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Research report

Effects of amyloid-beta on cholinergic and acetylcholinesterase-positive cells in cultured basal forebrain neurons of embryonic rat brain

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Abstract

The neurotoxic effects of amyloid-β_{1-42} and amyloid-β_{25-35} (Aβ) on cholinergic and acetylcholinesterase-positive neurons were investigated in primary cultures derived from embryonic 18-day-old rat basal forebrain. After various time intervals, the cultures were treated with 1, 5, 10 or 20 μM Aβ for different time periods. The cholinergic neurons and their axon terminals were revealed by vesicular acetylcholine transporter immunohistochemistry and the cholinoceptive cells by acetylcholinesterase histochemical staining. To assess the toxic effects of these Aβ peptides on the cholinergic neurons, image analysis was applied for quantitative determination of the numbers of axon varicosities/terminals and cells. The results demonstrate that, following treatment with 1 or 5 μM Aβ for 5, 10, 30, 60 or 120 min, no changes in vesicular acetylcholine transporter immunohistochemical staining were observed. However, after treatment for 30 min with 10 or 20 μM Aβ, the number of stained axon varicosities was reduced, and treatment for 2 h they had disappeared. In contrast, vesicular acetylcholine transporter-positivity could be seen in some of the neuronal perikarya even after 3 days after treatment. The acetylcholinesterase staining was homogeneously distributed in the control neurons. After Aβ treatment, the histochemical reaction end-product was detected in some of the neuronal perikarya or in the dendritic processes near to the soma. It is concluded that the neurotoxic effects of Aβ appear more rapidly in the cholinergic axon terminals than in the cholinergic and acetylcholinesterase-positive neuronal perikarya.

Theme: Disorders of the nervous system
Topic: Neurotoxicity

Keywords: Acetylcholinesterase; Alzheimer's disease; Amyloid-β; Neuronal culture; Neurotoxicity; Vesicular acetylcholine transporter

1. Introduction

There is considerable neurochemical and morphological evidence that amyloid-β_{1-42} ($A\beta_{1-42}$) plays a significant role in the pathogenesis of Alzheimer's disease (AD) (Ref. [44] and for reference, see Ref. [8]). It has also been shown that one of the primary features of AD is a cholinergic deficit (for reference, see Ref. [24]). In this devastating illness, the disturbance in the cholinergic neuronal transmission may be due to alterations in the elements of the cholinergic system (choline acetyltransferase, vesicular acetylcholine transporter (VAChT), acetylcholine (ACh), ACh receptors, and acetylcholinesterase (AChE). VAChT is present in the cholinergic nerve cells [15,41–43,52] and mediates the accumulation of ACh in the synaptic vesicles of the cholinergic neuron axon terminals. The cholinergic neurotransmission in the brain is therefore dependent on the appropriate functioning of the elements of the cholinergic system, including VAChT and AChE. A reduced cholinergic transmission is implicated in the memory impairment and cognitive dysfunction in AD.

Earlier studies revealed degeneration of neuronal perikarya as an important neuropathological hallmark of the disease. In AD, however, the most important early lesions may occur at the synaptic sites [33–35]. Loss of synapses may account for all the symptoms observed in AD [50]. Indeed, it has been demonstrated that the cellular substrate of AD pathology is synapto-axonal, and the loss of axon

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terminals and synaptic contacts is more important than the perikaryonal degeneration. Small et al. [47] suggested that in AD more attention should be paid to the effects of $A\beta_{1-42}$ on the synaptic function rather than on cell death.

The neurotoxic effects of Aβ and some of its fragments have been demonstrated in in vitro primary cultures from human [7] and rat cortical neurons [40], in hippocampal slice cultures [18] and following in vivo injection into various areas of the rat [28] and monkey brain [14]. The results demonstrate that various types of neurons can be affected by Aβ. In in vitro tissue cultures, the cytotoxic effect has been demonstrated for GABAergic cells [37]. There are no detailed data, however, on the toxic effects of $A\beta_{1-42}$ and its fragment $A\beta_{25-35}$ on the VAChT-immunoreactive cholinergic neurons, on their axon terminals or on the cholinoceptive AChE-positive neurons. To address this question, the effects of these peptides on VAChT-positive and AChE-stained neurons were studied in basal forebrain (BF) primary cultures derived from embryonic rat brain.

2. Materials and methods

2.1. Materials

The substances in this study were polyclonal anti-VAChT antibody (AB1578) from Chemicon International Inc. (Temecula, CA, USA); $A\beta_{1-42}$ (synthesized in the Department of Chemistry at our University); $A\beta_{35-25}$ from Bachem (Bubendorf, Switzerland), acetylthiocholine chloride, $A\beta_{25-35}$, ethopropazine hydrochloride, poly-L-lysine, normal sheep serum, normal rabbit serum, fluorescein diacetate (FDA), propidium iodide (PI); Triton X-100, and 3,3′ -diaminobenzidine.4HCl from Sigma (St. Louis, MO, USA); trypsin, Dulbecco's modified Eagle's medium (DMEM), Neurobasal™ Medium, N2 Supplement, B27 Supplement and fetal bovine serum (FBS) from GIBCO™ (Grand Island, NY USA), sheep-anti-mouse IgG-biotin and rabbit-anti-goat IgG-biotin from Jackson Immunores. Lab. (West Grove, PA, USA); streptavidin-horseradish peroxidase from Zymed Laboratories (South San Francisco, CA, USA); and Histoclear from National Diagnostics (Atlanta, GA, USA). All other reagents were of either laboratory or analytical grade from various suppliers.

