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Short communication

β-Amyloid$_{(1-42)}$ affects cholinergic but not parvalbumin-containing neurons in the septal complex of the rat

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Abstract

β-Amyloid$_{(1-42)}$ peptide (βAP$_{(1-42)}$) was injected into the medial septum of rats. After a 14-day survival time, neuronal alterations in the septal cholinergic and GABAergic systems were visualized by means of histo- and immunocytochemical methods. Neurons insulted by the peptide were primarily choline acetyltransferase-immunoreactive (ir), while only minor effects of βAP$_{(1-42)}$ were observed on parvalbumin-ir interneurons. These results indicate that the changes in intracellular Ca^{2+} level elicited by βAP$_{(1-42)}$ may contribute to β-amyloid neurotoxicity, and Ca^{2+}-binding proteins may play an important role in the protection against the neurotoxic effects of βAP$_{(1-42)}$.

Keywords: β-Amyloid; Calcium-binding protein; Choline acetyltransferase; Cholinotoxicity; Medial septum; Parvalbumin

The sole invariant hallmark of Alzheimer's disease (AD) is the presence and excessive accumulation of β-amyloid peptides (βAP) forming neuritic plaques [21]. Alternative processing of the amyloid precursor protein liberates potentially amyloidogenic βAP [22] that consist of 39–42 amino acid residues [12]. Small amounts of βAP exert trophic properties on neurons in culture [25], while treatment of neurons with high concentrations of these proteins causes neurodegeneration and cell death in vitro [16,25]. Recent studies have shown that in vivo administration of βAP elicits the malfunction of neurons, leading, for instance, to a reduced acetylcholine release of neurons from the medial septum (MS) of rats [1]. The precise mechanism of how βAP accumulation and deposition induce neuronal changes in the early stages of AD is still sketchy [17,21], but it has recently emerged that these peptides exhibit cholinotoxicity [1,13], contributing directly to the impairment of cognitive, learning and memory functions in AD. Contemporary results, however, support the hypothesis that βAP act by altering intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) [17,18], while a Ca^{2+} overload is known to be toxic to neurons [6]. Thus, βAP may also render neurons vulnerable (directly or indirectly) to different kinds of intracellular insults by disrupting normal intracellular Ca^{2+} homeostasis.

The MS plays an important role in learning and memory processes via the septo-hippocampal pathway [11]. Primarily cholinergic and γ-aminobutyric acid (GABA)ergic neurons, in approximately equal quantities, are situated in the MS of rats [23]. GABAergic neurons in the MS contain the Ca^{2+}-binding protein parvalbumin (PV) [9], which plays a principal role in buffering the cytosolic free Ca^{2+} levels [2,5]. While pathogenic changes in the cholinergic system can readily be demonstrated in the early stages of AD [3,21], PV-immunoreactive (ir) neurons represent a neuronal subset more resistant to degeneration in AD [14].

Since controversial results have recently been published as to whether βAP may have specific effects on different populations of neurons in vitro [15,20], we have determined the effects of one such peptide on septal cholinergic and GABAergic, PV-containing neurons in vivo.

The synthesis of human β-amyloid$_{(1-42)}$ peptide (βAP$_{(1-42)}$) has been previously described in detail [13]. In brief, βAP$_{(1-42)}$ was synthesized with amide at the C-terminal by a solid-phase technique involving Boc chem-

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istry. 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 CH_2Cl_2. 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 reverse-phase high pressure liquid chromatography (RP-HPLC) on an Astec-300-5 C_4 column. The purity was checked by RP-HPLC on a W-Porex-5 C_4 column. Amino acid analysis demonstrated the expected amino acid composition, and electrospray mass

