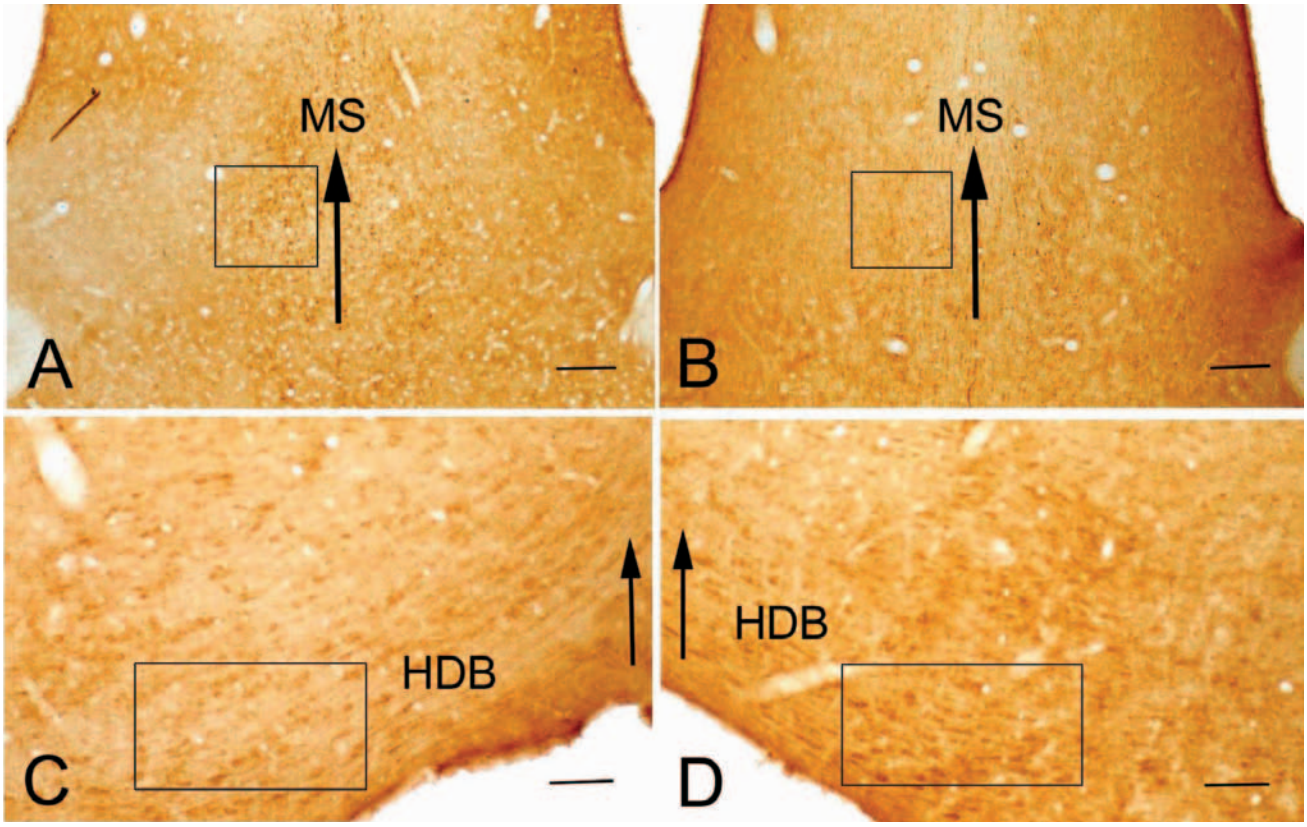

Figure 2. A,B: Alpha 7 subtype of nicotinic acetylcholine receptor (α7 nAChR)-immunostained coronal sections through the medial septal nucleus (MS) in an uninjected control animal (A) and following the injection of Aβ shown in Fig. 1C and the injection of PBS shown in Fig.1D (B). In (B), note moderate reduction in α7 nAChR-immunoreactive neurons in the MS of Aβ-injected animal when compared with the control animal (boxed area). The straight arrows in A and B indicate midline. **C,D:** α7nAChR-immunoreactive coronal sections through the horizontal nucleus of the diagonal band of Broca (HDB) following the injection of Aβ shown in Fig.1C and the injection of PBS shown in Fig.1D. Note a marked and focalized reduction in α7 nAChR-like-immunoreactive neurons in the ipsilateral HDB (C, boxed area) when compared with the contralateral HDB (D, boxed area), which correspond to the PBS-injected side. The straight arrows in C and D indicate the midline. Scale bars: 250 μm (A,B), 100 μm (C, D).

well as separately in small selected areas of both the left and right HDB, i.e., ipsilateral and contralateral to the injection of Aβ into retrosplenial cortex, and in selective corresponding areas of the HDB in uninjected control animals.