2.2. Neuronal cultures

All animal work carried out in these experiments was in accordance with the ethical guidelines for animal investigations of the Hungarian Ministry of Welfare.

Sprague–Dawley rats (bred in-house) were used in the experiments. Embryonic rat BF primary neuronal cultures were established according to data published previously [37]. In brief: the BF area was dissected from the brains of rat pups on embryonic day 18 (E18), incubated in 0.25% trypsin for 10 min and dissociated by gentle trituration. The suspension was settled for 10 min at $1000 \times g$. After resuspension, the cells were seeded at a density of $3.5–4 \times 10^{-4}$ cells/cm^2 on glass coverslips previously treated with 20 μg/ml poly-L-lysine. The neurons were cultured for the first 12 h in DMEM supplemented with 10% (vol/vol) FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml). After this period, the medium was changed to neurobasal medium supplemented with 2% B27. The neuronal cultures were grown in this medium in a humidified incubator at 37 °C in 5% CO_2 until treatment.

2.3. Treatment of neuronal cultures

Human $A\beta_{1-42}$ and its fragment $A\beta_{25-35}$ were dissolved in neurobasal medium supplemented with 1% N_2 for the treatment of cultures for various numbers of days in vitro (DIV). Since the peptides were not aged before application, their neurotoxic effect may be attributed to the soluble form rather than to the aggregated form. Such cultures were used to study the effects of the peptides on the VAChT immunoreactivity in the cholinergic neurons, and on the AChE histochemical staining of the cholinoceptive neurons. As the results obtained with $A\beta_{1-42}$ and $A\beta_{25-35}$ were similar, in the description only Aβ will be used to designate the peptides. The VAChT immunoreactivity was investigated on DIV7 in the controls and the samples, with the use of various doses (1, 5, 10 or 20 μM) of Aβ for 5, 10, 30, 60 or 120 min.

Other cultures were subjected to treatment with 20 μM Aβ on DIV14 for 3 days. On DIV17, some of the samples were incubated for 2 h. In this series of experiments, untreated samples and samples subjected to treatment for either 2 h or 3 days were stained for VAChT immunoreactivity. AChE histochemistry was also carried out on cultures treated similarly. The control samples were supplemented with 20 μM inactive reverse peptide $A\beta_{35-25}$.

The neurotoxicity of Aβ is revealed as morphological alterations in the cells, a reduction in the number of VAChT-positive varicosities, a diminution in the AChE staining and an increase in the number of PI-positive neurons.

2.4. VAChT immunohistochemistry

The VAChT-positive neurons and their axonic varicosities/terminals in the cultures were evaluated by immunohistochemistry. After fixation for 15 min in 4% paraformaldehyde solution, the appropriate control and the Aβ treated neuronal cultures (DIV5, DIV7, DIV14 and DIV17) were incubated for 30 min 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 in 0.05% 3,3′ -diaminobenzidine.4HCl, 0.3% $NiCl_2$ and 0.03% H_2O_2 in 0.05 M Tris–

HCl buffer (pH 7.6). After dehydration in alcohols and defatting in Histoclear, the samples were coverslipped in Histomount. The samples were counterstained with Safranin for better identification of the cellular structures.

2.5. AChE histochemistry

To study AChE positivity, a sensitive method [48] was used in a slightly modified form [25]. After fixation (described above), the neuronal culture samples (DIV5, DIV7, DIV14 and DIV17) were rinsed several times in 0.1 M sodium maleate buffer (pH 8.0). They were then pretreated for 15 min at room temperature in 2×10^{-4} M ethopropazine hydrochloride, rinsed in 0.1 M sodium maleate (pH 8.0) and incubated for 60 min in a medium consisting of 1.8 mM acetylthiocholine chloride, 0.1 M sodium citrate, 0.03 M copper sulfate, 0.005 M potassium ferricyanide and 0.1 M sodium maleate (pH 8.0). The reaction end-product was visualized by using 0.05% 3,3′ -diaminobenzidine.4HCl, 0.3% $NiCl_2$ and 0.03% H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.6). After dehydration in alcohols and defatting in Histoclear, the samples were coverslipped in Histomount. Safranin overstaining was applied for better visualization of the various cellular structures.