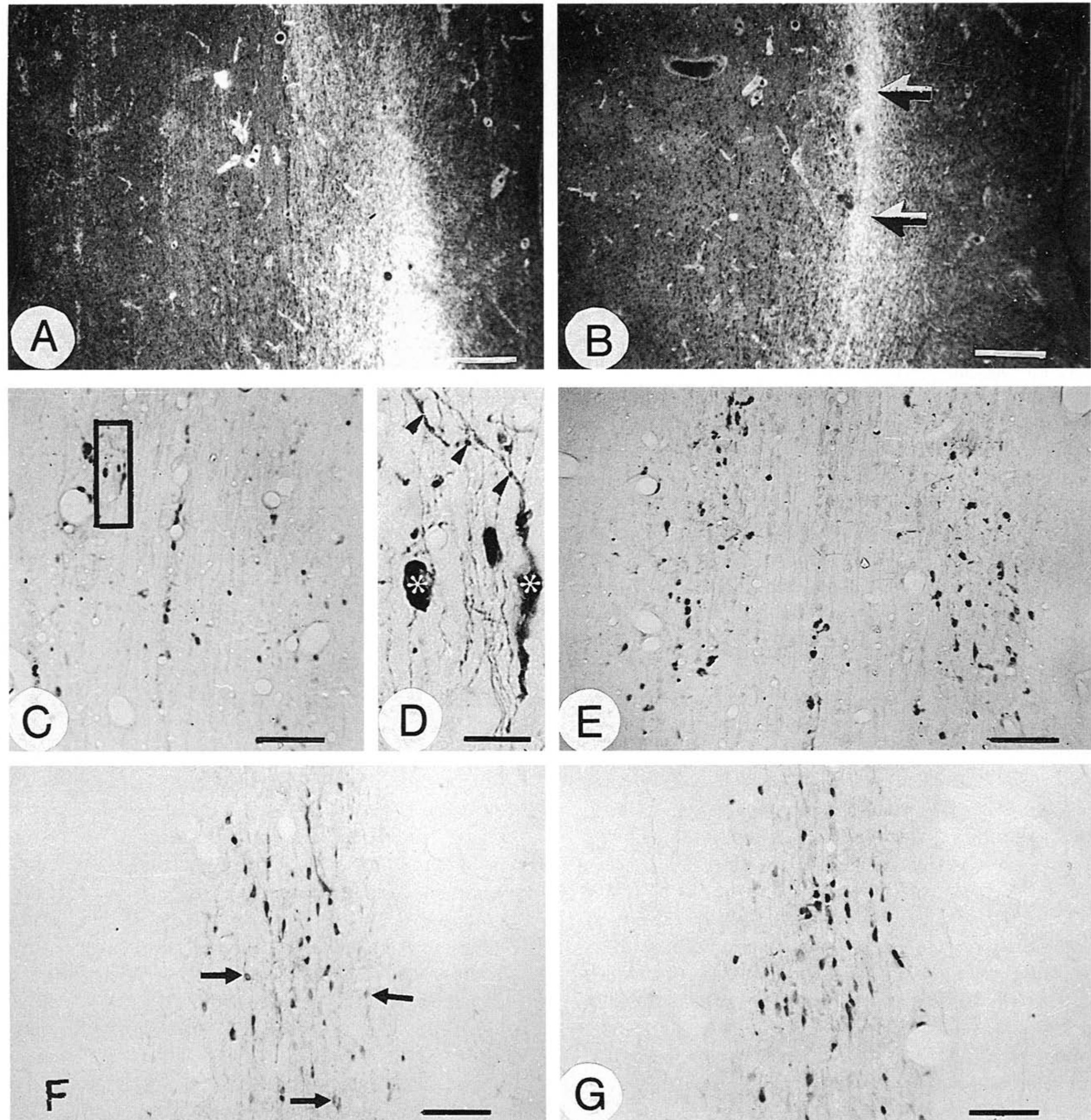

Fig. 1. Representative photomicrographs of $\beta AP_{(1-42)}$-injected (A,C,D,F) and control animals (B,E,G). Silver impregnation, specific for terminal neurodegeneration [10], revealed extensive damage to the neurons after $\beta AP_{(1-42)}$ treatment (A), while only the track of the needle (arrows) was labelled in control animals (B) as they are shown on dark-field photomicrographs. ChAT-immunolabelling demonstrated neuronal loss (C), perikaryal shrinkage (asterisk) and beaded fibres (arrowheads) of cholinergic neurons in the MS (D) after $\beta AP_{(1-42)}$ administration, while the PV-immunoreactive neurons remained unaffected (F), relative to controls (E,G; bright-field photomicrographs C–G, respectively). A slight decrease in PV-immunoreactivity was rarely observed after $\beta AP_{(1-42)}$ treatment (F, arrows). Enlargement (D) of the area from C is indicated by the box. Scale bars = 330 μm (A,B); 160 μm (C,E,F,G); 25 μm (D).

spectrometry gave the expected molecular ion. $\beta AP_{(1-42)}$ was dissolved in 30% acetonitrile containing 0.1% TFA when used in the experiments.

Ten male Wistar rats (320–370 g), divided into two groups (5 control and 5 treated animals), were used in these experiments. The animals were injected with 1 μl of 0.35 nmol/1 μl $\beta AP_{(1-42)}$ into the MS ($\sim$ 0.1 μl/min; AP = 0.2 mm, L = 0.3 mm, H = 6.0 mm from the bregma at an angle of 5°, [19]), under 0.12 ml 6% sodium pentobarbital (i.p.)/0.12 ml Hypnorm (i.m., Janssen, The Netherlands)/0.1 ml methyl atropine sulfate (i.p.) anaesthesia. Control animals received the same volume of the organic solvent only. After a 14-day survival time, all rats were deeply anaesthetized with 6% sodium pentobarbital and transcardially perfused with a 50 ml prerinse of heparinized saline. This was followed by 300 ml fixative containing 3% paraformaldehyde (PFA) and 0.2% picric acid solution. Brains were cryoprotected overnight at 4°C in 30% phosphate-buffered sucrose. Coronal cryostat sections (20 μm) were cut and collected in phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Silver staining was performed according to the method of Gallyas et al. [10]. Briefly, the cortical sections were postfixed overnight in 4% PFA, then pretreated in a solution of 2% NaOH and 2.5% NH_4OH, followed by impregnation in a mixture containing 0.5% $AgNO_3$, 0.8% NaOH and 2.5% NH_4OH. After the sections had been rinsed several times in a solution of 0.01% NH_4NO_3 and 0.5% Na_2CO_3 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 $Na_2S_2O_3$.

