β-AMYLOID, CHOLINERGIC TRANSMISSION, AND CEREBROVASCULAR SYSTEM - A DEVELOPMENTAL STUDY IN A TRANSGENIC MOUSE MODEL OF ALZHEIMER’S DISEASE

Dissertation

zur Erlangung des akademischen Grades Dr. med.

an der Medizinischen Fakultät der Universität Leipzig

eingereicht von: Dipl.-Med. Elena Kuznetsova

geboren am 06.11.1974 in Smela (USSR/Ukraine)

angefertigt an der: Universität Leipzig

Medizinische Fakultät
Paul-Flechsig-Institut für Hirnforschung
Abteilung für Neurochemie

Betreuer: Prof. Dr. rer. nat. habil. Reinhard Schliebs

Paul-Flechsig-Institut für Hirnforschung

Beschluss über die Verleihung des Doktorgrades vom: 19.02.2013
Elena Kuznetsova

β-AMYLOID, CHOLINERGIC TRANSMISSION, AND CEREBROVASCULAR SYSTEM - A DEVELOPMENTAL STUDY IN A MOUSE MODEL OF ALZHEIMER’ S DISEASE

Universität Leipzig, Dissertation

76 S., 100 Lit. 2, 29 Abb., 5 Tab.

Referat:


Am somatosensorischen Kortex werden beispielhaft die Expression des Glukosetransporters 1 oder Solanum tuberosum Lektin als Kapillarmarker und des vesikulären Acetylcholintransporters als Marker für cholinerge Fasern mittels Immunfluoreszenz und Laser-Scanning-Mikroskopie erfasst, einer semiquantitativen Computer-gestützten Bildanalytischen Auswertung unterzogen und mit dem Ausmaß der kortikalen Plaquebeladung korreliert. So konnte gezeigt werden, dass die Dichte der Blutgefäße und cholinergen Fasern im somatosensorischen Kortex von transgenen Tieren mit dem Alter im Vergleich zu nichttransgenen Kontrolltieren abnimmt, was mit einer Reduktion der perivaskulären cholinergen Innervation einhergeht.

<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>MEANING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abβ</td>
<td>β-amyloid</td>
</tr>
<tr>
<td>ABC</td>
<td>avidin–biotin-peroxidase complex</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>Bio-HRP</td>
<td>biotinylated horseradish peroxidase</td>
</tr>
<tr>
<td>BP</td>
<td>band-pass filter</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAA</td>
<td>cerebral amyloid angiopathy</td>
</tr>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine Tetrahydrochloride</td>
</tr>
<tr>
<td>DNS</td>
<td>donkey normal serum</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>FAD</td>
<td>familial Alzheimer's disease</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLUT1</td>
<td>glucose transporter 1</td>
</tr>
<tr>
<td>GNS</td>
<td>goat normal serum</td>
</tr>
<tr>
<td>hAPP</td>
<td>human amyloid precursor protein</td>
</tr>
<tr>
<td>HCh-3</td>
<td>[3H]hemicholinium-3</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>KPI</td>
<td>Kunitz Protease Inhibitor</td>
</tr>
<tr>
<td>LP</td>
<td>long-pass filter</td>
</tr>
<tr>
<td>LRP-1</td>
<td>lipoprotein receptor related protein 1</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser-Scanning-Mikroskop</td>
</tr>
<tr>
<td>mACHr(s)</td>
<td>muscarinic acetylcholine receptors</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
</tr>
<tr>
<td>nACHr(s)</td>
<td>nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>NBM</td>
<td>nucleus basalis of Meynert</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei antibody</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PHA-L</td>
<td>Phaseolus vulgaris leucoagglutinin</td>
</tr>
<tr>
<td>PHF</td>
<td>paired helical filaments</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>PS1</td>
<td>presenilin-1</td>
</tr>
<tr>
<td>PS2</td>
<td>presenilin-2</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>S1BF</td>
<td>somatosensory cortex 1 barrel field</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

S.E.M............................................. standard error of the mean
SF............................................... straight filaments
SMC............................................. smooth muscle cells
STL............................................... Solanum tuberosum lectin /agglutinin
TBS............................................... tris-buffered saline
TGF ............................................... transforming growth factor
VIP............................................... vasoactive intestinal polypeptide
VAChT ........................................ vesicular acetylcholine transporter
VEGF........................................... vascular endothelial growth factor
CHAPTER 1: INTRODUCTION

1.1 Alzheimer’s disease ................................................................. 1
1.2 APP processing and β-amyloid production ................................. 2
1.3 Cholinergic dysfunction in Alzheimer’s disease ......................... 5
1.4 Cerebrovascular abnormalities in Alzheimer’s disease ............. 8
1.5 Cholinergic innervation of intracortical cerebral microvessels ... 9
1.6 Transgenic Tg2576 mouse model of Alzheimer’s disease ........ 11
1.7 Aim of study ............................................................................. 14

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials ..................................................................................... 15
  2.1.1 Chemical reagents used ....................................................... 15
  2.1.2 Biological reagents used ...................................................... 15
  2.1.3 Preparation of solutions and buffers ................................. 15
  2.1.4 Antibodies and reagents used for immunohistochemistry .... 17
  2.1.5 Transgenic animals ............................................................. 19
2.2 Methods ..................................................................................... 20
  2.2.1 Tissue preparation and sampling of sections ....................... 20
  2.2.2 Immunohistochemistry ....................................................... 20
    2.2.2.1 Protocol of immunofluorescent labeling ....................... 20
    2.2.2.2 Protocol of immunoperoxidase labeling (ABC technique) 21
    2.2.2.3 Combination of primary and secondary antibodies .... 22
    2.2.2.4 Protocol of β–amyloid immunolabeling (Formic acid epitope retrieval method) .................... 23
  2.2.3 Histochemistry ................................................................. 23
    2.2.3.1 Thioflavin S staining .................................................. 23
    2.2.3.2 Nissl staining ............................................................ 23
    2.2.3.3 Solanum Tuberosum Lectin (STL) staining .................. 24
  2.2.4 Double and triple-coloured immuno-/ histochemical staining of brain sections .. 24
  2.2.5 Microscopy and digital image processing .............................. 25
  2.2.6 Morphological and morphometric analyses ......................... 25
    2.2.6.1 Cortical microvessels .................................................. 25
    2.2.6.2 Cortical cholinergic innervation ................................ 27
      2.2.6.2.1 Total density of VACht-immunoreactivity ............. 27
CHAPTER 3: RESULTS

3.1 Developmental and amyloid plaque-related changes in cerebral cortical capillaries in transgenic Tg2576 Alzheimer mice ........................................................... 31
   3.1.1 Morphological distribution of brain vessels in the cerebral cortex of wild type mice ..................................................................................................... 31
   3.1.2 Microvessel density under plaque burden .......................................................... 33

3.2 Developmental and amyloid plaque-related changes in cholinergic neurotransmission in cholinceptive target regions of transgenic Tg2576 mice ............ 39
   3.2.1 Visualisation of cholinergic nerve terminals in mouse brain ............................... 39
   3.2.2 VACHT-Expression in wild type and transgenic Tg2576 mice ............................ 40

3.3 Role of cholinergic system in β-amyloid-related changes in the cerebrovascular system of transgenic Tg2576 mice ................................................................................. 46
   3.3.1 *Solanum tuberosum* lectin (STL) histochemistry in visualisation of brain vessels, β-amyloid, and microglia ................................................................. 46
   3.3.1.1 Solanum tuberosum lectin and brain vessels ............................................. 46
   3.3.1.2 *Solanum tuberosum* lectin and β-amyloid plaques ................................... 47
   3.3.1.3 *Solanum tuberosum* lectin staining to visualize glial cells....................... 48
   3.3.2 Cholinergic perivascular innervation of cerebral cortical microvessels in transgenic Tg2576 and wild type mice ............................................................... 50

CHAPTER 4: DISCUSSION

4.1 β-Amyloid and brain vascular system: the vascular hypothesis of Alzheimer’s disease 55
   4.1.1 Evidences of a role of vascular mechanisms in Alzheimer’s disease .......... 55
   4.1.2 Effect of β-amyloid on brain vascular system .............................................. 57
   4.1.3 Effect of ischemia and hypoperfusion on APP processing .............................. 59
   4.1.4 Effect of β-amyloid on cholinergic function in brain vascular system .......... 59

4.2 Aim of study and main results obtained ............................................................... 61

4.3 Age-related changes in cerebral cortical microvessels in the presence and absence of β-amyloid plaque load ................................................................. 62

4.4 Age-related changes of cholinergic terminals in cholinceptive target regions in the presence and absence of β-amyloid plaque load ................................................. 64
4.4.1 VACHT – a reliable marker for detection of cholinergic terminals in cerebral cortex ........................................................................................................ 64
4.4.2 The barrel field of the somatosensory cortex 1 (S1BF) as a model region to reveal age-related changes in cholinergic innervation ........................................ 65
4.4.3 VACHT expression: morphological and morphometric studies ................................................................. 66
4.5 Age-related changes in cholinergic innervation of cerebral cortical microvessels in the presence and absence of β-amyloid plaque load .................................................. 69
  4.5.1 STL – a mono-marker for detection of cortical vessels, senile amyloid plaques and activated microglia in cerebral cortex ............................................................................. 69
  4.5.2 Cholinergic perivascular innervation of cerebral cortical microvessels in transgenic Tg2576 mice ........................................................................................................ 70
  4.5.3 Quantitation of cholinergic input on cerebral microvessels of mouse brain .................................................... 71
4.6 Summary and conclusions ............................................................................................................................... 75

REFERENCES .................................................................................................................................................. 77

ZUSAMMENFASSUNG DER ARBEIT
ERKLÄRUNG ÜBER DIE EIGENSTANDIGE ABFASSUNG DER ARBEIT
CURRICULUM VITAE
AKNOWLEDGEMENT
CHAPTER 1: INTRODUCTION

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of memory and cognition. There are estimates that in Germany approximately 1.2 million people suffering from dementia with about two thirds of these individuals representing dementia of AD type. Because of an expanding population and increasing life expectancy, the number of affected individuals is projected to rise to more than 2.6 million in Germany (Deutsche Alzheimer Gesellschaft e.V., 2011) and ~115 million worldwide by 2050 (Alzheimer’s Association, 2011: Wold Alzheimer report 2009, Alzheimer’s disease international). AD now afflicts 0.1 percent of individuals at age of 45-65 years and less than 3 percent at age of 70-74 years in Germany (Deutsche Alzheimer Gesellschaft e.V., 2011), with an approximate doubling of incidence for every 5 years of age afterwards (Alzheimer’s Association, 2011). By the age of 80 - 84 years, the risk reaches nearly 13 percent and approximately two thirds of people aged 90 and older have AD in Germany (Deutsche Alzheimer Gesellschaft e.V., 2011).

Two forms of AD are known: early-onset familial AD (familial form, FAD) and late-onset sporadic AD (sporadic form). The majority of AD is sporadic (≥ 95 percent).

In families with an autosomal dominant early-onset AD mutations in three genes, the amyloid precursor protein (APP) gene on chromosome 21, the presenilin-1 (PS1) on chromosome 14, and the presenilin-2 (PS2) on chromosome 1, have been found. About twenty missense mutations in APP gene have been described (Goedert and Spillantini, 2006), while more than 160 mutations in the presenilin genes have been identified (Goedert and Spillantini, 2006). Proteolytic processing of APP gives rise to the formation of β-amyloid, whereas presenilins are involved in the carboxy-terminal cleavage of APP (see chapter 1.2).

In AD two major histopathological hallmarks occur such as β-amyloid plaques deposed extracellularly in cerebral cortical and hippocampal areas, and neurofibrillary tangles that occupy much of the cytoplasm of select cortical pyramidal neurons.

β-Amyloid plaques

Amyloid plaques (synonym: neuritic plaques, senile plaques) are extracellular deposits which are mostly composed of β-amyloid (Aβ), a proteolytic fragment of the APP, in the gray matter of the brain. The deposits are associated with neuronal terminals and degenerative swollen neuritis, and surrounded by a web of astrocytic processes and microglia cells (Glenner et al.,
INTRODUCTION

1984; Masters et al., 1985). Senile plaques are visible in light or fluorescence microscopy after histological staining by silver, Congo red, Thioflavin S and cresyl violet, or by immunohistochemical detection.

