Alteration of cholinergic, excitatory amino acid and neuropeptide markers in the septum-diagonal band complex following injections of fibrillar β-amyloid protein into the retrosplenial granular cortex of the rat

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SUMMARY

The effects on cholinergic, excitatory amino acid and neuropeptide markers in the basal forebrain of an intracerebrally-injected synthetic peptide corresponding to the first 1-40 amino acids of beta-amyloid protein (Aβ1-40) was studied in rats. Focal deposition of Aβ in the retrosplenial granular cortex resulted in a significant loss of cholinergic and glutamate-immunoreactive neurons in the septum-diagonal band complex. The medial septal nucleus showed a reduction of 39% ± 1% in the number of cholinergic neurons and a significantly reduced number of cholinergic neurons (up to 22% ± 10%) was also found in the horizontal nucleus of the diagonal band of Broca in comparison with the same paramaters in uninjected controls. A markerd loss of glutamate-containing neurons was also found in the medial septal nucleus (up to 19% ± 6%) together with a decrease of approximately 22% ± 3% in the horizontal nucleus of the diagonal band of Broca when compared with uninjected control animals. Furthermore, a moderate reduction in fibres and structures such as terminals immunoreactive for glutamate, substance P, and neurotensin was observed in the septum-diagonal band complex. These results show that two different neurochemically defined populations of neurons in the basal forebrain are affected by the neurotoxicity of Aβ in vivo.

Key Words: Aβ-amyloid – Alzheimer's disease – Glutamate – Choline acetyltransferase – Neurotoxicity – Limbic system

INTRODUCTION

The retrosplenial cortex (caudal cingulate cortex) has attracted particular attention because it forms part of the cortical component of the classic Papez (1937) circuit and is thus intimately involved in cognitive processes such as attention, learning and memory (Gabriel et al., 1983; Sutherland and Hoesing, 1993; Valenstein et al.,

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1987). Other studies have shown that memory impairment is an early, prominent manifestation of Alzheimer's disease (AD), and the cerebral cortex, hippocampal formation, parahippocampal gyrus and basal forebrain are among the brain areas most consistently and heavily affected in this pathology (Braak and Braak, 1991; Duyckaerts et al., 1997; Stéphan et al., 2001).

In recent years, one current approach to the pathophysiology of AD has been to consider beta-amyloid (Aβ) deposition as a major pathogenetic event (Hardy and Allsop, 1991; Selkoe, 1991; Yankner et al., 1990). In particular, several studies have shown that Aβ, the major constituent of senile plaques in AD, accumulate abundantly in the brain areas subserving information acquisition and processing, and memory formation (Braak and Braak, 1991; Shinkai et al., 1997; Stéphan et al., 2001).

While conflicting data exist in the literature about the roles of Aβ in the brain, particularly in AD, several in vitro studies have provided firm experimental evidence for both neurotrophic (Whitson et al., 1989; Yankner and Mesulam, 1991) and neurotoxic effects in cultured neurons (Pike et al., 1993; Pike et al.,1991; Yankner and Mesulam, 1991; Yankner et al., 1990). However, in vitro models of selected cell lines of neuronal or glial origin have firm limitations (for review Harkany et al., 1999). Therefore, to resolve the limitations and difficulties of vitro studies, in vivo models have been introduced to determine the possible neurotoxic effect of the Aβ or its derivatives (Emre et al., 1992; Geula et al., 1998; Giovannelli et al., 1995, 1998; Gonzalo-Ruiz, 1999; Harkany et al., 1995, 1999; Kowall et al., 1991; Mckee et al., 1998).

Cortical cholinergic innervation shows one of the most severe pathological changes in AD (Coyle et al., 1983; Perry, 1986; Rossor et al., 1981), which may be correlated with the cognitive impairments involved in this disease (Bartus et al., 1982; Everitt and Robbins, 1997; Fibiger, 1991), and cholinergic deficits, from the basal forebrain to the cortex, have therefore received much attention (Rossor et al., 1981; Wenk et al., 1980; Whitehouse et al., 1981, 1982). However, apart from the general interest in the cholinergic forebrain system as a transmitter deficit in AD (Bartus et al., 1982; Lehericy et al., 1993; Perry, 1986; Whitehouse et al., 1981, 1982), little is known about other possible neurotoxic effects of in vivo administered Aβ on the basal forebrain neurons projecting to the cortex. Thus, the present study was carried out to investigate whether local administration of fibrillar Aβ1-40 into the retrosplenial granular cortex (RSg) affects the neurochemical markers in neurons of the basal forebrain, especially in the septum-diagonal band (nDBB/MS) complex, by combined with immunohistochemical detection of choline acetyltransferase (ChAT), excitatory amino acid and several neuropeptides [neurotensin (NT), substance P (SP) and leu-enkephalin (Enk)].

Material and Methods

Experimental animals and anaesthesia

Female Wistar albino rats (n=5) weighing between 250-300 g were used. They were kept under standard laboratory conditions (20°C ambient temperature, 12 h light/dark cycle, tap water and regular rat chow ad libitum). The animals were anaesthetized with Nembutal (45mg/kg, injected intraperitoneally) for the surgical procedure (injection of either fibrilar Aβ protein or the 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 all respects 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 EEC Directive 86/609. In addition to the neurotoxic material derived from these animals, sections from several other rats immunoreacted for single antigens only were also available as control material in this study. Thus, the control rats consisted of a group of uninjected rats (n=3) and rats injected with the vehicle solution to assess the potential effect of the toxicity induced by the vehicle solution alone (n=5).

