ALZHEIMER'S DISEASE

With special emphasis on *in vitro* and *in vivo* model systems and the effect of donepezil applied for treatment of the disease

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following publications that in the text are referred by their Roman numerals I-VI and can be found in the Appendix.

I. Kasa, P., Papp, H., Kasa, P.Jr., Torok, I. (2000) Donepezil dose-dependently inhibits acetylcholinesterase activity in various areas and in the presynaptic cholinergic and the postsynaptic cholinoceptive enzyme-positive structures in the human and rat brain. Neuroscience 101, 89-100.

II. Kasa, P., Papp, H., Kovacs, I., Forgon, M., Penke, B., Yamaguchi, H. (2000) Human amyloid-β1-42 applied *in vivo* inhibits the fast axonal transport of proteins in the sciatic nerve of rat. Neurosci. Lett. 278, 117-119.

III. Kasa, P., Papp, H., Pakaski, M. (2001) Presenilin-1 and its N-terminal and C-terminal fragments are transported in the sciatic nerve of rat. Brain Res. 909, 159-169.

IV. Pakaski, M., Farkas, Z., Kasa, P.Jr., Forgon, M., Papp, H., Zarandi, M., Penke, B., Kasa, P.Sr. (1998) Vulnerability of small GABAergic neurons to human β-amyloid pentapeptide. Brain Res. 796, 239-246.

V. Pakaski, M., Papp, H., Forgon, M., Kasa, P.Jr., Penke, B. (1998) Effects of β-amyloid on cholinergic, cholinoceptive and GABAergic neurons. Acta Biol. Hung. 49, 43-54.

VI. Papp, H., Kasa, P.Jr., Pakaski, M., Balaspiri, L., Kasa, P. (2001) Amyloid-β1-42 treatment does not have a specific effect on cholinergic neurons in *in vitro* basal forebrain neuronal cultures of rat. Acta Biol. Hung. (in press)

ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
Aβ	amyloid-beta
APP	amyloid precursor protein
ChAT	choline acetyltransferase
CNS	central nervous system
DAB	3,3'-diamino-benzidine tetrahydrochloride
DIV	days *in vitro*
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
mAChR	muscarine acetylcholine receptor
NBM	nucleus basalis of Meynert
NFT	neurofibrillary tangles
nAChR	nicotine acetylcholine receptor
NMJ	neuromuscular junction
OD	optical density
PBS	phosphate-buffered saline
PHF	paired helical filament
PNS	peripheral nervous system
PS-1	presenilin-1
PS-2	presenilin-2
SP	senile plaque
SYN	synaptophysin
TB	Tris-HCl buffer
VAChT	vesicular acetylcholine transporter

CONTENTS

1. INTRODUCTION

Alzheimer's disease (AD) is the most common form of senile dementia, affecting more than 20 million people worldwide. In Hungary the number of AD patients may be more than one thousand. With increasing life expectancy, this number will inevitably rise rapidly in the future, not only in the western world, but also in Hungary. Statistical data demonstrate that AD is the fourth leading cause of death after heart disease, cancer and stroke. It is the most common cause of dementia in the elderly, characteristically involving a progressive loss of cognition and changes in personality. The usual presenting symptom is a memory loss, which is often associated with language, visuospatial and attention problems.

Several theories have been put forward to account for the development of neuropathological changes in the AD brain. The real aetiopathological factor, however, remains a mystery. The results of the wide-ranging scientific research on AD prove the complexity of this disease. Various lines of evidence indicate that a number of factors may play a role in the development of the disease.

So many hypotheses have been put forward to explain the aetiology of AD, that the situation is far from clear. Our attention turned first towards the *amyloid cascade hypothesis*[28]. It has long been known that one of most important neuropathological changes in AD is the accumulation of Aβ-positive senile plaques (SPs) in the various areas of the central nervous system (CNS). The amyloid cascade hypothesis postulates that it is the Aβ in the SPs which exerts a neurotoxic effect on the different types of neurons[9,13,16,27,35,44,71] and results in their degeneration.

A large number of neurochemical data draw attention to lesions of the cholinergic system. The amyloid hypothesis has also attempted to shed light on the causal relations of these changes. It is well documented that a correlation can be demonstrated between the increase in the amount of Aβ and the alterations in the elements of the cholinergic system (choline acetyltransferase: ChAT, acetylcholinesterase: AChE, acetylcholine: ACh, the high-affinity choline uptake and the number of nicotinic and muscarinic ACh receptors (nAChRs and mAChRs). In consequence of the pathological accumulation of Aβ, a cholinergic hypofunction will develop in the CNS. Since the cholinergic system has been proved to be involved in the memory function, a *cholinergic hypothesis*[2] was formulated with respect to the

neuropathology of AD. The amyloid[28] and the cholinergic[2] hypotheses led to a need for further research to elucidate the role of Aβ in the pathogenesis of AD.

AChE inhibitors (donepezil, rivastigmin and galanthamine) have recently been introduced for the treatment of AD individuals in order to increase the efficacy of the remaining ACh and to ameliorate the memory problems of the patients. Although there have been a large number of neurochemical and clinical investigations on the effects of these drugs, there is no morphological evidence as to how the AChE inhibitors used for the treatment of AD affect the various areas of the CNS and within this the different cholinergic and cholinoceptive cells. This thesis summarizes our efforts to shed light on the aetiology and neuropathology of this terrible disease.

2. REVIEW OF THE LITERATURE

2.1. Neuropathological alterations in Alzheimer's disease

Macroscopically, the pathological hallmarks of AD, are found most frequently within the association regions of the cerebral cortex and within the medial temporal lobes, *i.e.* the hippocampus, amygdaloidal nuclei and parahippocampal gyri. Classically, the brains of individuals with AD are atrophic, with widened sulci, narrowed gyri and dilated ventricles (Fig. 1A; compare with Fig. 1B). In microscopic investigations, two pathognomonic lesions of AD can be revealed. These are the SPs (Fig. 1D and E) and neurofibrillary tangles (NFTs) (Fig. 1F).

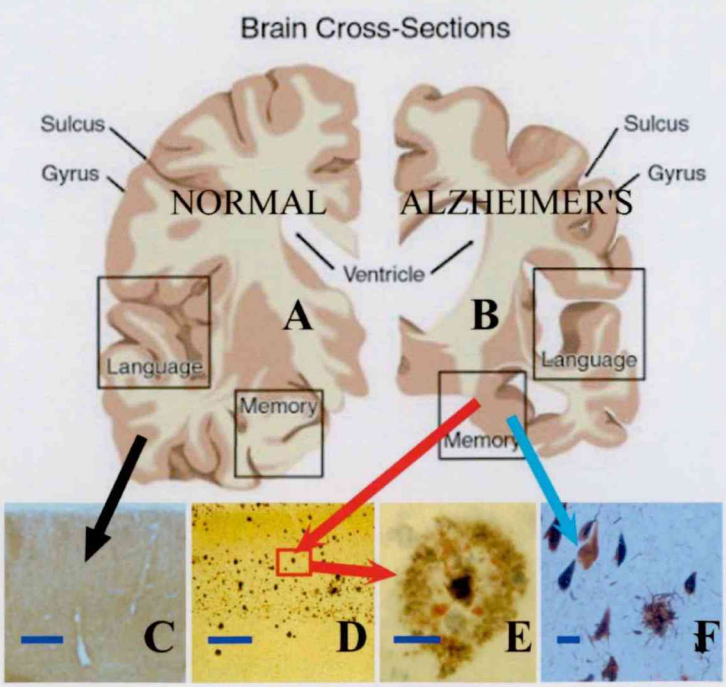

Fig. 1. Image A represents a control, while image B demonstrates an AD brain cross-section as seen from the front. In microscopic investigations, no neuropathological alterations can be observed in the control cortex (C), but there are a large number of SPs (D, E) and NFTs (F) in the cortex of AD individuals. (Panels C-F taken from my own immunohistochemical preparations.) Bar = 1mm (C,D), 50 μm (E), 15 μm (F)

The NFTs are composed primarily of paired helical filaments (PHFs), which may result from the intraneuronal accumulated neurotoxic Aβ. The subunit of the PHFs is the microtubule-associated protein tau, which exists in the PHFs in a hyperphosphorylated, insoluble form. Two major types of SPs are found in the AD brain: *diffuse plaques*, which are not accompanied by abnormal neurites (Fig. 2A) and Aβ-containing *neuritic plaques*, in which dystrophic neurites are present (Fig. 2B,C).

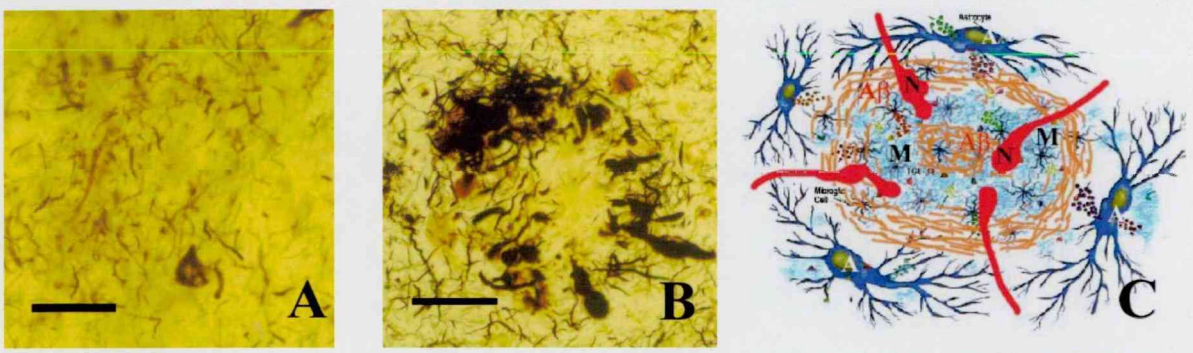

Fig. 2. Image A demonstrates a diffuse SP, image B represents an neuritic SP in AD brain sample. (Photos taken from my preparations.) The schematic drawing (C) represents a neuritic SP. The dystrophic neurites (N, red) inside the SP are unusually enlarged. Other components of the SP are microglia (M) and astrocytes (A); the deposition of Aβ is shown by brownish lines. (This SP is a modification from R&D Systems, 2001.) Bar = 50 μm (A,B)

The SPs are extracellular lesions approximately 100 μm in size, and can be stained with various techniques: silver impregnation, thioflavin-S, Congo red and immunohistochemistry. Biochemically, the insoluble cores of the SPs have been shown to be aggregated Aβ deposits, which consist of amyloid fibrils and are 39-43-amino acid Aβ peptides. The neuritic plaques may be more closely associated with AD since their presence correlates with regions of neuronal loss, and they are less frequently found in non-demented elderly patients. In addition to these correlative findings, the observation that microglial cells and reactive astrocytes are associated with virtually all neuritic plaques but with very few diffuse plaques, further suggests that these neuritic plaques may be critically important in the aetiology of AD.

Interestingly, neuritic and diffuse plaques may contain Aβ1-42, Aβ1-40, and N-terminally truncated Aβ and C-terminally truncated Aβ residues. Immunohistochemical studies of SPs with the use of antibodies specific for these Aβ forms demonstrate that almost all diffuse plaques stain for Aβ ending at residue 42 (Aβ1-42), while far fewer contain Aβ ending at

residue 40 (Aβ1-40). In contrast with the diffuse plaques, the neuritic plaques often contain a core of both Aβ1-40 and Aβ1-42, with Aβ1-42 present in the periphery of the plaque.

2.2. Relationship between the cholinergic system and Alzheimer's disease

The idea that some of the symptoms of AD are due to a deficiency of the neurotransmitter ACh in the brain was first demonstrated in 1976. Later, several groups of investigators reported that the activities of ChAT and AChE and the level of ACh were markedly reduced in autopsy brain tissue from patients with end-stage AD[35]. Many subsequent studies have confirmed these findings. It has also been shown that the extent of the hypocholinergic activity in autopsy brain tissue correlates with the severity of the disease[60] (as determined by the density of SPs, NFTs, or both). The *cholinergic hypothesis*[2] of AD that evolved from these studies postulates that at least some of the cognitive decline experienced by patients with AD results from a deficiency of ACh, or cholinergic neurotransmission. This hypothesis has been the stimulus for a great deal of effort in experimental pharmacology[3,17] and a large number of clinical trials.[8,14] Loss of the neurotransmitter ACh is also thought to play an instrumental role in the pathogenesis of AD. The degree of cholinergic deficit is correlated with the severity of dementia in AD. This hypothesis has led, in turn, to promising clinical interventions (see the results on AChE inhibitors below). It should be emphasized, however, that ACh is not necessarily the only neurotransmitter involved in AD. Neurochemical research has not ruled out the contributions of other substances in the pathogenesis of AD.[13,43]

2.3. Amyloid-β neurotoxicity and Alzheimer's disease

AD is characterized by the extracellular deposition of aggregated Aβ in the brain and its blood vessels. Aβ and its shorter fragments have been shown to be toxic to neurons in *in vitro*[79] tissue cultures and to rats and aged primates *in vivo.*[15] Aβ25-35 causes morphological alterations in the neuronal perikarya (atrophy and degeneration) and in the axons (fragmentation)[41], similar to those produced by the full-length Aβ1-42. It has been suggested

that this 11-amino acid internal sequence of Aβ is the biologically active fragment and may be responsible for the neurotoxic effect of Aβ1-42.

The toxic effect of Aβ has been demonstrated at micromolar concentrations in several tissue models.[19,79] Blusztajn and Berse[6] recently reported that synthetic Aβ at submicromolar concentrations does not cause cytotoxicity, but rather reduces the expression of cholinergic markers in neuronal cells. The interesting question arises, therefore, of whether the neurotoxic effect or the reduced expression of the cholinergic marker proteins is the primary manifestation of the Aβ effect. As mentioned earlier, AD may be characterized by a neuronal dysfunction in which the microtubule-associated tau-protein is abnormally hyperphosphorylated. If this is the case, the microtubule network is disrupted within the neuronal perikarya and the axonal transport may be severely affected. It may be assumed, therefore, that substances that are rapidly transported intraaxonally should accumulate in the axon after *in vivo* Aβ treatment. We have clarified this question and demonstrated that proteins, including AChE, amyloid precursor protein (APP), synaptophysin (SYN) and vesicular acetylcholine transporter (VAChT), accumulate in damaged nerves and are good markers of axonal lesions present in AD. We should emphasize that there has been no previous report of the *in vivo* effects of human Aβ1-42 on the axonal transport.

2.4. The amyloid precursor protein and presenilin in Alzheimer's disease

2.4.1. The amyloid precursor protein

The discovery that Aβ is the principal component of SPs prompted investigations into the origins of this peptide. Screening of cDNA libraries with oligonucleotide probes complementary to the Aβ sequence led to the cloning of the cDNA and gene for APP. A number of isoforms of APP are generated by alternative splicing of a 19-exon gene, with the most predominant isoforms encoding for a 695-amino acid form, a 751-amino acid form and a 770-amino acid form of APP (APP695, APP751 and APP770, respectively). Each isoform encodes a type-I integral membrane protein, with the Aβ domain embedded in the transmembrane and ectodomain. APP695 is the predominant APP isoform expressed in neurons, whereas APP751 expression predominates elsewhere. Although the normal function of APP remains to be further elucidated,[70] genetic studies suggest that, at least in a subset of

AD cases, an abnormal overproduction of Aβ rather than a loss of the normal function of APP may be critical for the pathogenesis. These genetic studies have prompted a detailed investigation into the cell biology of APP processing and Aβ production. APP is cotranslationally translocated into the endoplasmic reticulum (ER) before transport through the secretory pathway. APP matures in the ER and the Golgi apparatus. Some of the APP is eventually transported to the cell surface, and reinternalized via coated pit endocytosis.[45] During the transit of APP through the ER, the Golgi, cell surface and the endosomal/lyosomal system, a subset of APP molecules are subject to endoproteolysis.[4] Endoproteolytic cleavage of APP occurs at a variety of sites within the molecule (Fig. 3). The best-studied cleavage sites are between amino acids 16 and 17 of the Aβ region (α-secretase cleavage), immediately before Asp1 of Aβ (β-secretase cleavage) and after amino acid 40 or 42 of the Aβ domain (γ-secretase cleavages). Cleavage of APP by β- and γ-secretase releases Aβ, while α-secretase cleaves within the Aβ sequence and thus precludes amyloidogenesis. In a variety of non-neuronal cell types, the principal secretory cleavage of APP occurs at the α-secretase site. This cleavage generates sAPP (the secreted ectodomain of APP ending at the α-secretase cleavage site) and C83 (the 83-amino acid COOH tail of APP). Cell surface radioiodination experiments have shown that the α-secretase cleavage of APP can occur at the plasma membrane, and the resulting APP fragment is released (or secreted) into the extracellular space *in vivo* or into the incubation medium in *in vitro* tissue cultures.