Tissue sections processed for choline acetyltransferase (ChAT; EC 3.2.1.6) immunocytochemistry were rinsed several times in 0.01 M PBS, immersed in 0.3% H_2O_2 in PBS, rinsed again and incubated for 1 h at room temperature (RT) in 5% normal rabbit serum (NRS; Zymed, San Francisco, CA, USA) diluted in 0.2% Triton X-100 and azide-containing PBS under gentle movement of the incubation medium. Free-floating sections were incubated with goat anti-ChAT IgG, as primary antibody (kindly donated by Dr. L.B. Hersh [4]) diluted 1:2000 in 1% NRS and 0.2% Triton X-100 at 37°C for two days. After incubation, sections were thoroughly rinsed in PBS, preincubated for 1 h with NRS, followed by the second antibody incubation for 1.5 h at 37°C. The second antibody was rabbit anti-goat IgG (1:80; Sigma, St. Louis, MO, USA). Sections were rinsed overnight in PBS before the third incubation step (2 h at RT) in goat peroxidase-anti-peroxidase (PAP; 1:300; Dakopatts, Glosstrup, Denmark), diluted in Triton X-100-PBS. Finally, the peroxidase was visualized with 3,3′-diaminobenzidine (DAB) as chromogen (30 mg/100 ml) and 0.01% H_2O_2. Between incubation steps, brain samples immunostained for PV were always rinsed at least four times with PBS to which 0.5% Triton X-100 had been added. Prior to the first antibody incubation, sections were exposed to 0.1% H_2O_2 for 15 min and 5% normal sheep serum (NSS; Jackson, West Grove, PA, USA) for 30 min at RT. Sections were then incubated overnight at 4°C with the primary monoclonal antibody, mouse anti-PV IgG (1:2000; Sigma), which recognizes the Ca^{2+}-bound conformation of PV. After rinsing, sections were again incubated for 30 min in 5% NSS and exposed to biotinylated sheep anti-mouse IgG (1:200 in PBS, 2 h at RT; Amersham, Bucks, UK) and streptavidin-HRP (1:200 in PBS, 1 h at RT; Zymed, USA). Tissue-bound peroxidase was visualized by means of the same DAB-H_2O_2 reaction as described above. Standard control experiments were performed by omission of the primary antibodies, which did not yield any detectable immunoprecipitate (data not shown).

After a 14-day survival time silver staining of MS sections revealed extensive degenerating processes in $\beta AP_{(1-42)}$-treated animals as compared with the controls (Fig. 1A,B). Degenerating fibers were scattered throughout the MS (Fig. 1A) and they also appeared in efferent pathways (e.g. in the medial forebrain bundle; data not shown). In the control animals, only the needle-track was densely stained (Fig. 1B), revealing the effects of the operation trauma. The loss of the ChAT-ir neurons and a marked decrease in ChAT-immunoreactivity were observed after $\beta AP_{(1-42)}$ treatment, as compared with the control animals (Fig. 1C,D,E). As a result of neurodegenerative processes, the ChAT-ir neurons exhibited perikaryal shrinkage, while the degenerating and ChAT-ir processes had a beaded appearance, which is a characteristic feature of degenerating fibers (Fig. 1D). While the ChAT-ir neurons were clustered in the lateral zone and in the midline area of the MS, the PV-containing GABAergic interneurons were situated in its central portion. In spite of the fact that the central area of the MS was primarily damaged during the injection procedure, the PV-positive neurons displayed only a limited change after injection of $\beta AP_{(1-42)}$, relative to the controls (Fig. 1F,G). Alteration of the PV-ir neurites rarely occurred, and only a slight decrease in PV-immunoreactivity was observed after $\beta AP_{(1-42)}$ treatment. These changes were inconspicuous when compared to the characteristic degenerative changes in the ChAT-ir neurons.

Quantitative analysis of septal sections revealed a significant loss of ChAT-ir neurons after $\beta AP_{(1-42)}$ injections, as compared with the controls (46.71 ± 9.1 vs. 62 ± 6.4 neuron/MS, $P < 0.01$; Fig. 2). Analysis of our data revealed a slight decrease in the number of PV-ir septal neurons, albeit not significant in comparison with the controls (54.25 ± 2.9 vs. 56.63 ± 4.4 neuron/MS; Fig. 2).