2.6. Double staining for acetylcholinesterase and vesicular acetylcholine transporter and treatment with Aβ

The E18DIV12 samples were developed first for AChE, which was followed by VAChT immunohistochemistry as described above. For the VAChT staining, the peroxidase complex was visualized by incubating the samples in a DAB-$NiCl_2$ mixture. After double staining, a brownish colour could be detected at the site of the AChE; a brownish colour intermingled with a bluish-black indicates the localization of AChE and VAChT, bluish-black staining appearing where the VAChT reaction predominates.

To ascertain the effects of Aβ on the cholinergic-cholinoceptive neurons, tissue cultures were treated with 20 μM Aβ for 2 h, 2 or for 3 days. After treatment, the samples were subjected to AChE histochemistry and VAChT immunohistochemistry as described above. The samples were thereafter mounted, air-dried, dehydrated in an ethyl alcohol series, cleared in Histoclear, and finally coverslipped by using Histomount. Photos were taken under normal light field microscopy conditions (Nikon Microphot-FXA microscope).

2.7. Image analysis

The number of VAChT-immunoreactive axon varicosities/terminals and neurons in the BF cultures were evaluated with a Quantimet 500 MC Image Analysis System (Leica Cambridge) connected to a JVC-Color camera mounted on a Leica Laborlux "S" Leitz microscope. The immunohistochemical reactions in the axonal varicosities and cells were visualized, and the digitized image (256 gray levels) was displayed on a color monitor with 1024 × 768 pixel resolution. The axon varicosities were determined from 5-field/cover slips in randomly selected areas (25,750 μm^2/area, 1 pixel = 0.296 μm), while the numbers of stained cells were counted in larger areas (103,000 μm^2/area, 1 pixel = 0.593 μm) in control and $A\beta_{25-35}$ treated samples.

2.8. Neuronal viability studies

To study the neurotoxic effects on the cell bodies, BF cultures were exposed on DIV12 to 20 μM Aβ for 2 h or for 1, 2 or 3 days. Control samples were treated with a reverse Aβ peptide ($A\beta_{35-25}$). The neurotoxicity of Aβ was assessed by the use of FDA and PI staining [21]. FDA is taken up by viable (living) cells, while PI labels only dead/dying cells. After the various time intervals, the neurons were exposed for 5 min to PI (5 μg/ml) and FDA (15 μg/ml) as described [6]. The double-stained cultures were examined in an epifluorescence microscope (Microphot FXA, Nikon, Japan) at wavelengths appropriate for each fluorophore. The randomly selected areas (73,125 μm^2) were analyzed for live/dead cell counting. During the counting of the PI-positive cells, the FDA fluorescence faded in the same area; accordingly, the numbers of FDA-positive neurons were determined in a neighbouring area. The number of FDA-positive neurons was expressed as a percentage of the total number of stained cells. In each case, the number of PI-positive cells found in the appropriate control samples was subtracted.

2.9. Statistical evaluations

All evaluations were made under the same conditions. Results are expressed as means ± S.E.M.