The total number of single- and double-labelled cells within each nucleus of interest was calculated by multiplying the number of both types of cells counted in small selected areas of a series of sections (made up to 5-7 sections) by seven series of sections that had been collected from a given animal. The total number of double-labelled cells was compared to that of single-labelled cells to obtain a rough estimate of the relative proportions of each. In some cases the total number of double-labelled cells (e.g., ChAT/α7nAChR) in the Aβ-injected side was also compared to that in the contralateral side of the same section, corresponding to the PBS injected side. Since our previous study (Gonzalo-Ruiz and Sanz, 2002) had already reported the effect of Aβ on the survival of cholinergic and non-cholinergic neurons in the MS-nDBB complex, no attempt was made in this study to perform statistical evaluations of the cholinergic and non-cholinergic markers in the MS and HDB nuclei after Aβ injection.

Statistical analysis

Differences in the number of α7nAChR-immunoreactive cells in Aβ injected animals versus uninjected control animals were calculated by means of Student's t test for independent samples if a normal distribution can be assumed. If the normal distribution is not valid, the non-parametric Mann-Whitney U test was used. Differences in the number of α7nAChR-immunoreactive cells between Aβ and PBS injections were calculated by means of Student's t test for paired samples if the normal distribution can be assumed. If the normal distribution did not hold, the non-parametric Wilcoxon T-test was used. For the test of a normal distribution, we used the Shapiro-Wilk test. In Student's t test for inde-

pendent samples, Levine's Test for homogeneity of variances was employed.

We assumed the normal distribution of the variables on the basis of the test of normality and the Skewness and Kurtosis coefficients, which are less than two times their standard error. All tests were two-tailed. Statistical analysis was performed using the SPSS, version 11.01. Results were expressed as means ± Standard Deviation (SD).

RESULTS

Localization and deposition of Aβ injections

Based on the terminology and the mapping of Vogt and Peters (1981), the retrosplenial cortex of the posterior cingulate gyrus could be subdivided into two parts: the retrosplenial agranular (RSa) and the retrosplenial granular (RSg) cortex (Fig. 1A-F). The nine animals subjected to detailed examination received a single microinjection of Aβ1-40 in vehicle (PBS) into the left RSg or RSa (e.g., Fig. 1A, B, C and E) and a single microinjection of PBS alone into the corresponding regions of the right RSg or RSa (e.g., Fig. 1D and F). In one animal the injection was centred on the left RSg, also encroaching hippocampus. This animal was not included in further evaluations.

The presence of Aβ1-40 peptide injected into the retrosplenial cortex was detected with a monoclonal antibody against the human Aβ, which recognized rat Aβ in Western blot (data not shown), and as previously reported (Gonzalo-Ruiz and Sanz, 2002; Gonzalo-Ruiz et al., 2003), qualitative analysis revealed a dark staining at the level of Aβ injection site (e.g., Fig. 1E), whereas the injection of PBS alone showed a light background staining surrounding the injection site (e.g., Fig. 1F).

Effects of Aβ1-40 peptide on the α7 nAChR protein in neurons of the MS-nDBB complex

Qualitative analysis of sections immunostained for α7 nAChR revealed that all injections of Aβ, and even some PBS injections, centred either in the RSg (e.g., Fig. 1A, B and C) or in the RSa (e.g., Fig. 1D and E), resulted in a marked reduction in α7nAChR-immunoreactive neurons in different parts of the MS-nDBB complex (Fig. 2A-D). The most extensive reduction in the number of α7nAChR-immunoreactive neurons was found in the nuclei of the diagonal band of Broca (nDBB), especially in the horizontal nucleus of the DBB (HDB) (Fig. 2C and D). Although the decrease in α7nAChR-immunoreactive neurons was apparent throughout the rostrocaudal extent of the MS and HDB nuclei, the strongest reduction was located predominantly in the rostral two-thirds of these nuclei (Fig. 2A-D).

