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Short communication

Cholinotoxic effects of $\beta$-amyloid$_{1-42}$ peptide on cortical projections of the rat nucleus basalis magnocellularis

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Abstract

$\beta$-Amyloid$_{1-42}$ peptide (BAP) was injected into the right nucleus basalis magnocellularis (nbm) of rats. After a 14-day survival time, the acetylcholinesterase and choline acetyltransferase activities and the number of muscarinic receptors were found biochemically to be significantly reduced in the ipsilateral frontal cortices. Confirmation of these data with silver staining also revealed degeneration of the projective fibers of the nbm to the frontal cortex. These results demonstrate the cholinotoxicity of BAP in an in vivo animal model.

Keywords: $\beta$-Amyloid$_{1-42}$ peptide; Nucleus basalis magnocellularis; Frontal cortex; Cholinergic system; Cholinotoxicity

One of the neuropathological features of Alzheimer's disease (AD) is the extracellular accumulation of $\beta$-amyloid peptides forming neuritic plaques. The mature form of this peptide consists of 39–42 amino acid residues [7]. It is produced by an alternative proteolytic cleavage process from the amyloid precursor protein (APP), a receptor-like cell surface molecule [9] whose function remains obscure. APPs play important roles in numerous physiological functions, e.g., during neural development [15] or in coagulation [16]. Various $\beta$-amyloid peptide fragments are derived from APP during cleavage [1], differing in distribution and in physiological and chemical properties [1,13]. While $\beta$-amyloid$_{1-42}$ (BAP) is also produced during the normal cell metabolism [8], its physiological effects are controversial [6,19]. Many results indicate, for example, that BAP can exhibit direct neurotoxic properties, as detected both in vitro [11,19] and in vivo [12,13]. Yankner et al. [19] have reported that BAP in low concentrations exerts neurotrophic effects on neurons in culture, but above a threshold level the peptide acts as a toxin to neurons in a dose-dependent manner [12,19]. Deposits in the brains of AD patients may contain BAP in a concentration high enough to cause local neurodegeneration [1,7]. In spite of the abundance of BAP in neural tissues from patients with AD, its role in the etiology of the disease remains to be clarified.

The nucleus basalis magnocellularis (nbm), as part of the basal ganglia, plays an important role in cognitive and memory functions [2]. The nbm has extensive cholinergic projections to the cortical structures, and particularly to the frontal cortex [10]. Earlier studies have shown that the cortical cholinergic hypofunction characteristic of AD is primarily due to degeneration of the nbm [2]. Damage to different brain areas and transmitter systems is well characterized in AD [18].

The aim of this study was to determine whether BAP exerts neurotoxic effects on the neurons of the nbm, causing degeneration of the ascending cholinergic projections to the cortex, followed by neurochemical alterations similar to those observed in AD. Since the cortex has only a limited number of intrinsic cholinergic neurons [10], the effects of BAP injected into the nbm can be studied directly in frontal cortical samples. In vivo experiments, we measured the acetylcholinesterase (AChE, EC 3.1.1.7) and choline acetyltransferase (ChAT, EC 2.3.1.6) activities and the binding parameters of the muscarinic acetylcholine...
receptors (mAChR) in the frontal cortices of rats. We also employed a silver staining method specific for neurodegeneration [5] to visualize neurodegenerative processes in cortical sections after βAP treatment.

Human βAP was synthesized with amide at the C-terminal by a solid-phase technique involving Boc chemistry. Peptide chains were elongated on MBHA resin (0.6–0.8 mmol/g) and the syntheses were carried out manually. Couplings were performed with dicyclohexylcarbodiimide with the exception of Asn, which was incorporated in HOBT-ester form. The Boc group was removed by treatment with 50% trifluoroacetic acid (TFA) in CH₂Cl₂. After completion of the synthesis, the peptide was cleaved from the resin with liquid HF. Free peptides were solubilized in 95% TFA, filtered and lyophilized. The crude peptides were purified by RP-HPLC on an Astec-300 5 C₄ column. The purity was checked by RP-HPLC on a Water-Porex 5 C₄ column. Amino acid analysis demonstrated the expected amino acid composition and electrospray mass spectrometry (ES-MS) gave the expected molecular ion.