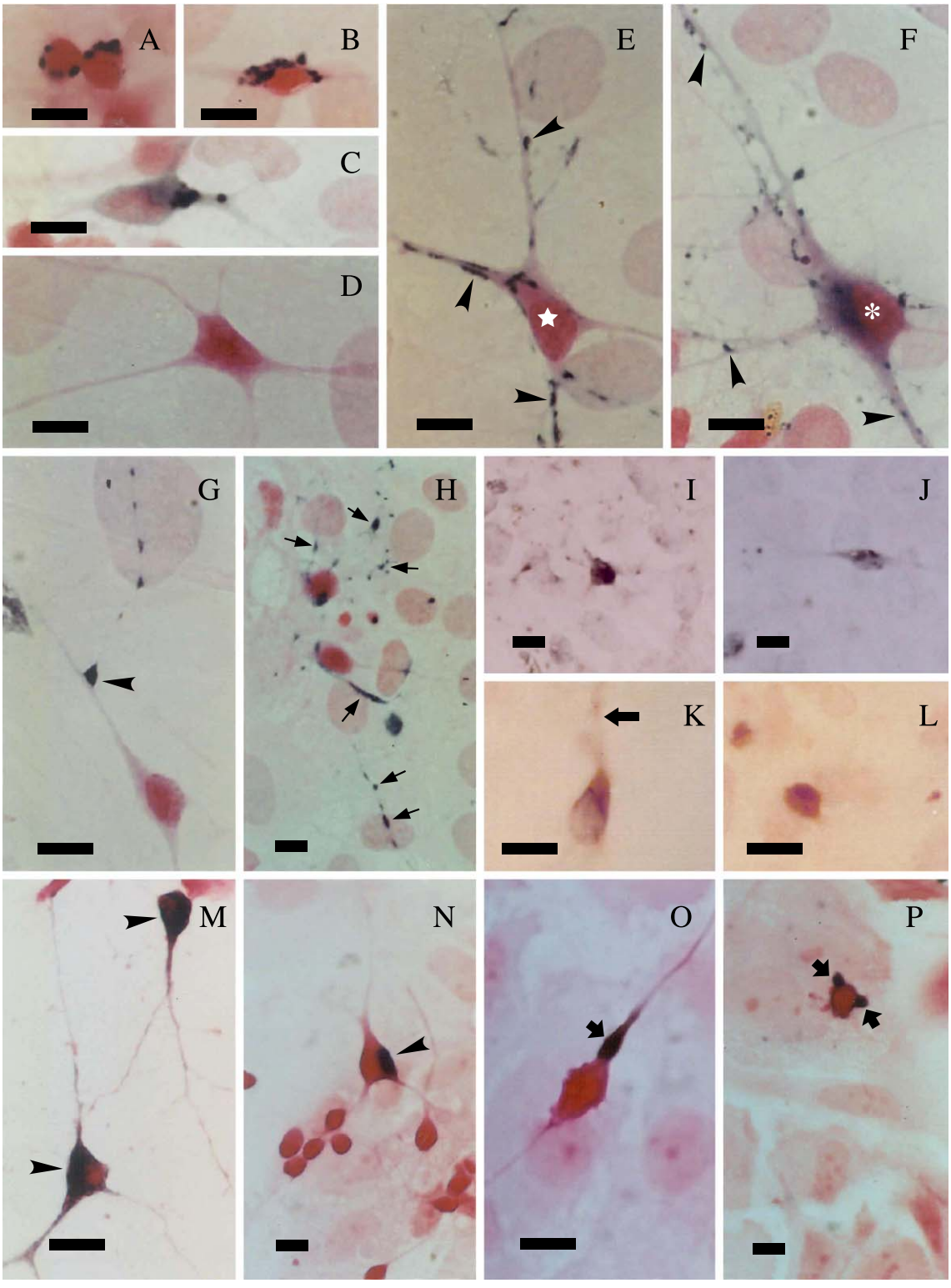
3. Results

3.1. Demonstration of VAChT-positive axon varicosities/terminals and the effect of Aβ

In the control cultures, no stained axon varicosities could be detected on DIV5. However, at this time the immunoreactivity first appeared within the cell body in the neurons of various sizes (10–20 μm). The staining was observed as small puncta and large dots. In the large multipolar neurons (20–30 μM), a diffuse staining was also evident. The VAChT immunopositivity appeared in the perikarya of some small round (Fig. 1A), bipolar (Fig. 1B) and multipolar (Fig. 1C) cells, while most of the small and large neurons were immunonegative (Fig. 1D). No staining could be revealed in the dendrites. By DIV7, VAChT-immunoreactive axon varicosities/terminals were present in the neuropil. Such immunopositive structures contacted the soma and dendrites of the immunonegative (Fig. 1E) and immunopositive (Fig.

1F) multipolar cells. In other cases, small bipolar neurons were innervated on their cell body (not demonstrated) or primary dendrites by VAChT-positive axon terminals (Fig. 1G). Most of the axon varicosities appeared scattered in the neuropil and among the neurons, without any sign of innervation of the neurons (Fig. 1H).

The numbers of cholinergic axon varicosities in the control and Aβ-treated samples were quantified by image analysis. The data revealed that the number of VAChT-positive varicosities increased continuously during development from DIV7 ($135 \pm 17/0.1\ mm^2$) to DIV17 ($320 \pm 27/0.1\ mm^2$).



When E18DIV7 samples were treated with 1 or 5 μM Aβ for 5, 10, 30, 60 or 120 min no changes were observed in the VAChT staining in the axon varicosities. Similarly, there were no alterations in the immunohistochemical reaction after treatment with 10 μM Aβ for 5 or 10 min. However, treatment with 10 μM Aβ for 30 min reduced (Fig. 1I) and that with 20 μM Aβ greatly attenuated the VAChT reaction in the axons (Fig. 1J). A longer incubation time with the peptide reduced the VAChT staining further. The results of these experiments, evaluated under microscope, are presented in Table 1.

After treatment with 20 μM Aβ for 2 h, nearly all the stained axon varicosities/terminals had disappeared, but staining was still observed in the neuronal perikarya (Fig. 1K). Similar VAChT staining was detected in the cell bodies when the cultures were treated with Aβ for up to 3 days (Fig. 1L).

Table 1
Microscopic evaluation of the effects of Aβ on the VAChT immunohistochemical staining in E18DIV7 basal forebrain neuronal tissue cultures

Dose of $A\beta_{1-42}$	Duration of treatment				
	5 min	10 min	30 min	60 min	120 min
0 μM	+	+	+	+	+
1 μM	+	+	+	+	+
5 μM	+	+	+	+	+
10 μM	+	+	↓	↓↓	↓↓
20 μM	+	+	↓↓	↓↓	–

The effects of various doses of $A\beta_{1-42}$ and times of treatment on the VAChT immunhistochemical staining are indicated: no effect: +; slightly reduced: ↓; greatly attenuated: ↓↓; disappeared: –.