Quantitative analysis revealed a statistically significant reduction of 20.47% (p=0.004, Mann-Whitney U) in the number of α7nAChR-immunoreactive cells in the MS ipsilateral to the Aβ-injected side as compared with the corresponding hemisphere of uninjected animals, while a difference of 7.06% (p<0.001) was observed in the MS ipsilateral to the Aβ-injected side as compared with the PBS-injected side (Table 1A, Fig. 3). In the HDB, we found a statistically significant reduction of 37.27% (p<0.001) in the number of α7nAChR-immunoreactive neurons in the HDB ipsilateral to the Aβ-injected side as compared with the corresponding hemisphere in uninjected animals (Table 1B, Fig. 3). A statistically significant reduction of 31.55% (p<0.001) was also found in the HDB ipsilateral to the Aβ-injected side as compared with the contralateral HDB, which corresponds to the PBS-injected side (Table 1B, Fig. 3).

Table 1. Differences in the number of α7nAChR-immunoreactive neurons in the MS (A) and HDB (B) nuclei following injection of Aβ1-40 into the left retrosplenial cortex and of vehicle-solution (PBS) alone into the right retrosplenial cortex.

Injection	Rats (n)	**(A)** MS α7nAChR-positive neurons	MS Difference 95% Confidence Interval	MS % Difference	MS p value	**(B)** HDB α7nAChR-positive neurons	HDB Difference 95% Confidence Interval	HDB % Difference	HDB p value
Aβ1-40 injected side	8	4047 ± 119	1040[a] 886 – 1194[a]	20.47%[a]	< 0.004[a,c]	4860 ± 119	2879[a] 2667 – 3092[a]	37.27%[a]	<0.001[a,d]
			306[b] 217 - 396[b]	7.06%[b]	< 0.001[b,e]		2237[b] 1858 - 2616[b]	31.55%[b]	< 0.001[b,e]
PBS-injected side	8	4353 ± 107				7098 ± 453			
Uninjected control animals	4	5087 ± 97				7740 ± 218			

α7nAChR-immunoreactive neurons were counted as described in Section 2 and the values of α7nAChR-immunoreactive neurons in the MS and HDB nuclei are expressed as the number of neurons per animal and represent the mean ± Standard Deviation (S.D).
a) Compared to control animals.
b) Compared to the PBS-injected site.
c) Mann-Whitney U test.
d) Student-t independent-samples test.
e) Student-t paired-samples test.