**Neurofibrillary tangles**

Neurofibrillary tangles, the second histopathological feature in AD, are pathological protein aggregates found within neurons in cases of AD (Grundke-Iqbal et al., 1986a, b; Iqbal et al., 1989). They occupy much of select cortical pyramidal neurons and mostly consist of hyperphosphorylated tau, a microtubule-associated protein. The tau protein is a highly soluble microtubule-associated protein. In humans, these proteins are mostly found in neurons. One of tau's main function is to modulate the stability and flexibility of axonal microtubules, which is mediated by its degree of phosphorylation. Hyperphosphorylation of tau depresses its microtubule assembly activity and binding to microtubules (Iqbal et al., 2005). Abnormal hyperphosphorylated tau demonstrates a high tendency to aggregate and to form paired helical filaments (PHF) and straight filaments (SF), thus causing the insoluble cytoplasmic inclusions as observed in AD (Alonso et al., 2001). The level of hyperphosphorylated tau in AD brain is about 4-8-fold higher as compared in age-matched normal brains. The precise mechanism of tangle formation is not completely understood. One of the possibilities is that the abnormal hyperphosphorylation of tau might be due to conformational changes in tau in the diseased brain, which might make it a better substrate for phosphorylation and/or a worse substrate for dephosphorylation (phospho-/dephosphorylation imbalance) (Iqbal et al., 2005; Mi and Johnson, 2006). In the adult human brain, six tau isoforms are expressed through alternative splicing of a single tau gene on chromosome 17 (Goedert et al; 1989a, b). In AD all of the six tau isoforms contribute to the formation of PHF by hyperphosphorylation. Tau pathology is also seen in several other human neurodegenerative disorders, associated with degeneration and dementia (Delacourte, 2005).

**1.2 APP processing and β-amyloid production**

β-Amyloid (Aβ) is formed from the APP by subsequent proteolytic activities of the β- and γ-secretase. Much research has focused on understanding the mechanisms by which APP is processed to generate Aβ, because pathological production of Aβ has been proposed as an initial event in the development of AD.

APP is a single transmembrane glycoprotein with 590-680 amino acid long extracellular N-terminal domain, transmembrane tail with 28 amino acids and an approximately 55 amino
acid short intracellular cytoplasmic C-terminal tail (Kang et al., 1987; Masters and Beyreuther, 1987; Selkoe, 2001; Goedert and Spillantini, 2006). Nine isoforms are produced from a single APP gene on chromosome 21 by alternative mRNA splicing and encode proteins ranging from 365 to 770 amino acids. Two of these isoforms (APP365 and APP563) do not contain Aβ peptides. APP expression occurs ubiquitously, and the primary isoform varies according to cell and tissue type.

In the nervous system, APP695 is expressed predominantly in neurons, whereas APP770 and APP751 are found in neuronal as well as non-neuronal cells (Golde et al., 1990; Kang and Muller-Hill, 1990; Koo et al., 1990; Wisniewski et al., 1997; Clippingdale et al., 2001; Selkoe, 2001), and additionally contain the Kunitz Protease Inhibitor (KPI) domain within their extracellular regions (Rohan de Silva et al., 1997; Kang et al., 1990). The levels of KPI-containing APP isoforms (APP770, APP751) are elevated in AD brain and associated with increased Aβ production and deposition (Menendez-Gonzalez et al., 2005; Bordji et al., 2010; Zhang et al., 2011). Following brain injury, expression of the APP751/770 isoforms is substantially increased in astrocytes and microglial cells (Siman et al., 1989; Van Den Heuvel et al., 2000; Zheng and Koo, 2011). All three isoforms share the same Aβ and are thus all potentially amyloidogenic. The functional significance of the tissue-specific alternative splicing of APP is still not understood.

APP is synthesized in the endoplasmic reticulum and transported through the Golgi apparatus to the trans-Golgi-network (Greenfield et al., 1999) where the highest concentration of APP is found in neurons. Then APP can be delivered in secretory vesicles to the cell surface where it is recycled via an endosomal-lysosomal pathway of degradation (Nordstedt et al., 1993; Caporaso et al., 1994), or may undergo a cleavage by the a-secretase pathway. Cell culture studies have suggested that most Aβ (1–40) /Aβ (1–42) is generated in the endosomal recycling pathway and the minority of Aβ (1–40) /Aβ (1–42) is produced in the secretory pathway, within the endoplasmic reticulum and Golgi apparatus (Haass et al., 1993a,b; Selkoe, 2001).

Picomolar and nanomolar concentrations of Aβ peptides are found constitutively in normal brain (Haass et al., 1992; Seubert et al., 1993; Kar et al., 2004). The normal physiological function of APP is currently unknown, although in neurons it has been demonstrated to be localized in synapses where it may play a role in extension of neuronal terminals and synaptogenesis. It has been suggested that APP demonstrates neurotrophic (Jaffar et al., 2001) and neuroprotective (Zhang, 2004) functions, as well as may play a role in neuronal migration and transcellular adhesion (Zheng and Koo, 2006). The APP intracellular domain contributes
to protein trafficking, metabolism, and presumably exerts signaling functions as a cell surface receptor (Zheng and Koo, 2011). Therefore, the effect of full-length APP on cellular activity may represent a composition of its cleavage products, depending on the ratio of APP metabolites generated (Zheng and Koo, 2011; Zhang et al., 2011). APP processing and Aβ generation are modulated by neuronal electrical activity (Nitsch et al., 1993; Kamenetz et al., 2003) and it has been proposed that Aβ-related peptides may act as a modulator of cholinergic function under normal conditions (Blusztajn and Berse, 2000; Hellstrom-Lindahl, 2000; Jhamandas et al., 2001; Kar, 2002; Dolezal and Kasparova, 2003; Wang et al., 2007).

Fig. 1: Processing of amyloid precursor protein (APP) by α-, β- and γ-secretase:
A – amyloidogenic pathway; B – non-amyloidogenic pathway
For more details, see description in the text.

APP can be cleaved by three proteolytic enzymatic activities termed α-, β-, and γ-secretase (Fig. 1). The α-secretase, a member of the ADAM (a disintegrin and metalloproteinase) family, represents a transmembrane zinc metalloproteinase, and is active at the cell surface (Sisodia, 1992; Roberts et al., 1994; Zhang et al., 2011). The α-secretase activity cleaves APP within the Aβ-sequence (between residues 16 and 17) releasing a large soluble ectodomain, the N-terminal sAPPα, and a P3-CT fragment. The P3-CT fragment is subsequently cleaved by γ-secretase into P3, and the CT fragment. Thus, the α-secretory pathway precludes Aβ (1-40/42) formation (Clippingdale et al., 2001; Selkoe, 2001). Non-neuronal cells preferentially process APP via α- and γ-secretase cleavage to generate sAPPα and the non-amyloidogenic
INTRODUCTION

fragment P3. Thus, non-neuronal cells are not a significant source of Aβ under normal conditions. However, neurons do not heavily rely on this pathway and produce very low levels of P3 (Chyung et al., 1997). sAPPα plays an important role in neuronal plasticity / survival, demonstrates neuroprotective properties against excitotoxicity, regulates neural stem cell proliferation and is important for early CNS development, implying that most of APP’s physiological function is mediated by sAPPα (Zhang et al., 2011).

Alternatively, APP can be cleaved by β-secretase pathway, which results in the formation of Aβ peptide, is mediated by the sequential actions of β-secretase (β-site APP cleaving enzyme, [BACE]) and γ-secretase enzymes). BACE1 is a membrane-bound aspartyl protease with a characteristic type I transmembrane domain near the C-terminus (Sinha et al., 1999; Vassar et al., 1999). Overexpressed BACE1 is mainly found in the Golgi endosomes and in the endosomes that provide an acidic environment in various cell lines (Zhang et al., 2011), BACE1 can be found also at the cell surface (Vassar et al., 1999; Walter et al., 2001; Huse et al., 2000; Huse et al., 2002). BACE2 is a homolog of BACE1 and cleaves substrates similar to BACE1, but its expression in neurons is basically lower than BACE1 (Bennett BD. et al., 2000). Interestingly, cellular BACE2 cleaves APP near the α-secretase site much more efficiently than at the β-secretase site, but do not exclude a potential contribution of BACE2 towards AD pathogenesis (Yan et al., 2001; Zhang et al., 2011). It was reported that sAPPβ can have function as a death receptor and mediate axonal pruning and neuronal cell death (Nikolaev et al., 2009). In addition to BACE1/ BACE2, cathepsin B has been proposed as a supplementary β-secretase (Zhang et al., 2011; Zheng and Koo, 2011). The β-secretase cleavage generates a truncated soluble sAPPβ and a membrane bound Aβ-containing C-terminal fragment. Further proteolysis of the C-terminal fragment by γ-secretase yields the Aβ peptide (Esch et al., 1990; Wisniewski et al., 1997; Vassar et al., 1999; Clippingdale et al., 2001; Selkoe, 2001).

Aβ is also produced under normal conditions and secreted in the brain as a soluble peptide, which raised the possibility that Aβ may also play a physiological role. Aβ appears to be a normal product of cellular metabolism throughout life and circulated in human biological fluids (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992).

1.3 Cholinergic dysfunction in Alzheimer’s disease

For more than 30 years, studies of the brains of those with advanced age and AD have consistently found damage or abnormalities in the cholinergic pathways that appeared to correlate well with the level of cognitive decline. Studies on aged individuals and AD
patients, as well as animal experiments suggest that many of cholinergic abnormalities including alterations in choline transport, acetylcholine release, nicotinic and muscarinic receptor expression, neurotrophic support, and axonal transport may all contribute to cognitive abnormalities in aging and AD. This has led to the formulation of the "cholinergic hypothesis of memory dysfunction in senescence and in AD" (Bartus et al., 1982; Coyle et al., 1983), which essentially states that a loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline. However, it is interesting to note, that cognitive deficits are detectable not earlier before at least 30% of total cholinergic basal forebrain cells have degenerated (for review, see, Schliebs and Arendt, 2006). This indicates a high capacity of the basal forebrain cholinergic system with regard to respond to neuronal degeneration. The selective cell loss in the nucleus basalis of Meynert (Whitehouse et al. 1981; Arendt et al. 1983) and severe neurofibrillary degenerations are associated with a dramatic loss of cholinergic input in many areas of the cerebral cortex (Geula and Mesulam 1989, Geula and Mesulam 1999; Mesulam, 2004). This cholinergic loss tends to correlate with the severity of the dementia (Perry et al. 1981; Francis et al. 1985) and with the classic neuropathological lesions such as neuritic plaques (Arendt et al. 1985). However, it is not yet clear whether the extensive loss of neurons and pre-synaptic terminals observed in AD is one of the primary features of this disease, or the consequences of the Aβ-pathology, or, for example, cerebrovascular dysfunction (see 1.4).

The progression of dementia is associated with reductions in a number of cortical cholinergic markers such as acetylcholine (ACh) content (Richter et al. 1980) and release (Nilsson et al. 1986), choline acetyltransferase (ChAT) activity (Bowen et al. 1976; Davies and Maloney, 1976), choline uptake (Rylett et al. 1983), activity of acetylcholinesterase (AChE) (Perry et al. 1978; Hammond et al. 1988). The later studies revealed a significant loss of nicotinic ACh receptors (Whithouse et al., 1986; Nordberg et al., 1986; Flynn and Mash, 1986; Nordberg, 1992; Perry et al., 1995) and select types of muscarinic ACh receptors (Katayama et al. 1990; Levey, 1996; Shiozaki et al. 2001; Mash et al.1985) in the cortical and hippocampal regions of AD brains. This was further supported by in vivo measurements of cholinergic receptor expression by positron emission tomography (PET) at different stages of AD, demonstrating receptor abnormalities that change with disease progression (Nordberg, 2001). Teaktong et al., found a selective increase in α7nAChR on astrocytes of AD brain, which has been suggested to play a role in Aβ mediated inflammatory processes in AD (Teaktong et al., 2003; Yu et al., 2005).
In contrast to observations in early-onset and aged AD cases, in patients with mild cognitive impairment (MCI, a prodromal stage of AD), and early forms of AD, no obvious basal forebrain cholinergic cell loss could be observed. Instead, mismatches in the expression of NGF, its precursor proNGF, both high and low NGF receptors, trkA and p75NTR, as well as changes in acetylcholine release, high-affinity choline uptake, alterations in muscarinic and nicotinic acetylcholine receptor expression have been detected, suggesting a loss of cholinergic function rather than cell death. These findings encourage the suggestion of an outstanding role of the cholinergic system in the functional processes that lead to AD. Malfunction of the cholinergic system may be solved pharmacologically by intervening in cholinergic as well as neurotrophic signaling cascades that have been shown to ameliorate the cholinergic deficit at early stages of the disease, and slow-down the progression (see, e.g. the review, Schliebs and Arendt, 2011).