3.2. Effect of Aβ on the number of VAChT-positive neurons

The dramatic effect of Aβ on the cholinergic axon varicosities/terminals prompted us to study the effects of Aβ on the number of VAChT-positive neurons. The number of stained perikarya was determined by image analysis on DIV14 and DIV17 in the control samples and after treatment with 20 μM Aβ for 2 h or for 2 days. Three areas (0.1 mm^2/area) were randomly selected at each time period and the stained cells were counted. The results showed that, in contrast with the axon terminals, the number of VAChT-immunopositive neurons was not decreased after treatment for either 2 h or 2 days. The variation in the number of stained neurons between DIV7 ($26 \pm 8/0.1$ mm^2) and DIV17 ($25 \pm 7/0.1$ mm^2) was insignificant (Fig. 2). The ratio of the unstained neurons ($225 \pm 32/0.1$ mm^2) and VAChT-positive cells ($25 \pm 7/0.1$ mm^2) was 9:1. This suggests that the cholinergic cells did not dominate in this primary neuronal culture derived from the BF of rat on E18.

3.3. Effect of Aβ on the AChE distribution

The localization of AChE activity in primary cultures of various types of neurons is well documented in earlier papers [17,19,45]. Here, we merely discussed the alterations caused in the histochemical staining on DIV5, DIV7, DIV14 and DIV17 by 20 μM Aβ treatment for 2 h or for 2 days.

In the control cultures the AChE staining was homogeneously distributed in the neuronal cell body and dendrites (Fig. 1M). After treatment for 2 h, no alteration in AChE staining could be discerned either in the neuronal perikarya or in their processes. However, the 2-day Aβ treatment resulted in the disappearance of most of the AChE positivity from the cell body and the dendrites (Fig. 1N). In some neurons, AChE staining could be observed mainly in the proximal parts of the dendrites (Fig. 1O). When a sample was treated at DIV14 for 3 days, the neurons underwent (apoptotic?) degeneration and AChE positivity was only in the remnants of the dendritic processes (Fig. 1P).

3.4. Demonstration of AChE and VAChT and the effects of Aβ on these double-stained neurons

To shed light on the subpopulation of VAChT-positive neurons related to the cells histochemically stained for AChE, double staining was carried out. E18DIV12 tissue cultures were stained first for the histochemical demonstration of AChE-positive cells, and thereafter for VAChT immunoreactivity. In these double-stained cholinergic-cholinoceptive cells, the presence of AChE was revealed as a brownish colour, while the VAChT reaction appeared as bluish-black staining. On the dendritic surface of the multipolar neurons, a large number of dots of various sizes were present; these reflect the VAChT-positive axon terminals (Fig. 3A). When such a neuronal culture was treated with 20 μM Aβ for 2 h, a large number of the VAChT axon varicosities disappeared from the surface of the dendrites (Fig. 3B). After treatment for 2 days, no stained axon varicosities could be detected, but the immunoreactivity

Fig. 1. Immunohistochemical demonstration of VAChT in basal forebrain cultures of embryonic rat brain (A–J). The reaction end-product is present in the perikarya of small round (A), bipolar (B) and larger multipolar (C) neurons. Other multipolar neurons are free of VAChT staining (D). After DIV7, the immunoreactivity appears on the axon terminals (arrowheads), which innervate a cholinoceptive neuron (white star) (E). In other instances, dendrites of a large multipolar cholinergic cell (white asterisk) (F) and a cholinoceptive bipolar neuron (G) are innervated by VAChT-positive axon terminals (arrowheads). In (H), the immunostained axon varicosities (arrows) are scattered among the various types of neurons. Treatment with 10 μM Aβ for 30 min reduced (I), 20 μM Aβ greatly attenuated the staining in the axons (J). After amyloid-β treatment for 2 h, the VAChT-positive synaptic terminals have disappeared from the dendrite (arrow) and weak staining can be observed in the neuronal perikaryon (K). Amyloid-β treatment for 2 days further reduces the staining and size of the immunostained cell (L). AChE histochemical staining of the cells in the basal forebrain cultures of embryonic rat brain (M–P). Note the presence of the reaction end-product in the perikarya (arrowheads) and dendrites in the control cultures (M), and the reduced staining in the cell body (arrowhead) (N) and a residual AChE in the dendrite (arrow) (O) after Aβ treatment for 2 days. After Aβ treatment for 3 days the neuron has degenerated and only a little enzyme activity remains in the fragments of the dendrites (arrows) (P). Scale bar = 10 μm.