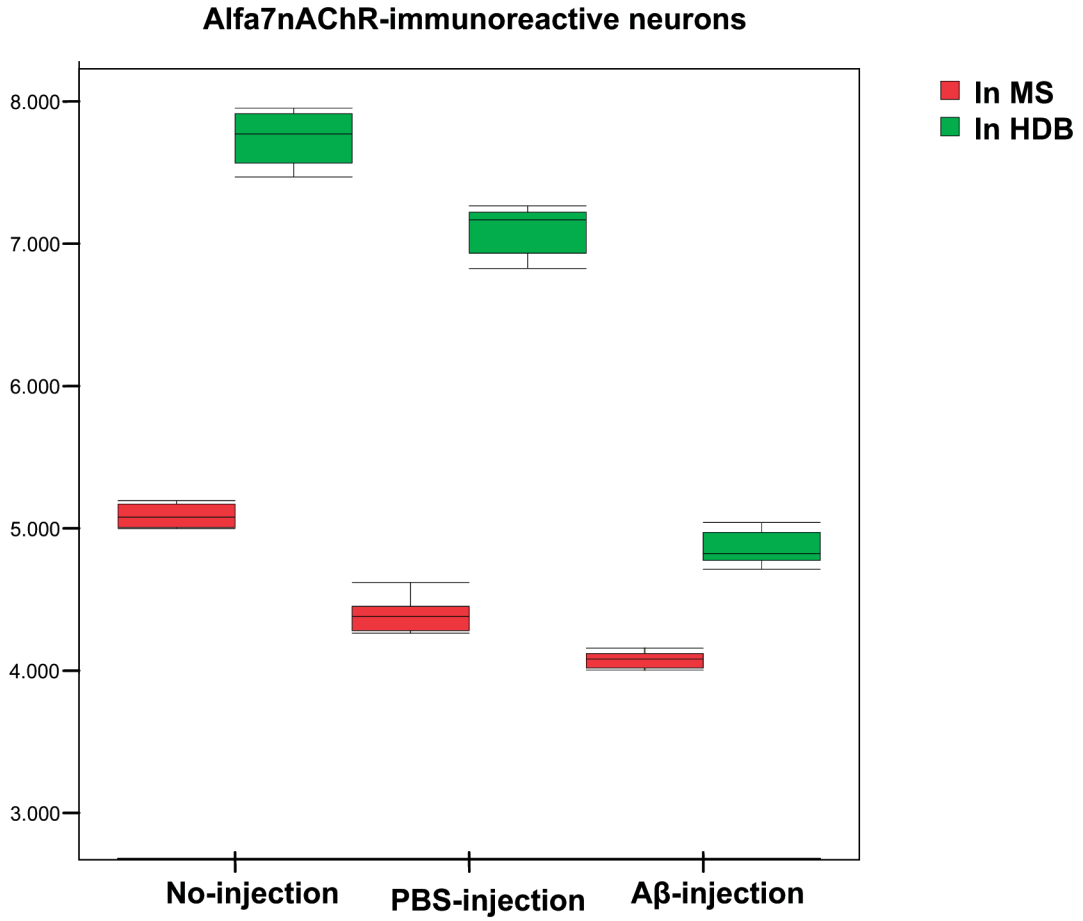

Figure 3. Injections of Aβ1-40 into the left retrosplenial cortex induce a significant reduction in the number of α7nAChR-containing neurons in the HDB ipsilateral to Aβ-injected side as compared with the same parameter in the corresponding hemisphere of uninjected control animals, and as compared with that seen in the PBS-injected side. A significant reduction in the number of α7nAChR-immunoreactive neurons was also observed in the MS ipsilateral to the Aβ-injected side as compared with the corresponding hemisphere in uninjected control animals and with the PBS-injected side. α7 nAChR-immunoreactive neurons were counted as described in Section 2. Total counts of α7nAChR-immunoreactive cells in the MS and HDB nuclei were calculated by multiplying the number of cells counted in a series of sections (made up to 5-7 sections per animal) by seven series of sections collected in each animal.

In order to address the cellular distribution of the α7 nAChR protein in MS-nDBB complex, tissue sections were subjected to double-labelling immunocytochemical analysis (e.g., Fig. 4A-L). In double-immunolabelling sections, α7nAChR- immunoreactivity was highly localized within the ChAT-positive neurons of MS (Fig. 4A and B), and HDB (Fig. 4C, D and E). Within ChAT-positive cells, α7nAChR-positive material appearing as densely packed granules, often occupied a substantial fraction of the cytoplasm and extended into their dendrites (e.g., Fig. 4D). In the MS, of the 2268 ChAT-positive cells studied, 956 (42.15%) were immunoreactive for α7nAChR and were located predominantly in the lateral compartments of the MS (e.g., Fig. 4A and B), while in the HDB at least 64.76% of the ChAT-positive neurons identified (2312 out of 3570 neurons) also expressed α7 nAChR-immunoreactivity. In addition, all injections of Aβ resulted in a marked reduction in the number of ChAT-positive neurons expressing α7 nAChR-immunoreactivity in different parts of the MS-nDBB complex, especially in the ipsilateral HDB (compare Fig. 4C and E).

Immunocytochemical analysis also revealed that α7 nAChR-positive material was localized within a subset of Parv-immunoreactive cells and on a small subpopulation of putative GABAergic cells of the MS-nDBB complex, especially in the MS (Fig. 4F, G, H and I). Within the putative GABAergic and PARV-immunoreactive cells, α7 nAChR-positive material appeared as dense granules distributed over the somata of these cells (e.g., Fig. 4G). By contrast, α7 nAChR-immunoreactivity was absent in a small subset of Calb-positive cells of the MS-nDBB complex (e.g., Fig. 4J, K and L).