Injection of either fibrillar Aβ1-40 protein or the vehicle solution

Anaesthetized 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) and the vehicle solution (0.01M phosphate buffered saline, PBS), in which these peptides were dissolved, were injected into the RSg. Microinjections of the fibrillar form of Aβ1-40 (2µg-4µg in 1µl-2µl of PBS, from BACHEM) were made in different parts of the left RSg using stereotaxic coordinates derived from the atlas of Paxinos and Watson (1986). The doses of Aβ1-40 were based on previous and concurrent studies with the same peptide (Sanz and Gonzalo-Ruiz, 2000). All microinjections were made using a 10µl Hamilton syringe, 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 over a period of 10 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 different

microsyringe, injections of vehicle (1µl-2µl of PBS) were made in different regions of the right RSg. After the injection, the scalp was sutured and the animal allowed to recover from the anaesthetic.

Fixation

Following postinjection survival periods of 4-14 days, based on previous and concurrent studies with the same peptide (Sanz and Gonzalo-Ruiz, 2000), the rats were reanaesthetized and killed by transcardiac 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 350-400ml of fixative solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB) at pH 7.2 (also perfused at 20°C).

Tissue preparation and immunohistochemical procedures

Immediately after perfusion, the brain was removed, trimmed and sectioned in the coronal plane at 40 µm, using a freezing microtome. Adjacent sections were collected as seven series. One series of sections was mounted on gelatinized slides and stained with 0.1% cresyl violet. The other six series of sections were processed for the immunohistochemical localization of Aβ (1-40), ChAT, glutamate (Glu), Enk, NT or SP, respectively. Sections processed for ChAT were first immersed for 1 h in 10% normal rabbit serum (NRS), whereas the serial sections processed for Aβ (1-40), Glu, NT, Enk, and SP were 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. Sections processed for the immunohistochemical localization of ChAT were then incubated in a solution containing a rat monoclonal antibody (1:4 dilution; Boehringer-Mannheim), while those for Aβ (1-40) were incubated in a solution containing a mouse monoclonal antibody against human Aβ (6E10; 1:1000 dilution; Senetek) for 18-24 h at 4°C. The series of sections processed for Glu, NT, Enk, and SP were incubated in a solution containing polyclonal rabbit antibodies against Glu (1:1000 dilution; Arnel), NT (1:2000 dilution; Eugene Tech. Inter), Enk (1:5000 dilution; INCSTAR), or SP (1:2000 dilution; Eugene Tech. Inter) for 18-24 h at 4°C. After incubation in the primary antibody, sections were washed either in 1% NRS (for ChAT) or in 1% NGS (for Aβ, Glu, NT, Enk, or SP) and then incubated in biotinylated 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 Aβ immunohistochemistry, and in biotinylated goat anti-rabbit IgG (Vector; 1:200 in PBS with 1% NGS) for the immunohistochemical localization of Glu, NT, Enk, and SP. Sections were then washed in PBS and immersed in an avidin-biotin-HRP complex (Vector 1:100) for 60 min. The immunoreaction product was demonstrated using 0.005% DAB and 0.01% hydrogen peroxide in PB (this chromogen produced a diffuse brown reaction product). Sections were rinsed through several changes of PB, mounted on gelatinized microscope slides, dehydrated, covered with Permount, and examined and photographed under bright-field illumination.

As a control, some sections from each series were incubated as described above but without the addition of primary antibody or after replacing the rat primary antibody with normal rat serum and the rabbit primary antibody with NRS. There was a complete absence of ChAT, Aβ, Glu, NT, Enk or SP-immunoreactive neurons or neuropil in such control sections.

Analysis of material and presentation of results

Sections stained with cresyl violet were used to identify each injection site, whereas those sections stained immunocytochemically with antibodies against Aβ1-40 peptide were used to show the Aβ1-40 deposit. In each of the five series stained immunocytochemically with antibodies against ChAT, Glu, SP, NT, or Enk, all of the sections through the basal forebrain, particularly through the nDBB/MS complex, were examined systematically. The specificity of the immunoreaction was checked by comparing sections stained either with ChAT, Glu, SP, NT, or Enk antiserum and control material respectively. Structures immunostained by antibodies but not seen in the control slides were considered to be specifically immunolabelled and are designated here as ChAT-, Glu-, SP-, NT-, or Enk-immunoreactive. All immunoreactive neurons were scanned systematically at x20 magnification using a calibrated eye-piece. The number of ChAT- and Glu-immunoreactive neurons was counted in selected small areas of the nDBB/MS complex in the Aβ injected side and compared with the number of neurons in selected corresponding small areas in untreated control animals and with those in the PBS-injected side. Values are expressed as % of neuron numbers in intracerebral injections of Aβ, in PBS control injections, and in uninjected animals, and represent means ± SEM (n=5 animals, except for control uninjected material, where n= 3).

RESULTS

Localization and deposit of Aβ injections

According to the terminology and the mapping of Vogt and Peters (1981), the retrosplenial cortex of the posterior cingulate gyrus was subdi-

vided into two parts: the retrosplenial agranular cortex (RSa) and the RSg (Fig. 1A-D). The findings in this study are based on all five animals that received a single injection of Aβ1-40 into the left RSg (e.g., Fig. 1A,B) and a single injection of vehicle into the right RSg (e.g., Fig. 1C). Immunocytochemical qualitative light microscopic analysis revealed the deposition of the synthetic Aβ1-40 peptide at the level of the injection site (e.g., Fig. 1D). Aggregated Aβ1-40 deposition was observed intimately surrounding the injection site, whereas peptide diffusion into the tissue only occurred rarely.