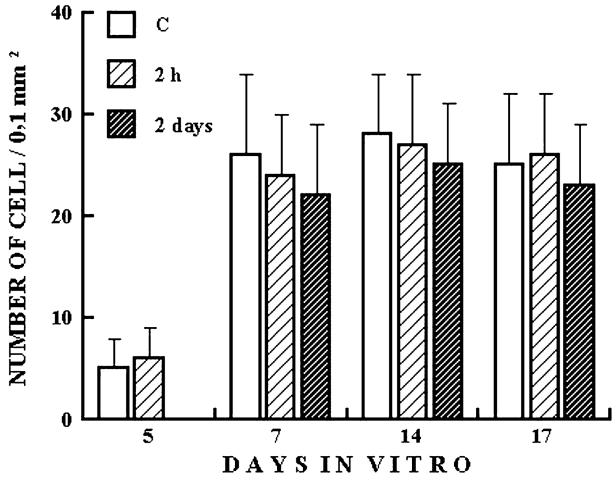


Fig. 2. Effect of 20 μM amyloid-β on the number of VAChT-positive neurons. Evaluation of the numbers of VAChT-positive cells in the control samples and after treatment with 20 μM Aβ for 2 h or 2 days on DIV5, DIV7, DIV14 and DIV17. The immunostained cells were counted in five-field per cover slips in randomly selected areas in untreated (C) and Aβ-treated samples. The numbers of cells are expressed per 0.1 mm^2. The data are mean values ± S.E.M.

was still present in the cell body. In these Aβ-treated double-stained neurons, some of the AChE reaction end-product remained in the perikaryon and in the dendrites near to the cell body (Fig. 3C). Longer treatment (3 days) resulted in the disappearance of the AChE staining in the dendrites, but the double staining remained in the perikaryon (Fig. 3D).

3.5. Quantitative evaluation of live and dead cells after Aβ treatment

The results presented above suggested that Aβ affected both the cholinergic and the AChE-positive neurons. To demonstrate whether Aβ affects other cell types, such as the non-cholinergic, non-cholinoceptive neurons in the BF cultures, we determined the percentage of dead/dying neurons relative to the live cells in the cultures treated on DIV12 with 20 μM Aβ for 2 h or for 1, 2 or 3 days. We found that the number of FDA-positive (live) cells decreased and the number of PI-stained (dead/dying) nuclei increased, in a time-dependent manner. Treatment with 20 μM Aβ for 2 h did not alter the number of PI-positive and FDA-stained cells as compared with the control samples.

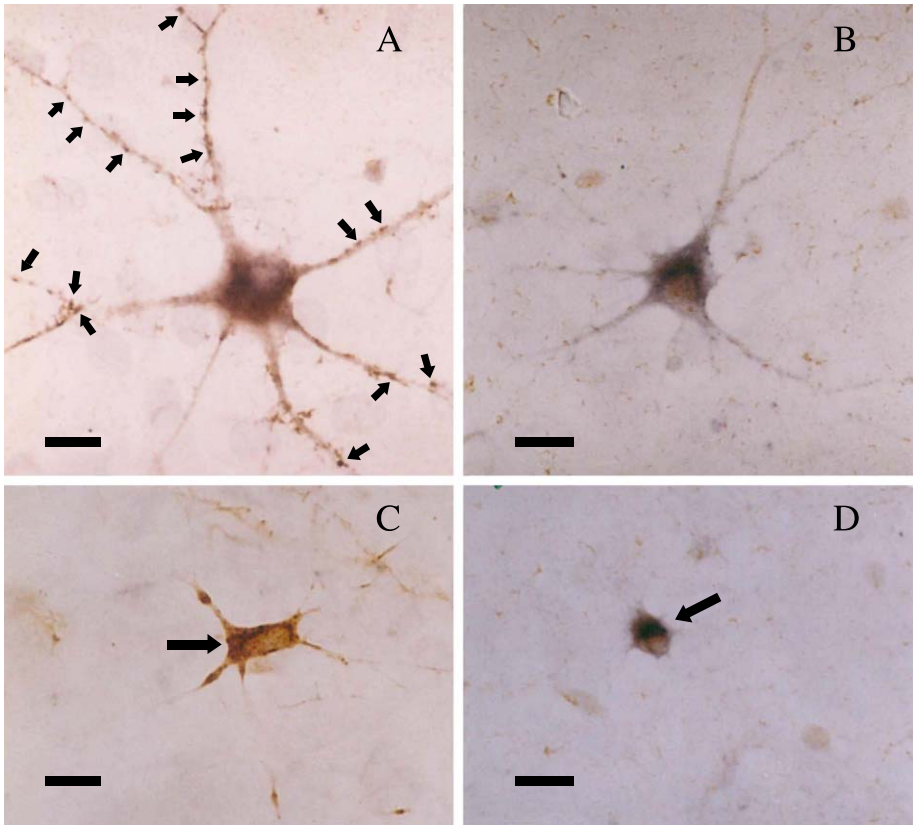


Fig. 3. Cells double-stained for AChE and VAChT in a rat basal forebrain tissue culture. The AChE staining appears in the dendrites and perikarya (brownish colour), while the VAChT is present in the axon varicosities (bluish-black, arrows) present on the surface of the dendrites (A). Two hours after treatment with 20 μM Aβ the number of VAChT-positive terminals is reduced (B). After 2 days, the AChE staining is still present in the main dendrite (arrowheads) near the cell body (arrow)(C), while after 3 days the double staining can be observed only in the perikaryon (arrow) (D). Scale bar = 10 μm.