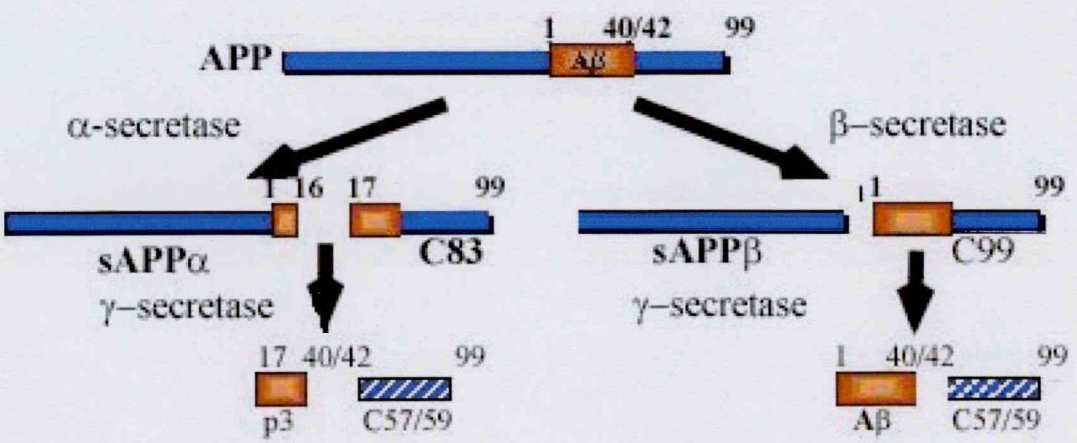

Fig. 3. Schematic structure of APP and its processing by α, β and γ-secretase.

Hypotheses regarding the abundance of a 4 kD Aβ species were based upon the demonstration that Aβ is an abnormal product of APP, which is restricted to the brains of aged or demented humans. Several studies have recently been conducted which revealed that Aβ might also be generated intracellularly from APP.[25] A substantial increase in intracellular Aβ suggests that Aβ production from APP may be more important than previously thought in the pathogenesis of AD.

2.4.2. The presenilins

In 1995 a presenilin gene (presenilin-1, PS-1) was discovered, and it was demonstrated that missense mutations of this gene cause familial AD.[72] This gene has been localized to chromosome 14, and presenilin-2 (PS-2) to chromosome-1. Analysis by *in situ* hybridization suggests that in the CNS, PS-1 and PS-2 mRNAs are expressed at the highest levels in neurons.[40] PS-1 and PS-2 are integral membrane proteins that contain multiple transmembrane domains (Fig. 4). These proteins have a C- and an N-terminal part, to which specific antibodies have been developed. It is therefore possible to study in detail the neuronal localization and the movement of PS-1 and PS-2 within a given neuron and from this to draw certain conclusions concerning their functions.

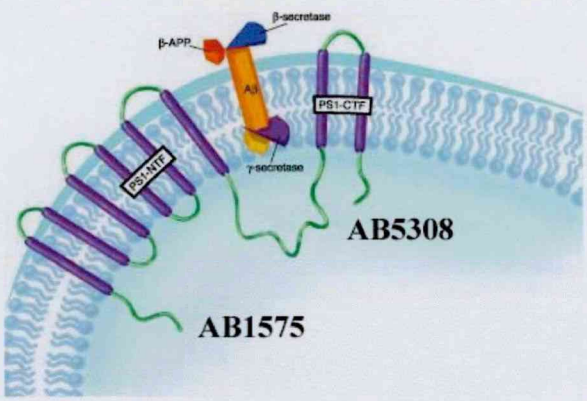

Fig. 4. Schematic representation and topological localization of PS-1 in a membrane. There are eight transmembrane domains and C-terminal and N-terminal endings. AB1517 and AB3214 represent the antibodies specific to the C and N-terminals used in our immunohistochemical and Western blot experiments. (Modified from R&D Systems, 2001.)

PS-1 and PS-2 are predominantly located within the ER and early Golgi apparatus. They are primarily expressed in neurons and are ubiquitously expressed within the brain. PS-1 has been localized in the SPs and NFTs in the brain of AD individuals.[7,29] In the human and rat CNS, PS-1 has been associated with different types of neurons and astrocytes. In the brain and *in vitro* tissue cultures, PS-1 has been found in the cell bodies and within these to be associated with the perinuclear envelope, the ER and the trans-Golgi network.[40] PS-1 has been detected in the dendrites and dendritic spines, the axons and the axon terminals.[7, 20] In

developing neurons, it can be observed in the axonal growth cone. In NT2N neurons, as a human neuronal model system,[10] and in hippocampal neuronal cultures derived from transgenic mice, however, PS-1 could not be found in the axons. The fragments of PS-1 have also been demonstrated in synaptic plasma membranes, synaptic contacts, neurite growth cone membranes, and clathrin-coated and small synaptic vesicles of the rat brain.[5] The existence of PSs in the axons is debated and further evidence is needed to clarify this question.

We were the first to demonstrate that the PSs are not only present in the neuronal perikarya, but also transported in the axons. Separation of the axoplasm fractions from the cerebral white matter of non-demented individuals revealed the N-terminal and C-terminal fragments of PS-1. This finding indicated that there the intracellular trafficking of PS-1 might occur through the axons in the human brain. In biochemical experiments, Ray *et al.*[66] demonstrated that PS-1 might reach the plasma membrane, while Beher *et al.*[5] provided evidence that fragments of PS-1 are present in the synaptic plasma membranes. They suggested that PS-1 may exit from the cell body and reach the synaptic organelles. The transport of PS-1 fragments in neuritic processes has also been suggested, but there was no direct morphological evidence for the axonal transport of PS-1.

Our studies revealed that PS-1 is present not only in the neuronal perikaryon but also in the axons. The presence of PS-1 in the motoric axon terminals at the neuromuscular junctions (NMJs) was similar to the localization of SYN, APP and VAChT.

2.5 Acetylcholinesterase inhibitors in Alzheimer's disease

Pharmacological treatment of AD is a promising new focus for interventions. A central goal in AD treatment research is the identification of agents that prevent the occurrence, defer the onset, slow the progression, or improve the symptoms of AD.[75] Progress has been made in this research arena, with several agents exerting beneficial effects in AD.

Recent attempts to treat AD have focused on enhancing the ACh function, using either cholinergic receptor agonists (*e.g.* nicotine) or, most commonly, AChE inhibitors (*e.g.* donepezil, rivastigmine, galanthamine and tacrine) to increase the availability of ACh in the synaptic cleft.[78] To study the effects of drugs on the cholinergic system, it is necessary to be

very familial with the regional distribution of the cholinergic neurons, their axon terminals, and synaptic sites in non-demented and in AD individuals. The main distribution of the cholinergic system in the control human brain is depicted in Fig. 5.

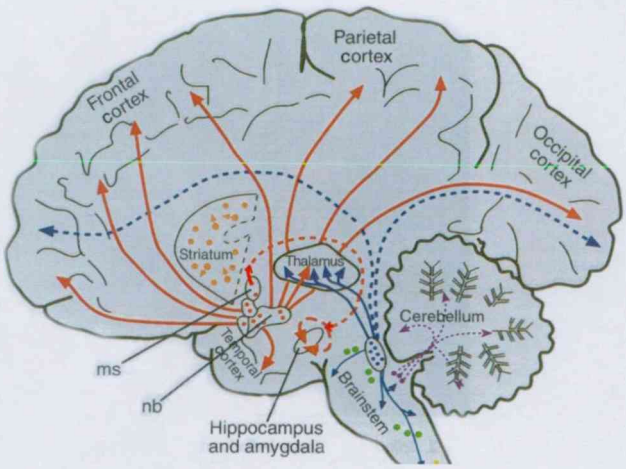

Fig. 5. Schematic illustration of the cholinergic systems in the human brain. Note the origin of the cholinergic axons from the nucleus basalis (nb) and the medial septum (ms). The cholinergic axons (red colour) innervate the hippocampus, the amygdala and the various cortical areas. (From Perry *et al.*, 1999.)

The first reported changes in reductions of the elements of the cholinergic system in autopsy samples of AD were observed in the neocortex, the hippocampus and many other brain regions.[35] Significant deficits of ChAT and AChE activities in AD were found in the frontal, parietal cortical and temporal cortical regions, the hippocampus and the amygdala, the decrease being most severe in younger AD patients. The reductions in these enzymes and the number of SPs in the cortex were correlated with the loss of neurons in the nucleus basalis of Meynert (NBM) and the cholinergic axons in the cortex. Via histochemical demonstration of the AChE-positive fibres and immunohistochemical demonstration of the Aβ-positive SPs (Fig. 6), the involvement of cholinergic neurons in AD can be well documented.

As stated above, the staining of AChE in the cortex is profoundly reduced in patients with AD. According to the cholinergic hypothesis of the disease, the inhibition of AChE activity present in the postsynaptic sites may be one of the most realistic approaches to the symptomatic treatment of AD.

Many neuropharmacological, pharmacokinetic and clinical studies have been performed on the effects of donepezil, rivastigmine and galanthamine on the cognitive functions.[17,26]

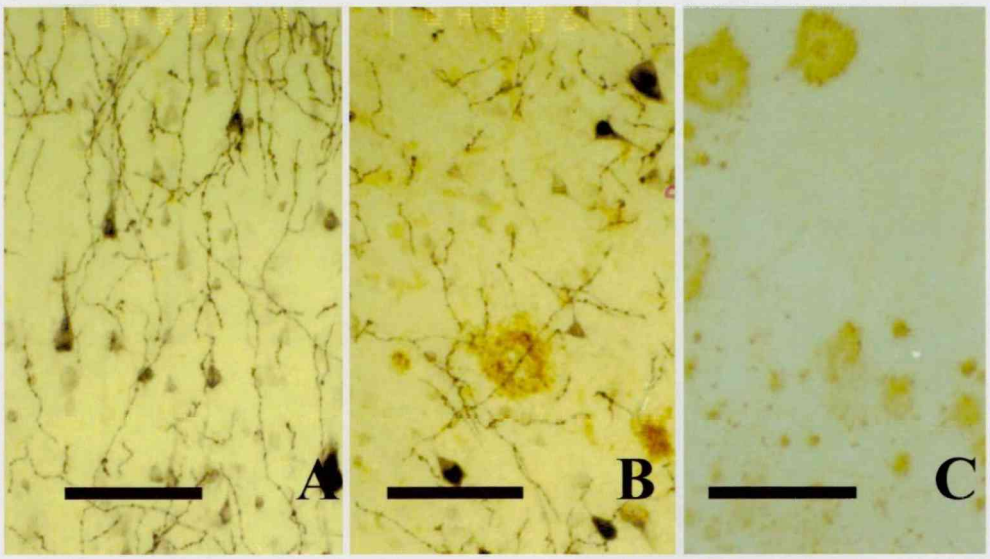

Fig. 6. Histochemical demonstration of AChE-positive structures in the aged non-demented brain (A), in the affected brain (B) and in the brain of an individual with AD (C). Note the gradual disappearance of the cholinergic fibres and the AChE-positive cells from the various cortical layers. Panel B is double-stained for AChE positivity and Aβ by histochemical and immunohistochemical means. (Photos taken from my preparations.) Bar = 150 μm

These drugs have been shown to be clinically effective and well tolerated in the treatment of the symptoms of mild to moderately severe AD[46,21] Although the use of AChE inhibitors is the most highly-developed approach for the treatment of AD, there has as yet been no demonstration of the precise morphological sites of its action. Nor is it known how AChE inhibitors with known central effects influence the enzyme activities in the peripheral nervous system (PNS). Biochemically, donepezil has been demonstrated to inhibit AChE activity in the cortex, the hippocampus, the striatum and the hypothalamus of the rat brain. Histochemical procedures, however, have advantages over biochemical methods because they allow detection of the effects of AChE inhibitors at the single cell level and at a single synaptic site, such as the peripherally located neuromuscular junction (NMJ). During recent years, therefore, we have a) investigated the regional selectivity of donepezil on the AChE activity in the non-demented human brain; b) compared the AChE inhibitory effects of donepezil in the non-demented human brain with those observed in the rat brain, and c) investigated the AChE-inhibitory effect of donepezil at the NMJ. It was also necessary to establish the effects of this agent on the intensity of AChE staining in the cholinergic axons innervating the extra- and intracerebral blood vessels.

3. AIMS OF THE STUDY

The present study was designed to investigate the neuropathological alterations caused by Aβ1-42 in *in vitro* neuronal cultures and to study the effect in the CNS of donepezil applied for the treatment of AD.

The specific aims were:

1. To identify different transmitter-containing neurons in *in vitro* neuronal cultures.
2. To reveal the effects of human Aβ1-42 on different transmitter-containing neurons in tissue cultures (cholinoceptive, cholinergic and GABAergic).
3. To study the *in vivo* neurotoxic effects of human Aβ1-42 on the axonal transport of various proteins.
4. To demonstrate the intraaxonal transport of APP and PS-1.
5. To investigate: a) the regional selectivity of donepezil on AChE activity in the non-demented human brain; b) the AChE-inhibitory effects of donepezil in the non-demented human brain in comparison with those observed in the rat brain; c) the AChE-inhibitory effect on the NMJ.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1. Autopsy samples from human brain

We examined autopsied brains from 16 patients aged between 68 and 92 years with AD, and from 12 patients aged between 50 and 81 years without neurological diseases. The interval between death and autopsy was in all cases less than 4 h.

The human brain was sliced coronally into approximately 1 cm slabs, which were fixed by immersion for 24-36 h in an ice-cold 0.1 M phosphate buffer (pH 7.4) solution containing 4% paraformaldehyde. After fixation, the samples were cryoprotected in 30% sucrose solution until they sank to the bottom of the container. The samples contained cortical areas, the hippocampal complex and the basal part of the brain. Forty-μm-thick sections were cut on a freezing microtome and collected in 0.9% sodium chloride buffered with 0.1 M sodium phosphate at pH 7.4 (PBS) containing 0.1% sodium azide.

4.1.2. Central nervous system of rat

These investigations were performed in accordance with the ethical guidelines for animal investigations of the Hungarian Ministry of Welfare. All experiments were carried out in

accordance with the European Communities Council Directive (24 November 1986; 86/609/EEC) and the Albert Szent-Györgyi Medical University Guidelines for Ethics in Animal Experiments. Every effort was made to minimize the number of animals used.

Adult Sprague-Dawley rats weighing 200-250 g were used in the experiments. The animals were perfused transcardially under ether anaesthesia. Blood was first removed from the vascular system with PBS; the brain was then perfusion-fixed with 0.1 M phosphate buffer, pH 7.4, containing 4% freshly prepared formaldehyde. Thereafter, the fixative was removed by perfusion with 150 ml PBS. The brains were immediately removed and immersed overnight in a similar fixative at 4 °C. This was followed by cryoprotection for 24 h in 30% sucrose in 0.05 M Tris-HCl buffer, pH 7.6 (TB), at 4 °C. Frozen sections were cut coronally at a thickness of 30 μm and collected in PBS and processed as free-floating sections for histochemical staining.

4.1.3. *In vitro* tissue cultures from embryonic rat brain

Primary basal forebrain cultures were prepared from 16-17-day-old (E16-17) rat embryos. The collected tissue samples were incubated for 10 min in 0.25% trypsin (Gibco) at 37 °C. The tissue was mechanically dissociated by trituration with a fire-polished Pasteur pipette. The resulting cell suspension was centrifuged for 10 min at 1000 x *g*. The pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated onto poly-l-lysine (Sigma)-coated coverslips ($3.5\text{-}4\text{x}10^4$ cells/cm^2) and were plated in a humidified 37 °C incubator in air containing 5% CO_2.

4.1.4. Sciatic nerve of rat

Adult male Sprague-Dawley rats (4-5 months old, 400-450 g) were used in the study of the axonal transport of APP and PS-1. The animals were anaesthetized with a cocktail of ketamine (70 mg/kg) and xylazine (10 mg/kg), administered intraperitoneally. The left sciatic nerve of the rat was exposed and ligated distally to the obturator internus muscle, using black silk thread. To visualize and quantify the anterograde and retrograde transport of PS-1 and the holoprotein of APP, a double-ligation procedure was applied. The right sciatic nerve was also exposed and a silk thread was placed loose around the nerve in the same location in the sham controls. The rats were allowed to recover for 6, 12 or 24 h prior to sacrifice. The ligated left sciatic nerve was removed; this contained a 4-mm segment above the proximal ligature, a 4-

mm middle segment, and a 4-mm segment below the distal ligature. Nerve segments that were similar in size were excised from the sham-operated animals. After different time intervals, the rats were anaesthetized with diethyl ether and perfused transcardially (200 ml) for 30 min with cold (4 °C) 4% paraformaldehyde solution, buffered with PBS. The sciatic nerves from the control and the ligated animals were immediately removed and immersed overnight in a similar fixative at 4 °C. After cryoprotection for 24 h in 30% sucrose solution, the sciatic nerves were cut at a thickness of 15 μm on a cryostat.

In the study of the neurotoxic effect of human Aβ1-42 on axonal transport, adult male Sprague-Dawley rats (250-300 g) were used. The animals were anaesthetized and 1.0 μl of freshly solubilized human Aβ1-42 (20 μM) solution was injected under the epineurium of the left sciatic nerve. The right sciatic nerve served as control and was injected with the Aβ1-42 solution vehicle. One day postinjection, the control and the Aβ1-42-injected nerves were dissected out and fixed by immersion for 12 h in 4% paraformaldehyde solution. Twenty-μm-thick longitudinal sections were cut on a freezing microtome.

4.1.5. Spinal ganglia of rat

Adult male Sprague-Dawley rats (4-5 months old, 400-450 g) were used in the study of the localization of APP and PS-1. The animals were anaesthetized with diethyl ether and perfused transcardially (200 ml) for 30 min with cold (4 °C) 4% paraformaldehyde solution, buffered with PBS. The spinal ganglia (L5 and L6) were removed and immersed overnight in a similar fixative at 4 °C. After cryoprotection for 24 h in 30% sucrose solution, the spinal ganglia were cut at 24 μm on a freezing microtome and processed as free-floating sections for histochemical staining.