General observations

Macroscopically, the brain of the Aβ-injected rats appeared normal. However, a moderate atrophy of the temporal, parietal and frontal cortices was noted as well as an enlargement of ventricular size (compare Figs. 2A, 3E and 3G with Figs. 2B, 3F and 3H). The immunoreactivity for ChAT, Glu, NT or SP in the different parts of the basal forebrain analyzed, particularly in the nDBB/MS complex, was similar in all uninjected control animals (Figs. 2A, 3A, C, E, G) and largely in agreement with previous descriptions (Armstrong et al., 1983; Emson et al., 1985; Finley et al., 1981; Ljungdahl et al., 1978; Ottersen and Storm-Mathisen, 1984). However, injection of Aβ1-40 into the RSg selectively affected the number of cholinergic and Glu-immunoreactive neurons, as well as the immunoreactivity in other neuronal systems, particularly the SP and NT, in the nDBB/ MS complex (Figs. 2B-H, 3B, D, F, H).

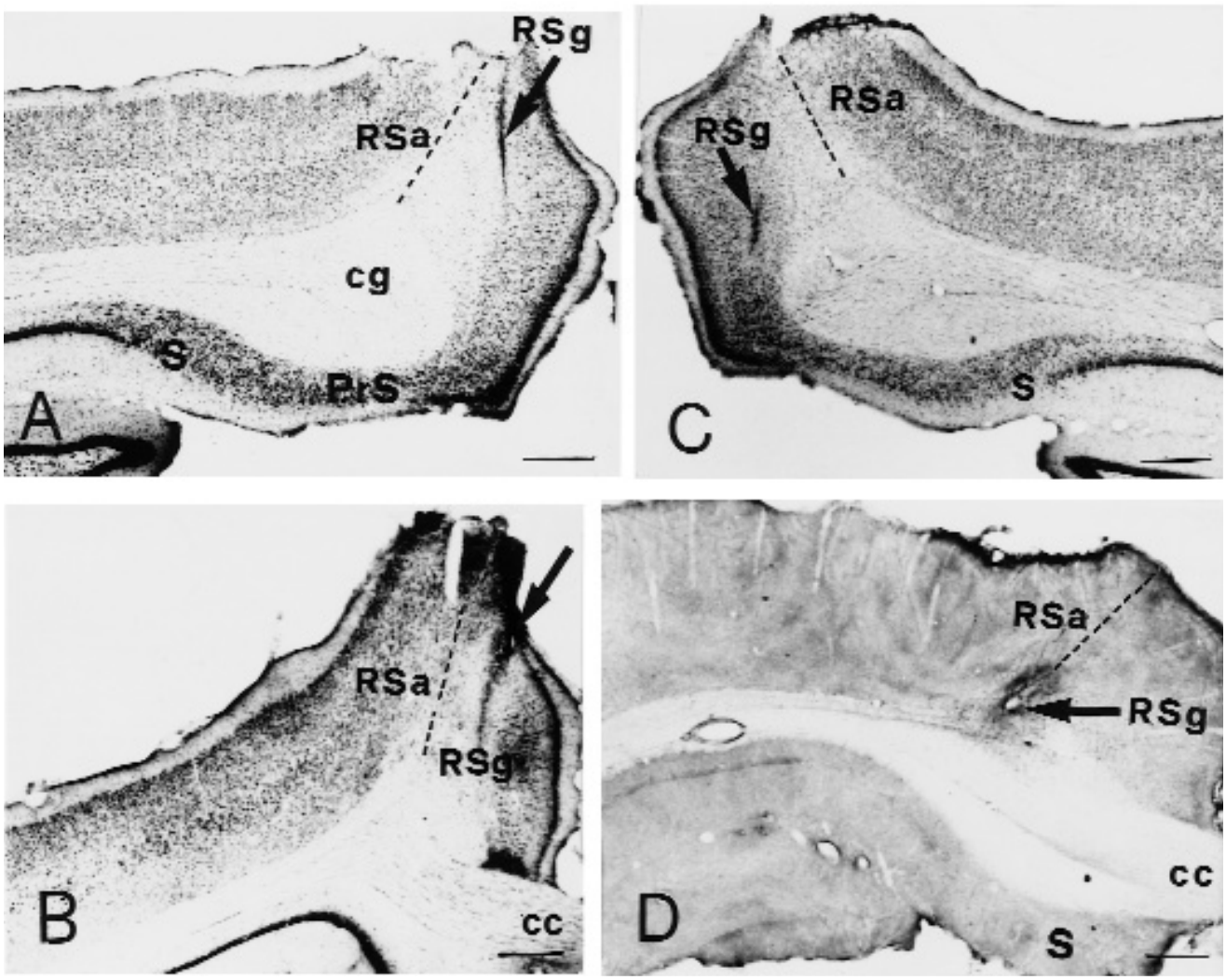

Fig. 1.- **A-C:** Low-magnification photomicrographs of coronal sections through the retrosplenial granular cortex (RSg) from cresyl violet-stained sections, showing representative injections of Aβ1-40 on the left RSg (A,B, arrows) and a representative injection of PBS centred on the right RSg (C, arrow). **D:** Photomicrograph of coronal section through the RSg from a section processed for Aβ-immunohistochemistry (using an antibody against Aβ1-40). The field shows Aβ deposition at level of the Aβ injection (arrow). Scale bars: 250 μm (A-D).