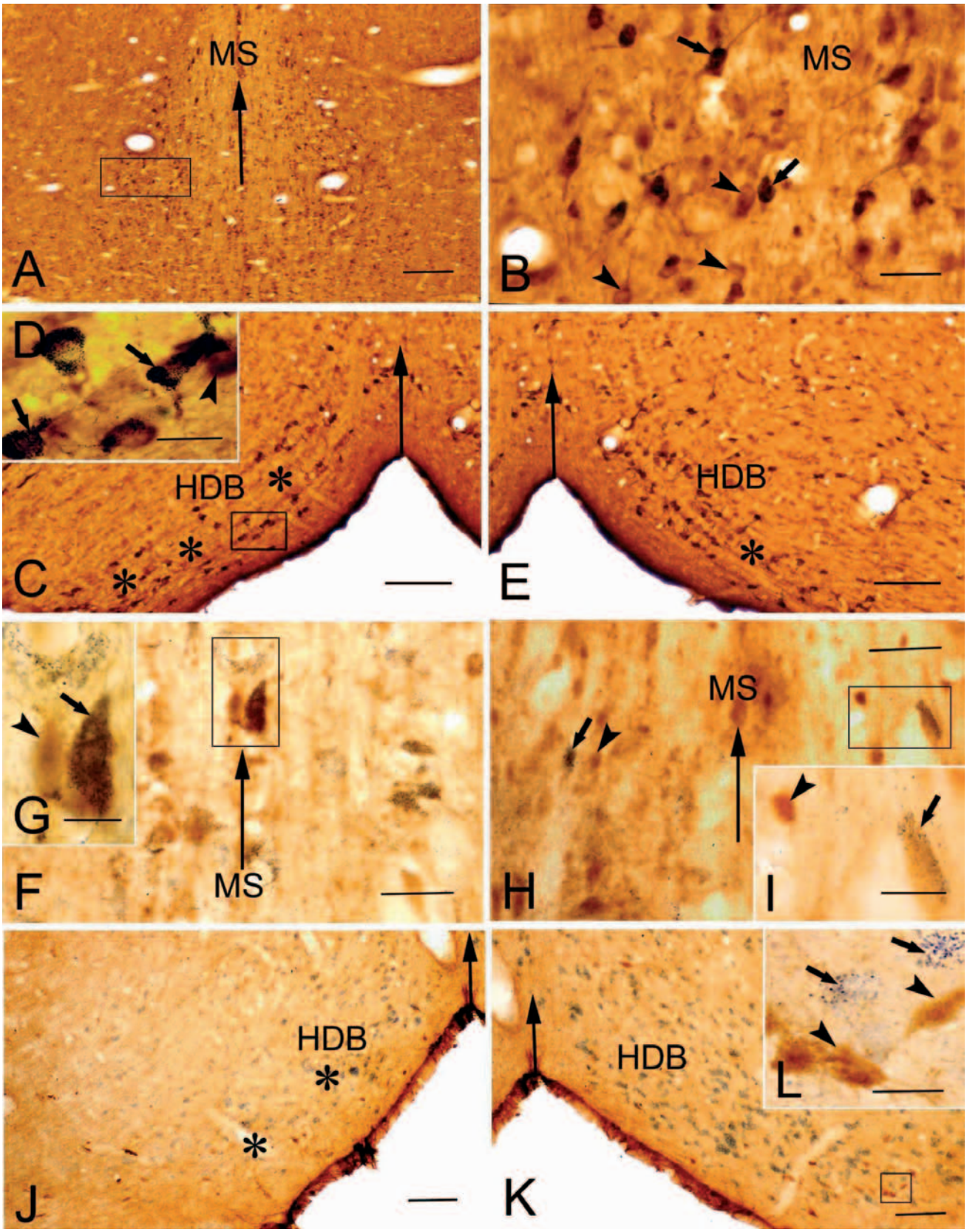

Figure 4. A: Photomicrograph of coronal section through the MS processed for the co-localization of choline acetyltransferase (ChAT) using DAB and α7 subtype of nAChR using BDHC, following an injection of Aβ into the left RSg and of PBS into the right RSg. The boxed area is enlarged in B. The straight arrow indicates midline. **B:** Higher magnification of boxed area in A, showing double-labelled neurons (arrows) and ChAT-immunoreactive neurons (arrowheads). **C-E:** Photomicrographs through the HDB following an injection of Aβ into the left RSg and of PBS into the right RSg. The sections were processed for co-localization of ChAT (using DAB) and α7 nAChR (using BDHC). The field in (C) shows a marked reduction in the number of immunoreactive neurons in the ipsilteral HDB (asterisks) as compared with the contralateral HDB (E, asterisk), which correspond to the PBS-injected side. The straight arrows indicate the midline. The boxed area in (C) is enlarged in D and shows a single- (arrowhead) and double-labelled (arrows) neurons. **F:** Coronal section through the MS processed for co-localization of parvalbumin (using DAB) and α7 nAChR (using BDHC) following an injection of Aβ into the left RSg and of PBS into the right RSg. The boxed area is enlarged in G. The straight arrow indicates the midline. **G:** Higher magnification of the boxed area in F, showing a single- (arrowhead) and a double-labelled cell (arrow). **H:** Photomicrograph through the MS in an Aβ-injected animal. The section

Effects of Aβ1-40 on the morphological profiles of α7 nAChR-immunoreactive neurons of the MS-nDBB complex

In Aβ-injected animals, most of the α7 nAChR-positive neurons of the MS-nDBB complex (e.g., Fig. 4K and L), as well as those that also expressed either cholinergic or GABA-related markers (e.g., Fig. 4D and G), did not show severe morphological changes when compared with controls, and cell size measurements of these neurons did not reveal significant differences in mean diameters between the Aβ-injected animals as compared with those in control animals. In the HDB, labelled cells appeared medium to relatively large in size (largest diameter of 30-40 μm), with somata that ranged in shape from fusiform to multipolar: the long axis of these cells was generally oriented in the coronal plane, parallel to the fibres of the diagonal band (e.g., . 4C, E and K). In the MS, single and double-labelled cells were of relatively small size (20-30 μm maximum diameter) with round to ovoid somata, and they were located along the midline and the lateral parts of the MS, with their long axes predominantly parallel to the dorsoventral axis of the nucleus (e.g. Fig. 4F).