4.2. METHODS

4.2.1. AChE histochemistry

For the visualization of AChE activity, a sensitive method[76] was used in a slightly modified form. After fixation, the tissue samples were rinsed several times in 0.1 M sodium maleate buffer (pH 6.0). Sections were pretreated for 20 min at 25 °C in ethopropazine hydrochloride (10^{-4} M), rinsed in 0.1 M sodium maleate (pH 6.0), incubated for 80-120 min in a medium consisting of acetylthiocholine chloride (1.8 mM), sodium citrate (0.1 M), copper

sulphate (0.03 M), potassium ferricyanide (5.0 μM) and sodium maleate (0.1 M; pH 6.0), and rinsed in TB. Finally, the sections were placed in a solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.15% nickel chloride ($NiCl_2$) and 0.03% hydrogen peroxide (H_2O_2) in TB. Sections were mounted on glass slides, dehydrated in a series of alcohol, cleared in xylene and coverslipped with DPX.

To determine the possible inhibitory effects of donepezil on the AChE histochemical staining, sections were preincubated for 20 min in PBS which contained different doses of donepezil ($5x10^{-9}$, $2x10^{-8}$, $5x10^{-8}$, $5x10^{-7}$ or $1x10^{-6}$ M). Thereafter, without washing, the control and treated samples were placed in an incubation medium that contained the same dose of donepezil as was present in the preincubation solution. Control samples were incubated in the absence of donepezil. The muscles were incubated for 7 min, the rat brain tissues for 60 min, and the human brain samples for 2 h at 25 °C, with constant agitation in a dark box.

4.2.2. Amyloid-β peptide immunohistochemistry

For the identification of Aβ the tissues were immunostained for Aβ. After fixation, tissue samples were pretreated with concentrated formic acid, washed in PBS, soaked for 10 min in 3% H_2O_2, and incubated with 2.5% Triton X-100 and then with 5% normal goat serum in PBS. The polyclonal antibody against the N/C-terminal of amyloid-β (1:30000) was applied for 24 h. This was followed by incubation with goat-anti-rabbit IgG-biotin (1:1000, Jackson Immunores. Lab.) for 90 min, and then with streptavidin-horseradish peroxidase (streptavidin-HRP) (1:1000, Jackson) for 90 min. The peroxidase reaction was developed by using DAB and $NiCl_2$.

When double-stained sections were prepared, the samples were developed first in DAB dissolved in 0.05 M TB. The staining resulted in a brownish colour. Thereafter, the sections were treated for 30 min with 0.6% H_2O_2 to eliminate the possibly persistent activity of the marker enzyme. After staining for the second antibody, the peroxidase complex was visualized by incubating the sections in a DAB-$NiCl_2$ mixture. This staining resulted in a bluish-black colour.

4.2.3. Amyloid precursor protein immunohistochemistry

For the identification of APP, the tissues were immunostained for APP. After fixation, the sections were soaked for 10 min in 3% H_2O_2, and tissue samples were incubated with 2.5% Triton X-100, and then with 5% normal sheep serum in PBS. The monoclonal antibody against APP (1:2000, Boehringer) was applied for 48 h. This was followed by incubation with sheep-anti-mouse IgG-biotin (1:1000, Jackson Immunores. Lab.) for 90 min, and then with streptavidin-HRP (1:1000, Jackson) for 90 min. The peroxidase reaction was developed by using DAB and $NiCl_2$.

4.2.4. Presenilin-1 immunohistochemistry

For the identification of PS-1, the tissues were immunostained for PS-1. The sections were soaked for 10 min in 3% H_2O_2, washed in PBS, and treated with 0.1 M PBS containing 0.3% Triton X-100 for 20 min, and then with 0.1 M PBS containing 5% normal rabbit serum for 60 min. The polyclonal antibody against PS-1 (1:45000, Chemicon) was applied for 48 h. This was followed by incubation with rabbit-anti-goat IgG-biotin (1:1000, Jackson Immunores. Lab.) for 90 min, and then with streptavidin-HRP (1:1000, Jackson) for 90 min. The sections were washed twice in 0.1 M PBS for 10 min between sera. When single staining was applied, the peroxidase complex was visualized by incubating the sections with 0.05 M TB containing 0.05% DAB, 0.15% $NiCl_2$ and 0.005% H_2O_2, resulting in a bluish-black colour.

When double-stained sections were prepared (stained either for PS-1 and APP or for AChE and PS-1), the samples were developed first in DAB dissolved in TB. The staining resulted in a brownish colour. Thereafter, the sections were treated for 30 min with 0.6% H_2O_2 to eliminate the possibly persistent activity of the marker enzyme. In the case of PS-1, the second staining was for APP, while the histochemical reaction for AChE was followed by either PS-1 or APP immunohistochemistry. After staining for the second antibody, the peroxidase complex was visualized by incubating the sections in a DAB-$NiCl_2$ mixture. In these double-stained sections, a brownish colour could be detected in some axons; in some others, the brownish colour was intermingled with a bluish-black colour; and in yet other axons, the bluish-black staining appeared as a separate entity.

4.2.5. Vesicular acetylcholine transporter immunohistochemistry

For a more exact identification of the cholinergic neurons, the tissues were immunostained for VAChT. After fixation, tissue samples 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, Chemicon) was applied for 48 h. This was followed by incubation with rabbit-anti-goat IgG-biotin (1:1000, Jackson Immunores. Lab.) for 90 min, and then with streptavidin-HRP (1:1000, Jackson) for 90 min. The peroxidase reaction was developed by using DAB and $NiCl_2$.

4.2.6. Synaptophysin immunohistochemistry

For the identification of neuronal synapses, the tissues were immunostained for SYN. After fixation, tissue samples were incubated with 2.5% Triton X-100, and then with 5% normal sheep serum in PBS. The polyclonal antibody against SYN (1:1000, Boehringer) was applied for 48 h. This was followed by incubation with sheep-anti-mouse IgG-biotin (1:1000, Jackson Immunores. Lab.) for 90 min, and then with streptavidin-HRP (1:1000, Jackson) for 90 min. The peroxidase reaction was developed by using DAB and $NiCl_2$.

4.2.7. GABA immunohistochemistry

For the identification of GABAergic neurons, the tissues were immunostained for GABA. After fixation, tissue samples were incubated with 2.5% Triton X-100, and then with 5% normal bovine serum in phosphate buffer. The polyclonal antibody against GABA (1:3000, Immunotech) was applied overnight at 37 °C. This was followed by incubation with goat-anti-rabbit secondary antibody (1:30, Jackson Immunores. Lab.) for 60 min, and then with rabbit peroxidase-antiperoxidase complex (1:200, Nordic Immun. Lab.) for 60 min. Staining was revealed with a solution containing DAB, $NiCl_2$ and H_2O_2 in TB.

4.2.8. Demonstration of amyloid-β peptide-positive structures and acetylcholinesterase activity in the same tissue section

For the visualization of AChE activity, a sensitive method[76] was used in a slightly modified form. After fixation, tissue samples were rinsed several times in 0.1 M sodium maleate buffer (pH 6.0). Sections were pretreated for 20 min at 25 °C in ethopropazine hydrochloride (10^{-3} M), rinsed in 0.1 M sodium maleate (pH 6.0), incubated for 80-120 min in a medium consisting of acetylthiocholine chloride (1.8 mM), sodium citrate (0.1 M), copper sulphate (0.03 M), potassium ferricyanide (5.0 μM) and sodium maleate (0.1 M; pH 6.0), and rinsed in TB solution. Finally, the sections were placed in a solution containing 0.05% DAB, 0.15% $NiCl_2$ and 0.03% H_2O_2 in TB. Non-specific cholinesterase activity was selectively inhibited continuously with ethopropazine hydrochloride ($1x10^{-4}$ M) introduced directly into the incubation medium.

After the demonstration of AChE-positive neurons, the tissues were immunostained for Aβ to identify the deposition sites of the peptide. After fixation, tissue samples were incubated with 2.5% Triton X-100, and then with 5% normal goat serum in PBS. The polyclonal antibody against Aβ (1:30000) was applied for 24 h. This was followed by incubation with goat-anti-rabbit IgG-biotin (1:1000, Jackson Immunores. Lab.) for 90 min, and then with streptavidin-HRP (1:1000, Jackson) for 90 min. The peroxidase reaction was developed by using DAB.

4.2.9. Western immunoblotting

Preparation of the sciatic nerve for SDS-PAGE

The control and ligated sciatic nerves were removed after ligation for 6, 12 or 24 h. Segments 4 mm in length of the sciatic nerves of the sham-operated control animals, and 4-mm segments from above the proximal ligature, from between the two ligatures, and from below the distal ligature of the sciatic nerve were excised on ice and immediately placed into a buffer solution which contained TB at pH 7.5, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 1 μg/ml pepstatin and detergents (1% Nonidet-P-40 and 0.1% sodium deoxycholate). Thereafter, the samples were homogenized. As the amount of PS-1 in the sciatic nerve was expected to be low, samples from two animals were pooled for PS-1 isolation. After centrifugation (10000 x g for 1 h at 4 °C), the proteins in the supernatant solutions were measured by the method of Hess. For analysis, 40 μg of protein was supplemented with Laemmli sample buffer and, after boiling for 5 min, loaded on 9.0% polyacrylamide gel for electrophoresis.

Western blot analysis

To ascertain whether only the C-terminal and N-terminal fragments, or the holoprotein of PS-1, or all of these, are transported in the sciatic nerve of rat, we used various polyclonal antibodies: AB5308 for the detection of the intact 45 kDa holoprotein and the C-terminal fragment [amino acids 275-367] and AB1575 to recognize the full-length and the N-terminal fragment [amino acids 14-33] of PS-1.

The separated proteins were transferred to nitrocellulose membranes by using the BioRad Mini-Protean II system. Membranes were blocked in 5% non-fat dry milk with 0.2% Tween-20 in 50 mM TBS for 1 h at room temperature. For the labelling of the fragments and the

holoprotein of PS-1, the antibodies were used in a dilution of 1:1000 in TBS containing 1% non-fat dry milk. The nitrocellulose membranes were incubated with the different primary antibodies overnight at room temperature. After the membranes had been washed five times in TBS, the filters were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) or horseradish peroxidase-conjugated rabbit anti-goat IgG (1:5000) as secondary antibody for 1 h at room temperature, followed by washing as described above. Bound antibodies were detected with the Supersignal Western blot chemiluminescence reagent, and this was followed by exposure to an autoradiography film.

Quantitation of blots

Optical densities (ODs) of immunoreactive bands were quantified by means of the NIH-Image program (NIH, USA). The intensity of the control band was taken as 100%, and changes were calculated with respect to this value. The reported data are the overall results of three different experiments. The error bar denotes the S.E. A probability level $P<0.05$ was taken to be statistically significant. Statistical evaluations were performed with Student's t-test.

4.2.10. Image analysis

In some of our experiments, image analysis was used. The OD in areas comprising the stained perikarya, dendrites and axons and the number of immunoreactive axonal varicosities was determined with a Quantimet 500MC Image Analysis System (Leica Cambridge) linked to a JVC-Color camera mounted on a Leica Laborlux "S" Leitz microscope.

The histochemical reactions were visualized and the digitized image (256 grey levels) was displayed on a colour monitor with 1024x768 pixel resolution. Five fields of each site were chosen at random. The fields of interest were measured and the OD measurements were averaged. All measurements were made under the same optical and light conditions.

5. RESULTS

5.1. *In vitro* experiments

5.1.1. Identification of cholinergic neurons and their axons in *in vitro* cell cultures (VI)

In the control tissue cultures (DIV7 to 17), the various types of cholinergic neurons (bipolar and multipolar) and the VAChT-immunoreactive axons, their varicosities and axon terminals could be revealed among many non-cholinergic cells. The VAChT immunoreactivity was observed mainly as puncta, and to a lesser extent as diffuse staining in

neurons of different sizes. The VAChT-immunoreactive axon terminals were present on the soma and dendrites of cholinoceptive and cholinergic cells (Fig. 7B-E).

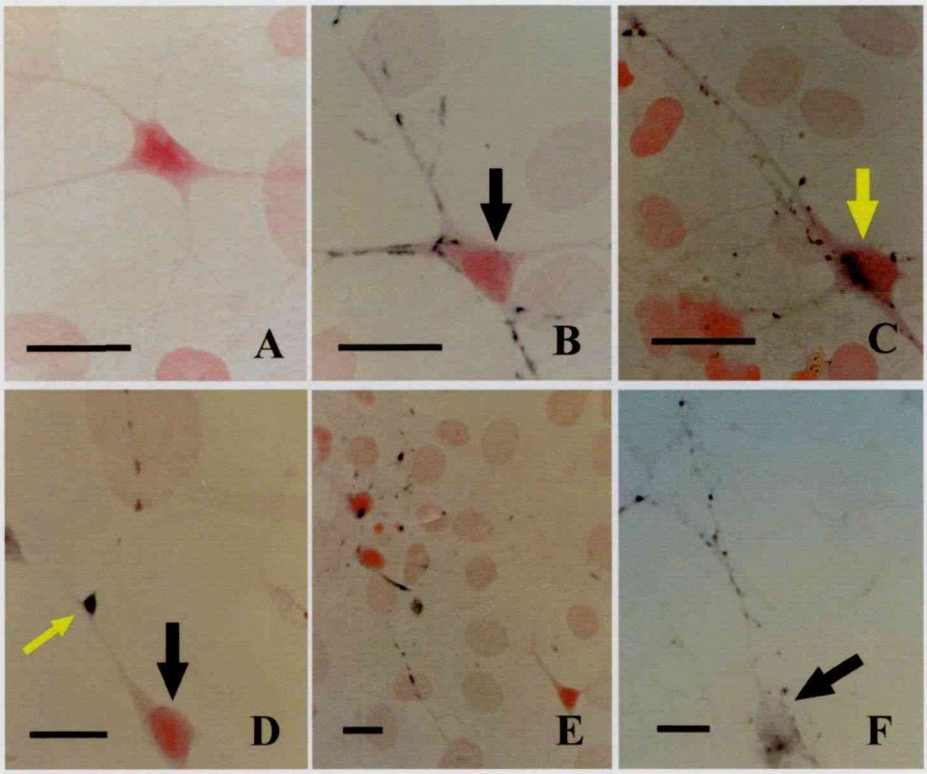

Fig. 7. Identification of cholinergic cells in *in vitro* tissue cultures with VAChT immunohistochemistry. Note the absence of immunostaining in a non-cholinergic multipolar cell (A), and the presence of VAChT-positive axon terminals on a cholinoceptive cell (B, arrow) and on a cholinergic neurons (C, arrow). In panel D, a VAChT-positive axon terminal innervates (yellow arrow) a dendrite of a non-cholinergic cell (black arrow). The distribution of VAChT-positive axon varicosities is presented in panel E. The SYN-positive axon terminals are present in a similar localization on the perikarya of a multipolar cell (F, arrow). Bar = 20 μm (A-D,F) or 10 μm (E) (Photos taken from my preparations.)

5.1.2. Neurotoxic effect of amyloid-β1-42 on cholinergic nerve cells in *in vitro* tissue cultures (VI)

After identification of the cholinergic neurons and their axon varicosities, we set out to study whether Aβ1-42 affects these neurons and their axon terminals. Using VAChT immunohistochemistry in control neuronal cultures derived from the basal forebrain on E18 and cultured for various time intervals (E18DIV7, E18DIV14 and E18DIV17), the density of the immunoreactive axons gradually increased (Fig. 8A-C). When similar neuronal cultures were treated for 2 days on days 5, 12 and 15 with 20 μM Aβ1-42, all the VAChT

immunoreactivity disappeared from the axon terminals (Fig. 8. D-F). This result suggests that Aβ in *in vitro* tissue cultures is very harmful to cholinergic neurons and their processes.

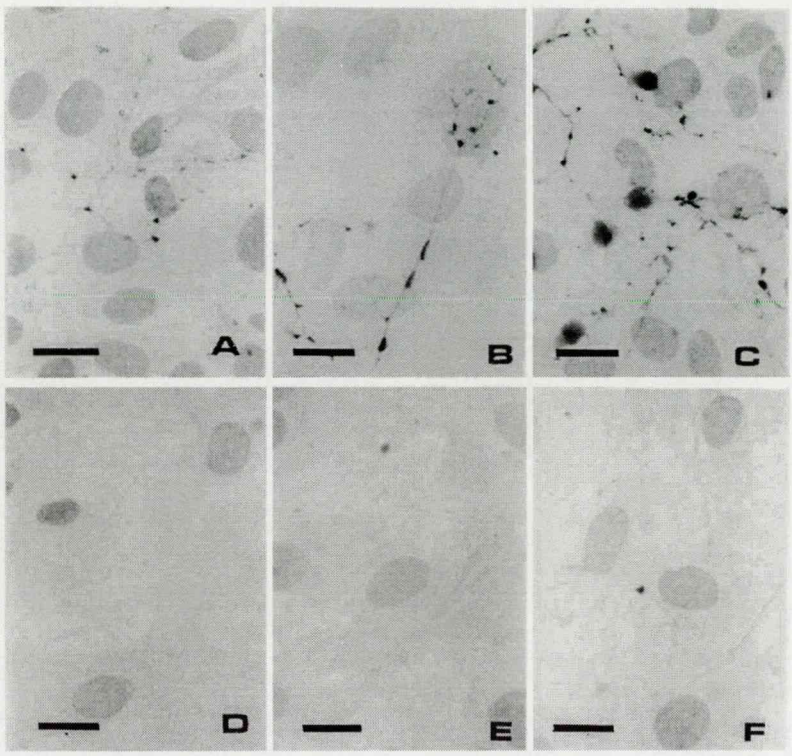

Fig. 8. Light microscopic localization of VAChT immunoreactivity in the cholinergic axon varicosities and in the axon terminals. Note that the numbers of small and large labelled puncta increase during the *in vitro* development of cholinergic neurons (A: DIV7; B: DIV14; and C: DIV17). After treatment with 20 μM Aβ1-42 for 2 days, the VAChT immuno-reactivity disappears in the basal forebrain tissue culture (D: DIV7; E: DIV14; and F: DIV17). Bar = 20 μm. (Papp *et al.*, 2001, in press)

Using image analysis, we next determined the number of VAChT axon varicosities of the cholinergic neurons during development in *in vitro* tissue cultures. Evaluation of the VAChT-positive axon varicosities and axon terminals in the control and Aβ1-42-treated samples is illustrated in Fig. 9.