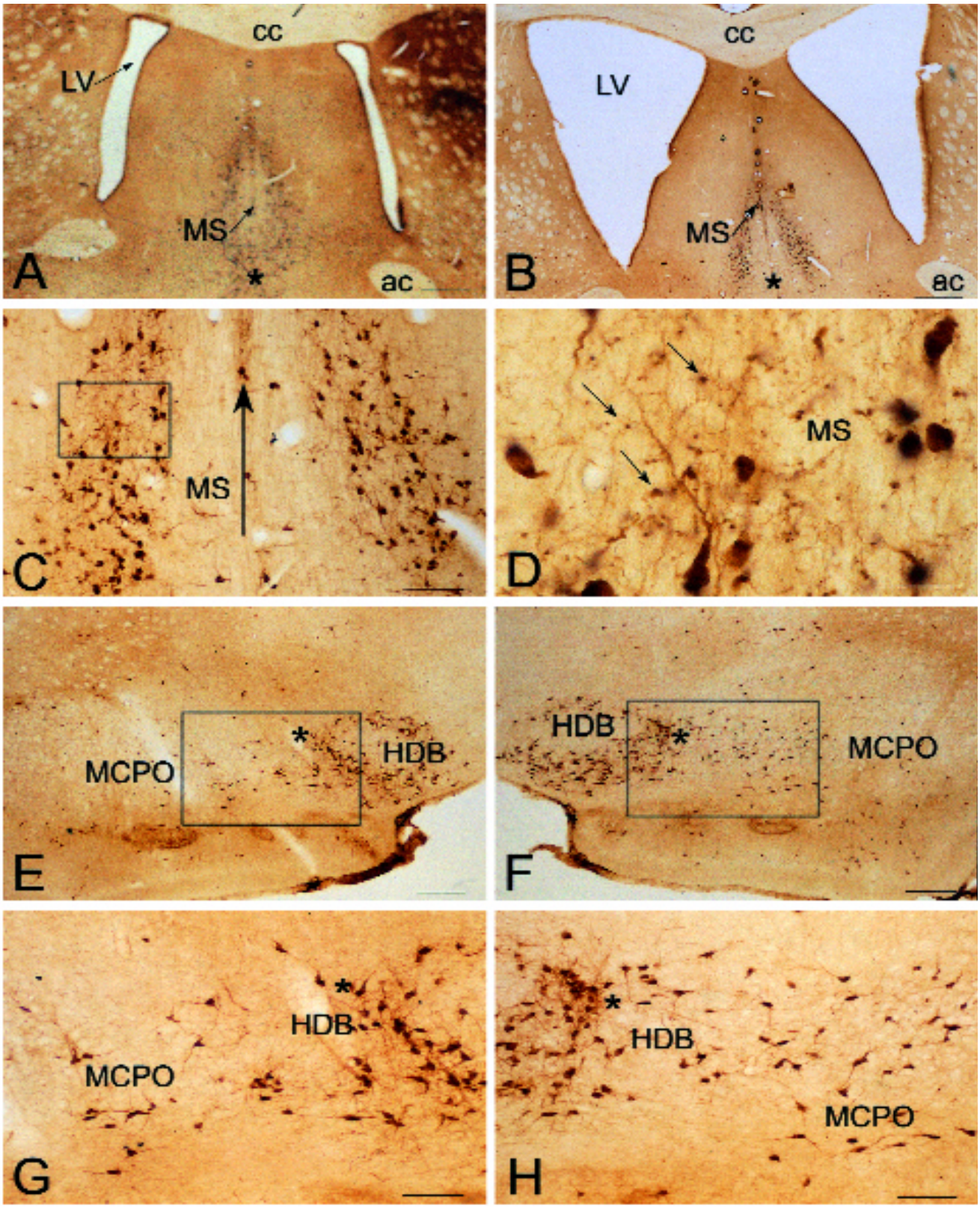

Fig. 2.- **A, B:** Choline acetyltransferase (ChAT)-immunostained coronal sections through the medial septal nucleus (MS) and the lateral ventricle (LV) in an uninjected control animal (A) and following an injection of Aβ into the left RSg and of PBS into the right RSg (B). In Fig. 1 B note the ventricular enlargement following the injection of Aβ in the RSg compared with that seen in the control animal (Fig. 1A). These photomicrographs also show moderate-to-large reductions in ChAT-immunoreactive neurons in the MS of Aβ-injected animal as compared with the control animal (A,B, asterisks). **C:** Part of Fig. 2B at higher magnification, showing a significant reduction in ChAT-immunoreactive neurons in the MS ipsilaterally to the injection of Aβ into the RSg as compared to the PBS control injection side. The boxed area is enlarged in D. Straight arrow indicates the midline. **D:** Higher magnification of boxed area in C, showing distorted ChAT-immunoreactive neurons and small unidentified immunoreactive profiles (arrows). **E, F:** ChAT-immunoreactive coronal sections through the horizontal nucleus of the diagonal band of Broca (HDB) and the magnocellular preoptic nucleus (MCPO) following the injection of Aβ shown in Fig. 1 A and the injection of PBS shown in Fig. 1C. Note the moderate reduction in cholinergic neurons in the ipsilateral HDB (E, asterisk) as compared with the contralateral HDB (F, asterisk), which correspond to the PBS-injected side. The boxed areas are enlarged in G and H respectively. **G, H:** High magnification of boxed areas in E and F, showing a significant reduction in ChAT-immunoreactive neurons in the HDB ipsilaterally to the injection of Aβ into the RSg (G, asterisk) as compared to the PBS control injection side (H, asterisk). Scale bars: 500 µm (A,B), 200 µm (E,F), 100 µm (C,G,H), 50 µm (D).

