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AMYLOID-β1-42 TREATMENT DOES NOT HAVE A SPECIFIC EFFECT ON CHOLINERGIC NEURONS IN *IN VITRO* BASAL FOREBRAIN NEURONAL CULTURES OF RAT

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The neurotoxic effect of amyloid-beta peptide (1-42) was investigated in cultures of neuronal tissue derived from the basal forebrain of embryonic rat. The axonal varicosities of the cholinergic cells were revealed by vesicular acetylcholine transporter staining, and the axonal varicosities in general by synaptophysin immunohistochemistry. The results demonstrate that the treatment of *in vitro* neuronal cultures with 20 μM amyloid-beta peptide (1-42) for 2 days on day 5, 12 or 15 exerted a neurotoxic effect on both the cholinergic and the non-cholinergic neurons. In the same cultures, the absolute number of synaptophysin-positive axon varicosities was reduced to greater extent (control: 203 ± 37/field vs treated: 101 ± 16/field) than the number of vesicular acetylcholine transporter-immunoreactive (control: 48 ± 4/field vs treated: 0/field) structures. It is concluded that amyloid-beta peptide (1-42) does not have a specific effect only on the cholinergic neurons, but affects non-cholinergic neurons as well.

Keywords: Amyloid-beta peptide – neurotoxicity – neuronal cultures – vesicular acetylcholine transporter – synaptophysin

INTRODUCTION

A cholinergic deficit is one of the major features of Alzheimer's disease (AD), where the hypofunction may be caused by amyloid-beta peptide (Aβ). Such a neurotoxic effect of Aβ has been demonstrated both in *in vivo* experiments [10] and *in vitro,* using neuronal cultures [11, 13, 15, 20].

The nervous system functions are dependent on the proper working of the synaptic apparatus. Diverse synaptic proteins such as synaptophysin (SYN) and the vesicular acetylcholine transporter (VAChT) that are distributed in various subcellular compartments in the axons and axon terminals may regulate the neurotransmission at the synaptic sites. In AD, the cholinergic deficit is well documented. It may be due to presynaptic alterations in the synthesis (choline acetyltransferase, ChAT) and the reduced release of acetylcholine (ACh), the decreased presence of receptors for ACh

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and the relative increased activity of acetylcholinesterase (AChE). VAChT is unique for the cholinergic neurons [2, 7]. It mediates the accumulation of ACh in the synaptic vesicles [3], which is largely built up from SYN. Reduced levels of these proteins may effectively influence the appropriate functioning of the synapse.

The cholinergic neurotransmission in the brain depends not only on the activities of ChAT and AChE, but also on the adequacy of the VAChT function and on the intactness of the synaptic vesicles, from which most of the ACh can be released. It remains to be elucidated how these proteins are damaged by the neurotoxic Aβ1-42 in the various transmitter-containing neurons. Through the use of [^{3}H]vesamicol quantitative autoradiography, the reduction of VAChT has been demonstrated in Aβ-infused rats [8]. Our present aim therefore was to shed more light on the *in vitro* effects of Aβ1-42 on the VAChT and SYN-immunoreactive structures present in the axon varicosities and axon terminals in cultures of neuronal tissue derived from embryonic rat basal forebrain.

MATERIALS AND METHODS

Materials

The substances used in this study were trypsin, Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) from Gibco; poly-L-lysine, normal sheep serum, normal rabbit serum and 3,3'-diaminobenzidine. 4HCl tetrahydrochloride from Sigma-Aldrich (St. Louis, MO, USA); polyclonal anti-synaptophysin (902314) from Boehringer (Mannheim, Germany); polyclonal anti-VAChT antibody (AB1578) from Chemicon International Inc. (Temecula, CA, USA); sheep-anti-mouse IgG-biotin and rabbit-anti-goat IgG-biotin from Jackson Immunores. Lab. (West Grove, PA, USA); and streptavidin-horseradish peroxidase from Zymed Laboratories Inc. (South San Francisco, CA, USA). All other reagents were of either laboratory or analytical grade from various suppliers.

Tissue cultures

Primary rat embryonic basal forebrain cultures were established via the protocol previously described [12]. In brief: the basal forebrain was dissected from the brains of embryonic rat pups on day 18 (E18), incubated in 0.25% trypsin for 10 min and dissociated by gentle trituration, and the suspension was settled for 10 min at 1000 × *g*. After resuspension, the cells were seeded at a density of $3.5–4.0\times10^{-4}$ cells/cm^2 on poly-L-lysine-coated glass coverslips in DMEM supplemented with 10% (vol/vol) FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml). The neuronal cultures were grown in a humidified incubator at 37 °C in 5% CO_2.