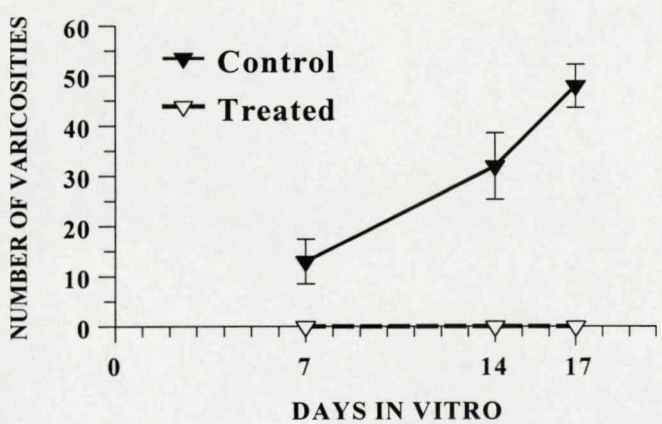

Fig. 9. Evaluation of VAChT-positive axon varicosities and/or axon terminals by image analysis in control and Aβ1-42-treated basal forebrain tissue cultures. The control samples were treated with the vehicle, and the other *in vitro* cultures with 20 μM Aβ1-42 for 2 days on days 5, 12 or 15. (Papp *et al.*, 2001, in press)

5.1.3. Neurotoxic effect of amyloid-β1-42 on synaptophysin-immunopositive axon terminals in *in vitro* tissue cultures. A comparative investigation with that of vesicular acetylcholine transporter immunostaining (VI)

In this comparative investigation, we were interested in whether Aβ1-42 acts only on the cholinergic neurons, or whether it exerts a more general effect and degenerates non-cholinergic nerve cells as well. If this is the case, we could demonstrate that the number of SYN-positive axon varicosities is reduced better than that of the VAChT-positive axon varicosities. 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. 10. A-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. 10. D-F).

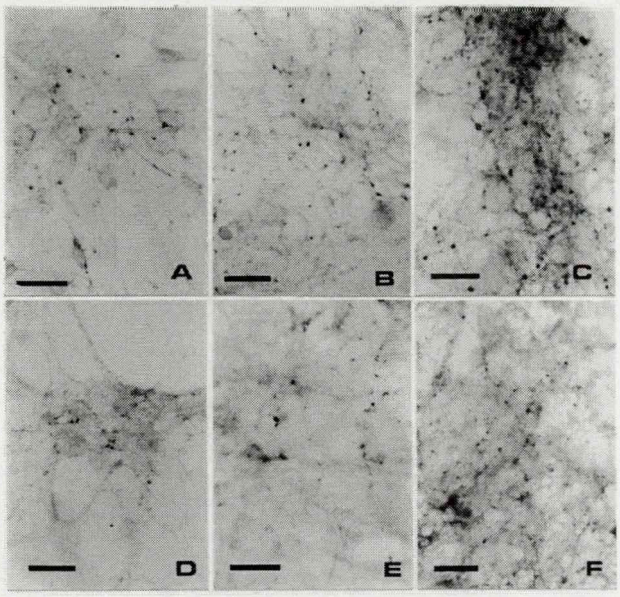

Fig. 10. Light microscopic localization of SYN immunoreactivity in the axon varicosities and in the axon terminals during development in the control basal forebrain neuronal cultures. Note the increased density of staining after the various time intervals. A: DIV7; B: DIV14; and C: DIV17. The SYN-immunore-active axon varicosities are reduced in the cultures after treatment with 20 μM Aβ1-42 for 2 days (D: DIV7; E: DIV14; and F: DIV17). Bar = 20 um. (Papp *et al.,* 2001, in press)

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. 11.

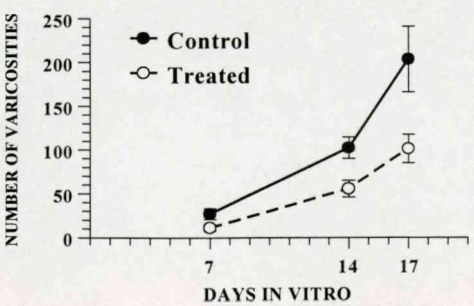

Fig. 11. Evaluation of SYN-positive axon varicosities and/or axon terminals by image analysis in control and Aβ1-42-treated basal forebrain tissue cultures. The control samples were treated with the vehicle, and the other *in vitro* cultures with 20 μM Aβ1-42 for 2 days on days 5, 12 or 15. (Papp *et al.,* 2001, in press)

5.1.4. Neurotoxic effect of amyloid-β1-42 on acetylcholinesterase-positive nerve cells in *in vitro* tissue cultures (V)

In earlier experiments, we demonstrated the neurotoxic effect of Aβ1-42 on the VAChT-positive cholinergic nerve cells (Section 5.1.2.) and on the SYN-positive axon varicosities (Section 5.1.3). These results suggested that Aβ1-42 acts not only on the cholinergic neurons, but also on other non-cholinergic and AChE-positive cells as well. Histochemical evidence in support of this suggestion is presented below.

In the control basal forebrain cultures (E16DIV4), the specific staining for AChE was evenly distributed in the perikarya and processes of the neurons (Fig. 12. A). After treatment with 20 μM Aβ1-42 for 24 h (E16DIV4+1), the localization of the staining pattern was changed. The ACh-positive reaction sites appeared to be present either dislocated to one area of the cell body (Fig. 12. B) or in one segment of the dendrite only (Fig. 12. C). After 3 days of *in vitro* treatment (E16DIV4+3), the AChE reaction end-product could be detected only in the degenerated dendrites (Fig. 12. D).

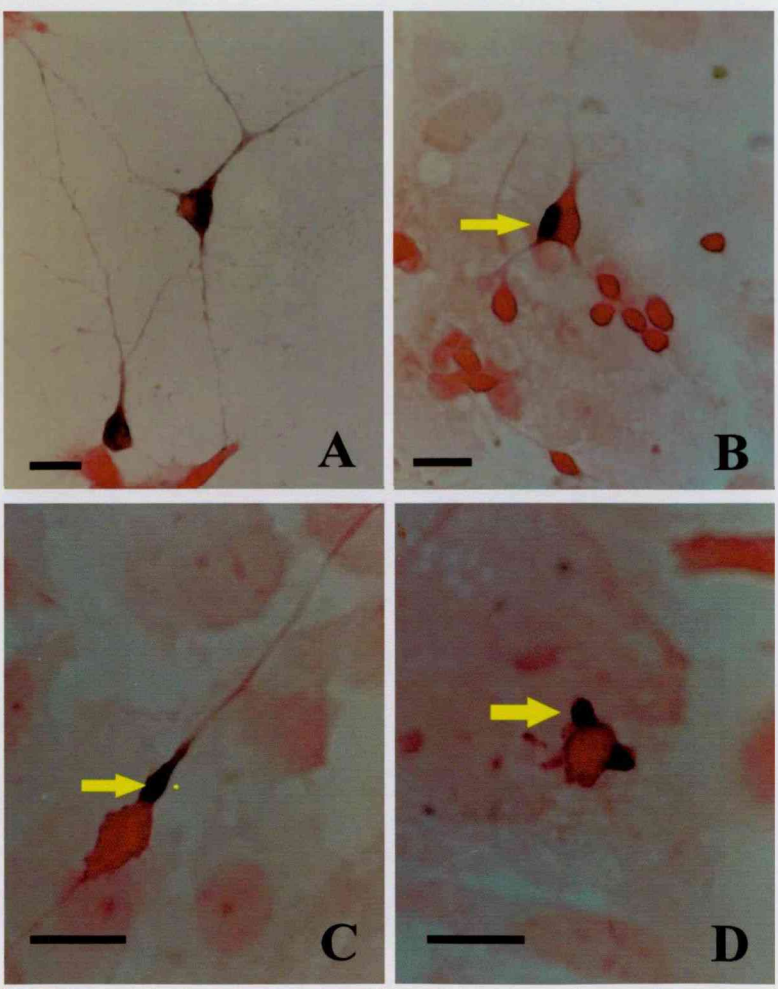

Fig. 12. AChE histochemistry in *in vitro* neuronal cultures. Note the pattern of enzyme staining in the control sample (A) and after Aβ1-42 treatment (B-D). Bar = 25 μm (Pakaski, Papp *et al.*, 1999.)

5.1.5. Neurotoxic effect of amyloid-β1-42 on GABAergic nerve cells in *in vitro* tissue cultures (IV)

In these experiments, we examined whether Aβ1-42 has a neurodegenerative effect on GABAergic nerve cells.

The staining pattern of the control cultures demonstrated a diffuse intracellular distribution of GABA in the perikarya and long processes of the neurons (Fig. 13. A-C). The neurons in the culture exposed to 20 μM Aβ1-42 revealed characteristic morphological changes: 1 day after Aβ1-42 treatment (DIV4+1), varicosities could be detected on the axons and terminal arborization of a large number of neurons (Fig. 13. D). By day 3 after Aβ1-42 incubation (DIV4+3), not only were varicosities visible on all the processes of the neurons (Fig. 13. E), but the number of GABA-immunoreactive neurons was also decreased. Five days after AβP1-42 exposure (DIV4+5), the GABA immunoreactivity was restricted to the perikarya in most of the positively stained neurons (Fig. 13. F), and the number of GABAergic cells was further decreased. The GABA-positive cells exhibited morphological indications of process degeneration.

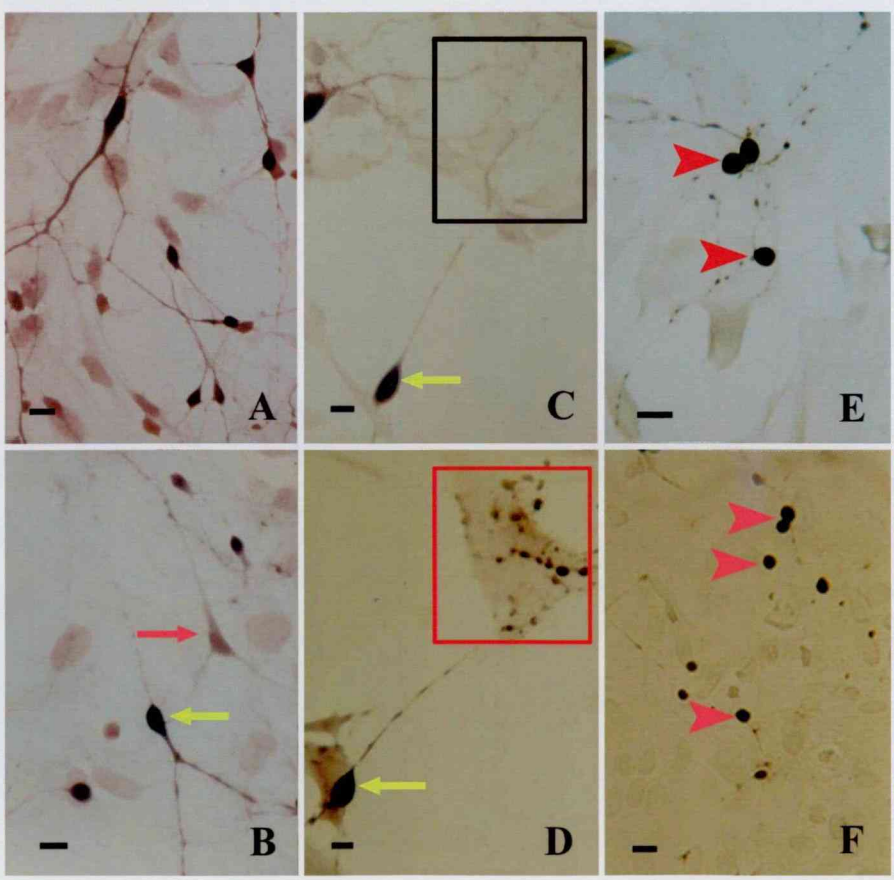

Fig. 13. Immunohistochemical staining of GABAergic neurons. Note the intactness of the neuronal perikarya (arrows in B and C) and axons of the GABA-positive cells (A-C and caged area in C). After 20 μM Aβ1-42 treatment, the neuronal perikarya are rounded (arrows in E and F) and the axons and axon terminals are fragmented (caged area in D). Bar = 25 μm (A) or 10 μm (C-F). (Pakaski, Papp *et al.*, 2000)

5.2. *In vivo* experiments

5.2.1. Axonal transport of presenilin-1 (III)

APP and PS-1 are not only of importance for the normal functioning of the various neurons, but also play central roles in the pathogenesis of AD.[4,12] First, we studied the axonal transport of PS-1 in a spinal cord-sciatic nerve-NMJ model system in rat.

PS-1 immunoreactivity was revealed in a discontinuous manner in a number of axons in the control sciatic nerve (Fig. 14. A). Similar axonal staining was seen in the sensory axons leaving the L5 and L6 dorsal root ganglia and in a number of Schwann cells around the myelinated axons. In the double-ligated samples, PS-1 was accumulated above the upper ligature (Fig. 14. D) and below the lower ligature (Fig. 14. E) after 6, 12 and 24 h. Between 6 and 24 h, however, the PS-1 staining gradually increased only on the proximal side. The staining was always more intensive in the segment proximal to the ligatures than below the lower ligature. Between the two ligatures, PS-1 staining was observed only in the cytoplasm of the Schwann cells (not demonstrated) after 24 h. The results of immunohistochemistry as concerns the anterograde and retrograde transport of PS-1 were verified by semiquantitative Western blot studies.

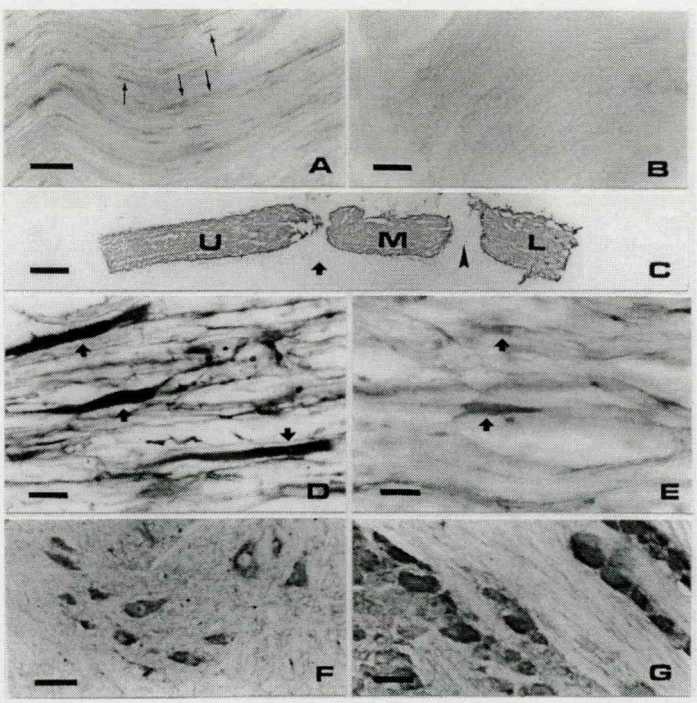

Fig. 14. Immunohistochemical demonstration of PS-1 in the various tissues. PS-1 staining appears in a discontinuous manner (arrows) in the axons of the control sciatic nerve bundle (A). No immunoreactivity was detected in the sciatic nerve samples without primary antibody (B). The arrangement of the double ligation on the sciatic nerve is demonstrated in C, where U indicates the segment above the proximal ligature (arrow), M the segment between the ligatures, and L the segment below the lower ligature (arrowhead). After 24 h of double ligation, a pronounced accumulation of PS-1 occurred above the proximal ligature (D, arrows). In the segment below the lower ligature, the staining for PS-1 was less expressed (E, arrows). PS-1 immunoreactivity occurred in the perikarya of the motoneurons (F) and the sensory nerve cell bodies of various sizes of the dorsal root ganglia (G). Scale bar = 50 μm (A, B, F, G), 1.0 mm (C) or 25 μm (D, E). (Kasa, Papp and Pakaski, 2001)

Western blotting confirmed the accumulation of the ~20-kDa C-terminal and ~25-kDa N-terminal fragments and the full-length 45-kDa holoprotein of PS-1 both above and below the ligature (Fig. 15).[33]