Effects of Aβ1-40 in the cholinergic system of the basal forebrain

In all animals, injections of Aβ1-40 confined to RSg (e.g., Fig. 1A, B, D) resulted in a significant loss of ChAT-immunoreactive neurons in different parts of the nDBB/MS complex. The most extensive reduction of cholinergic neurons was found in the medial septal nucleus (MS, Fig. 2B-D) and in the nuclei of the diagonal band of Broca (nDBB), especially in the horizontal nucleus of the nDBB (HDB, Fig. 2E-H), after 9-10 days postinjection,. Strong losses of ChAT-positive neurons were also found in the vertical nucleus of the nDBB and a less pronounced loss was seen in the magnocellular preoptic nucleus (MCPO, Fig. 2G). In the MS, we found a significant reduction (approximately 39% ± 1%) in the number of ChAT-immunoreactive neurons ipsilateral to the injection of Aβ into the RSg as compared with uninjected control animals (compare Fig. 2A with Fig. 2B) and a decrease of 22% ± 3% as compared with the PBS-injected side (e.g., Fig. 2C). Our analysis comparing the corresponding region of the MS in the Aβ-injected side with that in untreated control animals and with that in the PBS-injected side revealed 150-155 ChAT-immunoreactive neurons per section in uninjected control animals; 87-98 ChAT-positive neurons per section in the Aβ-injected side, and 102-127 ChAT-immunoreactive neurons per section in the PBS-injected side (Table 1). Furthermore, in all animals some immunostained neurons on the ipsilateral side appeared shrunken and showed a reduced dendritic arborization as compared with controls and with the PBS-injected side (Fig. 2C). In addition, in the MS we observed a number of very small round or irregularly shaped immunoreactive profiles, which we were unable to identified properly under the light microscope (Fig. 2D). Cell size analysis of ChAT-immunoreactive neurons in the MS revealed small differences between the Aβ-injected side (10-12 µm, 15 µm largest diameter) as compared with those in control animals and with those in the PBS-injected side (generally 10-15 µm, maximum diameter 20 µm). The injections of Aβ in the RSg also resulted in a significantly reduced number of ChAT-immunoreactive neurons (up to 22% ± 10%) in the ipsilateral HDB of the nDBB as compared with uninjected control animals, and a reduction of approximately 22% ± 1% as compared with the PBS-injected side (compare Fig. 2E, G with Fig. 2F, H). Our analysis comparing the corresponding region of HDB in the Aβ-injected side with that in untreated control animals and with that in the PBS-injected side, revealed 125-143 ChAT-immunoreactive neurons per section in uninjected control animals, 100-110 ChAT-positive neurons per section in Aβ-injected side, and 125-140 ChAT-immunoreactive neurons per section in the PBS-injected side (Table 1). In the HDB of the Aβ-injected animals the majority of ChAT-immunoreactive neurons did not seem to show severe morphological changes when compared with controls, and cell size measurements of ChAT-immunoreactive neurons in the HDB revealed small differences in the mean diameter between the Aβ-injected animals (20-22 µm, maximum diameter 30-35 µm) as compared with those in control animals (generally 20-25 µm, maximum diameter 30-40 µm).

Table 1.- Summary of cholinergic neurons in the MS and HDB of the nDBB following injections of Aβ1-40 in the left RSg and of vehicle (PBS) alone in the right RSg, and in uninjected control rats.

		Aβ1-40 in vehicle (n=5)		Vehicle (PBS) alone (n=5)		Uninjected animals (n=3)	
	n°	MS	HDB	MS	HDB	MS	HDB
ChAT	1	87	100	102	125	150	125
	2	98	110	127	140	155	143
	3	96	108	125	138	152	141
	4	94	104	123	136		
	5	89	102	120	132		

Effects of Aβ1-40 on glutamate immunoreactivity of the basal forebrain

Injections of Aβ1-40 confined to the left RSg (e.g., Fig. 1 A, B, D) also provided in vivo evidence for a loss of Glu-containing neurons in several parts of the basal forebrain, particularly in the MS and HDB of the nDBB (Fig. 3B, D). In the MS, we found a significant reduction (up 19% ± 6) in the number of Glu-positive neurons ipsilateral to the injection of Aβ in the RSg as compared with uninjected control animals (compare Fig. 3A with Fig. 3B), and a decrease of 16% ± 1% as compared with the PBS-injected side. Our analysis comparing the corresponding region of the MS in the Aβ-injected side with that in uninjected control animals and with that in the PBS-injected side revealed 127-137 Glu-immunoreactive neurons per section in uninjected control animals; 106-110 Glu-positive neurons per section in the Aβ-injected side and 122-132 Glu-immunoreactive neurons per section in the PBS-injected side (Table 2). A marked loss of Glu-positive neurons (up to 22% ± 3%) was also observed in the ipsilateral HDB of the nDBB in Aβ-injected rats as compared to untreated controls animals (compare Fig. 3 C with Fig. 3 D). Analysis comparing the corresponding region of the HDB in uninjected control animals with that in the Aβ-injected side and with that in the PBS-injected side revealed 130-140 neurons in the uninjected control animals; 98-110 neurons in the Aβ-injected side and 114-132 neurons in the PBS control injection side