Treatment of tissue cultures

Human Aβ1-42 (synthesized in the Department of Chemistry at our University) was dissolved, and the cultures were treated after dilution in DMEM. The Aβ1-42 (20 μM Aβ1-42) was added to cultures on DIV5, DIV12 or DIV15 and the treated samples were cultivated for a further 2 days in a serum-free condition. Control cultures were maintained under similar conditions, but supplemented with the vehicle alone. The numbers of stained varicosities and/or axon terminals were monitored on DIV7, DIV14 or DIV17.

Synaptophysin immunohistochemistry

For identification of axonal varicosities and axonal terminals, the tissue cultures were immunostained for SYN. After fixation, the control and Aβ1-42-treated samples were incubated with 2.5% Triton X-100, and then with 5% normal sheep serum in phosphate buffer. The polyclonal antibody against SYN (1 : 1000) was applied for 48 h. This was followed by incubation in sheep-anti-mouse IgG-biotin (1 : 1000) for 1.5 h, and then in streptavidin-horseradish peroxidase (1 : 1000) for 1.5 h. The peroxidase reaction was developed by using 3,3'-diaminobenzidine tetrahydrochloride and $NiCl_2$.

Vesicular acetylcholine transporter immunohistochemistry

The cholinergic neurons and their axonic varicosities and axon terminals in the cultures were evaluated by immunohistochemistry for VAChT. After fixation, the control and the Aβ-treated neuronal cultures were incubated with 2.5% Triton X-100, and then with 5% normal rabbit serum in phosphate buffer. The polyclonal antibody against VAChT (1 : 32000) was applied for 48 h. This was followed by incubation in rabbit-anti-goat IgG-biotin (1 : 1000) for 1.5 h, and then in streptavidin-horseradish peroxidase (1 : 1000) for 1.5 h. The peroxidase reaction was developed by using 3,3'-diaminobenzidine tetrahydrochloride and $NiCl_2$.

Image analysis

The cholinergic neurons and the numbers of VAChT and SYN-immunoreactive axon varicosities and/or axon terminals in the cultures were evaluated by image analysis. The number of immunoreactive axonal varicosities was determined with a Quantimet 500 MC Image Analysis System (Leica Cambridge) linked to a JVC-Color camera mounted on a Leica Laborlux "S" Leitz microscope.

The immunohistochemical reactions in the axonal varicosities were visualized and the digitized image (256 grey levels) was displayed on a colour monitor with

1024×768 pixel resolution. The neurotoxic effect of Aβ1-42 on the immunohistochemical staining was evaluated. Randomly selected areas of the control and treated cell cultures on coverslips were taken from 5 fields per cover slip. The numbers of immunoreactive axon varicosities in the control and the Aβ1-42-treated samples were counted. All measurements were made under the same optical light conditions.