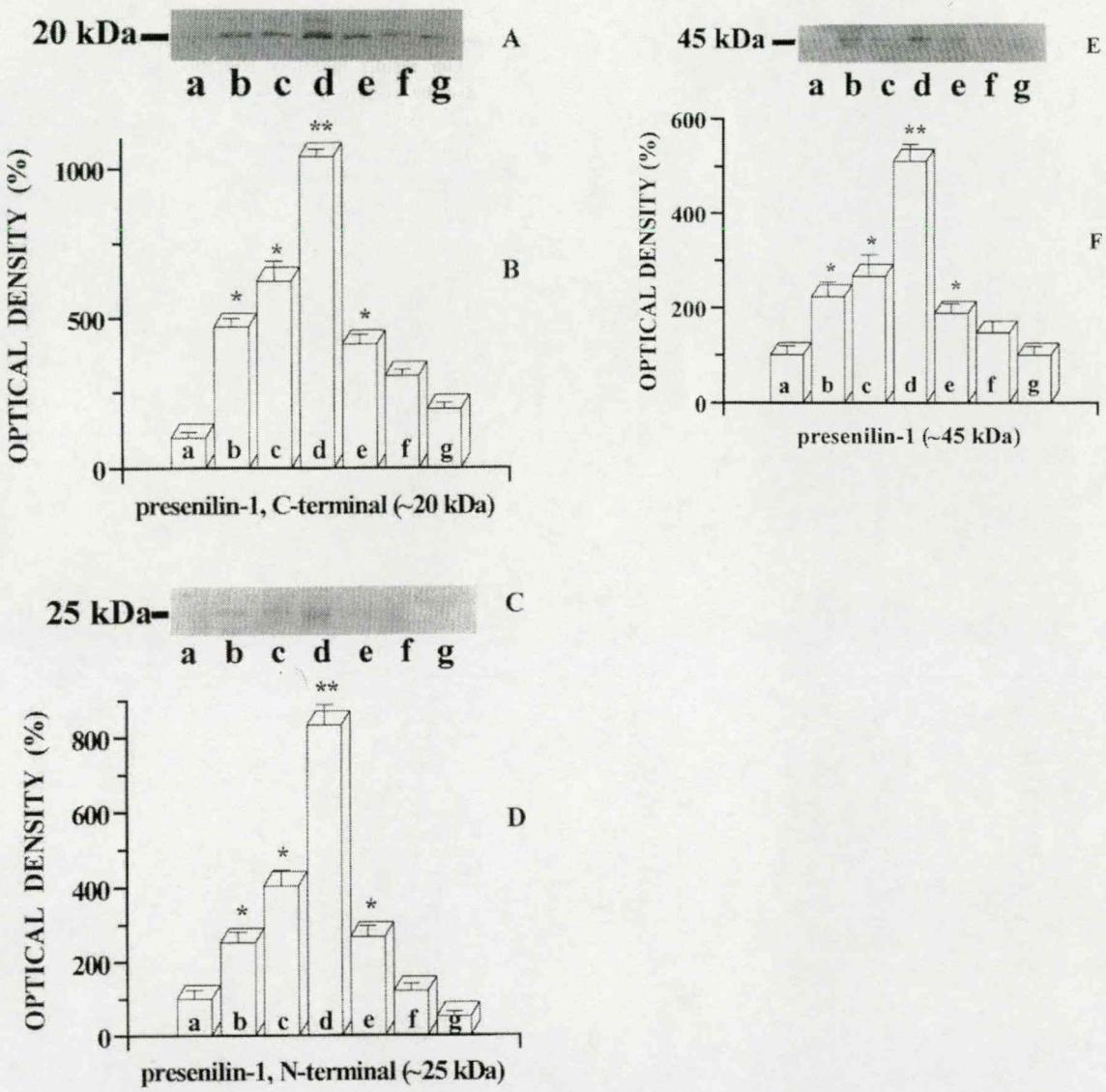

Fig. 15. Western blot of protein extracts from control samples (a) and from samples double-ligated for 6 h (b), 12 h (c) or 24 h (d) above the proximal ligature, or for 6 h (e), 12 h (f) or 24 h (g) below the distal ligature of the sciatic nerve. The polyclonal antibody AB5308 detected ~20-kDa protein (A) corresponding to the C-terminal PS-1 and intact ~45-kDa protein (E), while antibody AB1575 detected ~25-kDa protein (C) corresponding to the N-terminal PS-1. The immunoblot results in A, C and E were quantified and are demonstrated in histograms B, D and F, respectively. The OD is expressed as a percentage of that for the control sample (100%). The reported data are the overall results of three different experiments. The error bar denotes the S.E. Statistical evaluations were performed with Student's *t*-test. Significant difference from the control sample: * $P<0.05$, ** $P < 0.001$. (Kasa, Papp and Pakaski, 2001)

5.2.2. Axonal transport of amyloid precursor protein

Although the anterograde transport of APP in the sensory axons of the sciatic nerve has already been described[38], we re-examined the question of whether APP is transported only in an anterograde direction or similarly to that of PS-1. In the control nerve, the APP staining appeared in a similar localization and axonal distribution as for PS-1 (Fig. 16. A). When the nerves were removed after 6, 12 or 24 h, the accumulation of APP in the axons, similarly to that of PS-1, was revealed both proximally (Fig. 16. B) and distally (Fig. 16. C) to the ligatures. In a number of axons, the staining in the axoplasm was particularly intense on the proximal side, relative to the distal side.

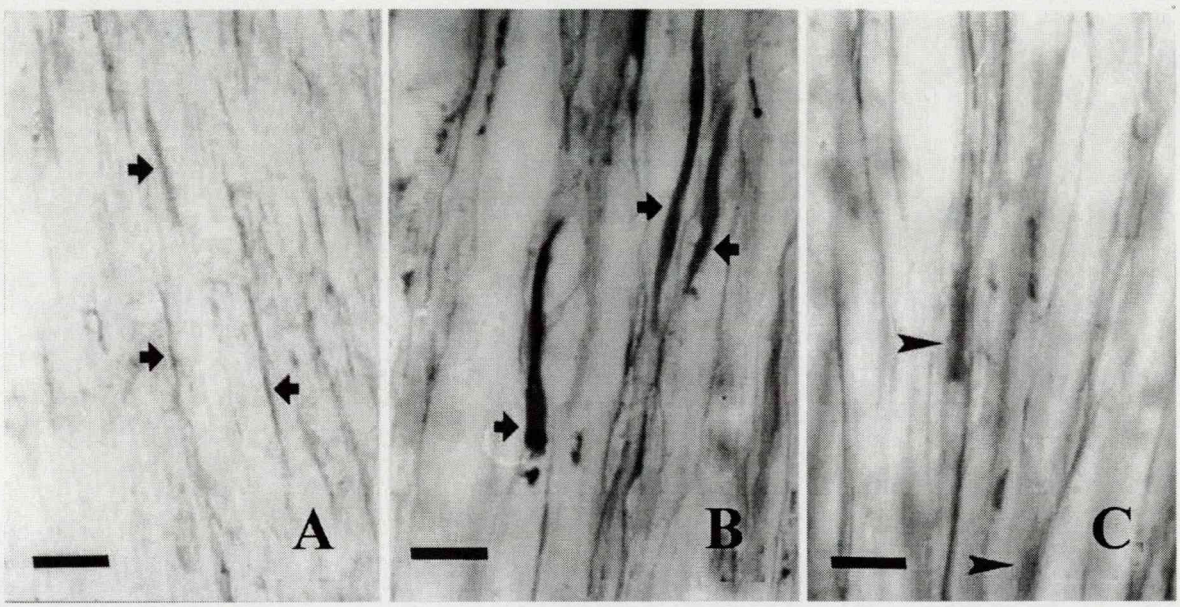

Fig. 16. Immunohistochemical demonstration of APP in the control (A, arrows) and double-ligated (B,C) sciatic nerve of rat. After 24 h of double ligation, a pronounced accumulation of APP occurred above (B, arrows) and below (C, arrowheads) the ligature.
Scale bar = 25 μm.

To quantify the immunohistochemical results, Western blot experiments were carried out in the control and the double-ligated sciatic nerve (Fig.17, panel A). Both 94-kDa and 116-kDa APP were present in the control sample (a) and the amounts of these forms accumulated above the proximal ligature up to 6 h (b), 12 h (c) and 24 h (d). After accumulation for 6 h (e), the amounts below the lower ligature decreased (f, g). The Western blot results were quantified and are demonstrated in histograms (panels B-D). The amounts of 94-kDa plus 116-kDa APP are depicted in panel B. The difference in the accumulation of 94-kDa and 116-

kDa APP is represented in panels C and D, respectively. These results[59] clearly demonstrate that APP is transported in both the anterograde and the retrograde direction in the sciatic nerve of rat.

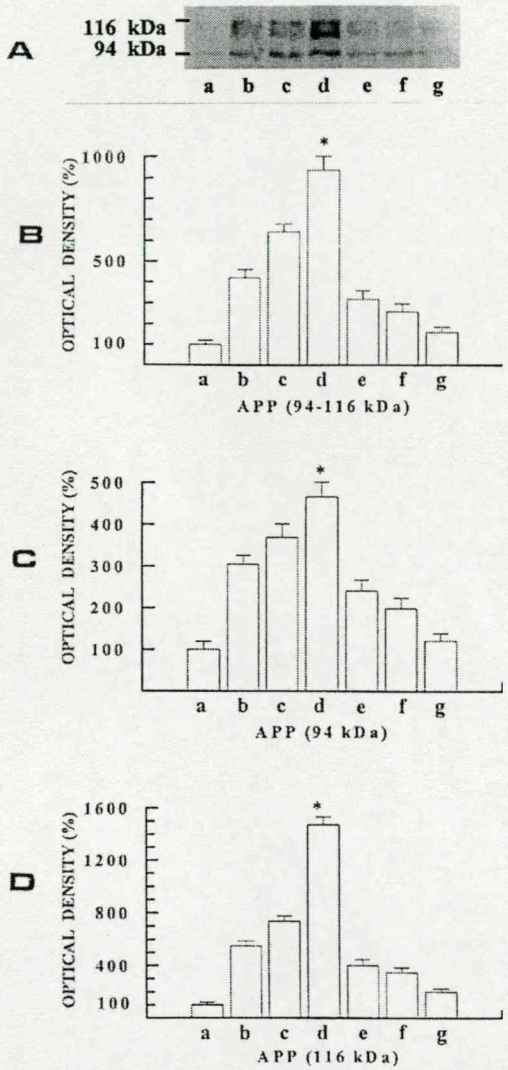

Fig. 17. The APP Western blot results on the ligated sciatic nerve (A). The OD in panels B-D is expressed as a percentage of that for the control sample (100%). Data are given as means ± S.E.M. Statistical evaluations were performed with Student's *t*-test. * signifies $p < 0.01$. Number of determinations = 3.

5.2.3. Transport of amyloid precursor protein and presenilin-1 in the motoric axons of the sciatic nerve of rat

The next question was to ascertain whether APP and PS-1 are transported in the motoric nerves or the sensory nerves or both. For this, double staining of the sciatic nerve for AChE and PS-1 or for AChE and APP was carried out in the sciatic nerve of rat (Fig. 18). The results[33] demonstrated overlapping immunoreactivity of APP and AChE and of AChE and PS-1 in the motoric axons, suggesting that both proteins reach the axon terminals and, after functioning there, return with information to the cell body.

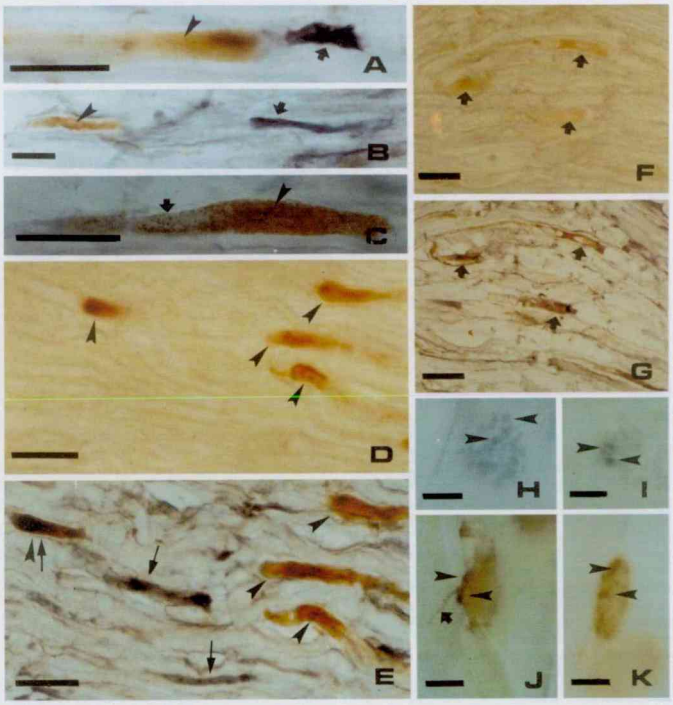

Fig. 18. Double-stained samples for AChE and PS-1 (A-C), for AChE and APP (D,E) and for PS-1 and APP (G). In the sciatic nerve double-ligated for 24 h and double-stained first for AChE (brown, arrowhead) and thereafter for PS-1 (bluish-black, arrow), the two types of staining appeared either in distinct regions within the same axon (A), or in different axons (B), or in the same areas of the fibres (C) above the proximal ligature. In C, the PS-1 immunoreactivity is present in dot-like structures (arrow) in the region where the AChE (arrowhead) is present. In samples first stained for AChE (D, arrowheads) and thereafter for APP, either the two reaction end-products appeared in the same some axon (E, arrowhead plus arrow), or the AChE positivity (arrowheads) and the APP immunoreactivity (arrows) were present in separate fibres (E). The immunoreactivity for PS-1 (F, arrows), PS-1 and APP (G, arrows) could be revealed above the ligature in the same areas of the same axon in the sciatic nerve double-ligated for 24 h. In the muscle samples, the APP (H) and PS-1 (I) immunoreactivity appears as discrete dots (arrowheads). In double-stained muscle samples, the postsynaptic sites were delineated by the AChE histochemical reaction (J, K, brown), while the PS-1 immunoreactivity appeared in the motoric axon (J, arrow) and in the presynaptic axon terminals (J, K, arrowheads) at the NMJ. (Kasa, Papp and Pakaski, 2001) Bar = 25 μm (A-G), 10 μm (H-K).

5.2.4. *In vivo* neurotoxic effect of human amyloid-β1-42 on axonal transport (II)

The neurotoxic effect of Aβ1-42 in the pathogenesis of AD is important. Here, we provide experimental evidence that such an effect *in vivo* may be due to the inhibition of axonal transport in the neurons.

Our results demonstrated that exogenously applied human Aβ1-42 could disturb the axonal transport of AChE, APP, VAChT and SYN in the sciatic nerve of rat (Fig. 19; a detailed description of the results can be found in paper II). The accumulation of immunoreactivity was restricted to the area where Aβ1-42 was present.

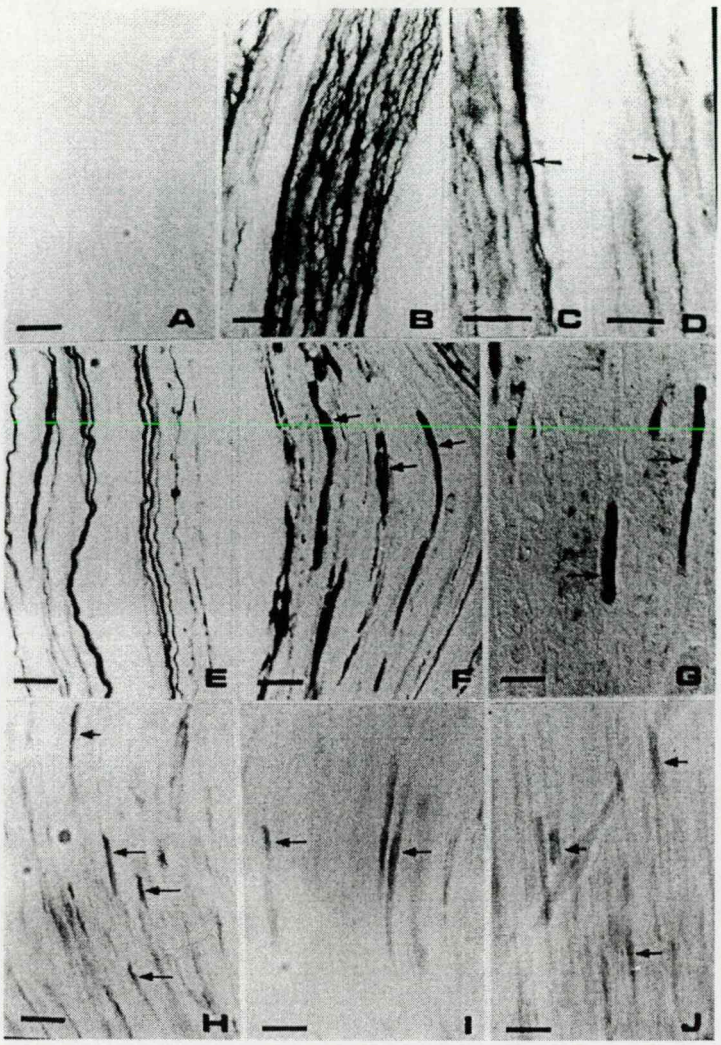

Fig. 19. Aβ staining could not be detected in the control nerve trunk of rat (A). After the application of human Aβ1-42, however, dark staining could be observed at the injection site among the myelinated axons (B) and at the node of Ranvier (C, D). In the control sciatic nerve, AChE staining was demonstrated in the cholinergic axons, as described earlier. In the nerve trunk treated with Aβ1-42 for 24 h, a considerable accumulation of AChE occurred at the injected site (F, G). In the control sciatic nerve, no immunohistochemical staining of APP, VAChT and SYN could be observed. 24 h after the injection of Aβ1-42, the APP (H), VAChT (I) and SYN (J) reactions were present in the axonal swellings, indicating local axonal injuries. (Kasa, Papp *et al.*, 2000)

5.3. Effects of acetylcholinesterase inhibitors

5.3.1. Effects of acetylcholinesterase inhibitors in autopsy human brain samples (I)

Although the use of AChE inhibitors is the most highly developed approach for the treatment of AD, the precise morphological sites of their action have as yet not been demonstrated. Nor is it known how AChE inhibitors with known central effects influence the enzyme activities in the PNS. This part of this thesis relates to the effect of donepezil on the AChE activity in human autopsy brain samples. Donepezil is used for the treatment of patients with AD. (A detailed description of the results obtained on donepezil can be found in paper I.)