Male Sprague-Dawley rats (140–160 g) were caged and kept on a normal diet and tap water ad libitum, in an air-conditioned room with a 12-h daylight cycle (light on at 07:00). They were anesthetized intraperitoneally with 6% Na-pentobarbital, and their heads were fixed in stereotaxic equipment. Three μl of 0.2 nmol/μl βAP was injected slowly (~0.15 μl/min) into the right nbm (AP -2.0 mm, lat. 3.5 mm, vent. 5.5 mm [17]) with a Hamilton microsyringe. Although the volume of βAP solution is slightly larger than used previously by others [6,12,13], it was necessary for the complete destruction of the nbm. The βAP was dissolved in 30% acetonitrile containing 0.1% TFA. The contralateral frontal cortices of βAP-treated animals were used as control areas from the same individuals. Control rats were kept under normal conditions without any operative insults, while sham-operated animals received 3 μl of organic solvent only.

For biochemical measurements, animals were decapitated after a 14-day survival time and the ipsi- and contralateral frontal cortices were removed. One set of cortical samples was frozen at −20°C for receptor binding experiments, while the others were kept on ice and used immediately for determination of enzyme activities. AChE activity was measured by the spectrophotometric method of Elman et al. [3]. Acetyltiiocholine (ACThCh) iodide was used as substrate. To inhibit nonspecific cholinesterase activity, ethopropazine-HCl (10⁻⁴ M) was added to the incubation mixture. The activity of AChE was expressed as nmol of ACThCh hydrolyzed/min/mg protein, as measured spectrophotometrically at 412 nm. ChAT activity was determined by the radiochemical method of Fonnum [4]. The final concentrations in the incubation mixture were: 0.6 mM [¹⁴C]acetyl-coenzyme A, 300 mM NaCl, 50 mM Na-phosphate buffer (pH 7.4), 10 mM choline-HCl, 20 mM EDTA and 0.1 mM physostigmine sulfate. The radioactivities of the samples were measured in a liquid scintillation counter.

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Fig. 1. AChE (A) and ChAT (B) activities in frontal cortical regions of control, sham-operated and βAP-treated animals. For the measurement of AChE activity, 5 control, 5 sham-operated and 10 βAP-treated animals were used. For the measurement of ChAT activity, 7 control, 10 sham-operated and 16 βAP-treated animals were used. Data on βAP-injected ipsilateral frontal cortical samples were compared with those on control and sham-operated ipsilateral, and with those on βAP-injected contralateral samples. βAP injections into the nbm resulted in a significantly (* P < 0.05, Student's t-test) decreased AChE activity in the ipsilateral frontal cortices as compared to the control group (16.38 ± 2.2 vs. 21.97 ± 4.5 nmol/min/mg protein), while the ChAT activity in the frontal cortices ipsilateral to the nbm injection was significantly reduced as compared to all other groups examined (24.48 ± 3.4, sham-operated, 23.8 ± 2.7 and βAP-injected contralateral side, 24.69 ± 2.6 nmol/h/mg protein, respectively, ** P < 0.01, Student's t-test). AChE and ChAT activities are expressed as nmol/min/mg protein and nmol/h/mg protein, respectively. Data are reported as means ± S.D.
scintillation counter. The ChAT activity was expressed as nmol of ACh synthesized/h/mg protein.

\((-\) Quinuclidinyl(phenyl)-4-[3H]benzilate ([3H]-QNB, NEN, Boston, MA, USA) binding assays were performed as modified from Yamamura and Snyder [20]. An aliquot of cortical homogenate (~0.1 mg of protein) was incubated for 120 min at 25°C with 0.05–3 nM [3H]-QNB in a total volume of 1 ml of 50 mM Na/K-phosphate buffer (pH 7.4 at 25°C) in the presence or absence of 1 μM atropine sulfate. Bound and free ligand were separated by rapid filtration of the mixture under vacuum through Whatman GF/C glass filters, which were then washed by 3 × 5 ml ice-cold physiological saline. The radioactivity on the filters was counted in a liquid scintillation counter at an efficiency of 44%. Receptor binding parameters (Kd and Bmax) were analyzed by using a non-linear regression program for an IBM AT (GraFit 3.0, Erithacus Software, Staines, Middlessex, UK).

The protein contents of the samples were measured by the method of Lowry et al. [14], with bovine serum albumin as standard. Statistical analyses were performed by Student’s t-test (Excel 5.0, Microsoft Co., Redmond, WA, USA). A P value less than 0.05 was considered significant.