Whereas in the brains of AD patients no tau mutations have been observed, pathogenic mutations in the tau genes cause frontotemporal dementia (Goedert and Jakes, 2005) suggesting that post-transcriptional alterations in tau gene expression may contribute to the cognitive deficits in AD presumably also by interacting with the cholinergic transmission. Several studies have demonstrated that activation of nAChRs results in a significant increase in tau phosphorylation, whereas mAChR activation may prevent tau phosphorylation (Hellström-Lindahl, 2000; Hellström-Lindahl et al., 2000; Wang et al., 2003; for reviews see: Buckingham et al., 2009; Bencherif and Lippiello, 2010). Nicotine was found to stimulate tau phosphorylation at those sites that were also hyperphosphorylated in AD, obviously induced through activation of the α7 subtype of nAChRs (Wang et al., 2003). This finding was further substantiated by observations in triple transgenic 3xTg-AD mice which develop age- and regional dependent accumulation of both plaques and tangles as well as progressive deficits in cognition (Oddo et al., 2003; Billings et al., 2005; Kitazawa et al., 2005). Chronic nicotine administration to one-month-old 3xTg-AD mice for five months did not change soluble Aβ protein levels but showed increases of phosphorylation and aggregation of tau, which appeared to be mediated by p38-MAPK (Oddo et al., 2005). Additionally, Aβ has relevant dual neuromodulatory effects on α7 and α4β2 nicotinic receptors, controlling the release of glutamate, aspartate and GABA, depending upon the concentration used (Mura et al., 2012). Interestingly, single cell gene expression profiling revealed a shift in the ratio of three-tandem repeat tau to four-tandem repeat tau in individual human cholinergic basal forebrain neurons within the nucleus basalis and CA1 hippocampal neurons during the progression of AD (Ginsberg et al., 2006). The meaning of it, however, awaits further elucidation.
1.4 Cerebrovascular abnormalities in Alzheimer's disease

There is a large body of evidence that neural activity is closely related to cerebral blood flow (CBF) (see for review Iadecola, 2004; Girouard and Iadecola, 2006). In several brain pathologies, the interaction between neural activity and cerebral blood vessels is disrupted, and the resulting homeostatic unbalance may contribute to brain dysfunction. Disruption of the regulation of the cerebral circulation deprives the brain of vital control mechanisms that ensure delivery of adequate amounts of substrate and control the homeostasis of the microenvironment in which the brain cells function. These alterations impair the ability of the brain to maintain CBF when the cerebral blood supply is compromised, exacerbating ischaemia and presumably promoting cognitive impairment. A growing body of evidence indicates that cerebrovascular dysfunctions and structural alterations occur differentially during normal ageing with pronounced effects in AD (Kalaria, 2009).

Cerebrovascular pathologies such as cerebral amyloid angiopathy and endothelial degeneration have been reported in many AD cases (de la Torre, 2002; Kalaria, 2000; Soffer, 2006; Thomas et al., 1996). Functional vascular abnormalities are one of the earlier clinical manifestations in AD (see, e.g., Farkas and Luiten, 2001), ischemic stroke increases the risk of the disorder (Kalaria, 2000; Kokmen et al., 1996; Snowdon et al., 1997), and the pathological hallmarks of AD and vascular ischemic changes have been found to coexist in more than 40 percent of elderly demented individuals (Jellinger, 2007; Jellinger, 2008). Currently, a growing number of epidemiological, clinical, pathological, and neuroimaging studies revealed a distinct association between vascular risk factors and AD (Milionis et al., 2008; Rocchi et al., 2009; Kalaria, 2009; de la Torre, 2010; Dickstein et al., 2010; Humpel 2011).

Structure and function of the neurovascular unit have been observed to be profoundly damaged in AD (Iadecola, 2004; Bell and Zlokovic, 2009; Humpel, 2011). Indeed, cerebrovascular abnormalities such as thickening of the microvascular basement membranes, decreased luminal diameter, reduction in number of cerebral capillaries, microvascular degeneration and diminished glucose transport across the blood-brain barrier have frequently been observed in Alzheimer patients (Mancardi et al., 1980 Kalaria and Pax, 1995; Kalaria and Hedera, 1995; Vinters et al., 1996, Claudio, 1996; Ellis et al., 1996; Farkas and Luiten, 2001; Drzezga et al., 2003; Hunt et al., 2007; Mosconi et al., 2010; Nicolakakis and Hamel, 2011), and the compromised cerebral hemodynamics in AD has been suggested to be associated with vascular oxidative stress (Faraci, 2006; Simpson et al., 2010) and inflammation (Paris et al., 2003).
Pathology of the blood brain barrier (BBB) is an early finding in white matter lesions associated with AD, and altered function of BBB transport may also have an impact on the accumulation of Aβ in the brain (Kalaria, 1992; Zlokovic, 2008; Farrall and Wardlaw, 2009). Serum inflammatory protein levels may alter the BBB endothelial tight junctions or actual transport mechanisms across the BBB, enough to allow amyloid or its precursors to leak out (Farrall and Wardlaw, 2009). The increased BBB permeability in AD may exacerbate further loss of BBB integrity thus escalating the cycle of Aβ protein accumulation and BBB damage (Farrall and Wardlaw, 2009). The downregulation of the BBB receptors LRP-1 (lipoprotein receptor related protein 1) and P-glycoprotein also may induce vascular deposition of Aβ (Bell et al., 2009).

A causal relationship between vascular mechanisms and the development of sporadic AD has already been hypothesized in the 1990s of the last century (de la Torre and Mussivand, 1993), suggesting that sporadic AD may represent a vascular disorder caused by impaired cerebral perfusion (for reviews, see de la Torre, 2008; Isingrini et al., 2009; Iadecola, 2010; Humpel, 2011). Indeed, during the last decades, a vast number of studies have been accumulated providing strong clinical and experimental evidence of an important role of vascular mechanisms in the development and progression of sporadic AD, which will be discussed in more detail in chapter 4.1.

1.5 Cholinergic innervation of intracortical cerebral microvessels

Neuronal terminals are closely associated with cerebral blood vessels. Pial arteries at the surface of the brain are densely innervated by perivascular nerves that originate from autonomic and sensory ganglia ("extrinsic nerves") and contain many vasodilators (NO, acetylcholine, vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), substance P and cholecystokinin neurokinin A) and vasoconstrictors (noradrenaline, neuropeptide Y (NPY) and serotonin) (Hamel, 2006). Intracerebral arterioles and capillaries (microcirculations vessels) are innervated by "intrinsic nerves" that originate from subcortical neuronal centers as well as from local cortical interneurons (see Fig. 2). These axons contain many neurotransmitters, as the extrinsic nerves (Hamel, 2006; Van Beek and Claassen, 2011). Cortical microvessels receive a cholinergic input that originates primarily from basal forebrain neurons which, upon electrical or chemical stimulation, induce significant increases in cortical
INTRODUCTION

perfusion together with a dilation of intracortical microvessels. Physiological evidence indicates that central cholinergic pathways are involved in the regulation of CBF (Biesold et al., 1989; Lacombe et al., 1989; Kurosawa M. et al., 1989; Hamel, 2004). The involvement of ACh as a neurotransmitter in the control of regional CBF was demonstrated by the administration of cholinergic drugs, such as blockers of mAChR, which decreased CBF and ACh-esterase inhibitors, which led to increased CBF (see for review Farkas and Luiten, 2001).

In vivo and in vitro studies have shown that intracortical microvessels dilated in response to stimulation with ACh (Thomas et al., 1997; Elhusseiny and Hamel, 2000). This response is dependent on nitric oxide (NO) production, and mediated by muscarinic receptor activation (mAChR), presumably through the M5-mAChR subtype (Elhusseiny and Hamel, 2000). Basalo-cortical perivascular nerve terminals were detected by light and electron microscopic immunocytochemistry of anterogradely transported Phaseolus vulgaris leucoagglutinin (PHA-L) following its injection in the basal forebrain (substantia innominata), and were compared to cortical perivascular cholinergic (immunoreactive for choline acetyltransferase (ChAT))

---

**Fig. 2:** Schematic presentation of different types of perivascular cholinergic innervation of intracerebral microvessels, modified after Hamel, 2006. For more details, see description in the text.
terminals. It was established that cholinergic neurons project not only to the cortical neuropile, but also to the arterioles and capillaries within the cerebral cortex (Vaucher and Hamel, 1995).

Cholinergic afferents also project to intracortical neurons that synthesize vasodilatators NO and vasoactive intestinal peptide (VIP) (Vaucher et al., 1997; Cauli et al., 2004). These correspond to distinct sub-population of GABA interneurons which were found to send numerous projections to local microvessels (Estrada and DeFelipe, 1998). Basal forebrain cholinergic fibers can directly affect the cortical microvascular bed, but further suggest that specific population of GABA interneurons could serve as a functional relay to adapt perfusion to locally increased neuronal activity. GABA interneurons translate incoming neuronal signals from various sub-cortical afferents into adapted local neuronal and vascular responses, including those originating from basal forebrain cholinergic neurons (Kawaguchi, 1997; Cauli et al., 2004; Hamel, 2004). Cortical microvessels are endowed with heterogeneous mAChRs (Grammas et al., 1983; Luiten et al., 1996; Elhusseiny et al., 1999). Immunohistological studies indicate that the total intensity of mAChR decreases along the vascular tree from large conducting vessels to capillaries (Badaut et al., 1997). ECs cultures from arterioles and capillaries of human brain expressed M1, M2 and M5 receptors; SMCs have all subtypes except the M4-mAChR; perivascular astrocytes have all five mAChR (Elhusseiny et al., 1999). Binding sites for mAChR were also found in cultured retinal pericytes (Ferrari-Dileo et al., 1991).

In conclusion, cholinergic dysfunctions of the basal forebrain as observed in AD may also contribute to the cerebrovascular abnormalities in a large portion of AD patients. To study this aspect of AD in more detail, appropriate animal models of AD are required.

1.6 Transgenic Tg2576 mouse model of Alzheimer’s disease

The understanding that mutations in three genes (APP, PS1 and PS2) are directly associated with early onset AD and enhanced Aβ formation has provided a powerful rationale for studying pathogenetic aspects of AD in vivo by creating transgenic animals carrying these mutations.

Over the past few years a variety of transgenic mouse lines that overexpressed mutant APP, PS1 or both APP and PS1 as transgenes have been developed and extensively examined (see for review Games, 2006).
One of the most widely used transgenic mice in the AD field represents the Tg2576 mouse developed by Karen Hsiao and co-workers in 1996. This mouse overexpresses the Swedish double mutation of the hAPP695 (hAPP<sub>695</sub> K670N/M671L), that favours the formation of the Aβ peptide (Hsiao et al., 1996). Transgenic APP levels in the brain are 5-6 fold higher as compared to the endogenous mouse APP (Hsiao, 1998). Aging related increases in the levels of soluble and insoluble fibrillar Aβ (1-40) and Aβ (1-42) as well as the ratio of Aβ (1-42)/Aβ (1-40) have been found in Tg2576 mouse (Hsiao et al., 1996; Apelt et al., 2004). In Tg2576 mice Aβ (1-42) is most abundant in plaques, whereas Aβ (1-40) predominantes in vessels (Kawarabayashi et al., 2001). In addition, this model develops cerebral amyloid angiopathy from 10 months onwards (Hsiao, 1998; Calhoun et al., 1999; Christie et al., 2001; Robbins et al., 2006), while a profound alteration in the regulation of the cerebral blood circulation occurs already at 2–3 months of age (Niwa et al., 2000a and 2002a; Iadecola, 2004). In the old Tg2576 mice reduction in CBF is associated with corresponding reductions in cerebral glucose uptake and activity of enzymes of brain glycolysis (Niwa et al., 2002b; Bigl et al., 2003). From the age of 3 months onwards these mice demonstrate cognitive deficits (King et al., 1999). However, the most behavioural and memory abnormalities such as hippocampally-mediated spatial memory tasks (different maze tests, contextual fear learning, operant learning) and non-spatial memory tasks (auditory startle, eye blink conditioning, object recognition), deficits in working memory retention and non-memory sensorimotor perturbations (hyperactivity, reductions in open field activity, disturbed sleep and wake patterns) have been observed in Tg2576 mice at the age of 5 to 6 months (for review, see Games et al., 2006). Cognitive deficits in Tg2576 mice correlate to impaired hippocampal long-term potentiation, and these disturbances are accompanied by minimal or no loss of presynaptic or postsynaptic structural elements in the hippocampus (Chapman et al., 1999). Moreover, electrophysiological investigations revealed age-related impairments in basal synaptic transmission in hippocampus of transgenic Tg2576 mice (Fitzjohn et al., 2001).

Analysis of the cholinergic system revealed subtle and differential changes in cholinergic synaptic markers in aged transgenic Tg2576 mice (Gau et al., 2002; Apelt et al., 2002; Klingner et al., 2003) as well as degeneration of ChAT-immunoreactive fibres in the environment of β-amyloid plaques and activated glial cells by means of ultrastructural investigations (Aucoin et al., 2005; Tomidokoro et al., 2000; Cha et al., 2001; Hernandez et al., 2001; Lüth et al., 2003). While no significant differences in size, shape or immunostaining profile of cholinergic neurons in the basal forebrain of Tg2576 mice were reported
(Tomidokoro et al., 2000; Apelt et al., 2002; Wong et al., 1999), an increased p75\textsuperscript{NTR} protein level in medial septal cholinergic neurons were observed in 12-month old Tg2576 mice (Jaffar et al., 2001).