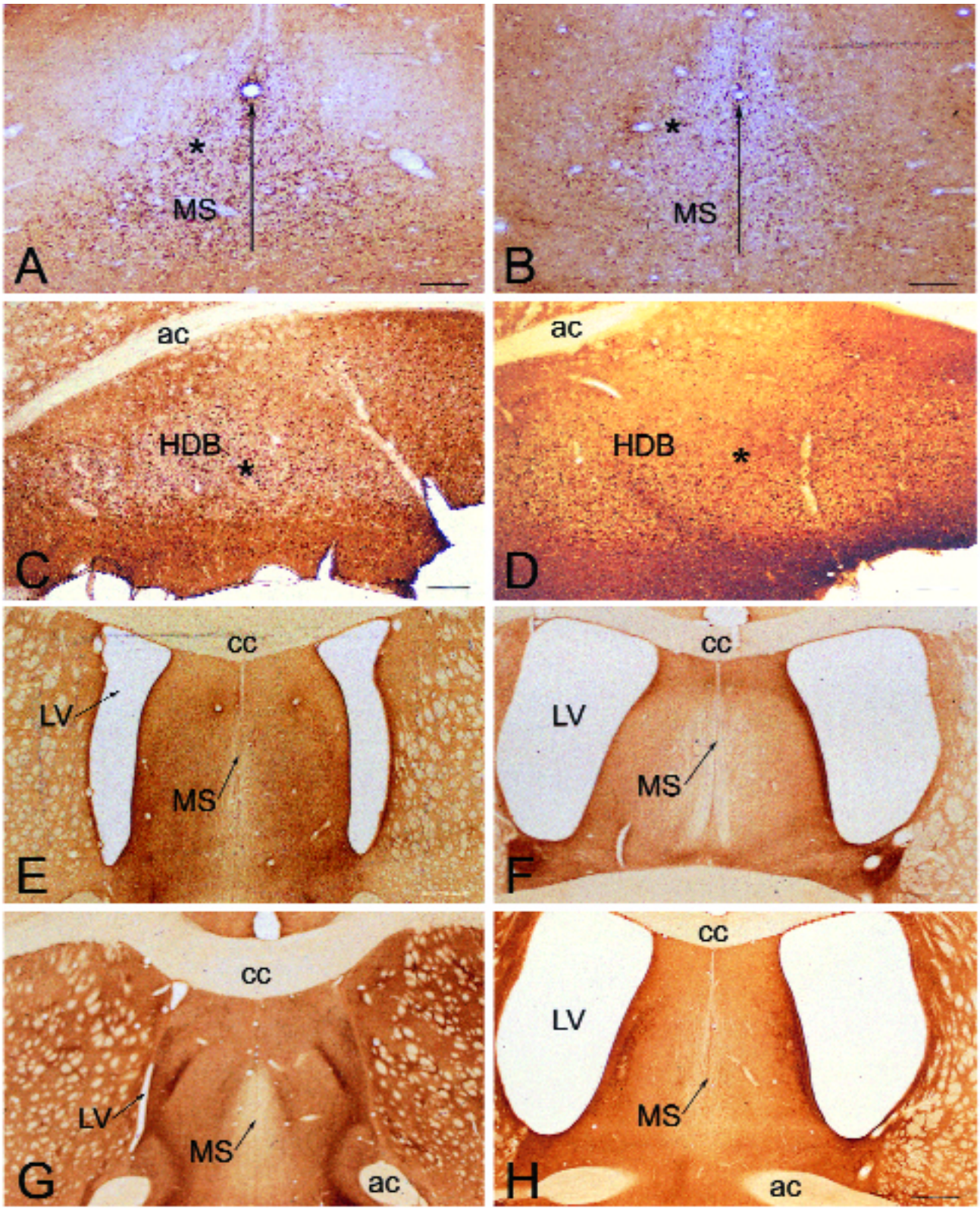

Fig. 3.- **A, B:** Glutamate (Glu)-immunostained coronal sections through the medial septal nucleus (MS) in an uninjected control animal (A) and following an injection of Aβ into the left RSg and of PBS into the right RSg (B). Note the moderate-to-large reductions in Glu-immunoreactive neurons in the MS of Aβ-injected animal as compared with the control animal (asterisks). Straight arrows indicate the midline. **C, D:** Glu-immunostained sections through the horizontal nucleus of the diagonal band of Broca (HDB) in an uninjected animal (C) and following an injection of Aβ into the left RSg in Fig. 3D. The fields show a moderate reduction in Glu-immunoreactive neurons and a moderate decrease in Glu-immunoreactivity in the HDB of an Aβ-injected animal (D, asterisk) as compared with the control uninjected material (D, asterisk). **E, F:** Neurotensin (NT)- immunostained coronal sections through the medial septal nucleus (MS) and the lateral ventricle (LV) in an uninjected control animal (E) and following the injection of Aβ shown in Fig. 1A (F). Note the ventricular enlargement following the injection of Aβ into the RSg (F) as compared with that in the control animal (E). The field also shows a moderate-to-light decrease in NT-like immunoreactivity in the septal region of an Aβ-injected animal (F) as compared with that in an uninjected control animal (E). **G, H:** Substance P (SP)-immunostained coronal sections through the medial septal nucleus (MS) in an uninjected control animal (G) and following the injection of Aβ in the RSg shown in Fig. 1A (H). Note moderate decrease in SP-like immunoreactivity in the MS of the Aβ-injected animal (H) as compared with the corresponding region of the MS in the untreated control animal (G). The field in Fig. 3G also shows ventricular enlargement following the injection of Aβ into the RSg (H). Scale bars: 500 µm (E-H), 200 µm (C,D), 150 µm (A, B).

(Table 2). The loss of Glu-positive neurons in the HDB of the nDBB, however, appeared to be a "focal" localization within this nucleus (Fig. 3 D). In the MS and HDB of the Aβ-injected animals, most of the Glu-immunoreactive neurons did not show severe morphological changes as compared with controls and cell size analysis of Glu-positive neurons did not reveal significant differences in the mean diameter between Aβ-injected animals and those of control animals.

Table 2.- Summary of glutamate immunoreactive neurons in the MS and HDB of the nDBB after injections of Aβ 1-40 in the left RSg and of vehicle alone in the RSg, and in uninjected control rats.

		Aβ1-40 in vehicle (n=5)		Vehicle (PBS) alone (n=5)		Uninjected animals (n=3)	
	n°	MS	HDB	MS	HDB	MS	HDB
Glu	1	106	98	122	114	127	130
	2	110	110	132	132	137	140
	3	108	104	130	128	135	132
	4	108	106	128	126		
	5	106	100	126	118		

Alterations of neuropeptide markers in the basal forebrain

Following injections of Aβ1-40 into the rat RSg, several neuropeptides systems, especially the SP and NT, also appeared to be a selectively vulnerable to Aβ. Using immunocytochemical staining methods a slight decrease was observed in NT-immunoreactive fibres and structures such as terminals in the basal forebrain, particularly in the MS, lateral septal nucleus (LS) and the HDB of the nDBB of Aβ-injected animals as compared with uninjected control animals (compare Fig. 3E with Fig. 3F). In addition, qualitative analysis of SP-immunostained sections of the basal forebrain revealed a slight decrease in the SP-immunoreactive fibres and structures such as terminals in the MS and HDB of the nDBB of Aβ-injected animals as compared with the corresponding hemispheres in untreated control animals (compare Fig. 3 G with Fig. 3 H). However, in Enk-immunostained sections of the nDBB/MS complex of Aβ-injected animals, we failed to observe any significant differences as compared to untreated control animals (not illustrated).