For histochemistry, all rats were deeply anesthetized with 6% Na-pentobarbital and perfused transcardially with 50 ml of heparinized physiological saline followed by 300 ml of fixative containing 3% paraformaldehyde (PFA) and 0.2% picric acid. Brains were cryoprotected overnight in 30% buffered sucrose. Twenty-μm thick coronal sections were cut on a cryostat microtome and collected in phosphate-buffered saline.

Silver staining was performed by a modified method of Gallyas et al. [5]. Briefly, cortical sections were postfixed overnight in 4% PFA, then pretreated in a solution of 2% NaOH and 2.5% NH₄OH, followed by impregnation in a mixture containing 0.5% AgNO₃, 0.8% NaOH and 2.5% NH₄OH. After the sections had been rinsed several times in a solution of 0.01% NH₄NO₃ and 0.5% Na₂CO₃ in 30% ethanol, staining was developed in a solution containing 0.01% citric acid, 0.5% formaldehyde and 10% ethanol. After washing of the sections several times in 0.5% acetic acid, the reaction was stopped with Na₂S₂O₃.

βAP injections into the nbm resulted in a significantly (P < 0.05) decreased AChE activity in the ipsilateral frontal cortices as compared to the control group (16.38 ± 2.2 vs. 21.97 ± 4.5 nmol/min/mg protein; Fig. 1A), while the ChAT activity in the frontal cortices ipsilateral to the nbm injection was significantly reduced as compared to all other groups examined (19.31 ± 1.8 vs. control, 24.88 ± 3.4, sham-operated, 23.8 ± 2.7 and βAP-injected contralateral side, 24.69 ± 2.6 nmol/h/mg protein, respectively, P < 0.01: Fig. 1B). Ipsilateral cortical samples from control and sham-operated animals, and also βAP-treated contralateral samples, displayed enzyme activities not significantly different from each other. The slight reduction observed in the frontal cortices of sham-operated animals may be an effect of the injected organic solvent of the βAP as it was previously demonstrated [12,13,21]. The reduction in ChAT activity, localized presynaptically, resembled the loss of projective fibers originating from the nbm, while the decrease in AChE activity may reflect a loss in activity of the cortical cholinergic system as well. As a further result of the βAP treatment, the number of [3H]-QNB binding sites (Bmax) in the βAP-treated ipsilateral cortical samples decreased significantly (P < 0.05) as compared to the control value (Table 1), while the dissociation constant (Kd) remained unchanged. Analysis of the binding of the M₁ receptor subclass-selective antagonist [3H]pirenzepine revealed no change in the cortical (mostly) postsynaptic M₁ AChR population (data not shown). We assume that the loss measured by means of the non-selective antagonist [3H]-QNB indicates a specific decrease in the presynaptic M₁ AChRs.

A silver histochemical procedure specific for neurodegeneration revealed extensive fiber degenerations in the frontal cortex after βAP treatment, as compared with both sham-operated and control animals (Fig. 2A–C). In response to the organic solvent in sham-operated animals, some degenerating neurites were observed ipsilateral to the injection.

In summary, our results clearly demonstrate that βAP exerts neurotoxic effects on the cholinergic neurons of the

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**Table 1**

<table>
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<tr>
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<th>Bmax (fmol/mg protein)</th>
<th>Kd (nM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>504.57 ± 32.8</td>
<td>0.73 ± 0.2</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>499.14 ± 153.5</td>
<td>0.68 ± 0.3</td>
</tr>
<tr>
<td>βAP injection, ipsilateral sample</td>
<td>402.00 ± 94.8</td>
<td>0.58 ± 0.2</td>
</tr>
<tr>
<td>βAP injection, contralateral sample</td>
<td>465.83 ± 121.7</td>
<td>0.70 ± 0.4</td>
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Changes in Bmax and Kd for [3H]-QNB binding to frontal cortical membranes of control, sham-operated and βAP-injected animals. Each value is the mean ± S.D. of the results on eight samples. * P < 0.05 (Student’s t-test), as compared to the control group. Binding parameters were calculated with the computer program GraFit 3.0 (Erithacus Software, Staines, Middlessex, UK).
In this model, the toxic effects of βAP can be studied directly, and some of the neurochemical parameters of a central cholinergic hypofunction can be modelled.

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