Discussion

The purpose of this study was to assess the toxicity of intracerebrally injected Aβ1-40 peptide on the amount of α7 nAChR protein in neurons of the MS-nDBB complex. In order to evaluate the specificity of this toxicity, the ipsilateral hemisphere to the Aβ-injected side was compared with the contralateral hemisphere in the same animal, corresponding to that receiving the vehicle-solution (PBS)-injection alone, and with the corresponding hemisphere of uninjected animals as controls. Our quantitative analyses revealed a significant decrease in the number of α7 nAChR-immunoreactive neurons in the MS and HDB ipsilateral to the Aβ–injected side as compared with that observed in the corresponding hemisphere in uninjected control animals, and with that observed in the contralateral hemisphere corresponding to the PBS-injected side.

Methodological considerations

Since the specificity and selectivity of immunocytochemical methods is often a matter of concern, several issues arise in relation to the procedures used in this study. The first involves the presence of α7 nAChR-immunoreactivity in the cell bodies of the rat MS-nDBB complex. The rabbit anti-α7 nAChR and the goat anti-α7 nAChR antibodies used here are well characterized (Santa Cruz Biotech., Inc. Catalogue nº sc-5547 and sc-1447, respectively), and both antibodies resulted in similar patterns of immunostaining, consistent with the functional α7 nAChR recently described in the rat MS-nDBB complex (Henderson et al., 2005; Thinschmidt et al., 2005). However, the significance of the specificity of α7 nAChR-immunolabelling has recently been questioned (Herber et al., 2004; Jones and Wonnacott, 2005). Herber et al. (2004) found that α7 nAChR antibodies displayed comparable levels of immunoreactivity in brain tissue from α7-knockout mice and wild-type animals. However, the knockout studies were necessarily limited to a comparison of immunoreactivities in mouse tissue. Furthermore, the mAB306 antibody, tested by Herber et al. (2004), has previously been shown to cross-react with a 44-kDa mitochondrial protein (Fabian-Fine et al., 2001), which could explain the labelling seen in brain tissue from α7 nAChR-subunit knockout mice. Therefore, extrapolation of these findings to other mammalian species is risky because there are likely to be differences in the epitope sequences recognized by the antibodies, and in overall protein expression profiles. On the other hand, the specificity of the α7 nAChR immunoreactivity was supported by the results of the control experiments. Thus, α7 nAChR-immunolabelling was completely absent in sections incubated without the addition of primary antibody, or after the primary antibody had been replaced with the respective normal serum, or when the antibody was first pre-absorbed with α7 peptide prior to immunostaining. These observations make it

was processed for co-localization of GABA (using DAB) and α7 nAChR (using BDHC) and shows a double-labelled cell (arrow) and a putative GABAergic cell (arrowhead) The boxed area is enlarged in I. The straight arrow indicates the midline. The field in **(I)** shows a double-labelled neuron (arrow) and a putative-GABAergic cell (arrowhead). **J,K:** Photomicrographs through the HDB in an Aβ-injected animal. The section was processed for co-localization of calbindin (calb) (using DAB) and α7 nAChR (using BDHC). The field in (J) shows a marked reduction in the number of α7-like-immunoreactive neurons in the ipsilateral HDB (asterisks) as compared with the contralateral HDB (K), which correspond to the PBS-injected side. The boxed area in K is enlarged in L. The straight arrows indicate the midline. **L:** Higher magnification of the boxed area in K, showing single Calb-immunoreactive neurons (arrowheads) and single α7nAChR-like-immunoreactive neurons (arrows). Scale bars: 200 μm (A,C,E); 100 μm (F,H,J,K), 50 μm (B, D), 25 μm (G,I,L).

unlikely that our findings would reflect non-specific α7 nAChR-immunolabelling.