The AChE-positive structures in the different areas of the human brain proved to be sensitive to donepezil in a dose-dependent manner. The most important finding in this

experiment was that donepezil inhibited the AChE activity in the postsynaptic cholinoceptive neurons more effectively than that in the presynaptic cholinergic axons. Donepezil ($2x10^{-8}$ M) inhibited most of the AChE-positive neurons and reduced some of the axonal staining in the temporal cortex (Fig. 20).

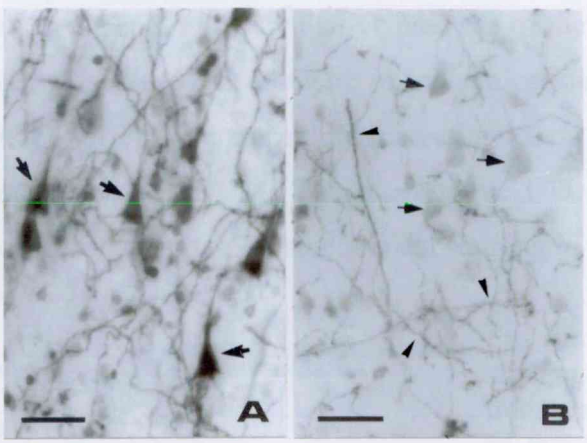

Fig. 20. Photographs showing the differences in AChE activity between the control (A) and the donepezil-treated (B) human temporal cortex. Note the presence of intensely-stained pyramidal cells (arrows) and the AChE-positive nerve fibres in the control sample (A), and the disappearance of AChE activity from most of the neuronal perikarya (arrows) in the AChE-positive nerve fibres in the control sample (A), and the disappearance of AChE activity from most of the neuronal perikarya (arrows) in the $2x10^{-8}$ M donepezil-treated sample (B). A stained neuron can rarely be observed in the donepezil-treated sample, though some AChE positivity is still present in many cholinergic nerve fibres (arrowheads) (B). Scale bar = 50 μm. (Kasa, Papp *et al.,* 2000)

For quantitative analysis of the inhibitory effect of donepezil, we measured the changes in AChE histochemical staining in the AD brain. The results revealed a dose-dependent inhibitory effect of donepezil in the various areas of the cortex, the hippocampus, the caudate-putamen and the NBM of the human brain (Fig. 21).

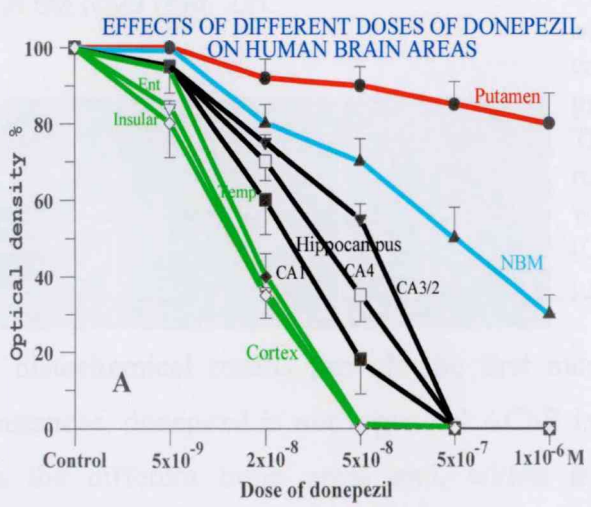

Fig. 21 Changes in OD in the AChE histochemical staining in the various areas of the human brain after treatment with different doses of donepezil. The OD of the AChE histochemical reaction in the control sample is taken as 100%. Five fields of each area of interest were measured (n = 5) and the OD results were averaged. Symbols are as follows: putamen (●), NBM (▲), hippocampus CA1 (■), hippocampus CA3 and CA2 (▼), hippocampus CA4 (□), temporal cortex (◆), entorhinal cortex (▽) and insular cortex (◇). (Kasa, Papp *et al.,* 2000)

5.3.2. Effects of acetylcholinesterase inhibitors in rat brain (I)

Similarly as in the human brain (see above), various concentrations of donepezil ($5x10^{-9}$ M, $2x10^{-8}$ M, $5x10^{-8}$ M, $5x10^{-7}$ M and $1x10^{-6}$ M) dose-dependently inhibited the AChE staining in the various areas of the rat brain. The detailed results of these experiments can be found in paper I. For quantitative analysis of the inhibitory effect of donepezil, we measured the changes in the OD of AChE histochemical staining. The results revealed a dose-dependent inhibitory effect of donepezil in the various areas of the cortex, the hippocampus, the caudate-putamen and the NBM. The quantitative OD results relating to the inhibitory effects of donepezil on the AChE histochemical staining in the rat brain are presented in Fig. 22.

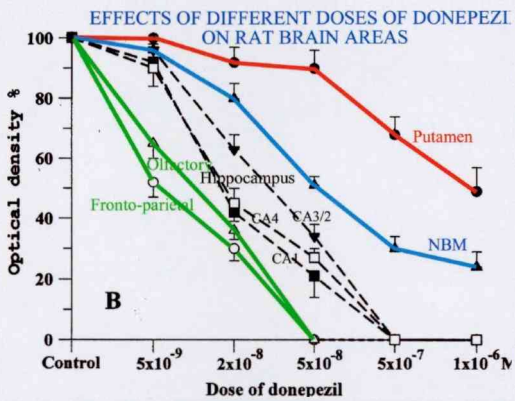

Fig. 22. Changes in OD in the AChE histochemical staining in the various areas of the rat brain after treatment with different doses of donepezil. The OD of the AChE histochemical reaction in the control sample is taken as 100%. Five fields of each area of interest were measured (n = 5) and the OD results were averaged. Symbols are as follows: caudate putamen (●), NBM (▲), hippocampus CA1 (■), hippocampus CA3 and CA2 (▼), hippocampus CA4 (□), fronto-parietal cortex (○) and primary olfactory cortex (△). (Kasa, Papp *et al.*, 2000)

During the treatment of human patients with donepezil, the fasciculation of different muscles can occur. Among others, we studied the inhibitory effect of donepezil on AChE activity at the NMJ. The results showed that donepezil has not only an AChE-inhibitory effect in the various areas and in the various neuronal structures in the CNS, but also an undesired effect at the NMJ (Fig. 22).

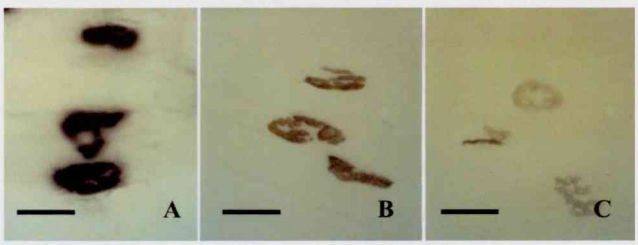

Fig. 23. Photographs showing the AChE activity of the NMJ in the diaphragm of a control rat (A). Heavy AChE reaction end-product is present in the control sample (A). The application of $2x10^{-8}$ M donepezil reduced the enzyme staining slightly (B), whereas $5x10^{-7}$ M donepezil did so strongly (C) in the motor end-plate. Scale bar = 50 µm. (Kasa, Papp *et al.*, 2000)

These histochemical results provide the first morphological evidence that, under *in vitro* circumstances, donepezil is not a general AChE inhibitor in the CNS, but rather selectively affects the different brain areas and, within these, the cholinergic and cholinoceptive structures.

6. DISCUSSION

6.1. Toxicity of amyloid-β1-42 in *in vitro* cell cultures

To explain the massive neuronal loss in the brain of AD patients, several models have been proposed, Aβ being implicated as the principal culprit.[30,41] Indeed, according to the *amyloid cascade hypothesis*,[28] Aβ may play key roles in the AD pathology, although the mechanism by which it becomes involved in the neurodegeneration is still unclear. In this context, it is worthwhile to mention that Aβ is not a pathological product, but a physiological product found in the culture-conditioned medium or cerebrospinal fluid. This suggests that, besides its well-known pathological function, Aβ has a physiological function too.

The earlier neurochemical and neuropathological data emphasized the relatively selective vulnerability of the cholinergic system to Aβ, which led to the *cholinergic hypothesis.*[2] However, data accumulated which indicated that, besides the cholinergic neurons[1,16], other transmitter-containing neurons undergo degeneration in AD.[9,27,43,77]

The establishment of *in vitro* systems that can recapitulate some aspects of the pathogenesis of AD is of utmost importance for an advancement of our understanding of the disease mechanism. Although the neurotoxicity of Aβ has been observed in various cell cultures,[19,79] no *in vitro* or *in vivo* data were earlier available relating to comparisons of the vulnerability of cholinergic, cholinoceptive and GABAergic neurons to Aβ toxicity. Our study for the first time identified the cholinergic neurons in *in vitro* tissue cultures[53,54] and revealed that Aβ1-42 and its fragments (Aβ31-35 and Aβ34-39) are toxic not only to cholinergic neurons, but also to cholinoceptive and GABAergic neurons in basal forebrain cultures.[51,52,55-58] The toxic effect predominated mainly on small, bipolar neurons, while the large multipolar neurons displayed more resistance to Aβ. Our results are in contradiction with the data of Pike and Cotman,[62] who found that GABAergic neurons are resistant to Aβ treatment. We hypothesize that the apparent controversy may arise from differences in the solvent and in the concentrations of the peptides applied in the different experiments. Another difference lies in the age and origin of the cultures used by our group and by Pike and Cotman. It is unclear how Aβ may have a toxic effect on the various types of neuronal cultures. It has been suggested that it is not the soluble form,[47] but rather the aggregated form of Aβ which is toxic in *in vitro* tissue cultures. However, it seems unlikely that aggregated Aβ

peptide may interact with specific binding sites of the neuronal perikarya and axonal membrane. A more plausible explanation would be direct surface interactions between Aβ and the lipid bilayer present in the membranes. In support of this suggestion, Aβ was also found to be toxic when it was applied as a fresh solution. The concentration of the peptide applied in *in vitro* tissue cultures is important. It has been found that a low concentration (below 1 μM) may have a trophic effect, while at high concentrations (above 10 μM) it has a neurotoxic effect.[80] In our experiments, 20 μM Aβ was found to be neurotoxic to cholinergic, cholinoceptive and GABAergic neurons. Our *in vitro* tissue culture data are therefore in close relationship with data found on AD patients: besides the cholinergic neurons, other neurotransmitter systems may be involved in the aetiopathology of the disease.[27,77]

6.2. *In vivo* effect of amyloid-β1-42 on axonal transport

The typical neuropathological features of AD include the cerebral deposition of Aβ1-42 in the SPs and the degeneration of cholinergic nerve cells.[35] The disease is characterized by a neuronal dysfunction in which the microtubule-associated tau-protein is abnormally hyperphosphorylated, which in turn may cause a disruption of the microtubule network and affect the axonal transport. If this suggestion is correct, it may be assumed that substances that are rapidly transported axonally should accumulate in the axon after Aβ treatment. It was earlier demonstrated that proteins, including AChE,[69] APP,[38,61,73] SYN and VAChT,[42] accumulate in a nerve trunk when it is damaged by mechanical lesions. There had been no previous report of the *in vivo* effects of human Aβ1-42 on the axonal transport of these proteins. We therefore used membrane-bound proteins to demonstrate the neurotoxic effects of Aβ1-42 on axonal transport. A new experimental set-up was introduced to study the possible *in vivo* effect of Aβ on the axonal transport of various enzymes and axonal proteins. It was found that under *in vivo* circumstances Aβ very effectively inhibited the transport of different proteins.[32]

The mechanism whereby Aβ can block the fast axonal transport of AChE, APP, VAChT and SYN is not clear at present. Several hypotheses have been put forward to explain the neurotoxic effect of Aβ.[19] It has been amply demonstrated that the aggregated and soluble forms of the peptide are very harmful to neurons and endothelial cells both *in vitro*[36,37,52] and *in vivo*.[18,24] Extracellularly applied Aβ was internalized selectively by hippocampal CA1

neurons and caused the accumulation of amyloidogenic carboxy terminal fragments of APP. In our experiments, the immunohistochemical localization of the injected peptide suggested that it could be internalized into the axoplasm at the nodes of Ranvier. If this is the case, Aβ may inhibit the transport inside the axon by affecting dynein, the dynactin complex and the kinesin system, known to be responsible for the fast axonal transport. When Aβ is applied exogenously, the accumulation of APP is regulated by tau-protein kinase I/glycogen synthase kinase-3β, resulting in an impairment of axonal transport and in the cytoplasmic accumulation of APP. The results of this study support the suggestion that AD may be initiated by a failure in axonal transport, which is followed by neuronal degeneration.

6.3. Effect of donepezil on acetylcholinesterase activity in central and peripheral nervous tissues

In biochemical experiments, donepezil has been shown to inhibit AChE activity in the human brain and in human erythrocytes. Neurochemical studies have demonstrated that presynaptic cholinergic markers such as ChAT, the ACh level and the high-affinity choline uptake are significantly reduced in the cortex, the hippocampus and the NBM in AD.[35] Since the neocortex and the hippocampal formation are known to be major sites of the neuropathologic changes in AD, inhibition of the residual AChE activity in these areas might well be beneficial in alleviating the cholinergic hypofunction in AD. Indeed, in clinical applications, donepezil is used for the treatment of memory loss and the behavioural deterioration associated with the ACh deficit in AD.[3,14]

Biochemical experiments have demonstrated that AChE and its different molecular forms (soluble and membrane-bound) are reduced in the brain in AD[64,65] and anomalous AChE activity appears in the SPs and cerebrospinal fluid. This histochemical study revealed the noteworthy finding that the AChE activity present in the postsynaptic cholinoceptive cortical pyramidal cell body is more sensitive than the presynaptic cholinergic axons to donepezil. (It is interesting to note that the cholinergic neurons present in the NBM, which receives cholinergic innervations and may therefore be regarded as cholinoceptive, are not as sensitive to donepezil as the purely cholinoceptive pyramidal cells within the cortical layers.[31]) Our results therefore provide the first morphological evidence of a difference in the effects of donepezil on the presynaptic cholinergic and postsynaptic cholinoceptive sites in the human brain. Similarly to our results, in biochemical experiments where donepezil was added to

soluble and crude mitochondrial fractions obtained from non-demented or AD brain, the AChE activity was inhibited in the soluble and the particulate fractions at a low concentration of the inhibitor. Donepezil inhibits the postsynaptic AChE activity better than the presynaptic AChE activity, which is quite remarkable. By slowing down the hydrolysis of ACh at the synaptic sites, donepezil will enhance the effect of the residual cholinergic presynaptic elements in AD. These results are similar to those observed for heptyl-physostigmine.

In biochemical experiments, donepezil has been shown to inhibit the AChE activity in the brain, the cortex and the hippocampus of rat. It elevates the extracellular levels of catecholamines and ACh in the cerebral cortex and the hippocampus. *In vitro* experiments[49] revealed that the IC_{50} of donepezil on the AChE activity present in the homogenate of the rat brain cortex is around 10^{-8} M, which is somewhat less than suggested by our OD experiments. The differences can be explained, however, in terms of the methods used in the various studies. In *in vivo* animal studies, donepezil has been demonstrated to improve the working memory.[22]

The *in vivo* inhibitory effect of donepezil on AChE activity has also been investigated in the frontal cortex, the hippocampus, the striatum and the hypothalamus of rat. Biochemical studies suggested that the different doses of donepezil inhibited the AChE activity in the frontal cortex more markedly than that in the striatum. Our histochemical results clearly demonstrated that the different neuronal elements (cell bodies and axonal processes) in the various brain regions are affected differently, and even within the same region, such as the cortex, the hippocampus, the NBM and the striatum. This therefore provides firm morphological evidence that the cholinoceptive pyramidal neuronal perikarya are more sensitive than the cholinergic presynaptic axons in the cortex and the hippocampus of rat to a given concentration of donepezil. On the other hand, the neuronal cell bodies in the NBM, from where the cholinergic axons emanate to various areas of the cortex, are moderately resistant to donepezil inhibition. We found that the most resistant AChE-positive neuronal perikarya are located in the putamen, the NBM, the diagonal band of Broca and some cells in the hypothalamus. As an alternative interpretation, higher concentrations of donepezil are needed to inhibit AChE in the basal forebrain neurons and the striatum because of their higher AChE activity. Via the AChE-inhibitory effect of donepezil in neuropharmacological experiments, a microdialysis technique has shown that the oral administration of donepezil leads to an increase in the level of ACh in the cerebral cortex of rats.