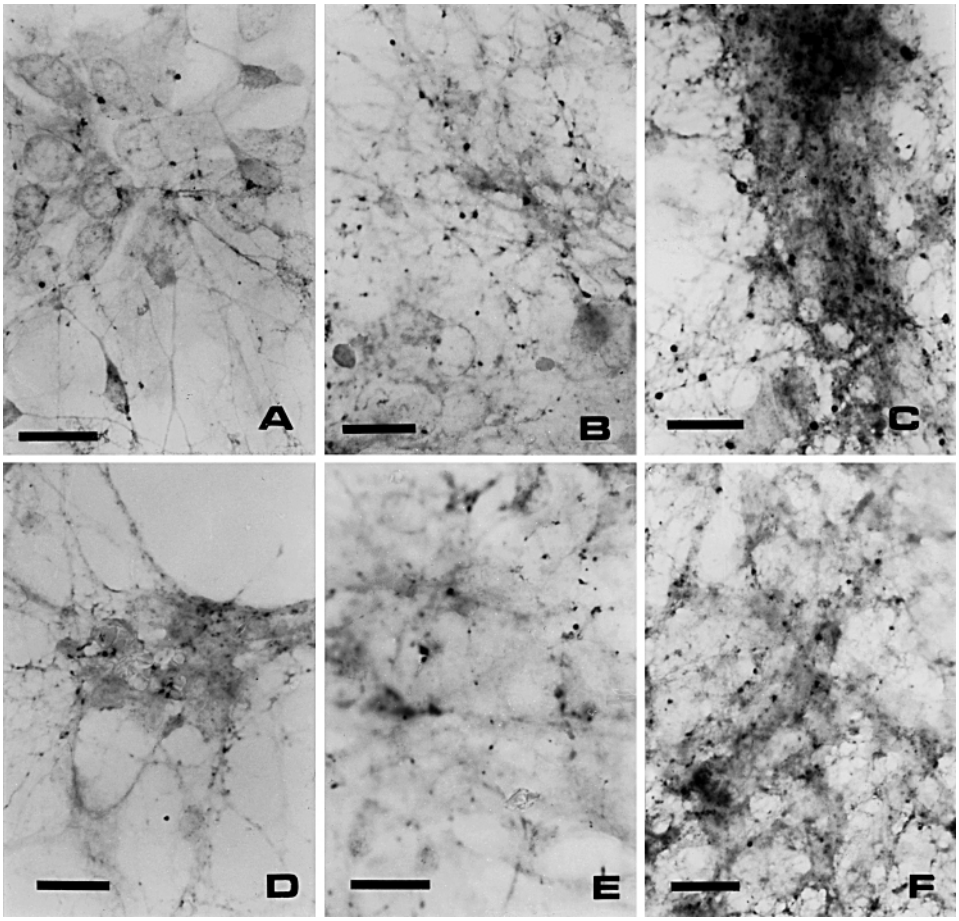

Fig. 1. Light microscopic localization of synaptophysin immunoreactivity in the axon varicosities and in the axon terminals during development in the control basal forebrain neuronal cultures. Note the increased density of staining after the various time intervals. A: DIV7, B: DIV14 and C: DIV17. The synaptophysin-immunoreactive axon varicosities are reduced in the cultures after treatment with 20 μM Aβ1-42 for 2 days (E: DIV7, F: DIV14 and G: DIV17). Bar = 20 μm

RESULTS

Effects of amyloid-beta peptide on the number of synaptophysin-positive axon terminals

In control tissue cultures, the SYN-positive immunoreactive axon varicosities and the axon terminals could be revealed diffusely distributed among neurons of various sizes. They were present on the soma of the neurons and closely related to some dendrites. The SYN-immunopositive structures differed considerably in size. The number of axonal varicosities increased as the duration of cultivation progressed (Fig. 1A–C). After 20 μM Aβ1-42 treatment of the basal forebrain tissue cultures for 2 days, the numbers of SYN-positive structures were diminished on DIV7, DIV14 and DIV17 (Fig. 1D–F).

For quantitative determination, the numbers of SYN-positive axon varicosities in the control and the Aβ-treated samples of the cultures were investigated by means of image analysis at various times (DIV7, DIV14 and DIV17). The results of the analyses of the control and Aβ-treated samples are summarized in Fig. 2.

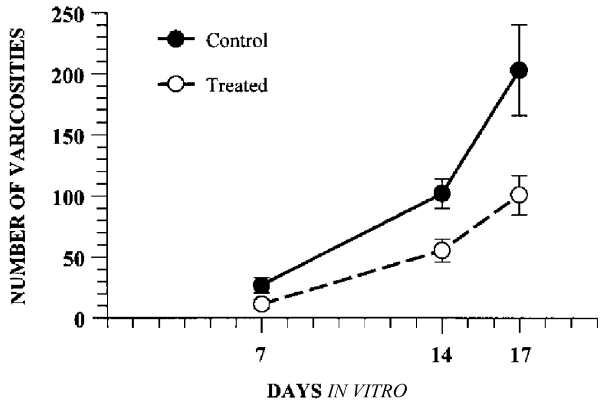

Fig. 2. Evaluation of synaptophysin-positive axon varicosities and/or axon terminals by image analysis in control and Aβ1-42-treated basal forebrain tissue cultures. The control samples were treated with the vehicle, and the other *in vitro* cultures with 20 μM Aβ1-42 for 2 days on days 5, 12 or 15

Effects of amyloid-beta peptide on the number of vesicular acetylcholine transporter-positive axon terminals

In the control tissue cultures, besides the various types of cholinergic neurons (bipolar and multipolar), the VAChT-immunoreactive axon varicosities and axon terminals were distributed similarly to the SYN (Fig. 3A–C), but their number was much lower, as revealed by qualitative analysis of the stained samples. The immunoposi-

tive axon varicosities were among the VAChT-negative cell bodies and near the positively stained neuronal perikarya. VAChT immunoreactivity could also be revealed mainly as puncta and to a lesser extent as diffuse staining in neurons of different sizes.