Quantitation of [\textsuperscript{3}H]hemicholinium-3 (HCh-3) binding to high-affinity choline uptake sites has been used as a marker to detect loss of cortical cholinergic fibres. Transgenic mice with no significant plaque load demonstrated reduced HCh-3 binding to choline uptake sites in anterior brain regions as compared to nontransgenic littermates, while in aged transgenic mice with high number of plaque deposits decreased HCh-3 binding levels were accompanied by increased vesicular acetylcholine transporter binding in selected cortical brain regions (Wong et al., 1999; Hernandez et al., 2001; Klingner et al., 2003). Receptor binding studies reported either no change in mAChR binding sites in the brain of Tg2576 mice (Cha et al. 2001; Bednar et al., 2002), or a reduction in M1- and M2- mAChR binding sites in some neocortical regions in aged animals (Apelt et al., 2002). Further, an age-related increase in \(\alpha\)7 nAChR expression were observed in Tg2576 mouse brain (Dineley et al., 2001; Bednar et al., 2002), which have been suggested to play a role in A\(\beta\) mediated inflammatory processes (Teaktong et al., 2003; Yu bet al., 2005). In aged transgenic mice GABA\(\alpha\)\textsubscript{1}, NMDA, AMPA, kainate, and beta-adrenergic as well 5-HT\textsubscript{1A} and 5-HT\textsubscript{2A}-receptor binding levels were hardly affected, whereas \(\alpha\)1- and \(\alpha\)2-adrenoceptor binding was increased in selected cerebral cortical regions as compared to non-transgenic littermates (Klingner et al., 2003).

The development of changes in both cholinergic and non-cholinergic markers in transgenic Tg2576 mouse brain already before the onset of progressive plaque deposition provides \textit{in vivo} evidence of a modulatory role of soluble \(\beta\)-amyloid on cortical neurotransmission and may be referred to the deficits in learning and memory observed in these mice also before significant plaque load.

A\(\beta\) depositions in transgenic Tg2576 mouse brain have been shown to induce micro- and astrogliosis which is accompanied by the induction of both pro- and anti-inflammatory cytokines (Irizarry et al., 1997; Frautschy et al., 1998; Benzing et al., 1999; Apelt and Schliebs, 2001). Astrocytes were found in close proximity to both fibrillar and diffuse A\(\beta\) deposits and were detectable at very early stages of plaque development, while activated microglia appeared in and around fibrillar A\(\beta\) plaques only.

Markers of oxidative stress as well as of the antioxidant defense enzymes heme-oxygenase and superoxide dismutase were increased in these transgenic animals (Hsiao, 1998). Apelt et al. demonstrated that the activities of superoxide dismutase and glutathione peroxidase in cortical tissue from Tg2576 mice steadily increased from postnatal age 9–12 months; the
levels of cortical nitric oxide, and reactive nitrogen species demonstrated peak values around 9 months of age (Apelt at al., 2004).

1.7 Aim of study

As outlined in the Introduction part, a large number of AD patients demonstrate cerebrovascular pathology such as cerebral amyloid angiopathy, microvascular degeneration, and impaired vascular regulation coupled with impaired glucose metabolism in cholinceptive target regions. These pathological changes are accompanied by the occurrence of dysfunctions in cortical cholinergic neurotransmission, an early and consistent feature of AD. Therefore, the question arises of whether changes in cerebral vessels, cortical cholinergic denervation, and β-amyloid formation and deposition are interrelated.

The present study intends to contribute to the following questions:

1) Do cholinergic and vascular dysfunctions interact with each other, i. e. cortical cholinergic dysfunction affects the vascular system and/or vice versa.

2) Does the formation and deposition Aβ–peptides during aging interact with the cholinergic innervation of cerebral cortical blood microvessels.

To in vivo characterize the developmental relationship of amyloid formation and deposition, cortical cholinergic innervation and cerebrovascular abnormalities, transgenic Tg2576 mice that express the Swedish double mutation of human APP and progressively develop Alzheimer’s-like amyloid deposits were considered as an appropriate in vivo animal model. Serial cortical cryocut sections, obtained from mice at ages ranging between 4 and 18 months, were subjected to immunohistochemistry to label vascular endothelial cells, cortical cholinergic nerve terminals and β-amyloid plaques, followed by a thorough quantitative evaluation of the age-related spatial relationship between cerebral cortical capillaries, amyloid plaques and cholinergic terminals, using computer-assisted imaging analysis.
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical reagents used
Alkylphenylpolyethyleneglycol (Triton X-100) (FERAK Laborat GmbH; Berlin Germany); Cresylviolet; 3,3'-Diaminobenzidine Tetrahydrochloride (DAB) (Sigma-ALDRICH, Inc; St. Louis, USA); Di-sodiumhydrogenphosphate Dihydrate (Na$_2$HPO$_4$.2H$_2$O; M=177.99) (Carl Roth GmbH, Karlsruhe, Germany); Ethanol (C$_2$H$_5$OH M= 46,07); Entellan (Merck, Darmstadt, Germany); Formic acid; 85% solution (HCOOH; M= 46,03) (VEB Jenapharm-Laborchemie APOIDA, Germany); Nickel Ammonium Sulphate (II) Hexahydrate (Ni(NH$_4$)$_2$(SO$_4$)$_2$.2x6H$_2$O; M=395) (Merck, Darmstadt, Germany); Hydrogen Peroxide, 30% solution (H$_2$O$_2$); Paraformaldehyde (HO(CH$_2$O)$_n$H) (VEB LABORCHEMIE APOIDA, Germany); Sucrose (C$_{12}$H$_{22}$O$_{11}$; M=342.30) (Carl Roth GmbH, Karlsruhe, Germany); Sodium Azide (NaN$_3$; M= 65.01); Sodium Chloride (NaCl; M= 58.442) (Carl Roth GmbH, Karlsruhe, Germany); Sodium Dihydrogenphosphate Monohydrate (NaH$_2$PO$_4$.H$_2$O; M=138) (Carl Roth GmbH, Karlsruhe, Germany); Thioflavin S (Sigma Chemical Co; St. Louis, USA); Tris (C$_4$H$_{11}$NO$_3$; M=121.14) (Carl Roth GmbH, Karlsruhe, Germany).

2.1.2 Biological reagents used
Biotinylated horseradish peroxidase (Bio-HRP); Bovine serum albumin (BSA) (SERVA Electrophoresis GmbH, Heidelberg, Germany); Heparin Sodium (Hofmann-La Roche AG, Grenzach-Wyhlen, Germany); Normal Goat Serum (GNS), Donkey Normal Serum (DNS) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA); Streptavidin (native protein) (Molecular Probes, Inc, Eugene, USA).

2.1.3 Preparation of solutions and buffers
Perfusion buffer:

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>9.0 g</td>
<td>0.9%</td>
</tr>
<tr>
<td>Heparin Sodium</td>
<td>5000 IE (1.0 ml)</td>
<td>5000 IE/l</td>
</tr>
</tbody>
</table>

Ad 1 liter of bidistilled water.

Phosphate buffer (PB):

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$.2H$_2$O</td>
<td>17.8 g/l</td>
<td>0.1 M</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$.1H$_2$O</td>
<td>13.8 g/l</td>
<td>0.1 M</td>
</tr>
</tbody>
</table>
adjust to pH value of 7.4

**Fixative:**

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraformaldehyde</td>
<td>40.0 g/l</td>
<td>4 %</td>
</tr>
</tbody>
</table>

Ad 1 liter of 0.1 M PB-buffer pH 7.4.

**Postfixation solution:**

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>300.0 g</td>
<td>30%</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.5 g</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

Ad 1 liter of 0.1 M PB-buffer, pH 7.4.

**Tris-buffered saline (TBS buffer):**

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>12.14 g</td>
<td>0.1 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.77 g</td>
<td>0.15 M</td>
</tr>
</tbody>
</table>

Ad 1 liter of bidistilled water. Adjust pH to 7.4 with HCl.

**Tris-buffered saline with bovine serum albumin (TBS-BSA buffer):**

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>2 g</td>
<td>2%</td>
</tr>
</tbody>
</table>

Ad 100 ml of 0.1 M TBS buffer, pH 7.4

**Avidin–biotin-peroxidase complex (ABC):**

<table>
<thead>
<tr>
<th></th>
<th>Stock solution of reagents</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin</td>
<td>1 mg/ml</td>
<td>12.5 μg/ml</td>
</tr>
<tr>
<td>Biotinylated horseradish peroxidase (Bio-HRP)</td>
<td>0.5 mg/ml</td>
<td>2.5 μg/ml</td>
</tr>
</tbody>
</table>

Reagents were dissolved in 1.0 ml of 0.1 M TBS, pH 7.4, containing 2% bovine serum albumin, under steadily stirring at room temperature for 45 minutes allowing to form the ABC complex.

**DAB-Nickel buffer (buffer for immunoperoxidase labeling):**

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>6.057 g</td>
<td>0.05 M</td>
</tr>
</tbody>
</table>

Ad 1 litre of bidistilled water. Adjust pH to 8.0 with HCl.

**DAB-Nickel staining solution:**

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni(NH₄)₂(SO₄)₃ x 6 H₂O</td>
<td>0.04 g</td>
<td>0.4%</td>
</tr>
<tr>
<td>DAB</td>
<td>0.002 g</td>
<td>0.02%</td>
</tr>
<tr>
<td>H₂O₂ 30%</td>
<td>5 μl</td>
<td>0.015%</td>
</tr>
</tbody>
</table>
Ad 10 ml of 0.05 M DAB-Nickel buffer, pH 8.0.

1% Thioflavin S staining solution:
Thioflavin S.........................................- 0.05 g
Bidistilled water ..........................- 5.0 ml

3% Cresyl violet solution:
Cresyl violet ........................................- 3.0 g
Bidistilled water ..........................- 100.0 ml

Nissl staining solution:
Cresyl violet 3% ....................................- 5.0 ml
Sodium acetate 0.1 N .................- 8.0 ml
Acetic Acid 0.1 N ...............................- 92.0 ml

Dehydration solutions for Nissl staining:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>Ethanol 70%</td>
</tr>
<tr>
<td>No. 2</td>
<td>Ethanol 80%</td>
</tr>
<tr>
<td>No. 3</td>
<td>Ethanol 85%</td>
</tr>
<tr>
<td>No. 4</td>
<td>Ethanol 95%</td>
</tr>
<tr>
<td>No. 5</td>
<td>Ethanol 100%</td>
</tr>
</tbody>
</table>

2.1.4 Antibodies and reagents used for immunohistochemistry

In Table 1 all antibodies, both primary and secondary, used in this study for immunohistochemistry, are listed including their specificities, manufacturer, and the final concentration at which the particular antibody was applied for immunostaining, while in Table 2 the most important reagents for histochemistry are summarized.

Table 1: Antibodies used for immunohistochemical staining.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Manufacturer</th>
<th>Final dilution/concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1</td>
<td>Rat (mouse)</td>
<td>CALBIOCHEM- NOVABIOCHEM International, Inc.; catalog No 400055</td>
<td>1:3000</td>
</tr>
<tr>
<td>VAChT</td>
<td>Rat (mouse)</td>
<td>Phoenix Pharmaceuticals, Inc., Belmont, USA.; catalog No H-V006; lot No 420206</td>
<td>1:500</td>
</tr>
<tr>
<td>ChAT</td>
<td>Avian, Chicken, Guinea Pig, Human, Mouse, Monkey, Rat, Opposum.</td>
<td>CHEMICON® International, Inc.; catalog No AB 144 P; lot No 22090335</td>
<td>1:25 20µg/ml</td>
</tr>
<tr>
<td><strong>NeuN</strong></td>
<td>Monoclonal Mouse Anti-Neuronal Nuclei IgG1, clone P19</td>
<td>Mouse, rat, human, chick, salamander</td>
<td>CHEMICON® International, Inc.; catalog No MAB377; lot number 25070259</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>4G8</strong></td>
<td>Biotinylated monoclonal Mouse Anti-Human Amyloid-Beta Protein IgG, isotype IgG2α, clone 4G8</td>
<td>Human, mouse</td>
<td>SIGNET Laboratories, Inc.; Dedham Massachusetts, USA. catalog No 9240-02</td>
</tr>
<tr>
<td><strong>GFAP</strong></td>
<td>Polyclonal Rabbit Anti-Cow glial fibrillary acidic protein</td>
<td>Human, mouse, rat, sheep, cow, cat, dog</td>
<td>DAKO, A/S Denmark; catalog No MAB 3418; lot No 096(701)</td>
</tr>
<tr>
<td><strong>CD11b</strong></td>
<td>Monoclonal rat anti mouse CD11b Protein, isotype IgG2b; clone 5C6</td>
<td>Mouse, rat</td>
<td>SEROTEC Ltd., Oxford, England; catalog No MCA711</td>
</tr>
</tbody>
</table>