Another important issue concerns the toxicity of Aβ peptide. Recent *in vitro* studies suggest that in the toxic effects of Aβ peptide it may became important to consider the different Aβ forms; monomeric, oligomeric or polymeric (for review, see Bell et al., 2004). Other *in vitro* studies, however, have reported that soluble Aβ peptide can polymerize spontaneously into fibrils (Guan et al., 2001; Dahlgren et al., 2002). Since *in vitro* models of selected cell lines of neuronal or glial origin have strong limitations (for a review, see Harkany et al., 1999), in recent years several *in vivo* studies have been performed and have provided strong evidence that both the Aβ1-40 (Kowall et al., 1991; Giovannelli et al., 1995; 1998; Shin et al., 1997; Gonzalo-Ruiz and Sanz, 2002; Gonzalo-Ruiz et al., 2003; Reyes et al., 2004) and Aβ1-42 (Harkany et al., 2001) peptides are neurotoxic in the rat brain. Thus, as no significant differences were observed between the toxic effects of Aβ1-40 (Kowall et al., 1991; Giovannelli et al., 1995, 1998; Gonzalo-Ruiz and Sanz, 2002; Reyes et al., 2004) or Aβ1-42 (Harkany et al., 2001), and as in the rat brain the key one is Aβ1-40 and not Aβ1-42 (Shin et al.,1997), in the present study we used Aβ1-40 peptide to investigate the toxic effects of this peptide.

Under our experimental conditions, the neurotoxicity of Aβ1-40 amyloid was observed a few days after unilateral injection of this peptide. Other studies have reported that Aβ1-40 induces toxicity up to four months post-injection (Giovannelli et al., 1995; Shin et al., 1997). Concomitantly, complete recovery is observed six months post-injection (Winkler et al., 1994; Giovannelli et al., 1998). Since no significant differences were observed in the toxic effects of Aβ1-40 at any time-point (Giovannelli et al., 1995; Shin et al., 1997; Gonzalo-Ruiz and Sanz, 2002; Gonzalo-Ruiz et al., 2003; Reyes et al., 2004), in the present study we established short post-injection survival periods (four days - two weeks) to investigate acute-to-subchronic responses to Aβ peptide.

Aβ-toxicity on the alpha 7 subtype of nicotinic acetylcholine receptor (α7 nAChR) protein in the MS-nDBB complex

The present results show that all injections of Aβ peptide into the retrosplenial cortex, and even some injections of vehicle-solution (PBS) alone, induced a selective reduction of α7nAChR-immunoreactivity in the cell bodies of the MS-nDBB complex. The loss of α7nAChR-immunoreactive neurons in the basal forebrain, however, may to a large extent depend on the chemical structure of the particular Aβ fragments injected, and this might also involve several other experimental variables, such as age or biological variations in the response of individual animals to the injected substances. The extensive decrease in α7 nAChR-immunoreactive neurons in the MS-nDBB is congruent with the localization of the lesion in the retrosplenial cortex, as well as the cholinergic projections from the basal forebrain to different cortical areas, including to the retrosplenial cortex (Mesulam et al., 1983; Gonzalo-Ruiz and Morte, 2000). Such results are supported by recent studies in transgenic models, indicating that cholinergic deficits are caused by both the loss of cholinergic basal forebrain neurons and, locally, by cerebral amyloidosis in the neocortex (Apelt et al., 2002; Hu et al., 2003; Hartmann et al., 2004). Taken together with the results from previous studies pointing to an indirect and bidirectional mechanism of the cytotoxic effects of Aβ (Harkany et al., 2001; Walsh et al., 2002; Gonzalo-Ruiz et al., 2003), it seems reasonable to assume that intracerebrally-injected Aβ peptide would induce the degeneration of α7 nAChR-containing neurons in the MS-nDBB complex.

The present findings are also consistent with a large number of electrophysiological and pharmacological studies that support the detrimental effects of Aβ peptide with regard to the down-regulation of α7 nAChR (Guan et al., 2001; Liu et al., 2001; Lee and Wang, 2003). On the other hand, significant reductions in the levels of α4 or α7 subtype proteins have also been observed in several regions of the brains of AD patients (Lee et al., 2000; Wevers et al., 2000; Court et al., 2001). However, there is evidence that some α7 nAChRs are up-regulated in the brains of AD patients (Hellström-Lindahl et al., 1999) and in a mouse model of AD overexpressing Aβ (Dineley et al., 2002b; Spencer et al., 2005), although it is presently unknown whether this increase is associated with neurons and/or astrocytes (Teaktong et al., 2003; Gonzalo-Ruiz et al., 2004a; Xiu et al., 2005; Yu et al., 2005). Together, the above findings indicate that the interaction between Aβ and α7 nAChR is controversial since both the inhibi-

tion and activation of the α7nAChR receptor has been reported.