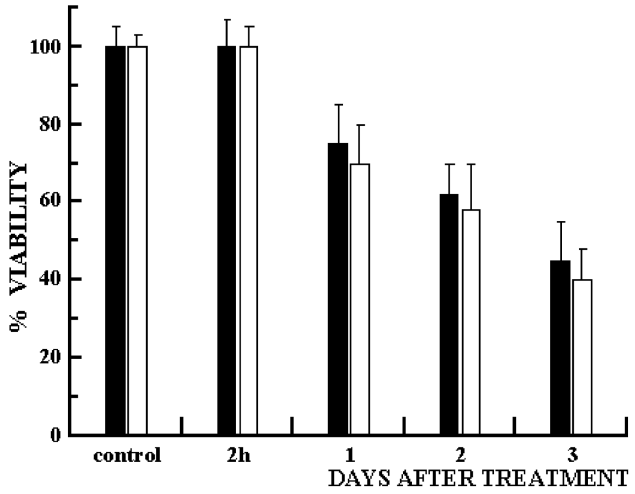


Fig. 4. Viability of neurons after treatment with 20 μM amyloid-β on DIV12 for 2 h, or for 1, 2 or 3 days. After the various time intervals, the neurons on the cover glass were exposed for 5 min to PI (5 μg/ml) and FDA (15 μg/ml). The survival of the neurons is expressed as a percentage of the total number of stained cells. In each case, the number of PI-positive cells found in the corresponding control sample (supplemented with the reverse peptide $A\beta_{35-25}$) was subtracted. The stained neurons were counted in five different microscopic fields from at least three experiments. Each column indicates the mean ± S.E.M. for viable neurons (filled columns). Similar samples were stained for VAChT and the cells were counted. The histogram (open columns) demonstrate that the Aβ treatment affected the cholinergic neurons similarly to the non-cholinergic cells.

The number of surviving, FDA-fluorescent neurons was reduced to 75 ± 10% after 1 day, to 62 ± 8% after 2 days and to 45 ± 10% after 3 days of treatment. Image analysis of similarly treated samples stained for VAChT immunoreactivity disclosed that the number of these neurons decreased in nearly the same proportion as that for the FDA-fluorescent cells (Fig. 4).

4. Discussion

The present immunohistochemical study has established that VAChT-positive axon varicosities and terminals are more sensitive to $A\beta_{1-42}$ or $A\beta_{25-35}$ than are the perikarya of cholinergic cells in primary neuronal cultures of BF. Our data also suggest that, in the histochemically stained AChE-positive neurons the Aβ reduce the reaction end-product in a time-dependent manner. These observations are supported by a live/dead cell viability assessment, where the quantitative studies demonstrated that Aβ treatment affects not only the cholinergic neurons, but other neurons too, which may degenerate after a longer period.

4.1. Neurotoxic effects of Aβ on VAChT-positive nerve cells and axon terminals

It is well documented that one of the primary features of AD is a cholinergic deficit (for reference, see Ref. [24]). The reduction of ACh in AD brain samples has been attributed to the neurotoxic effect of $A\beta_{1-42}$ on the cholinergic neurons, and especially on the cholinergic axon terminals. Indeed, when a vesamicol analog selective ligand meta-[^{125}I] iodobenzyltrozamicol(+)-was used as probe as a selective ligand to demonstrate a reduction in the density of binding sites for VAChT, a reduction was observed with aging and age-related neuropathology in the temporal cortex [11], though a significant reduction in the number of cholinergic neurons has not been reported in the early stages of AD [16]. Such results on human brain underlined the necessity for experiments designed to study the effects of $A\beta_{1-42}$ and $A\beta_{25-35}$ in in vivo and in vitro model systems in order to gain more insight via neuropathological alterations that can mimic those found in AD.

In an in vivo experiment, we have demonstrated that $A\beta_{1-42}$ inhibits the fast axonal transport of VAChT in the sciatic nerve of rat [26]. Similarly, when $A\beta_{1-40}$ or $A\beta_{25-35}$ was injected into the rat and monkey cerebral cortex, it produced localized necrosis at the injection site, which was surrounded by a zone of neuronal loss and gliosis, indicating a neurotoxic effect of $A\beta_{25-35}$ [28]. In other in vivo experiments, $A\beta_{25-35}$ resulted in a marked decrease in cholineacetyltransferase immunoreactivity in the BF medial septum [9]. In the parietal cortex of rats infused with Aβ, the reduction of [^{3}H]-vesamicol binding to VAChT was also revealed [20]. These in vivo animal studies support the suggestion that $A\beta_{1-42}$ in the human brain may cause a cholinergic hypofunction and memory impairment.