**Secondary antibodies**

<table>
<thead>
<tr>
<th>Polyclonal Biotin-SP-conjugated AffiniPure Goat Anti-Rabbit IgG</th>
<th>Human, mouse, rat</th>
<th>Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA, USA. catalog No 11-065-144</th>
<th>1:500</th>
<th>2 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal Cy&lt;sup&gt;TM3&lt;/sup&gt;-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L)</td>
<td>Rabbit; minimal cross-reaction to human, mouse and rat serum proteins</td>
<td>Jackson ImmunoResearch Laboratories, Inc.; catalog No 111-165-144; lot No 67223</td>
<td>1:75</td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>Polyclonal Cy&lt;sup&gt;TM2&lt;/sup&gt;-conjugated AffiniPure Goat Anti-Mouse IgG</td>
<td>Mouse; minimal cross-reaction to rat, human, bovine and horse serum proteins;</td>
<td>Jackson ImmunoResearch Laboratories, Inc.; catalog No 115-225-100; lot No 47110</td>
<td>1:50</td>
<td>26 μg/ml</td>
</tr>
<tr>
<td>Polyclonal Cy&lt;sup&gt;TM3&lt;/sup&gt;-conjugated AffiniPure Goat Anti-Mouse IgG (H+L)</td>
<td>Mouse; Minimal cross-reaction to rat, human, bovine, horse and rabbit serum proteins</td>
<td>Jackson ImmunoResearch Laboratories, Inc.; catalog No 115-165-166; lot No 68848</td>
<td>1:300</td>
<td>(5 μg/ml)</td>
</tr>
<tr>
<td>Polyclonal Cy&lt;sup&gt;TM2&lt;/sup&gt;-conjugated AffiniPure Donkey anti-Goat IgG (H+L)</td>
<td>Goat; Minimal cross-reaction to chicken, guinea pig, Syrian hamster, horse, human, mouse and rat serum proteins</td>
<td>Jackson ImmunoResearch Laboratories, Inc.; catalog No 705-225-147; lot No 57922</td>
<td>20 μg/ml</td>
<td>---</td>
</tr>
<tr>
<td>Polyclonal Cy&lt;sup&gt;TM5&lt;/sup&gt;-conjugated AffiniPure Goat Anti-Mouse IgG</td>
<td>Mouse</td>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
<td>20 μg/ml</td>
<td>---</td>
</tr>
<tr>
<td>Polyclonal Cy&lt;sup&gt;TM5&lt;/sup&gt;-conjugated AffiniPure Goat Anti-Rabbit IgG</td>
<td>Rabitt</td>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
<td>20 μg/ml</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 2: List of reagents, used for histological staining.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated-Solanum Tuberosum (Potato Lectin)</td>
<td>Vector Laboratories, Inc., Burlingame, USA; Catalog No.B-1165; Lot No.Q0923</td>
<td>30 μg/ml</td>
</tr>
<tr>
<td>Streptavidin, Cyanine, Cy²M conjugated</td>
<td>purchased from Dianova, Hamburg</td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>Streptavidin, Cyanine, Cy³M conjugated</td>
<td>purchased from Dianova, Hamburg</td>
<td>20 μg/ml</td>
</tr>
<tr>
<td>Thioflavin S</td>
<td>Sigma Chemical Co, St. Louis, USA</td>
<td>1%</td>
</tr>
<tr>
<td>Cresylviolet</td>
<td></td>
<td>3%</td>
</tr>
</tbody>
</table>

2.1.5 Transgenic animals

The transgenic mice used in this study contained the human APP695 with the double mutation (K670N, M671L), which was found in a large Swedish family with early onset of Alzheimer's disease, inserted into a hamster prior protein (PrP) cosmid vector in which the PrP open reading frame was replaced by that for the variant APP (Tg(HuAPP695,K670N-M671L)2576), as developed and described previously by Hsiao et al. (Hsiao et al., 1996; Hsiao, 1998). The transgene is expressed in C57B6/SJL F1 mice (kindly provided by Dr. Karen Hsiao, University of Minnesota), backcrossed to C57B6 breeders. N2 generation mice were studied at ages of 4, 6, 8, 10, 12, 18 months (n=3-5; see Table 3). Age-matched non-transgenic littermates served as controls. The transgenity was determined in 2-month-old animals in tail biopsy material by PCR (Hsiao et al., 1996), and further cross-checked after sacrifice.

The animals were housed in a 12h light-dark cycle, with ad libitum access to food and water under the care of the Animal House of the Leipzig Medical Faculty (Medizinisch-Experimentelles Zentrum) according to the guidelines of Animal Care of the European Union.

Table 3: List of animal experimental groups studied including size of group (no of mice).

<table>
<thead>
<tr>
<th></th>
<th>4 month</th>
<th>6 month</th>
<th>8 month</th>
<th>10 month</th>
<th>12 month</th>
<th>18 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg2576</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Wild Type</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Tissue preparation and sampling of sections

Mice were deeply anaesthetized by an overdose of CO\textsubscript{2} and transcardially perfused with the perfusion buffer (50 ml) followed by the fixative (50 ml, for composition, see 2.1.3). Brains were removed from the skull and post-fixed in the same fixative overnight (for 24 h) at room temperature. After cryoprotection of the tissue by equilibrating in 30% sucrose in PB buffer, series of coronal sections were cut at 30 $\mu$m thickness on a freezing microtome (Leica, Heidelberg, Germany) and collected in 0.1 M TBS, pH 7.4, with 0.005% sodium azide as additive.

2.2.2. Immunohistochemistry

Depending on the properties of the secondary antibody used to visualize the immunoreactivity, either by fluorescent or non-fluorescent dyes, different staining protocols were applied as described below.

2.2.2.1 Protocol of immunofluorescent labeling:

1. Free-floating brain slices were washed in 0.1 M TBS pH 7.4, three times for 10 minutes.
2. Non-specific binding sites in the tissue were blocked with either 5% normal goat (GNS) or donkey serum (DNS) and 0.3% Triton X-100 in TBS (GNS / DNS-T-TBS) for one hour.
3. After blocking sections were incubated overnight at room temperature with either GNS-T-TBS or DNS-T-TBS containing the particular primary antibody of interest (see, Table 1).
4. Washing out the unbound primary antibody with TBS, pH 7.4, three times for 10 minutes.
5. Incubation of slices for one hour at room temperature with the appropriate Cy-conjugated secondary antibody (see, Table 1), prepared in TBS containing 2% bovine serum albumin (TBS-BSA) and used at concentrations as stated in Table 1.
6. To wash out the unbound secondary antibody, sections were treated three times for 10 min with TBS, briefly dipped in distilled water, mounted on fluorescence-free slides, air-dried and coverslipped with Entellan.
2.2.2.2 Protocol of immunoperoxidase labeling (ABC technique):

1. Free-floating sections were washed in 0.1 M TBS, pH 7.4, three times for 10 minutes.
2. To remove endogenous peroxidase activity sections were incubated for 30 min in TBS containing 0.6% hydrogen peroxide.
3. To wash out remaining hydrogen peroxide slices were bathed in 0.1 M TBS, pH 7.4, three times for 10 minutes.
4. After blocking of non-specific binding sites in the tissue either with GNS-T-TBS or DNS-T-TBS for one hour, sections were further incubated overnight at room temperature with either GNS-T-TBS or DNS-T-TBS containing the particular primary antibody of interest (see, Table 1).
5. Washing out the unbound primary antibody with TBS, pH 7.4, three times for 10 minutes.
6. Incubation of slices for one hour at room temperature with the appropriate biotinylated secondary antibody (see, Table 1), prepared in TBS-BSA and used at concentrations as stated in Table 1.
7. To wash out the unbound secondary antibody, sections were treated three times for 10 min with TBS, pH 7.4, followed by incubation with avidin-biotin-peroxidase complex (ABC) at room temperature for one hour.
8. Sections were rinsed in 0.1 M TBS, pH 7.4, twice for 10 minutes, and once in 0.05 M Tris-buffer, pH 8.0, for 10 minutes.
9. For visualization of the immunoreactivity, sections were shortly preincubated in 0.05M Tris-buffer, pH 8.0, containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.4% nickel–ammonium sulphate (II) hexahydrate (Tris-DAB-Nickel), followed by further incubation for x min in Tris-DAB-Nickel solution additionally containing 0.015% hydrogen peroxide.
10. To wash out non-reacted dye, sections were treated for 10 min in 0.05 M Tris-buffer, pH 8.0, and twice for 10 min in 0.1 M TBS, pH 7.4.
11. Finally, sections were briefly dipped in distilled water mounted onto gelatine-coated glass slides, air-dried and coverslipped with Entellan.

Immunolabeling of sections by omission of primary antibodies were considered as control in both protocols.
2.2.2.3 Combination of primary and secondary antibodies

In Table 4 the primary antibodies used in this study are listed together with the secondary antibodies applied to visualize the immunoreactivity including the antigen that should be detected in the brain tissue.

**Table 4:** Combinations of primary and secondary antibodies used to visualize a particular immunoreactivity including the cellular markers to be detected in brain tissue.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Visualization of immunoreactivity</th>
<th>Detection of</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1</td>
<td>Biotin-SP-conjugated AffiniPure Goat Anti-Rabbit IgG or Polyclonal Cy&lt;sub&gt;TM3&lt;/sub&gt;-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L)</td>
<td>DAB-Ni or fluorescent staining</td>
<td>Vascular endothelial cells/ brain vessels</td>
</tr>
<tr>
<td>VAChT</td>
<td>Polyclonal Biotin-SP-conjugated AffiniPure Goat Anti-Rabbit IgG or Polyclonal Cy&lt;sub&gt;TM3&lt;/sub&gt;-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L)</td>
<td>DAB-Ni or fluorescent staining</td>
<td>Cortical cholinergic terminals and extracortical cholinergic neurons</td>
</tr>
<tr>
<td>NeuN</td>
<td>Polyclonal Cy&lt;sub&gt;TM2&lt;/sub&gt;-conjugated AffiniPure Goat Anti-Mouse IgG or Polyclonal Cy&lt;sub&gt;TM5&lt;/sub&gt;-conjugated AffiniPure Goat Anti-Mouse IgG</td>
<td>fluorescent staining</td>
<td>Neuronal body</td>
</tr>
<tr>
<td>ChAT</td>
<td>Polyclonal Cy&lt;sub&gt;TM2&lt;/sub&gt;-conjugated AffiniPure Donkey anti-Goat IgG</td>
<td>fluorescent staining</td>
<td>Cortical cholinergic terminals and extracortical cholinergic neurons</td>
</tr>
<tr>
<td>4G8</td>
<td>Polyclonal Cy&lt;sub&gt;TM3&lt;/sub&gt;-conjugated AffiniPure Goat Anti-Mouse IgG</td>
<td>fluorescent staining</td>
<td>Neuritic (senile) and diffuse plaque (total plaque load)</td>
</tr>
<tr>
<td>CD11b (Mac-1)</td>
<td>Polyclonal Cy&lt;sub&gt;TM3&lt;/sub&gt;-conjugated AffiniPure Goat Anti-Rat IgG</td>
<td>fluorescent staining</td>
<td>Microglia</td>
</tr>
<tr>
<td>GFAP</td>
<td>Polyclonal Cy&lt;sub&gt;TM5&lt;/sub&gt;-conjugated AffiniPure Goat Anti-Rabbit IgG</td>
<td>fluorescent staining</td>
<td>Astroglia (GFAP-positive macroglia)</td>
</tr>
</tbody>
</table>
2.2.2.4 Protocol of β-amyloid immunolabeling (Formic acid epitope retrieval method):
To break the protein cross-links of β-amyloid and to unmask the antigens, a pretreatment of brain sections by formic acid is recommended to enhance staining intensity (Kitamoto et al., 1987).

1. Free-floating sections were washed in 0.1 M TBS, pH 7.4, three times for 10 minutes.
2. Incubation of brain sections with 85% formic acid for 10 minutes at room temperature.
3. To wash out the formic acid, slices were bathed in 0.1 M TBS, pH 7.4, three times for 10 minutes.

For immunofluorescent detection of β-amyloid, steps 2 - 6 of the protocol of immunofluorescent labeling (2.2.2.1) were applied, while for visualization β-amyloid by the ABC technique, steps 2 -11 of the protocol of immunoperoxidase staining (2.2.2.2) were used.

2.2.3 Histochemistry

2.2.3.1 Thioflavin S staining
Fluorescence labeling of β-amyloid by Thioflavin S was performed after DAB-Nickel immunochistochemical staining of brain vessels with GLUT1 antibody in order to demonstrate fibrillar β-amyloid (senile plaque) in the brain tissue sections of mice. Immunolabeling was also performed to establish co-localisation of total β-amyloid loading (immunohistochemistry with 4G8 antibody) and fibrillar component of β-amyloid deposits (Thioflavin S-positive aggregates). For this reason the brain sections were covered with 1 % Thioflavin S solution for 20 minutes in darkness, followed by washing the slides for one minute with bidistilled water, and by immersion into 80% ethanol for 40 minutes. Finally the slides were washed two times with bidistilled water, air-dried, and coverslipped with Entellan.