Two days after 20 μM Aβ1-42 treatment, the VAChT immunoreactivity in the axon varicosities in the DIV7, DIV14 and DIV17 samples was dramatically reduced or even eliminated (Fig. 3D–F). Evaluation of the VAChT-positive axon varicosities and axon terminals in the control and Aβ1-42-treated samples by means of image analysis is illustrated in Fig. 4.

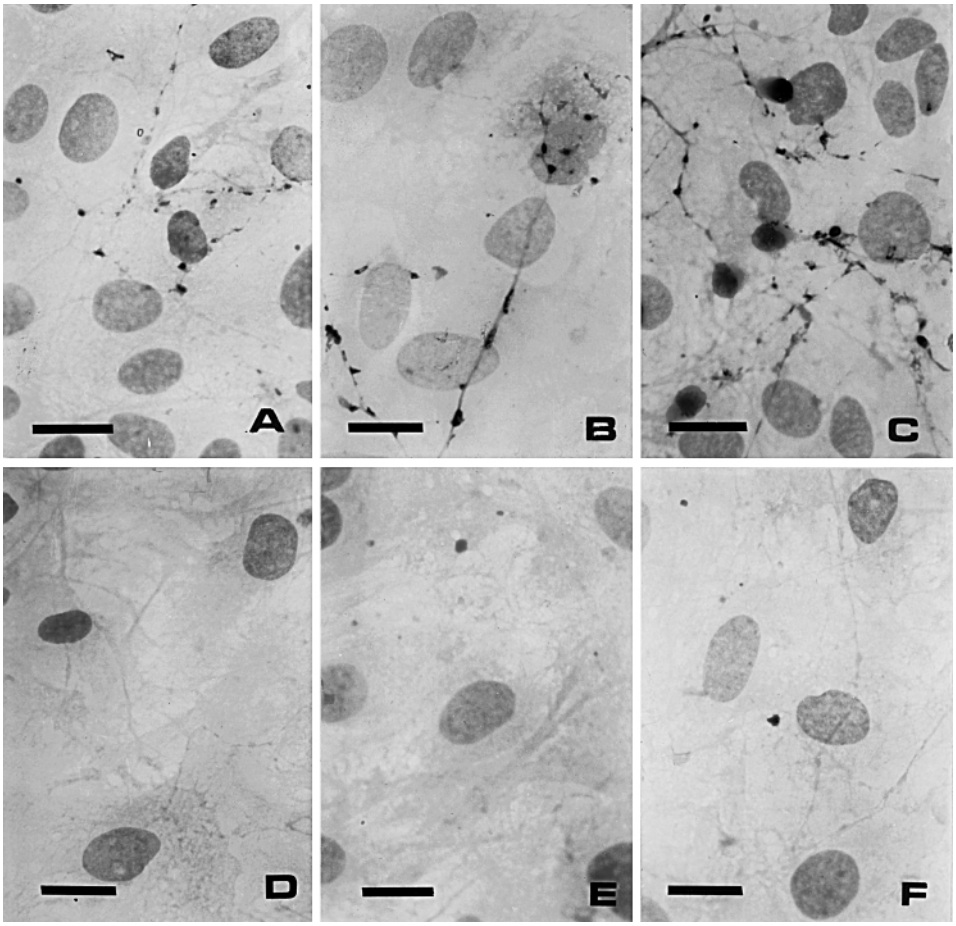

Fig. 3. Light microscopic localization of vesicular acetylcholine transporter immunoreactivity in the cholinergic axon varicosities and in the axon terminals. Note that the number of small and large labelled puncta increase during the *in vitro* development of cholinergic neurons (A: DIV7; B: DIV14 and C: DIV17). After treatment with 20 μM Aβ1-42 for 2 days, the vesicular acetylcholine transporter immunoreactivity disappears in the basal forebrain tissue culture (E: DIV7; F: DIV14 and D: DIV17). Bar = 20 μm