At the present stage of investigation, it is still unclear whether the Aβ-mediated reduction in α7 nAChR protein in the MS-nDBB complex is due to degeneration of α7nAChR expressing neurons or to a lower expression of α7 nAChR in neurons that are still alive. Our previous findings (Gonzalo-Ruiz et al., 2004a) showed that Aβ1-40 intracerebrally injected into the retrosplenial cortex induces a marked increase in α7 nAChR-immunoreactivity at the centre of the deposit of Aβ peptide. Thus, if Aβ1-40, like Aβ1-42 (Nagele et al., 2002), binds to the α7 receptor, then α7 nAChR-immunoreactivity will be translocated from the cell surface to discrete cytoplasmic granules within the neurons in just a short period of time. Even if cells increase the rate of synthesis of α7 nAChR in an effort to replace the Aβ-mediated receptor loss, the chronic presence of Aβ1-40 in the surrounding extracellular space would counteract this tenency and finally maintain reduced levels of α7 nAChR protein. The chronic decrease in α7nAChR receptors may represent a molecular mechanism through which the degeneration of cholinergic neurons in the basal forebrain is mediated. In light of the above observations, we suggest that the reduction in α7nAChR-immunoreactive neurons observed in this study might be due to a loss of these neurons, although the expression of the α7 gene and/or the amount of α7 nAChR protein within these neurons might also be severely affected.

Aβ/α7 nAChR interaction and its role in cholinergic and GABAergic cells of the MS-nDBB complex

Despite several lines of evidences, including our own, indicating the possibility of a direct interaction between Aβ and the α7 nAChR, the mechanisms underlying such an interaction have not been well characterized. Since α7 nAChR is highly permeable to calcium (Seguela et al., 1993; Quick et al., 1997), it has been suggested that the interaction between Aβ and α7 nAChR would comprise, at least in part, calcium (Dineley et al., 2002a). On the other hand, consistent with previous studies (Dominguez del Toro et al., 1994), the present results show that the α7 nAChR protein is highly expressed in cholinergic neurons of the MS-nDBB complex. It may therefore be suggested that the high affinity of Aβ to α7 nAChR may alter calcium homeostasis in a particular cholinergic neuron, thus affecting its proper physiological function. Such physiological alterations may lead to neurodegeneration and/or may even be responsible for neuronal death in the MS-nDBB complex (Giovannelli et al., 1995; Harkany et al., 2001; Gonzalo-Ruiz and Sanz, 2002). In turn, they would be responsible for the cholinergic dysfunction in the cortical areas that are innervated by the cholinergic neurons of the MS-nDBB complex, including the retrosplenial cortex (Mesulam et al., 1983; Gonzalo-Ruiz and Morte, 2002).

In addition, it is also well established that the MS-nDBB complex provides a crucial input to the hippocampus (Kohler et al., 1984; Freund and Antal, 1988), and that both GABAergic and cholinergic neurons of the MS-nDBB play an essential role in generating hippocampal theta oscillation (Vertes and Kocsis, 1997). Since both GABAergic and cholinergic MS-nDBB neurons express α7 nAChR (Dominguez del Toro et al., 1994; Yang et al., 1996, and present study), it has recently been suggested that these neurons could also contribute to the hippocampal effects of α7 nAChR agonist (Sick et al., 2006). Because hippocampal theta activity (Ji et al., 2001; Ge and Dani, 2005) and retrosplenial cortex (Gabriel et al., 1983; Sutherland and Hoesing, 1993) are associated with several cognitive functions and since the decrease in the numbers of nAChRs, including the α7 nAChR, has been well correlated with cognitive dysfunction observed in AD patients (Nordberg, 1994; Whitehouse and Kalaria,1995), the present findings therefore, suggest that the Aβ-mediated depletion of α7 nAChR in both cholinergic and GABAergic cells of the MS-nDBB complex may contribute to such cognitive dysfunction. Thus, the use of drugs blocking the effects of Aβ on α7 nAChR and increasing the number of nAChRs, may be a promising therapeutic strategy to prevent brain degeneration and the consequent intellectual decline that occurs in AD, as previously reported (Kem, 2000; Levin and Rezvani, 2000; Sabbagh et al., 2002).

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Abbreviation for figures

cc	corpus callosum
cg	cingulum
HDB	horizontal nucleus of the diagonal band of Broca
MS	septum medial
PrS	presubiculum
RSa	retrosplenial agranular cortex
RSg	retrosplenial granular cortex

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