2.2.3.2 Nissl staining
Nissl staining (cresyl violet) was used to stain all neuronal (partially glial cells) cell bodies in a particular brain section. Sections were immersed in Nissl staining buffer for 30 minutes, followed by rinsing in bidistilled water. The slices were dehydrated by bathing in a series of ethanol solutions with increasing concentrations (70 – 100%), air-dried and coverslipped with Entellan.
2.2.3.3 Solanum Tuberosum Lectin (STL) staining

_Solanum tuberosum_ lectin (STL) was used to label cerebral blood vessels. For STL labeling sections were washed in 0.1 M TBS pH 7.4 and preincubated with 5% normal goat serum and 0.3 %Triton x-100 in TBS (NGS-T-TBS) for one hour by room temperature. Sections were incubated in a cocktail of antibody and biotinylated _Solanum tuberosum_ lectin (dilution 30 μg/ml) in NGS-T-TBS overnight by room temperature (see, Table 5). Afterwards, sections were extensively rinsed in TBS and incubated with a mixture of carbocyanine (Cy)-conjugated secondary antibodies (accordingly to primary antibodies) and Cy2-conjugated Streptavidin (dilution 20 μg/ml) (for visualisation of reactivity from lectin) in TBS with 2% BSA (TBS-BSA) for one hour at room temperature. Finally, section were rinsed in TBS and in distilled water, mounted onto slides, dried and coverslipped with Entellan.

2.2.4 Double and triple-coloured immuno- / histochemical staining of brain sections

To check for cellular co-localization of selected protein markers, brain sections were subjected to subsequent staining with particular antibodies and/or dyes, as listed in Table 5.

_Table 5:_ Cocktails used for double and triple staining of antigens in one and the same brain section.

<table>
<thead>
<tr>
<th>Cocktail of antibodies/dyes</th>
<th>Tissue markers to be detected</th>
<th>Type of staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Anti-GLUT1 Thioflavin S</td>
<td>Brain vessels and fibrillar β-amyloid load (neuritic plaque)</td>
<td>Double staining</td>
</tr>
<tr>
<td>2 Anti-GLUT1 Solanum Tuberosum Lectin</td>
<td>Co-localisation of staining from brain vessels; by transgenic mice β-amyloid load (Potato Lectin)</td>
<td>Double staining</td>
</tr>
<tr>
<td>3 Anti-VACTh Anti-ChAT</td>
<td>Co-localisation of cortical cholinergic terminals</td>
<td>Double staining</td>
</tr>
<tr>
<td>4 Anti-VACTh Anti-NeuN</td>
<td>Cortical cholinergic terminals and neuronal cells in the cortex</td>
<td>Double staining</td>
</tr>
<tr>
<td>5 Anti-VACTh Anti-NeuN Solanum Tuberosum Lectin</td>
<td>Cortical cholinergic terminals, neuronal cells body β-amyloid load and vessels</td>
<td>Triple staining</td>
</tr>
<tr>
<td>6 4G8* Thioflavin S</td>
<td>Co-localisation of total β-amyloid and fibrillar β-amyloid</td>
<td>Double staining</td>
</tr>
<tr>
<td>7 Solanum Tuberosum Lectin Thioflavin S</td>
<td>Co-localisation of blood vessels and β-amyloid plaque</td>
<td>Double staining</td>
</tr>
<tr>
<td>8 Solanum Tuberosum Lectin Thioflavin S 4G8*</td>
<td>β-amyloid plaque labeling by three markers</td>
<td>Triple staining</td>
</tr>
<tr>
<td>9 Solanum Tuberosum Lectin CD11b (Mac-1)</td>
<td>Co-localisation of microglia and blood vessel</td>
<td>Double staining</td>
</tr>
<tr>
<td>10 Solanum Tuberosum Lectin</td>
<td>Co-localisation of β-amyloid plaque</td>
<td>Triple staining</td>
</tr>
</tbody>
</table>
2.2.5 **Microscopy and digital image processing**

The DAB-stained brain sections were analysed using a Zeiss Axioplan – Axio Vision two light microscope including a Sony DXC-930P colour video camera system. A microscope equipped with a ×2.5, ×10, ×20 without immersion plan achromatic objectives and a ×10 projection lens was used. Double-labeled sections (DAB-Nickel and Thioflavin S) were investigated using a double light (fluorescents and normal light) microscopy.

Immunofluorescent preparations were examined with a Zeiss LSM 510 Confocal Laser Scanning Microscope Axioplan 2. A microscope equipped with a ×40 water immersion and ×100 oil immersion plan achromatic objectives and a ×10 projection lens was used. Confocal images of Cy2 fluorescence were obtained with the Argon laser (488 nm) and emission band-pass filter (BP) 505-550. The Helium-Neon laser (543 nm) and emission filter BP 560-615 were used to detect Cy3 fluorescence. The second Helium-Neon Laser (633 nm) and emission long-pass filter (LP) 650 was used for Cy5 fluorescence detection. In case of triple immunohistochemistry, Cy5-labeled structures were color-coded in blue. The slides histochemical labelled with Thioflavin S were examined using the Argon laser (488 nm) and emission filter BP 505-550. To avoid overlap in the excitation spectrum of the different fluoroprobes a separate track and channel was used for each laser. Multi-slice imaging was obtained by optical sectioning of the stained tissue with confocal laser scanning microscope. In this manner, appointed volume was systematically scanned across an x-y plane, and through multiple z planes. The deep-tissue volume scans produced images from slice thickness of 0.98 µm.

*Adobe Photoshop 5.0* software (Adobe Systems, Mountain View, CA) was used to process the original images with minimal alterations to the background (brightness and contrast).

2.2.6 **Morphological and morphometric analyses**

2.2.6.1 **Cortical microvessels**

The amount of immunostained capillaries was semiquantitatively estimated from the area covered by GLUT1-immunoreactivity (as corresponding reaction product) and referred to the

<table>
<thead>
<tr>
<th>4G8** Anti-GFAP markers (Solanum Tuberosum Lectin and 4G8**) and GFAP positive macroglial cells</th>
<th>4G8** Anti-GFAP markers (Solanum Tuberosum Lectin and 4G8**) and GFAP positive macroglial cells</th>
</tr>
</thead>
</table>

*no formic acid pretreatment; **with formic acid pretreatment (see 2.2.2.4)*
MATERIALS AND METHODS

The total area of the corresponding cerebral cortical region, using a video camera-based, computer assisted imaging device and the software package of Imaging Research Inc., MCID 4.0. Thresholds for object segmentation (of reaction product) were established in standard slides and remained constant throughout the analysis session. Regions routinely analyzed comprised parietal cortex (bregma –1.46–1.58): barrel field in somatosensory cortex 1 (S1BF). Capillary density was defined as percentage of the area covered by GLUT1-immunoreactivity as compared to the total area of a distinct cerebral cortical region. Densities of brain capillaries were estimated either in the vicinity of plaques (capillaries located within a circle with varying diameter, see Figs. 2.1) or in distinct cortical layers (see Figs. 2.2). To analyze the laminar distribution of capillaries, the cerebral cortex was subdivided into boxes of 200µm x 50µm from layer I to VI (Figs. 2.2), and the capillary density in each box was graphed against cortical depth. Cortical layers were differentiated by Nissl staining of subsequent sections.

For analysing the spatial relationship between cerebral cortical capillaries and amyloid plaques brain sections double stained for both brain capillaries and β-amyloid plaques were used. Around a particular β-amyloid plaque circles with increasing diameters were drawn (Figs. 2.1), and the vessel density per circle area estimated as described above. This procedure allows to estimate the capillary density against the distance from senile plaques.

Fig. 2.1: Brain sections double stained for both GLUT1-immunoreactivity (labeled in black) and for β-amyloid (green fluorescence (core region appears white)), were used to estimate the capillary density against the distance from senile plaques. Circles with increasing diameter were drawn around the plaque core (white), and the density of capillaries per particular circle area is determined. Scale bar 100 µm.

A. GLUT1-immunoreactivity (highlighted in red colour) detected in circle a.
B. GLUT1-immunoreactivity (highlighted in red colour) detected in circle b.

With increasing distance from the plaque core GLUT1-immunoreactivity enhances.
2.2.6.2 Cortical cholinergic innervation

2.2.6.2.1 Total density of VACHT-immunoreactivity

The cholinergic innervation was studied in brain slices containing the barrel field area of somatosensory cortex (bregma -1.70-1.82mm, according to the brain atlas of Franklin and Paxinos, 1997), subjected to immunohistochemistry to label VACHT. Photomicrographs of the immunostained sections were obtained using a confocal laser scanning microscope equipped with a ×40 water immersion plan achromatic objective and zoom mode 0.7 (final magnification 28x). A total of four random of depth scans (one photomicrograph on each side from two adjacent sections per mouse) were taken from each of the following layers: I, II-III (taken together), IV, V, VI. The delimitation of individual cortical layers was based on known morphological criterias visualized in sections counterstained for NeuN (see Figs. 2.3).

The total VACHT-immunopositive labeling (comprising varicosities and cholinergic fibers) was analysed using the software package Soft Imaging System analySIS® (Soft imaging system GmbH, Muenster, Germany), particularly designed for color grain counting. The area occupied by VACHT-immunoreactivity (VACHT-IR) was measured in each cortical layer, corrected for background staining by setting a detection threshold, referred to the total area of the respective layer, and expressed as percentage of VACHT-immunoreactivity of total area of each layer.
Fig. 2.3: Representative images of coronal mouse brain slices obtained at bregma -1.70 mm with schematic drawings of the cortical layers selected (red and blue frameworks) for semiquantification of the cortical cholinergic fibre networks (VAcHt-IR) of barrel field area in the somatosensory cortex (S1BF) (A). The photomicrographs of VAcHt-immunostaining (B) were obtained by laser scanning microscopy (LSM 510, Zeiss). Five adjacent fields were used to sample cortical layers I-VI within the hemisphere in each section. Illustration of the analytical protocols used to determine total VAcHt-IR (B red fluorescence, D) in each layer of cortex (F) using the software package Adobe Photoshop 5.0 and Soft Imaging System analySIS® according NeuN staining (B blue, C, E). Scale bar 50 μm.
2.2.6.2.2 Estimation of the density of varicosities on cholinergic fibres

Brain sections were immunostained for VACHT-immunoreactive cholinergic fibres as described above. Using the software package *Adobo Photoshop 5.0* and *Soft Imaging System analySIS®*, the total length of fibres of a selected area was measured, and the total number of varicosities pertinent to VACHT-immunoreactive fibres was counted. The ratio of total length of fibres and the total number of varicosities per section allowed to calculate a “mean distance between varicosities” on cholinergic terminals of a particular area (see Figs. 2.4).

![Fig. 2.4](image)

**Fig. 2.4**: Representative example to demonstrate the estimation of the density of varicosities on cholinergic fibres. Laser scanning micrographs of brain sections (A) immunostained for VACHT-immunoreactive cholinergic fibres (red fluorescence) were used to estimate the length of cholinergic fibres (B, indicated by yellow lines), and the number of varicosities (C, indicated by yellow dots) using the software package *Adobo Photoshop 5.0* and *Soft Imaging System analySIS®*, as indicated by yellow drawings. The ratio of total length of fibres and the total number of varicosities per section was calculated and used as an entity to estimate the mean distance between varicosities on cholinergic terminals of the area shown.

2.2.6.3 Estimation of cholinergic perivascular innervation of cortical microvessels

Brain sections double-stained for VACHT and STL were used to characterize cholinergic innervation of cerebral cortical microvessels. Using the image software package *analySIS®*, the length of microvessels and number of perivascular VACHT-positive terminals in close vicinity (within a distance of 3 µm) of vessel basal lamina were estimated in laser scanning micrographs of brain sections, immunohistochemically stained for VACHT-immunoreactive cholinergic fibres and STL-positive cerebral cortical vessels, as representatively demonstrated in Figs. 2.5. The number of perivascular VACHT terminals in close vicinity to the vessel basement membrane (≤3 µm), counted in a fixed area of individual cortical layers (I, II-III, IV, V and VI) of S1BF, were related to the corresponding total length of vessels present in the same area. The data expressed as ratio of number of cholinergic terminal endings located within a 3-µm-distance range from the cortical vessels basal lamina to the total length of microvessel, represent the “mean contacts/endings per 100 µm length of blood vessel” assessed in a particular cortical area.
Fig. 2.5: Representative examples to demonstrate estimation of cholinergic perivascular innervation of cortical microvessels. Laser scanning micrographs of brain sections (A), double immunohistochemically stained for VACHT (red fluorescence) and STL (green fluorescence), were used to estimate length of microvessels (B, indicated by yellow lines) and number of endings of perivascular VACHT-positive terminals in close vicinity (≤3 µm) to the vessel basal lamina (C, indicated by yellow dots) by means of the image software package analySIS® as indicated by yellow drawings.

2.2.6.4 Three-dimensional-imaging of vessels innervation

The three-dimensional image processing was used to visualize the morphological parameters of perivascular cholinergic terminals (VACHT-positive) projecting to brain capillaries and larger vessels.

A series of 2D images were collected through the whole thickness of a particular brain section (z-level) by Laser Scanning microscopy, and later assembled to provide a 3D image (see also 2.2.5).

2.2.7 Statistical analysis

The corresponding data obtained from animals in each experimental group were averaged, and the corresponding standard deviations calculated. Differences between parameters quantitatively assessed in this study were tested using one-way analysis of variance (ANOVA), followed by a Student’s t-test analysis.