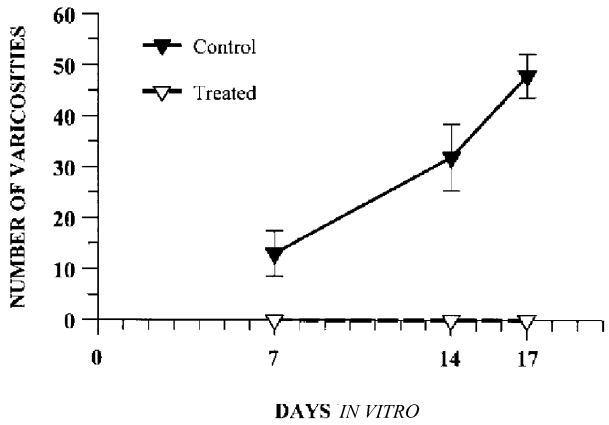

Fig. 4. Evaluation of vesicular acetylcholine transporter-positive axon varicosities and/or axon terminals by image analysis in control and Aβ1-42-treated basal forebrain tissue cultures. The control samples were treated with vehicle, and the other *in vitro* cultures with 20 μM Aβ1-42 for 2 days on days 5, 12 or 15

DISCUSSION

The results presented here support earlier findings that VAChT immunohistochemistry is a unique and useful means for the demonstration of cholinergic neurons, their axon varicosities and axon terminals both in the adult central and peripheral nervous systems [2, 18] and during development in tissue cultures [14].

Our immunohistochemical study resulted in three principal findings. First, various types of cholinergic neurons (small bipolar and larger multipolar) can be revealed in the primary neuronal cell culture. Second, the VAChT-positive axon varicosities are more sensitive than the SYN immunoreactive neuronal structures to this Aβ treatment. Third, the use of embryonic basal forebrain neuronal tissue cultures and their treatment with the neurotoxic Aβ provides a good *in vitro* cellular model with which to investigate the effects of various chemical agents on the cholinergic neurons and also to study the pathomechanism related to AD.

One of the primary features of AD is a cholinergic deficit, which may be caused by the neurotoxic Aβ. A reduction in the cholinergic terminal integrity has been demonstrated through use of a vesamicol analogue [^{125}I]MIBT [5], which is known to block the uptake of ACh into the cholinergic synaptic vesicles [1]. It has been suggested that the demonstration of VAChT can be a useful marker for assessment of the loss of cholinergic structures in AD. Indeed, the VAChT immunoreactivity in the axon varicosities furnishes a good possibility to detect pathophysiological alterations in the cholinergic neurons. Such alterations, as demonstrated here, can be induced not only under *in vivo* circumstances, but *in vitro* as well.

The toxic effect of Ab has already been demonstrated at micromolar concentrations in several tissue models [see refs 6, 16, 19]. More recently, however, Blusztajn

and Berse [4] reported that synthetic Aβ at submicromolar concentrations does not cause cytotoxicity, but rather reduces the expression of cholinergic markers in neuronal cells. This is supported by an earlier experiment in which we observed that Aβ affects the cholinergic neurons within 2 h in primary neuronal cultures [15]. At present, however, we are unable to decide whether the neurotoxic effect or the reduced expression of the cholinergic marker proteins was revealed in our experiments. The neurotoxic effect has been revealed in an *in vivo* experiment, demonstrating that Aβ1-42 inhibits the fast axonal transport of various types of proteins (including VAChT) in the sciatic nerve of rat [9].

A reduced level of presynaptic vesicle protein SYN occurs in the hippocampus of individuals with AD [17]. In our tissue culture model, a reduction in the SYN-immunoreactive structures was similarly observed after Aβ1-42 treatment. However, the decrease in the SYN staining is probably due not only to a reduction in the cholinergic axonal varicosities, but rather to the neurotoxic effect of Aβ on other neurotransmitter-containing neurons, too. This is supported by experimental evidence. Pákáski et al. [12] demonstrated that Aβ1-42 *in vitro* not only affects the AChE-positive neurons, but also a neurodegenerative effect on GABAergic neurons. Further studies are required to clarify whether the reduction in the SYN-positive axon varicosities is due to the degeneration of cholinergic and GABAergic neurons or the degeneration of other transmitter containing neurons. Such investigations are currently in progress in our laboratory.

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