It is well documented that the intra- and extracerebral blood vessels and capillaries are innervated not only with aminergic, but also with cholinergic nerve fibres, in both the human and the rat brain.[68] The brain cortical blood flow is largely dependent on the normal function of cholinergic neurons located in the basal forebrain and projecting their axons to the cortex. A dysfunction of these cholinergic neurons or their axon terminals innervating the blood vessels may therefore lead to pathology of the cortical microcirculation.[48] Indeed, a disturbed cerebral blood flow has been demonstrated in AD.[63,74] The abnormalities may be dependent on cerebral endothelial cells, profound irregularities in the course of the vessels, thickening of the vascular basement membrane and a reduction or loss of innervations in the cerebral microvasculature. These factors may play significant roles in the dysfunction of the blood-brain barrier, in the cerebral microcirculation and in the pathogenesis of AD.

Factors believed to stabilize or improve the blood circulation may result in an amelioration of the symptoms in AD.[11] On the other hand, modulation of the cholinergic neurotransmission may result in changes in memory performance in AD patients.[23] Indeed, if AChE inhibitors (*e.g.* donepezil) are used to reduce the AChE activity in the cholinergic nerves, it might be suspected that the ACh remaining in the cholinergic nerves innervating the intra- and extracerebral blood vessels may regulate the blood circulation in AD more effectively than without treatment. In our histochemical experiments, the AChE activity was inhibited by $2x10^{-8}$ M donepezil, and totally abolished by $5x10^{-8}$ M donepezil. Application of this drug and/or other AChE inhibitors to ameliorate the blood circulation in the treatment of AD is therefore an important possibility.

Both human and animal *in vivo* studies have demonstrated a peripheral effect of donepezil. The oral administration of donepezil inhibited the pectoral muscle cholinesterase activity in young rat in a dose-dependent manner.[39] Donepezil at or above 2.5 mg/kg caused fasciculation, a peripheral cholinergic sign of the activation of neuromuscular transmission.[50] In nerve-hemidiaphragm preparations, an intracellular recording technique revealed that the application of $1x10^{-9}$ M donepezil increased the amplitude, the time-to-peak and the half-decay time of miniature end-plate potentials. In our *in vitro* experiments, the AChE activity in the NMJ was reduced by $2x10^{-8}$ M donepezil, suggesting a local effect of donepezil on the postsynaptic membrane of the NMJ. In human studies,[8,67] muscle cramps were registered as a side-effect in patients receiving a high dose of donepezil (10 mg daily during a 24-week trial); this could be attributed to the inhibition of AChE activity in the postsynaptic membrane

folding at the NMJ. When the effects of donepezil on the cholinergic structures in the CNS and PNS are compared, however, the drug is observed histochemically to be more selective for the brain cholinoceptive neuronal perikarya than for the neuromuscular junction. In the cortex, the AChE positivity in the pyramidal cells can be abolished by $2x10^{-8}$ M donepezil, while at the neuromuscular junction a similar level of inhibition can be achieved only with $5x10^{-7}$ M. The results of this study reveal the importance of the regional, cellular and also tissue specificity of the effects of donepezil on AChE activity.[34]

7. SUMMARY AND CONCLUSIONS

7.1. Experiments in *in vitro* basal forebrain neuronal cultures:

1. We have provided the first identification of the cholinergic and cholinoceptive neurons and their synaptic contacts in an *in vitro* basal forebrain neuronal culture system.
2. We have demonstrated that Aβ1–42 and its fragments Aβ25-35 and Aβ31-35 are toxic not only to cholinergic, but also to the cholinoceptive and GABAergic neurons.
3. Characteristic morphological changes have been revealed in the different transmitter-containing neurons after Aβ treatment:
   a.) The VAChT-immunoreactive neurons lost their processes after Aβ treatment and the cells become rounded.
   b.) In the AChE-positive cells, the intracellular localization of AChE was altered.
   c.) After Aβ1-42 treatment, the enzyme was translocated either to the perikaryonal plasma membrane or to a segment of the dendrite.
   d.) In GABAergic neurons, the processes were pruned and fragmented, and finally the cells were rounded and lost their processes.
4. The VAChT-positive axon varicosities proved more sensitive than the SYN-immunoreactive neuronal structures to Aβ treatment.

Conclusion: 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 relating to AD.

7.2. *In vivo* experiments on the axonal transport in the sciatic nerve of rat:

1. PS-1 is present in the axons and can be transported in both the anterograde and the retrograde direction in the motoric and sensory axons of the sciatic nerve of rat.
2. We have discovered that not only the C-terminal and N-terminal fragments of PS-1, but also the holoprotein may be transported bidirectionally in the axons.
3. By means of Western blot studies, we have presented evidence that APP is conveyed bidirectionally and may carry information both from the cell body to the nerve terminal and from the axonal terminal to the neuronal perikarya.
4. It has been discovered that Aβ can disturb the fast axonal transport of AChE, APP, VAChT and SYN.

Conclusion: The *in vivo* neurotoxic effect of Aβ1–42 on the axonal transport of various proteins has been demonstrated.

7.3. *In vitro* effect of donepezil in autopsy human and rat brain:

1. It has been demonstrated in *in vitro* experiments that donepezil (used for the treatment of AD patients) exerts a dose-dependent inhibitory effect on AChE activity both in the autopsy human brain and in rat brain samples.
2. Donepezil selectively affects the different brain areas and, within these, the cholinoceptive and cholinergic structures.
3. The most sensitive areas are the cortex and the hippocampal formation.
4. Within the different layers of the cortex, the cholinoceptive AChE-positive postsynaptic pyramidal cell bodies proved more sensitive than the presynaptic cholinergic axonal processes.
5. The most resistant cholinergic fibres are present in the putamen, the basolateral nucleus of the amygdala, the olfactory tubercle and the lateral habenular nucleus.
6. In the rat brain, the postsynaptic cholinoceptive and presynaptic cholinergic structures are inhibited by nearly the same dose of donepezil as in the human brain.
7. Donepezil can not only affect the AChE activity in the CNS, but also exert an inhibitory effect in the PNS tissue. It effectively inhibits the AChE activity at the NMJ and in the intra- and extracerebral blood vessel innervation.

Conclusion: Donepezil used for the treatment of AD individuals may inhibit the AChE activity not only in the brain, but also in the PNS tissue.

8. REFERENCES

1. Arvidsson, U., Riedl, M., Elde, R., Meister, B. (1997) Vesicular acetylcholine transporter (VAChT) protein: A novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. J. Comp. Neurol. 378, 454-467.
2. Bartus, R. T., Dean, R. L., Pontecorvo, M. J., Flicker, C. (1985) The cholinergic hypothesis: a historical overview, current perspective, and future directions. Ann. N. Y. Acad. Sci. 444, 332-358.
3. Barner, E. L., Gray, S. L. (1998) Donepezil use in Alzheimer disease. Ann. Pharmacother. 32, 70-77.
4. Bayer, T. A., Wirths, O., Majtényi, K., Hartmann, T., Multhaup, G., Beyreuther, K., Czech, C. (2001) Key factors in Alzheimer's disease: β-amyloid precursor protein processing, metabolism and intraneuronal transport. Brain Pathol. 11, 1-11.
5. Beher, D., Elle, C., Underwood, J., Davis, J. B., Ward, R., Karran, E., Masters, C. L., Beyreuther, K., Multhaup, G. (1999) Proteolytic fragments of Alzheimer's disease-associated presenilin 1 are present in synaptic organelles and growth cone membranes of rat brain. J. Neurochem. 72, 1564-1573.
6. Blusztajn, J. K., Berse, B. (2000) The cholinergic neuronal phenotype in Alzheimer's disease. Metab. Brain Dis. 15, 45-64.
7. Busciglio, J., Hartmann, H., Lorenzo, A., Wong, C., Baumann, K., Sommer, B., Staufenbiel, M., Yankner, B. A. (1997) Neuronal localization of presenilin-1 and association with amyloid plaques and neurofibrillary tangles in Alzheimer's disease. J. Neurosci. 17, 5101-5107.
8. Burns, A., Rossor, M., Hecker, J., Gauthier, S., Petit, H., Moller, H. J., Rogers, S. L., Fiedhoff, L. T. (1999) The effects of donepezil in Alzheimer's disease - results from a multinational trial. Dement. Geriatr. Cogn. Disord. 10, 237-244.
9. Chu, D. C. M., Penney, J. B. Jr., Young, A. B. (1987) Quantitative autoradiography of hippocampal $GABA_B$ and $GABA_A$ receptor changes in Alzheimer's disease. Neurosci. Lett. 82, 246-252.
10. Cook, D. G., Sung, J. C., Golde, T. E., Felsenstein, K. M., Wojczyk, B. S., Tanzi, R. E., Trojanowski, J. Q., Lee, V M.-Y., Doms, R. W. (1996) Expression and analysis of presenilin 1 in a human neuronal system: Localization in cell bodies and dendrites. Proc. Nat. Acad. Sci. (USA) 93, 9223-9228.
11. Crawford, J. G. (1998) Alzheimer's disease risk factors as related to cerebral blood flow: additional evidence. Med. Hypotheses 50, 25-36.
12. Czech, C., Tremp, G., Pradier, L. (2000) Presenilins and Alzheimer's disease: biological functions and pathogenic mechanisms. Progr. Neurobiol. 60, 363-384.
13. Deutsch, S. I., Morihisa, J. M. (1988) Glutamatergic abnormalities in Alzheimer's disease and a rationale for clinical trials with L-glutamate. Clin. Neuropharmacol. 11, 18-35.
14. Doody, R. S. (1999) Clinical profile of donepezil in the treatment of Alzheimer's disease. Gerontology 45, Suppl. 1. 23-32.
15. Emre, M., Geula, C., Ransil, B. J., Mesulam, M.-M. (1992) The acute neurotoxicity and effects upon cholinergic axons of intracerebrally injected β-amyloid in the rat brain. Neurobiol. Aging 13, 553-559.
16. Efange, S. M., Garland, E. M., Staley, J. K., Khare, A, B., Mash, D. C. (1997) Vesicular acetylcholine transporter density and Alzheimer's disease. Neurobiol. Aging 18, 407-413.

17. Farlow, M. R. (2001) Pharmacokinetic profiles of current therapies for Alzheimer's disease: implications for switching to galantamine. Clin. Therap. 23, A13-A24.
18. Fezoui, Y., Hartley, D. M., Harper, J. D., Khurana, R., Walsh, D. M., Condron, M. M., Selkoe, D. J., Lansbur, P. T. Jr., Fink, A. L., Teplow, D. B. (2000) An improved method of preparing the amyloid β-protein for fibrillogenesis and neurotoxicity experiments. Amyloid 7, 166-178.
19. Forloni, G. (1993) β-amyloid neurotoxicity. Funct. Neurol. 8, 211-225.
20. Fraser, P. E., Yang, D-S., Yu, G., Lévesque, L., Nishimura, M., Arawaka, S., Serpell, L. C., Rogaeva, E., George-Hyslop, P. S. (2000) Presenilin structure, function and role in Alzheimer disease. Biochim. Biophys. Acta 1502, 1-15.
21. Frisoni, G. B. (2001) Treatment of Alzheimer's disease with acetylcholinesterase inhibitors: bridging the gap between evidence and practice. J. Neurol. 248, 551-557.
22 Furey, M. L., Pietrini, P., Haxby, J. V., Alexander, G. E., Lee, H. C., VanMeter, J., Grady, C. L Shetty, U., Rapoport, S. I., Schapiro, M. B., Freo, U. (1997) Cholinergic stimulation alters performance and task-specific regional cerebral blood flow during working memory. Proc. Natl. Acad. Sci. (USA) 94, 6512-6516.
23. Geaney, D. P., Soper, N., Shepstone, B. J., Cowen P. J. (1990) Effect of central cholinergic stimulation on regional cerebral blood flow in Alzheimer disease. Lancet 335, 1484-1487.
24. Giovannelli, L., Scali, C., Faussone-Pellegrini, M.S., Pepeu, G., Casamenti, F. (1998) Long-term changes in the aggregation state and toxic effects of β-amyloid injected into the rat. Neuroscience 87, 349-357.
25. Gulya, K. (1998) Biological activities of amyloid precursor protein. Acta Biol. Hung. 49, 7-27
26. Hake, A. M. (2001) Use of cholinesterase inhibitors for treatment of Alzheimer disease. Cleve. Clin. J. Med. 68, 608-609.
27. Hardy, J., Cowburn, R., Barton, A., Reynolds, G., Dodd, P., Wester, P., Carroll, O', A-M., Lofdahl, E., Winblad, B. (1987) A disorder of cortical GABAergic innervation in Alzheimer's disease. Neurosci. Lett. 73, 192-196.
28. Hardy, J. A., Higgins, G. A. (1992) Alzheimer's disease – the amyloid cascade hypothesis. Science 256, 184-185.
29. Huynh, D. P., Vinters, H. V., Ho, D. H., Ho, V. V., Pulst, S. M. (1997) Neuronal expression and intracellular localization of presenilins in normal and Alzheimer disease brains. J. Neuropathol. Exp. Neurol. 56, 1009-1017.
30. Ikeda, E., Shiba, K., Mori, H., Ichikawa, A., Sumiya, H., Kuji, I., Tonami, N. (2000) Reduction of vesicular acetylcholine transporter in β-amyloid protein-infused rats with memory impairment. Nucl. Med. Commun. 21, 933-937.
31. Kasa, P. (1986) The cholinergic systems in brain and spinal cord. Prog. Neurobiol. 26, 211-272.
32. Kasa, P., Papp, H., Kovacs, I., Forgon, M., Penke, B., Yamaguchi, H. (2000) Human amyloid-β1-42 applied in vivo inhibits the fast axonal transport of proteins in the sciatic nerve of rat. Neurosci. Lett. 278, 117-119.
33. Kasa, P., Papp, H., Pakaski, M. (2001) Presenilin-1 and its N-terminal and C-terminal fragments are transported in the sciatic nerve of rat. Brain Res. 909, 159-169.
34. Kasa, P., Papp, H., Kasa, P. Jr., Torok, I. (2000) Donepezil dose-dependently inhibits acetylcholinesterase activity in various areas and the presynaptic cholinergic and the postsynaptic cholinoceptive enzyme-positive structures in the human and rat brain. Neuroscience 101, 89-100.

35. Kasa, P., Rakonczay, Z., Gulya, K. (1997) The cholinergic system in Alzheimer's disease. Prog. Neurobiol. 52, 511-535.
36. Kasa, P., Pakaski, M., Penke, B. (1993) Synthetic human β-amyloid has selective vulnerable effects on different types of neurons. In.: Alzheimer's Disease and Related Disorders, Communications (Ed.: M. Nicolini, P. F. Zatta, B. Corain), pp. 311-312, Pergamon Press.
37. Kasa, P., Pakaski, M., Penke, B. (1992) Synthetic human beta-amyloid has selective vulnerable effects on different types of neurons and glial cells in in vitro cultures derived from embryonic rat brain cortex. Neurobiol. Aging. 13, 410
38. Koo, E. H., Sisodia, S. S., Archer, D. R., Martin, L. Y., Weidemann, A., Beyreuther, K., Fischer, P., Masters, C. L., Price, D. L. (1990) Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. Proc. Nat. Acad. Sci. (USA) 87, 1561-1565.
39. Kosasa, T., Kuriya, Y., Matsui, K., Yamanishi, Y. (1999) Inhibitory effects of donepezil hydrochloride (E2020) on cholinesterase activity in brain and peripheral tissues of young and aged rats. Eur. J. Pharmacol. 386, 7-13.
40. Kovacs, D. M., Fausett, H. J., Page, K. J., Kim, T. W., Moir, R. D., Merriam, D. E., Hollister, R. D., Hallmark, O. G., Mancini, R., Felsenstein, K. M., Hyman, B. T., Tanzi, R. E., Wasco, W. (1996) Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. Nature Med. 2, 224-229.
41. Kowall, N. W., McKee, A. C., Yankner, B. A., Beal, M. F. (1992) In vivo neurotoxicity of β-amyloid [β(1-40)] and the β(25-35) fragment. Neurobiol. Aging 13, 537-542.
42. Li, J-Y., Dahlström, A-M., Hersh, L. B., Dahlström, A. (1998) Fast axonal transport of the vesicular acetylcholine transporter (VAChT) in cholinergic neurons in the rat sciatic nerve. Neurochem. Int. 32, 457-467.
43. Lowe, S. L., Francis, P. T., Procter, A. W., Palmer, A. M., Davison, A. N., Bowen, D. M. (1988) Gamma-aminobutyric acid concentration in brain tissue at two stages of Alzheimer's disease. Brain 111, 785-799.
44. Marczynski, T. J. (1998) GABAergic deafferentation hypothesis of brain aging and Alzheimer's disease revisited. Brain Res. Bull. 45, 341-379.
45. Marquez-Sterling, N. R., Lo, A. C. Y., Sisodia, S. S., Koo, E. H. (1997) Trafficking of cell-surface β-amyloid precursor protein: Evidence that a sorting intermediate participates in synaptic vesicle recycling. J. Neurosci. 17, 140-151.
46. McGleenon, B. M., Dynan, K. B., Passmore A. P. (1999) Acetylcholinesterase inhibitors in Alzheimer's disease. Br. J. Clin. Pharmacol. 48, 471-480.
47. Monji, A., Yoshida, I., Tashiro, K., Hayashi, Y., Matsuda, K., Tashiro, N. (2000) Inhibition of A β fibril formation and A β-induced cytotoxicity by senile plaque-associated proteins. Neurosci. Lett. 278, 81-84.
48. Nebu, A., Ikeda, M., Fukuhara, R., Shigenobu, K., Maki, N., Hokoishi, K., Komori, K., Yasuoka, T., Tanabe, H. (2001) Relationship between blood flow kinetics and severity of Alzheimer's disease: assessment of severity using a questionnaire-type examination, Alzheimer's disease assessment scale, cognitive sub-scale (ADAS(cog). Dement. Geriatr. Cogn. Disord. 12, 318-325.
49. Ogura, H., Kosasa, T., Kuriya, Y., Yamanishi, Y. (2000) Comparison of inhibitory activities of donepezil and other cholinesterase inhibitors on acetylcholinesterase and butyrylcholinesterase in vitro. Methods Find. Exp. Clin. Pharmacol. 22, 609-613.