In in vitro studies, VAChT-like immunoreactivity was demonstrated in cell lines such as pheochromocytoma (PC12) cell lines regulated by NGF [3,30] or retinoic acid [5], in BF-derived SN56 cells [4] and in cell line NG108-15 treated with retinoic acid [10]. The expression of VAChT positivity was also revealed in primary neuronal cultures derived from the septum of embryonic rat [49] and the septum and cortex of mouse brain [27]. In these earlier studies, the presence of VAChT immunostaining was observed mostly in the perikarya of the neurons [10,30]. Our immunohistochemical study has for the first time demonstrated a detailed distribution of VAChT-positive axon terminals, and the probable synaptic relationships between the cholinergic (VAChT-positive) and cholinoceptive cells (neurons innervated by VAChT-positive axon terminals) in BF neuronal cultures. If the contacts between these cells are indeed the synaptic sites, then we have demonstrated that, in addition to the VAChT-negative cells, the cholinergic neurons on their soma and dendrites may likewise be innervated. This observation is important because Aβ affects first the VAChT-positive axon terminals and therefore possibly the synaptic sites as well. In the AD brain, such a synaptic effect of Aβ has already been suggested [29,32,47]. In other in vitro studies, primary neuronal cultures have similarly been extensively used to study the effects of $A\beta_{1-42}$ on GABAergic [22,37] and cholinergic markers including VAChT [12,38] and ACh release [23]. In neuronal cultures, the toxic effect of

Aβ has been demonstrated [7,13,31,40,53]. However, the rapid effect of $A\beta_{1-42}$ or $A\beta_{25-35}$ on the VAChT-positive neurons was not reported in earlier immunohistochemical studies. The reason for this fast effect on the axon terminals and for the slower effect on the perikarya is not yet clear and demands further investigations. In electrophysiological and neurochemical studies, however, the rapid effect of Aβ has already been demonstrated. In voltage clamp recording, it has been shown that on single neurons $A\beta_{25-35}$ affects the membrane current within minutes [46]. Similarly, in neurochemical studies, fibrillar $A\beta_{1-40}$ has been shown to enhance the phosphorylation of tau protein as early as after 1 h in cortical neuronal cultures of rat [2]. Caspase-3 activity has been revealed to be increased in synaptosomes within 2 h by $A\beta_{25-35}$ and $A\beta_{1-42}$ [35].

4.2. Neurotoxic effect of Aβ on AChE-positive nerve cells

In AD, the loss of AChE-positive cholinoceptive neurons in the various areas of the human brain is well documented (for reference, see Ref. [24]). Stable complexes of AChE and Aβ may play a role in this pathological process [1]. Indeed, such complex formation of AChE and $A\beta_{1-40}$ or AChE and $A\beta_{1-42}$ is known to increase the neurotoxicity of Aβ [36]. Hence, it is conceivable that, in our in vitro tissue cultures, the cholinergic and AChE-positive neurons will pickup more $A\beta_{1-42}$ or $A\beta_{25-35}$ from the incubation medium during treatment and these enzyme-positive neurons will be more sensitive than cells which lack this enzyme. The findings of our in vitro AChE histochemical and VAChT immunohistochemical studies are in good accordance with the neuropathological results obtained on human AD brain samples. Both human and in vitro tissue culture studies have demonstrated that both cholinergic and AChE-positive neurons are affected by $A\beta_{1-42}$ and $A\beta_{25-35}$ peptide. However, the neurotoxic effects these peptides on the VAChT-positive neurons appear more rapidly (within 2 h) than those on the AChE-stained cells (24 h or later). The difference in the effects of $A\beta_{1-42}$ or $A\beta_{25-35}$ on the cholinergic and AChE-positive neurons awaits for further elucidation. More recently, we have demonstrated that in vitro treatment of in vitro neuronal cultures with 20 μM $A\beta_{1-42}$ for 2 days on day, 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 than the number of VAChT-immunoreactive structures, suggesting that $A\beta_{1-42}$ does not have a specific effect only on the cholinergic neurons, but affects non-cholinergic neurons as well [39]. The question arises as to whether the soluble or the aggregated form of Aβ causes the neurotoxic effect. Originally, it was thought that the aggregated fibrillar form was responsible. However, subsequent studies revealed that the toxic effect is retained in samples where the protofibrils (soluble oligomers) are present. This was supported by the finding by Walsh et al. [51] that the administration of non-fibrillar Aβ oligomers to live animals produced deficits in long-term potentiation, a phenomenon thought to be related to memory formation. It is plausible that the early effect (30 min) on the VAChT-positive axon varicosities in our experiment may be due to the soluble form, while the late effect (2 days) on the AChE is rather due to the aggregated fibrillar form of Aβ.

In summary, it seems likely that the effects of $A\beta_{1-42}$ or $A\beta_{25-35}$ appear most rapidly on the axon terminals and the neuronal cell body undergoes degeneration only later. Accordingly, the $A\beta_{1-42}$ neurotoxicity in AD may be ascribed to a rapid synaptic (or trans-synaptic) effect, followed by structural and neurochemical alterations in the postsynaptic neuron. This view is supported by the fact that in AD some of the neurons degenerate, while others may undergo atrophy rather than neurodegeneration [16].

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