In conclusion the above results can be summarized as follows:

1. Aβ injection in the rat RSg provides in vivo evidence of an extensive loss of cholinergic neurons in several parts of the basal forebrain, particularly in the MS and HDB of the nDBB.

2. There also appears to be an increase in the sensitivity to Aβ toxicity of a subpopulation of Glu-immunoreactive neurons of the basal forebrain neurons projecting to the RSg, as well as a moderate reduction in Glu-immunoreactivity in fibres and structures such as terminals in the MS and HDB of the nDBB.

3. We found a significant decrease in the SP- and NT-like immunoreactivity in the nDBB/MS complex of Aβ-lesioned animals as compared to untreated controls, whereas Enk-immunostained sections of the basal forebrain of Aβ-injected animals did not show any significant differences between or within the two groups of animals investigated. Thus, the decrease in SP - and NT-like immunoreactivity in the basal forebrain may reflect a response to Aβ toxicity, whereas Enk-like immunoreactivity exhibited resistance to Aβ neurotoxicity.

DISCUSSION

The purpose of this study was to assess the toxicity of intracerebrally injected Aβ1-40. In order to evaluate the specificity of this toxicity, animals receiving the vehicle (PBS) in which this peptide was dissolved and uninjected animals were used as controls. Each hemisphere served as the control for the contralateral hemisphere in the same animal. The results revealed that the neurotoxicity of intracerebrally injected Aβ1-40 peptide exceeds that of the vehicle in which it is injected and that of uninjected control material.

Although there has been considerable debate about the toxicity of Aβ toxicity in the rat brain (Clemens and Stephenson, 1992; Stein-Behrens et al., 1985; Winkler et al., 1994), our data show that unilateral injections of Aβ1-40 into the rat RSg result in a significant loss of cholinergic as well as of Glu-immunoreactive neurons and neuritic degeneration, accompanied by immunohistochemical changes in several neuropeptide systems such as SP and NT within the nDBB/MS complex.

Taken together, the present findings confirm and extend the results of a previous studies describing a neurotoxicity effect of synthetic Aβ1-40 peptide after intracerebral injection in the rat (Emre et al., 1992; Giovannelli et al., 1994,1998; Gonzalo-Ruiz, 1991; Harkany et al., 1995; Kowall et al., 1991; O´Mahony et al., 1998), mouse (Flood et al., 1991, 1994) or primate (Geula et al., 1998; McKee et al., 1998). However, to the best of our knowledge this study is the first report showing an interaction between damage to the RSg and the neurotoxic pathway of Aβ action elicited in definite but limited neurotransmitter system-specific alterations, such as a loss of cholinergic and Glu-positive neurons in the basal forebrain. Therefore, the present data provide in vivo evidence for the pivotal nature of acetylcholine and glutamate in the neurotoxicity of Aβ. From this point, we shall focus our dis-

cussion on the effect of Aβ on the cholinergic and glutamatergic systems. In addition, some comments will be also made on the vulnerability of several neuropeptide systems, particularly in the SP and NT, to Aβ.

Aβ neurotoxicity in the cholinergic system

Consistent with our ealier observations (Gonzalo-Ruiz, 1999), the present results show that unilateral injections of Aβ induce a significant and systematic loss of cholinergic neurons in the basal forebrain, particularly in the nDBB/MS complex. The extensive loss of cholinergic projection neurons in the nDBB/MS is congruent with the localization of the lesion in the RSg, as well as the basal forebrain connections of the RSg. Indeed, there is evidence from previous studies that the nDBB/MS complex contains a large population of ChAT-immunoreactive neurons and that these nuclei are the major sources of cholinergic projections to various cortical areas, including the RSg (Armstrong et al., 1983; Gonzalo-Ruiz and Morte, 2000; Mesulam et al., 1983; Wenk et al., 1980). On the other hand, previous studies have reported persistent behavioral deficits and an extensive loss of ChAT-positive cholinergic projection neurons following Aβ infusion into the basal forebrain (Giovannelli et al., 1995; Harkany et al., 1998; O'Mahony et al., 1998). Therefore, the findings in our study are consistent with the view that intracerebrally-injected Aβ induces a reduction in cholinergic neurons in the nDBB/MS complex, and in turn, that it would lead to an indirect cholinergic denervation on circumscribed cortical areas connected with the basal forebrain, including the RSg.

Despite the above, however, the functional significance of a selective decline in cholinergic markers in the basal forebrain after intracerebrally injected Aβ into the RSg remains to be determined. Several studies have suggested that the RSg is intimately involved in cognitive functions such as learning and memory (Gabriel et al., 1983; Sutherland and Hoesing, 1993; Valenstein et al., 1987). Furthermore, there is also evidence that the cholinergic system plays a crucial role in learning and memory processes (Bartus et al., 1982; Everitt and Robbins, 1997; Fibiger, 1991; Van der Zee and Luiten, 1999) and cholinergic deficiency has also been implicated in the cognitive and behavioral manifestation of AD (Coyle et al., 1983; Decker and McGaugh, 1991; Perry, 1986). Therefore, in light of these findings, we suggest that the pathological disturbance in the cholinergic afferents to the RSg associated with injected Aβ could be correlated with a decrease in cognitive processes, as previously reported in Aβ-treated rats (Giovannelli et al., 1995, 1998; Stéphan et al., 2001).