50. Ogura, H., Kosasa, T., Kuriya, Y., Yamanishi, Y. (2001) Central and peripheral activity of cholinesterase inhibitors as revealed by yawning and fasciculation in rats. Eur. J. Pharmacol. 415, 157-164.
51. Pakaski, M., Farkas, Z., Kasa, P. Jr., Forgon, M., Papp, H., Zarandi, M., Penke, B., Kasa, P. Sr. (1998) Vulnerability of small GABAergic neurons to human β-amyloid pentapeptide. Brain Res. 796, 239-246.
52. Pakaski, M., Papp, H., Forgon, M., Kasa, P. Jr., Penke, B. (1998) Effects of β-amyloid on cholinergic, cholinoceptive and GABAergic neurons. Acta Biol. Hung. 49, 43-54.
53. Papp, H., Pakaski, M., Kovacs, I. Kasa, P. (1998) Characterization of the developmental expression of the vesicular acetylcholine transporter and acetylcholinesterase in cultured neurons. Clin. Neurosci. 51, 57.
54. Papp, H., Pakaski, M., Kasa, P. (1999) Identification of cholinergic neurons and their synaptic connections in rat embryonic neuronal culture and effects of human β-amyloid-42 on the cholinergic neurons. Neurobiology 7, 368-369.
55. Papp, H., Pakaski, M., Penke, B., Kasa, P. (1996) Effects of β-amyloid and its fragments on cholinergic and cholinoceptive neurons in vitro. Clin. Neurosci. 49, 49-50.
56. Papp, H., Pakaski, M., Kasa, P. (2000) *In vitro* effects of human amyloid-β peptide 1-42 on different transmitter-containing neurons in tissue culture. MIET 33. Nemzeti Nagygyűlés (Budapest), 50-51.
57. Papp, H., Kasa, P. Jr., Pakaski, M., Balaspiri, L., Kasa, P. (in press) Amyloid-β1-42 treatment does not have a specific effect on cholinergic neurons in *in vitro* basal forebrain neuronal cultures of rat. Acta Biol. Hung.
58. Papp, H., Pakaski, M., Balaspiri, L., Kasa, P. (2000) In vitro effects of human amyloid-β peptide (Aβ1-42) and its fragments on the vesicular acetylcholine transporter and synaptophysin immunoreactive axon terminals. Neurobiology 8, 379-380.
59. Papp, H., Pakaski, M., Kasa, P. (2001) Presenilin-1 and the amyloid precursor protein are transported bidirectionally in the sciatic nerve of adult rat. Neurochem. Int. (submitted).
60. Perry, E., Walker, M., Grace, J., Perry, R. (1999) Acetylcholine in mind: a neurotransmitter correlate of consciousness? Trends Neurosci. 22, 273-280.
61. Piccini, A., Ciotti, M. T., Vitolo, O. V., Calissano, P., Tabaton, M., Galli, C. (2000) Endogenous APP derivatives oppositely modulate apoptosis through an autocrine loop. NeuroReport 11, 1375-1379.
62. Pike, C. J., Cotman, C. W. (1993) Cultured GABA-immunoreactive neurons are resistant to toxicity induced by β-amyloid. Neuroscience 56, 269-274.
63. Postiglione, A., Lassen, N. A., Holman, B. L. (1993) Cerebral blood flow in patients with dementia of Alzheimer's type. Aging 5, 19-26.
64. Rakonczay, Z., Kovacs, I. (1998) Cholinesterases in Alzheimer's disease and cholinesterase inhibitors in Alzheimer therapy. Acta Biol. Hung. 49, 55-70.
65. Rakonczay, Z., Torok, I., Kasa P. (1999) Preferential inhibition of acetylcholinesterase molecular forms in normal human brain. Neurobiology 7, 75-77.
66. Ray, W. J., Yao, M., Mumm, J., Schroeter, E. H., Saftig, P., Wolfe, M., Selkoe, D. J., Kopan, R., Goate, A. M. (1999) Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. J. Biol. Chem. 274, 36801-36807.
67. Rogers, S. L., Farlow, M. R., Doody, R. S., Mohs, R., Friedhoff, L. T. (1998) A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Donepezil Study Group. Neurology 50, 136-145.

68. Saito, A., Wu, J. Y., Lee, T. J. (1985) Evidence for the presence of cholinergic nerves in cerebral arteries: an immunohistochemical demonstration of choline acetyltransferase. J. Cereb. Blood Flow Metab. 5, 327-334.
69. Schlaepfer, W. W. (1968) Acetylcholinesterase activity of motor and sensory nerve fibers in the spinal nerve roots of the rat. Z. Zellforsch. 88, 441-456.
70. Schubert, W., Prior, R., Weidemann, A., Dircksen, H., Multhaup, G., Masters, C. L., Beyreuther, K. (1991) Localization of Alzheimer β A4 amyloid precursor protein at central and peripheral synaptic sites. Brain Res. 563, 184-194.
71. Seidl, R., Cairns, N., Singewald, N., Kaehler, S. T., Lubec, G. (2001) Differences between GABA levels in Alzheimer's disease and Down syndrome with Alzheimer-like neuropathology. Naunyn Schmiedebergs Arch. Pharmacol. 363, 139-145.
72. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-F., Bruni, A. C., Montesi, M. P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R. J., Wasco, W., Da Silva, H. A. R., Haines, J. L., Pericak-Vance, M. A., Tanzi, R. E., Roses, A. D., Fraser, P. E., Rommens, J. M., George-Hyslop, P. H. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375, 754-760.
73. Sisodia, S. S., Koo, E. H., Hoffman, P. N., Perry, G., Price, D. L.(1993) Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. J. Neurosci. 13, 3136-3142.
74. Skoog, I., Kalaria, R. N., Breteler, M. M. (1999) Vascular factors and Alzheimer disease. Alzheimer Dis. Assoc. Disord. 13, 106-114.
75. Sramek, J. J., Cutler, N. R. (1999) Recent developments in the drug treatment of Alzheimer's disease. Drugs & Aging 14, 359-373.
76. Tago, H., Kimura, H., Maeda, T. (1986) Visualization of detailed acetylcholinesterase fiber and neuron staining in rat brain by a sensitive histochemical procedure. J. Histochem. Cytochem. 34, 1431-1438.
77. Vogt, B. A., Crino, P. B., Volicer, L. (1991) Laminar alterations in γ-aminobutyric $acid_A$, muscarinic, and β adrenoceptors and neuron degeneration in cingulate cortex in Alzheimer's disease. J. Neurochem. 57, 282-290.
78. Weinstock, M. (1995) The pharmacotherapy of Alzheimer's disease based on the cholinergic hypothesis: An update. Neurodegeneration 4, 349-356.
79. Yankner, B. A., Dawes, L. R., Fisher, S., Villa-Komaroff, L., Oster-Granite, M. L., Neve, R. L. (1989) Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. Science 245, 417-420.
80. Yankner, B. A., Duffy, L. K., Kirschner, D. A. (1990) Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. Science 250, 279-282.

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APPENDIX

List of in extenso publications

Kasa, P., Farkas, Z., Forgon, M., **Papp, H.,** Balaspiri, L. (1998) Effects of different galanins on the release of acetylcholine in the various areas of rat brain. Ann. New York Acad. Sci. 863, 435-437. **IF: 1,381**

Kasa, P., **Papp, H.,** Kasa, P.Jr., Torok, I. (2000) Donepezil dose-dependently inhibits acetylcholinesterase activity in various areas and in the presynaptic cholinergic and the postsynaptic cholinoceptive enzyme-positive structures in the human and rat brain. Neuroscience 101, 89-100. **IF: 3,563**

Kasa, P., **Papp, H.,** Kovacs, I., Forgon, M., Penke, B., Yamaguchi, H. (2000) Human amyloid-β1-42 applied *in vivo* inhibits the fast axonal transport of proteins in the sciatic nerve of rat. Neurosci. Lett. 278, 117-119. **IF: 2,091**

Kasa, P., **Papp, H.,** Pakaski, M. (2001) Presenilin-1 and its N-terminal and C-terminal fragments are transported in the sciatic nerve of rat. Brain Res. 909, 159-169 **IF: 2,526**

Pakaski, M., Farkas, Z., Kasa, P.Jr., Forgon, M., **Papp, H.,** Zarandi, M., Penke, B., Kasa, P.Sr. (1998) Vulnerability of small GABAergic neurons to human β-amyloid pentapeptide. Brain Res. 796, 239-246. **IF: 2,526**

Pakaski, M., **Papp, H.,** Forgon, M., Kasa, P.Jr., Penke, B. (1998) Effects β-amyloid on cholinergic, cholinoceptive and GABAergic neurons. Acta Biol. Hung. 49, 43-54. **IF: 0,291**

Pakaski, M., Rakonczay, Z., Fakla, I., **Papp, H.,** Kasa, P. (2000) *In vitro* effects of metrifonate on neuronal amyloid precursor protein processing and protein kinase C level. Brain Res. 863, 266-270. **IF: 2,526**

Papp, H., Kasa, P.Jr., Pakaski, M., Balaspiri, L., Kasa, P. (2001) Amyloid-β1-42 treatment does not have a specific effect on cholinergic neurons in *in vitro* basal forebrain neuronal cultures of rat. Acta Biol. Hung. (in press) **IF: 0,291**

Rakonczay, Z., **Papp, H.** (2001) Effects chronic metrifonate treatment on cholinergic enzymes and the blood-brain barrier. Neurochem. Int. 39, 19-24. **IF: 2,662**

List of Abstracts

Balaspiri, L., **Papp, H.,** Langel, Ü., Kasa, P. (2000) Synthesis of some segments of β-amyloid precursor peptide (ßAPP) and presenilins containing "difficult sequences". Clin. Neurosci. 53, 16.

Fakla, I., Kovacs, I., Torok, I., Rakonczay, Z., **Papp, H.,** Kasa, P. (1999) Alzheimer's disease: Is there any relationship between the reduction in cholinergic elements β-amyloid deposition, and APP expression in the human temporal cortex? Neurobiology 7, 303-304.

Forgon, M., Kovacs, I., **Papp, H.,** Pakaski, M., Kasa, P. (2000) Is there any relationship between experimentally induced cholinergic hypoactivity and amyloid precursor protein expression? Clin. Neurosci. 53, 15-16.

Forgon, M., **Papp, H.,** Pakaski, M., Balaspiri, L., Kasa, P. (2000) Modulation of GABA release by Aß1-42 peptide in rat basal forebrain tissue slices. MIET 33. Nemzeti Nagygyűlés (Budapest), 51-53.

Kasa, P., Farkas, Z., Pakaski, M., Forgon, M., Gulya, K., **Papp, H.,** Soos, K. (1998) Aged amyloid β peptide (1-42) and its fragments induce direct acetylcholine release, but do not affect high-affinity choline uptake *in vitro*. Fifth International Geneve/Springfield Symposium on Advances in Alzheimer Therapy

Kasa, P., Pakaski, M., Zarandi, M., Forgon, M., **Papp, H.,** Rakonczay, Z. (1998) Alterations in the distribution of acetylcholinesterase within the cholinergic and cholinoceptive neurons in *in vitro* tissue cultures in response to human amyloid-β peptide and some of its fragments. Neurobiol. Aging 19, 201.

Kasa, P.Jr., **Papp, H.,** Pakaski, M., Kovacs, I., Kasa, P. (1998) Amyloidβ1-42 induces reduction of vesicular acetylcholine transporter and synaptophysin-immunoreactive axon varicosities in *in vitro* tissue cultures, as revealed by image analysis. Clin. Neurosci. 51, 55-56.

Kasa, P., **Papp, H.,** Pakaski, M. (2000) Expression of presenilin-1, presenilin-2 and amyloid precursor protein in different neurons and their processes in the central- and peripheral nervous system. Effects of mechanical lesion on the axonal transport of these proteins. Neurobiology 8, 343-344.

Kasa, P., **Papp, H.,** Pakaski, M. (2000) Visualization of the effects in the central and peripheral nervous systems of acetylcholinesterase inhibitors used for the treatment of Alzheimer's disease. MIET 33. Nemzeti Nagygyűlés (Budapest), 41-45.

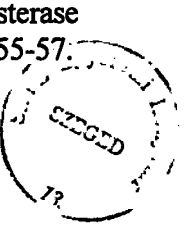
Kasa, P., **Papp, H.,** Pakaski, M. (2001) Presenilin-1 is transported from the motoneurons to their axon terminals. The 5th Int. Conf. Progr. in Alzheimer's and Parkinson's Disease, Kyoto (Japan), 69.

Pakaski, M., **Papp, H.,** Fakla, I., Rakonczay, Z., Kasa, P. (1998) Modulation of Alzheimer's β-amyloid precursor protein levels both by mechanical damage and pharmacologically. Clin. Neurosci. 51, 47.

Pakaski, M., **Papp, H.,** Kovacs, I., Fakla, I., Kasa, P. (1998) *In vivo* and *in vitro* metabolism of amyloid precursor after neuronal damage. Neurobiol. Aging 19, 245.

Pakaski, M., **Papp, H.,** Fakla, I. Soos, K., Checler, F., Kasa, P. (1999) Effect of human amyloid peptide on rat cortical endothelial cells. Neurobiology 7, 365-366.

Pakaski, M., **Papp, H.,** Rakonczay, Z., Fakla, I., Kasa, P. (2001) Effects of acetylcholinesterase inhibitors on the metabolism of amyloid precursor protein *in vitro*. Neurobiology 9, 55-57.



Papp, H., Pakaski, M., Penke, B., Kasa, P. (1996) Effects of β-amyloid and its fragments on cholinergic and cholinoceptive neurons *in vitro*. Clin. Neurosci. 49, 49-50.

Papp, H., Pakaski, M., Kovacs, I., Kasa, P. (1998) Characterization of the developmental expression of the vesicular acetylcholine transporter and acetylcholinesterase in cultured neurons. Clin. Neurosci. 51, 57.

Papp, H., Pakaski, M., Kasa, P. (1999) Identification of cholinergic neurons and their synaptic connections in rat embryonic neuronal culture and effects of human β-amyloid1-42 on the cholinergic neurons. Neurobiology 7, 368-369.

Papp, H., Pakaski, M., Rakonczay, Z., Kasa, P. (2000) Differences in the *in vivo* and *in vitro* effects of metrifonate on the acetylcholinesterase activity in the human and the rat brain. A histochemical study. Clin. Neurosci. 53, 13-14.

Papp, H., Pakaski, M., Kasa, P.Jr., Balaspiri, L., Kasa, P. (2000) *In vitro* effects of human amyloid β peptide (Aβ1-42) on the vesicular acetylcholine transporter and synaptophysin immunoreactive axon terminals. Neurobiology 8, 379-380.

Papp, H., Pakaski, M., Kasa, P. (2000) *In vitro* effects of human amyloid-β peptide 1-42 on different transmitter-containing neurons in tissue culture. MIET 33. Nemzeti Nagygyűlés (Budapest), 50-51.

Rakonczay, Z., **Papp, H.**, Kasa, P. (2000) Inhibition of cholinesterases by metrifonate does not change the blood-brain barrier. Neurobiology 8, 389.

Sebok, L., Farkas, Z., **Papp, H.**, Balaspiri, L., Kasa, P. (1996) The role of human galanin (hGAL1-30), hGAL1-19 and hGAL17-30 in the release of acetylcholine in the striatum: A neuropharmacological study. Neurobiology 4, 237-239.

Torok, I., **Papp, H.**, Pakaski, M., Kasa, P. (2000) There is no relationship between the density of cholinergic fibers and the amount of amyloid precursor protein in the entorhinal and temporal cortices in the non-demented and Alzheimer's disease brain. Clin. Neurosci. 53, 24-25.

Torok, I., **Papp, H.**, Zombori, J., Kasa, P. (2000) There is no relationship between the accumulation of amyloid-β peptide and the formation of neurofibrillary tangles in human nerve cells. Pathol. Int. 50, 175.

Torok, I., **Papp, H.**, Zombori, J., Mayer, P., Kasa, P. (2001) Amyloid-β peptide may not play the main role in the formation of neurofibrillary tangles in the Alzheimer's disease. 4th Conf. Hung. Soc. Neuropathol. Budapest

Zombori, J., **Papp, H.**, Kasa, P. (2000) Intraaxonal and intraneuronal presence of amyloid-β peptide in Alzheimer's disease brain samples. Clin. Neurosci. 53, 25-26.