The observations yielded by silver- and ChAT-immunolabelling in the present study are consistent with previous in vivo data of ourselves [13] and others [1], suggesting that βAP exert toxic effects on cholinergic neurons when injected into the rat brain. Analogous with the present results, Dong et al. [7] reported that intracere-

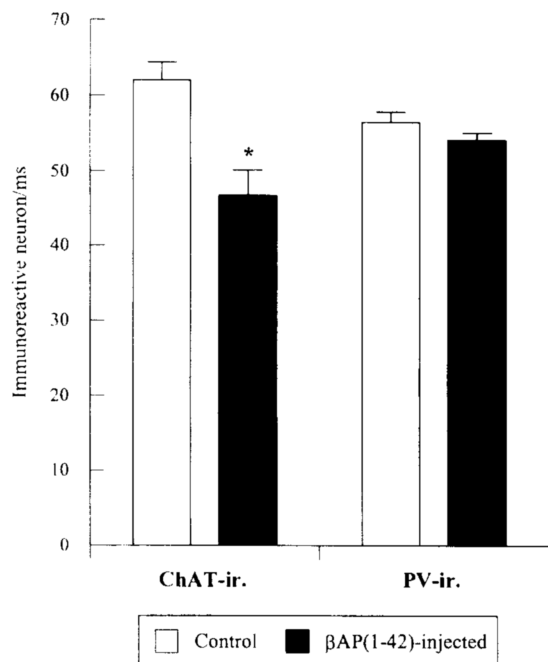

Fig. 2. Quantitative analysis of ChAT-ir and PV-ir neurons in the MS after $\beta AP_{(1-42)}$ treatment. $\beta AP_{(1-42)}$ injection decreased the number of ChAT-ir neurons significantly ($P < 0.01$), while the number of PV-ir neurons was reduced only slightly. For quantitative analysis, sections throughout the MS were collected (at the AP coordinate 0.5–0.1 mm [19]). ChAT-ir and PV-ir neurons were counted per MS ($n = 4$ animals per group in at least two adjacent sections). Data were evaluated with Student's t-test, with statistical significance defined as $P < 0.05$, with Microsoft Excel 5.0 (Microsoft Co., Redmond, WA, USA).

broventricular infusion of AF64A reduced the number of ChAT-ir neurons in the MS, while PV-ir interneurons remained unaffected. Moreover, it has recently been reported that GABAergic neurons are resistant to βAP neurotoxicity in vitro [20]. Histochemical analysis of AD brains corroborates these results, as PV-containing populations of neurons seemed to be preserved in AD [14]. For example, Ferrer et al. [8] reported that PV-ir dystrophic neurites and aberrant terminal sprouts had been found associated with senile plaques, while the density of the PV-ir neurons in the cerebral cortex remained unaffected in AD. These data support our present observations in rats, as the PV-ir neurons of the MS seemed to be less affected after $\beta AP_{(1-42)}$ treatment, although some changes occurred in the PV-ir neurites. It is interesting to speculate as to the role of the Ca^{2+}-binding protein PV in the prevention of βAP neurotoxicity. We have therefore focused our attention on the role of PV in the protection of neurons against βAP neurotoxicity, via buffering of the $[Ca^{2+}]_i$. As outlined earlier [17], βAP toxicity is mediated by alterations in the Ca^{2+} homeostasis of the neurons. In addition, the Ca^{2+} channel blocker nimodipine was able to attenuate the effects of βAP [24], suggesting the principal role of the Ca^{2+} overload in βAP-induced neurotoxicity. PV is known to regulate $[Ca^{2+}]_i$ through the active binding of free, cytosolic Ca^{2+}. This regulatory role of the Ca^{2+}-binding protein may contribute to the prevention of neurodegeneration which results form $[Ca^{2+}]_i$ overload. It is noteworthy, however, that PV-ir neurons may also be affected by $\beta AP_{(1-42)}$ but changes of PV gene expression may mask this neuronal damage by upregulation of the synthesis of the peptide.

In summary, the present report provides histochemical evidence that $\beta AP_{(1-42)}$, the major amyloid component of neuritic plaques, induces a specific cellular injury of septal cholinergic neurons, whereas the GABAergic, PV-positive neurons were relatively spared. As PV has been shown to be present within most of the GABAergic interneurons of the ms [9], our in vivo results support the observations that GABAergic neurons are resistant to βAP neurotoxicity [20]. Since the GABAergic neurons are so richly provided with the Ca^{2+}-binding protein, this characteristic supports the hypothesis of deranged $[Ca^{2+}]_i$ homeostasis in βAP induced cell damage. For that reason the cholinergic neurons in the MS are more sensitive to βAP induced damage.

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