The Kolmogorov-Smirnov test was applied to test the sample distribution against a normal distribution. All calculations were performed with SPSS software (version: 11.5 and 14.0). P < 0.05 was considered to be statistically significant.
CHAPTER 3: RESULTS

Using a transgenic animal approach the effect of aging-related β-amyloid deposition on cerebral cortical blood vessels, and their cholinergic innervation was studied by means of morphometric analyses of brain sections immunostained for brain vessel, cholinergic nerve terminals, and β-amyloid.

3.1 Developmental and amyloid plaque-related changes in cerebral cortical capillaries in transgenic Tg2576 Alzheimer mice

3.1.1 Morphological distribution of brain vessels in the cerebral cortex of wild type mice

To reveal the distribution of blood capillaries in the mouse brain during postnatal development, cryocut sections throughout the brain obtained from mice at ages ranging between 4 and 18 months, were immunostained to label the glucose transporter type 1 (GLUT1), which is used as a marker of vascular endothelial cells.

Representative images of immunostained section from normal adult mice are presented in Figs. 3.1, demonstrating a region-dependent distribution of GLUT1-immunoreactivity with higher densities of brain capillaries in cerebral cortical regions such as motor and somatosensory cortices as well as hippocampus, while in order brain regions including piriform cortex, striatum, thalamic areas, and corpus callosum, a somewhat lower immunostaining for vascular endothelial cells was observed.
Fig. 3.1 A-F: Representative examples of immunohistochemistry to label the glucose transporter GLUT1, a marker of vascular endothelial cells, in brain sections of a 8-month-old transgenic mouse (A), including hippocampus (B), somatosensory cortex (C), piriform cortex (D), corpus striatum (E) and thalamic area (F). Scale bars: A, 800 μm; B-D, 200 μm; E-F, 100 μm.

Moreover, in most cerebral cortical areas, the distribution of brain capillaries demonstrated a laminar pattern as representatively shown for the somatosensory cortex in Fig. 3.2. Densitometric evaluation of GLUT1-immunoreactivity over all layers of the somatosensory cortex revealed somewhat higher densities of brain vessels in cortical layers III, IV and V (Fig. 3.3).
To reveal whether deposition of amyloid plaques may effect cerebral cortical blood supply or blood-brain barrier, a quantitative analysis of the spatial relationship between cerebral cortical capillaries and amyloid plaques was performed using dual immunohistochemistry to label both brain capillaries and β-amyloid plaques. Cortical cryocut sections, obtained from transgenic Tg2576 mice that overexpress the Swedish double mutation of human amyloid precursor protein and progressively develop Alzheimer-like β-amyloid deposits at ages of 10 month onwards, were immunostained with anti-GLUT1 antibody and counterstained with Thioflavin S. The combination of light and fluorescence microscopy revealed different
distribution patterns of brain capillaries around senile plaques, as shown in Fig. 3.4. Around large senile plaques the capillary density was lower and the size of vessels appeared smaller as compared to those located more distal, while diffuse plaques demonstrated a close association of capillaries with no signs of any damage Fig. 3.4).

Cortical brain sections from 18-month-old transgenic Tg2576 mice which demonstrate a high plaque load, were immunostained for GLUT1 and counterstained with Thioflavin S, and used
RESULTS

35

to estimate the densities of brain capillaries around large senile plaques (≥ 50 µm in diameter). Applying quantitative computer-assisted image analysis (for methodological details, see 2.2.6), the GLUT1-immunoreactivity was determined in dependence on the distance from the plaque core. As graphed in Fig. 3.5, the capillary density in the vicinity of senile plaques is lower by about 60% as compared to that detected more distal from the plaque core.

![Density of cortical capillaries around large senile plaques](image)

**Fig. 3.5:** Plot of density of capillaries against their distance from senile plaques which are larger in size by more than 50 µm. Data are given as percentage of capillary load per area and represent the mean ± S.E.M. of five animals. Friedman Test, *P ≤ 0.05, **P ≤ 0.01 vs. value detected at a distance of 60 µm.

**Inserts:** A, B representative image of a large Thioflavin S-positive senile plaque (white lesion) in parietal somatosensory cortex of 18-month-old transgenic mice. Scale bar: 100 µm.

Furthermore, cerebral brain vessel distribution in cortical areas with low plaque load was compared with that demonstrating a high plaque burden. In the somatosensory cortex of 18-month-old Tg2576 mice with high plaque deposits, the density of brain vessels was significantly lower as compared to neighboured areas with low plaque burden. This effect was
RESULTS

mainly observed in cortical layer IV (Fig. 3.6). The capillary density in low plaque areas of the somatosensory cortex did not significantly differ from that estimated in non-transgenic, wild type mice (Fig. 3.6).

**Fig. 3.6A:** Comparison of laminar density distribution of brain capillaries in cortical areas with high (black columns) and low (white columns) β-amyloid plaque load. Immunostaining for GLUT1 was evaluated in the somatosensory cortex of 18-month-old transgenic mice. Data are given as percentage of capillary load per area and represent the mean ± S.E.M. of five animals. ** P < 0.001 vs. corresponding control value, two-tailed Student's t-test.

In Tg2576 mouse brain, cortical levels of soluble β-amyloid steadily increase with age, but significant deposition of fibrillar, senile β-amyloid plaques in cortical areas does not occur before postnatal age of 10 month (Apelt et al., 2004). To reveal whether already the accumulation of soluble β-amyloid during early postnatal life may have consequences on the brain capillary system, the density of brain vessels estimated in selected cortical regions from transgenic Tg2576 at ages ranging between 4 and 18 months was compared with that observed in non-transgenic littermates.

**Fig. 3.6B:** Representative brain section used for estimation of capillary densities in areas with high and low plaque load (white colour staining). Scale bar: 200 μm.
In the somatosensory cortex of non-transgenic mice the capillary density steadily increased with age between 4 and 10 months, reaching the highest level at the age of 10 month and persisting up to the age of 18 months (Fig. 3.7).

Fig. 3.7: Comparison of the developmental pattern of capillary densities estimated in cortical layers (I, II-III, IV, V and VI) of the somatosensory cortex of transgenic Tg2576 (black squares) mice and non-transgenic littermates (grey triangles) at ages ranging between 4 and 18 months. Data are given as percentage of capillary load per area and represent the mean ± S.D. of three to five animals in each group of age. *P ≤ 0.05 vs. control value, two-tailed Student's t-test.
The developmental profile of brain capillary in transgenic Tg2576 mouse brain observed between ages of 4 and 10 months did not differ to that of normal mice, indicating that soluble β-amyloid production does not have any impact on blood-brain supply. However, following deposition of cortical β-amyloid plaques from age of 12 months onwards, the density of brain capillaries in the somatosensory cortex decreased with further aging in cortical layers II to V but being statistically significant in cortical layer IV only (Fig. 3.7).
3.2 Developmental and amyloid plaque-related changes in cholinergic neurotransmission in cholinceptive target regions of transgenic Tg2576 mice

Based on studies in transgenic Tg2576 mice demonstrating changes in cortical cholinergic neurotransmitter function in Tg2576 mice already before any amyloid plaque deposits (Klingner et al., 2003), the present study was undertaken to reveal whether age-related accumulation of cortical soluble and fibrillar β-amyloid peptides may affect cholinergic nerve terminals. The vesicular acetylcholine transporter (VACChT) was used as a marker of cholinergic nerve terminals and semiquantitative immunohistochemistry was applied to reveal β-amyloid-related changes in cortical cholinergic fibre density.

The study was designed to include young adult 4-month-old transgenic animals that display enhanced levels of cortical soluble human β-amyloid but still no plaque deposition, and aged 18-month-old transgenic mice with high load of both soluble and plaque-forming fibrillar β-amyloid peptides.

3.2.1 Visualisation of cholinergic nerve terminals in mouse brain

Immunohistochemical visualisation of the VACChT in cholinergic nerve terminals was compared with immunostaining for choline acetyltransferase (ChAT), the synthesizing enzyme of acetylcholine, another marker for cholinergic fibres.
Double immunofluorescence using polyclonal rabbit anti-VACHT antibody and the polyclonal goat anti-ChAT antibody showed virtually complete overlap of cholinergic fibre staining (Fig. 3.8). Staining for either VACHT or ChAT revealed similar numbers of immunoreactive fibres in the cerebral cortex. However, VACHT staining was much more intense than that for ChAT, indicating that immunohistochemistry for VACHT is more useful to detect cholinergic terminal fields as that for ChAT.

### 3.2.2 VACHT-Expression in wild type and transgenic Tg2576 mice

The VACHT immunostaining was exemplarily assessed in brain sections comprising the barrel field of somatosensory cortex 1 (S1BF) from 4-month-old wild type mice. Microscopic inspection of VACHT immunohistochemistry revealed the presence of VACHT-expressing lattice-like fibre network in the cortical tissue. The VACHT-positive fibres were distributed in all directions, but primarily horizontally in the most superficial layers with a vertical orientation of the fibres starting in deeper cortical layers. Fine varicose VACHT-immunopositive fibres pervaded all layers of the somatosensory cortex, as representatively shown in Fig. 3.9.

Semiquantitative evaluation of the density of VACHT-immunoreactivity over all somatosensory cortical layers of brains from wild type mice revealed a somewhat higher cholinergic fibre density in layers I to III, followed by layers V to VI, while the lowest density of VACHT-immunoreactivity was observed in layer IV, regardless of the two ages studied (Fig. 3.10).
In SB1F of wild type mice, the cholinergic fibre density did not change with aging regardless of the cortical layer examined (Fig. 3.10C). In contrast, in cortical layers II/III and IV of S1BF from transgenic Tg2576 mice, age-related decreases in cholinergic fibre densities were observed. VAChT-immunoreactivity in layers II/III and IV of S1BF of 18-month-old Tg2576 mice was lower by 12%, and 34%, respectively, as compared to corresponding values obtained in 4-month-old Tg2576 animals (Fig. 3.10D, and Fig. 3.11B, D).

When comparing VAChT-immunoreactivity in S1BF of wild type and transgenic Tg2576 mice, no transgene-related changes in laminar cholinergic fibre densities were observed in mice at the age of 4 months (Fig. 3.10A, and Fig. 3.11A, B), while significantly lower VAChT densities were found in layers II/III and IV by 11% and 22%, respectively, of S1BF from 18-month-old Tg2576 mice as compared to age-matched wild type animals (Fig. 3.10B, and Fig. 3.11C, D).

Preliminary experiments revealed no differences in size and thickness of somatosensory and parietal cortex between wild type and transgenic mice (data not shown).
**Fig. 3.10 A-D:** Density of VACHT-immunoreactivity in different layers of S1BF of wild type and transgenic Tg2576 mice at ages of 4- and 18-months. Data represent the mean ±SD (N=3-5), and are expressed as percentage of VACHT-immunoreactivity per area of the respective layer. Statistically significant differences are designated by asterisks.

* P ≤ 0.05, vs. control as indicated, ANOVA followed by two-tailed Student's t-test.
To further reveal whether the transgene-related reductions in density of VACHT in layers II to IV of S1BF are accompanied by changes in number of cholinergic synapses, the number of axonal varicosities and boutons of VACHT-immunoreactive fibres in cortical layers II to IV were estimated by using high-power Laser scanning images as representatively shown in Fig. 3.12. The fine structural analysis showed that cholinergic fibres in tissues of 18-month-old transgenic and wild type mice have many swollen varicosities on the terminals, as compared to young 4-month-old animals. The elderly animals of both wild type and transgenic mice have varicosity, which appear larger in size as compared to those detectable in younger ones.
**RESULTS**

Fig. 3.12 A-D: High-power laser scanning microscopic images of VACHT-Immunostained fibres and boutons in layer IV of the somatosensory cortex (S1BF) of 4-month-old wild type (A) and transgenic Tg2576 mice (B), as well as 18-month-old wild type (C) and Tg2576 mice (D).

Note there are similar densities of VACHT-positive axons and boutons in 4-month-old wild type (A) and transgenic mice (B), while diminished densities of boutons were observed in 18-month- transgenic mice (D) as compared to age-matched wild type animals (C).

There are also structural abnormalities as increased size of VACHT-positive varicosities in aged mice (C, D) compared to 4-month-old mice (A, B). Some terminals in aged tissues are comparable in size to those of elderly controls, but others are clearly reduced, or swollen and hypertroph (D). Scale bar 10 µm.

To substantiate these observations a morphometric study of presynaptic boutons in single axons in layer II/III and IV of the S1BF was performed by estimating the distances between varicosities (boutons) present at particular axons and calculating a mean distance between varicosities per each layer (Fig. 3.13).

In both 4-month-old wild type and transgenic Tg2576 mice the mean distance between varicosities of cholinergic fibres in S1BF did not differ regardless of the cortical layer studied.
RESULTS

(Fig. 3.13). However, the mean distance between varicosities of cholinergic axons increased in layers II-III and IV of S1BF of both 18-month-old wild type and Tg2576 mice, as compared to corresponding data obtained in 4-month-old wild type mice (Fig. 3.13).

The loss of varicosities assessed in S1BF of 18-month-old mice was slightly less in transgenic Tg2576 mice than in age-matched wild-type animals, detectable both in layers II-III (age-related increase in mean distance between varicosities of 108.1 ±3.8% in Tg2576-mice vs. 113.2±2.9% in wild type animals), and in layer IV (112.8±3.9% vs. 120.57±5.39%, respectively; Fig. 3.13).