Neurotoxic effect of Aβ on excitatory amino acid transmitter systems

The present study has also shown that injections of Aβ into the rat RSg result in a moderate and systematic loss of Glu-immunoreactive neurons and fibres in the basal forebrain, particularly in the nDBB/MS complex. There is also previous evidence for Glu as major neurotransmitter candidate for cortical pyramidal cells and hippocampal pathways, as well as a possible third component in basal forebrain neurons projecting to cerebral cortex (Fleck et al., 1993; Gonzalo-Ruiz and Morte, 2000; Gritti et al., 1997; Ottersen, 1991; Ottersen et al., 1990). Since lesiones to the cortex produce significant reductions in Glu concentrations in the terminal fields of some corticofugal projections (Hassler et al., 1982; Kim et al., 1977) and since there is evidence from animal experiments that lesioning the cortex can cause pathological changes in the cells of the nucleus basalis of Meynert (which innervates the cortex) that are similar to changes seen in AD (Sofroniew and Pearson, 1985) and that this nucleus receives glutamatergic input (Davies et al., 1984), the present findings support the notion that injection of Aβ into the RSg would induce glutamate abnormalities in the nDBB/MS complex and would in turn lead to glutamatergic dysfunction in cortical areas connected with the basal forebrain. However, it is unclear how far these results represent a loss of glutamatergic terminals in the cerebral cortex because the validity of this marker for such terminals has been questioned in the literature (Fonnum, 1984; Ottersen, 1991; Ottersen and Storm-Mathisen, 1984; Ottersen et al., 1990).

There is now evidence that glutamatergic deficits are associated with the pathophysiology of AD. However, there is a controversy about the alterations in glutamate content in the cerebral cortex, hippocampus and subcortical areas of AD patients (Greenamyre et al., 1987; Hyman et al., 1987; Maragos et al., 1987; Tarby et al., 1980) as well as about the role of excitatory amino acid receptors in AD (Greenamyre et al., 1985, 1987; Maragos et al., 1987a; Penney et al., 1990). This apparent conflict may be due to the fact that in addition to having a neurotransmitter role Glu is involved in many different biochemical and metabolic processes (Fonnum, 1984; Ottersen et al., 1984; Ottersen et al., 1990). As a result, it is unclear whether the alterations of glutamate neurotransmission systems is a consequence of excitotoxic changes produced by glutamatergic overactivity or result from a decrease in glutamatergic function. According to this view, the excitotoxicity produced by glutamate or related excitatory amino acid receptors as a possible contributor to neurodegeneration in AD has been proposed in several studies (Chalmers et al., 1990; Greenamyre and Young, 1989; Hardy and Cowburn, 1987; Procter et al., 1988; Procter

et al., 1986) that found a positive correlation between glutamatergic excitotoxicity and the degree of severity of the pathological signs of AD. In addition, Mattson et al. (1992) have shown that accumulation of Aβ protein in AD can potentiate excitotoxic degeneration. In contrast, there is now some evidence that suggests that glutamatergic hypoactivity may also contribute to the spread of pathological processes in AD (Cross et al., 1987; Francis et al., 1993; Hardy et al., 1987). Therefore, although the relevance of these studies to the slowly developing changes that occur in the brain in AD remains to be elucidated, we suggest that a change in the balance of glutamate neurotransmission systems affecting pyramidal neurons may contribute to the spread of disease via well-established anatomical pathways. On the other hand, since there is considerable evidence linking glutamatergic transmission to learning and memory functions (Advokat and Pellegrin, 1992; Collingridge and Singer, 1990; Danysz et al., 1995; Ridel, 1996; Ridel and Reymann, 1996), the present findings also support the notion that a substantial loss of glutamatergic pathways in the cerebral cortex might play a pivotal role in neural systems underlying memory and impaired learning and that this may contribute to cognitive dysfunction in AD.

Aβ neurotoxicity in neuropeptide markers in the basal forebrain

We have shown that injections of Aβ into the RSg result in a selective decrease in SP- and NT-like immunoreactivity in the nDBB/MS complex.There has also been a report of reduced substance P (Beal and Mazurek, 1987; Crystal and Davies, 1982) as well as of neurotensin (Ferrier et al., 1983; Rossor et al., 1984) contents in AD disease, although this is not a consistent finding (Nemeroff et al., 1983).

Substance P may be of interest in AD, since it co-localizes with acetylcholine and also with several neuropeptides, such as somatostatin (SS) (Crawley et al., 1985; Sutin and Jacobowitz, 1988). Armstrong and Terry (1985) observed swollen SP-immunoreactive processes in the senile plaques of AD patients and the co-localization of SP with somatostatin-like-immunoreactive processes has also been observed in same senile plaques (Armstrong et al., 1989). In addition, Benzing et al. (1993) noted that the greatest density of senile plaques in AD occurred in brain regions showing a marked depletion of both SS and SP. Thus, the present findings suggest that decreased in SP-immunoreactivity associated with injected Aβ might be correlated with the pathological signs of AD. Furthermore, previous evidence has indicated that several neuropeptides, such as SP and NT, appear to play a role in memory processes and to interact with the cholinergic system (Decker and McGaugh, 1991; Schlesinger et al., 1986). Thus, in the light of these anatomical data, together with pharmacological (Fort et al., 1998) and electrophysiological findings (Alonso et al., 1996), we suggest that both cholinergic and non-cholinergic neurons in the basal forebrain may play a role in the cognitive decline associated with AD.

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Abbreviations for figures

ac: anterior commissure
cc: corpus callosum
cg: cingulum
HDB: horizontal nucleus of the diagonal band of Broca
LV: lateral ventricle
MCPO: preoptic nucleus
MS: medial septal nucleus
PrS: presubiculum
Rsa: restrosplenial agranular cortex
RSg: retrosplenial granular cortex
S: subiculum

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