![Graphs showing mean distance between varicosities in layers II-III and IV of S1BF from wild type and transgenic Tg2576 mice at ages of both 4 and 18 months.](image)

**Figs. 3.13:** Mean distance between varicosities present on VACHT-immunostained fibres determined in layers II-III, and IV of S1BF from wild type and transgenic Tg2576 mice at ages of both 4 and 18 months. Data are expressed as percentage of the mean distance detected in S1BF of 4-month-old wild type mice (=100%), and represent the mean ±SD (N=3-5). Statistically significant differences are designated by asterisks. * P≤0.05, ** P≤0.01, vs. control as indicated, ANOVA followed by two-tailed Student's t-test.
3.3 Role of cholinergic system in β-amyloid-related changes in the cerebrovascular system of transgenic Tg2576 mice

There is strong evidence that central cholinergic pathways are involved in the regulation of cerebral cortical blood flow. Using an immunohistochemical approach, the question should be addressed whether the basal forebrain cholinergic innervation of cortical microvessels may be affected by accumulation of β-amyloid deposits. To reveal any relationship of cerebral blood vessels, cholinergic innervation, and β-amyloid plaque deposition, the parietal cortex of 18-month-old transgenic Tg2576 mice was examplarily selected.

In preliminary experiments, the usefulness of *Solanum tuberosum* lectin (Potato lectin) as a marker to label both vessels, β-amyloid plaques, and activated microglial cells, was tested (chapter 3.3.1).

3.3.1 Solanum tunerosum lectin (STL) histochemistry in visualisation of brain vessels, β-amyloid, and microglia

3.3.1.1 Solanum tunerosum lectin and brain vessels

To reveal whether STL histochemistry labels brain capillaries, which can also be detected by immunohistochemistry for GLUT1, brain sections comprising the parietal cortex of 18-month-old Tg2576 mice were subjected to histochemistry for STL followed by immunostaining for GLUT1. Visualization of lectin and immunoreactivity by Laser scanning microscopy demonstrates a co-localization of STL and GLUT1-immunoreactivity of cerebral blood brain vessels (Fig. 3.14), indicating the usefulness of STL to label cerebral capillaries.

Fig. 3.14 A-C: Laser scanning photomicrograph of double staining with *Solanum tuberosum* lectin (STL, Potato Lectin) histochemistry (green fluorescence, A), and GLUT1 immunohistochemistry (red fluorescence, B) in parietal cortex of 18-month-old transgenic Tg2576 mouse, demonstrating overlapping signals of STL binding and GLUT1-immunoreactivity (merge, C). Magnification: 40x, zoom mode 0.7; scale bar: 50 μm.
3.3.1.2 *Solanum tuberosum* lectin and β-amyloid plaques

To answer the question which kind of β-amyloid (diffuse or fibrillar plaques), is stained by *Solanum tuberosum* lectin (STL), brain sections were stained with STL and immunostained with 4G8, a monoclonal anti-human amyloid beta protein, and counterstained with Thioflavine S, which labels fibrillar plaques. No labeling was observed in non-transgenic, age-matched littermates (data not shown).

Sections immunolabeled for β-amyloid (4G8) and counterstained with Thioflavin S, demonstrated the presence of both diffuse (Thioflavine-S-negative) and fibrillar (Thioflavine-S-positive) β-amyloid deposits in the parietal cortex of 18-month-old Tg2576 mice (Fig. 3.15). In brain sections that did not receive a pretreatment with formic acid, the antibody 4G8 stained the amyloid core, and weakly the halo of the senile plaques (Fig. 3.15C).

**Fig. 3.15 A-F:** Laser scanning photomicrographs of brain sections comprising the parietal cortex of 18-month-old transgenic mouse Tg2576, stained with *Solanum tuberosum* lectin, STL (red fluorescence, **A**), Thioflavin S (green fluorescence, **B**), and immunostained with 4G8 (blue fluorescence, **C**). Images D to F represent merged presentations of two markers: **D:** Dual staining with STL and Thioflavine S. **E:** Dual staining with 4G8 and Thioflavin S. **F:** Triple staining with STL, Thioflavin S and 4G8. Scale bar: 50 μm.

Labeling of β-amyloid by 4G8 and Thioflavin S in one and the same brain section allows to differentiate between fibrillar, Thioflavine-S positive β-amyloid, and the total amount of β-amyloid immunostained by 4G8. The presence of amyloid fibrils within a plaque was independent of its size. While some small plaques clearly contained amyloid fibrils, numerous
large 4G8-positive plaques were found to be Thioflavin-S-negative (data not shown). The comparison of plaque staining pattern by STL and 4G8 revealed a strong correlation suggesting that STL labels both diffuse and fibrillar β-amyloid in brain sections that were not pretreated with formic acid (Fig. 3.15 A,C,F).

In brain sections that received a pretreatment with formic acid before starting immunohistochemistry, the intensity of 4G8 immunostaining of senile plaques was significantly increased, which allowed to visualize even small primitive diffuse plaques (Fig. 3.16). The formic acid pretreatment of brain sections appeared to apparently increase density, and mean size of β-amyloid deposits (Fig. 3.16) as compared to analysis of sections not pretreated by formic acid (Fig. 3.15).

3.3.1.3 Solanum tuberosum lectin staining to visualize glial cells
In order to reveal which kind of glia (micro-or astroglial cells) is stained by STL, brain sections were subjected to either immunostaining with anti-CD11b antibody, that labels
microglial cells, or glial fibrillary acidic protein (GFAP, that labels astroglial cells), and in each case counterstained with STL.

**Fig. 3.17 A-C:** Laser scanning photomicrographs of brain sections comprising the parietal cortex of 18-month-old transgenic mouse Tg2575, labeled with *Solanum tuberosum* lectin, STL (green fluorescence, A), and immunostained for microglial cells (anti-CD11b antibody, red fluorescence, B). Note the co-localization of STL signals in A, (labels both plaques and microglial cells surrounding the plaques), and CD11b immunoreactivity in B (labeled microglial cells), as presented in C (merge of A and B). Scale bar: 20 μm.

In brain sections comprising the parietal cortex of 18-month-old transgenic Tg2576 mice, both diffuse and fibrillary β-amyloid deposits induced prominent glial responses in close proximity to the sites of plaques (Fig. 3.16 C, E, F; Fig 3.17 A-C; and Fig. 3.18 B, C). Activated microglia as visualized by CD11b-immunohistochemistry (Fig. 3.17B), appeared in clusters that are localized upon or very close to β-amyloid plaques. Interestingly, not all clusters of CD11b-positive cells are associated with an amyloid core, indicating that both diffuse and compact plaques were surrounded by activated microglia. This finding was further validated by double immunofluorescent staining for both β-amyloid (STL) and CD11b (Fig. 3.18 A-C: Laser scanning photomicrographs of brain sections comprising the parietal cortex of 18-month-old transgenic mouse Tg2576, labeled with *Solanum tuberosum* lectin (STL, red fluorescence, A), and immunostained for astroglial cells (anti-GFAP antibody, blue fluorescence, B). Note the presence of reactive astrocytes (B) in close proximity of STL-positive β-amyloid deposits (A), which are not co-localised with lectin staining (C, merge of A and B). Scale bar: 50 μm.

**Fig. 3.18 A-C:** Laser scanning photomicrographs of brain sections comprising the parietal cortex of 18-month-old transgenic mouse Tg2576, labeled with *Solanum tuberosum* lectin (STL, red fluorescence, A), and immunostained for astroglial cells (anti-GFAP antibody, blue fluorescence, B). Note the presence of reactive astrocytes (B) in close proximity of STL-positive β-amyloid deposits (A), which are not co-localised with lectin staining (C, merge of A and B). Scale bar: 50 μm.
3.17 A, B), using confocal laser scanning microscopy, showing a virtually complete overlap of STL staining with immunoreactivity for microglial cells (Fig. 3.17C). The data indicate the usefulness of STL to detect microglial cells in brain sections of Tg2576 mice. Immunolabeling of brain sections for astroglial cells (anti-GFAP antibody; Fig. 3.18B) and counterstained with STL (Fig. 3.18A) demonstrated that presence of reactive astrocytes clustered around the plaques, but were not observed upon the cores. There are also clusters of activated astrocytes that were not associated with amyloid plaque cores, being presumably around diffuse plaques (Fig. 3.18B, C). STL fluorescence was not co-localized with immunoreactivity for astroglial cells suggesting that STL does not label astroglial cells (Fig. 3.18C).

3.3.2 Cholinergic perivascular innervation of cerebral cortical microvessels in transgenic Tg2576 and wild type mice

To reveal any differences in perivascular cholinergic innervation of cerebral cortical microvessels in transgenic Tg2576 and wild type mice during aging, a semiquantitative immunohistochemical study was performed, using the S1BF region as a representative brain region. Cortical perivascular cholinergic nerve fibres were demonstrated by immunohistochemistry, using a polyclonal anti-VAChT antibody. Determination of brain blood vessels was performed with Solanum tuberosum lectin (STL) staining. Neuronal marker NeuN was used as a neuronal marker for recognition of cortical layers in the triple staining of mouse brain slices.

As representatively shown in Fig. 3.19, a rich network of VAChT-immunoreactive cholinergic fibres has been observed in the S1BF from 8-month-old mice. In all cortical layers studied cholinergic neurovascular associations surrounding intraparenchymal blood vessels of varying sizes (capillaries, arterioles and larger vessels) were observed (Fig. 3.19).

High power Laser scanning microsopy demonstrated that some cholinergic fibres did not have close contacts with the blood vessels but ended within a distance of 3 µm of the vessels, while other terminals were found attached on or in the immediate vicinity of the vessels wall (Fig. 3.20).
Fig. 3.19: Representative example of a double fluorescent Laser scanning micrograph demonstrating the cholinergic perivascular innervation (red fluorescence) of capillaries and large penetrating arteries (green fluorescence) in the S1BF area of a 8-month-old transgenic Tg2576 mouse. VAChT-positive terminals (red) are visible directly opposed to the vascular basement membrane, or located within the immediate vicinity of cortical vessels (green). Scale bar represents 50 μm.
RESULTS

Fig. 3.20: Representative series of 2D high power Laser scanning photomicrographs along the z-axis (A1-3, B1-3) of double immunofluorescent labeling of brain sections from 8-month-old Tg2576 mouse comprising the S1BF region, illustrating by virtual three-dimensional-imaging in more detail the distribution of perivascular cholinergic terminals (red fluorescence) around blood vessels (green fluorescence). VACHT-positive nerve fibres (red) are closely associated and partly attached on the wall of the large vessel (green; A, see white arrows). The cholinergic nerve fibres appear to overlay the vessel wall, or being apposed directly to the wall (B, indicated by white arrows) and located within the immediate vicinity of small microvessels. Scale bar: 10 μm.

To reveal any transgene-mediated changes in the cholinergic innervation of cerebral cortical blood vessels, the number of cholinergic terminal endings / vessel contacts within a 3-μm-distance range from the vessel was estimated and correlated with the total length of blood vessels present in a particular cortical layer of the S1BF region. The ratio of perivascular cholinergic terminal endings / vessel contacts and total length of blood vessels indicates the perivascular density of cholinergic innervation of blood vessels in a particular region/layer.

At ages of 6 and 8 months the cholinergic innervation of blood vessels in the S1BF region did not significantly differ between transgenic and wild type mice regardless of the cortical layer examined (Fig. 3.21).

However, during further postnatal development of transgenic Tg2576 mice up to the age of 18 months a significant loss of density of cholinergic innervation of blood vessels was observed in all S1BF cortical layers except layer VI as compared to the corresponding innervation level at the age of 6 months (Fig. 3.21). In layer I of the S1BF from 18-month-old Tg2576 mice the reduction in vascular cholinergic innervation amounted to about 30%, in layer II-III by 30%,
RESULTS

Fig. 3.21: Developmental pattern of perivascular cholinergic innervation estimated in cortical layers I, II-III, IV, V and VI of the S1BF region of transgenic Tg2576 mice (black column) and non-transgenic littermates (grey column) at ages ranging between 6 and 18 months. The data are expressed as ratio of cholinergic terminal endings/vessel contacts (located within a 3-µm-distance range from the vessels) and the total length of microvessels assessed in a particular cortical layer of S1BF region and represent the mean ± S.D. (N=3-5).
* P ≤0.05  **P ≤ 0.01, ***P ≤ 0.001, ANOVA followed by two-tailed Student's t-test.
in layer IV by 26%, and in layer V by 22% as compared to the corresponding levels estimated in 6-month-old Tg2576 animals (Fig. 3.21). In contrast, in wild type mice, decreases in perivascular cholinergic innervation during aging were only observed in layer I (by 27%), and layer V (by 16%) of the S1BF from 18-month-old mice as compared to the corresponding values calculated in 6-month-old wild type mice (Figs. 3.21).

However, in 18-month-old Tg2576 mice a significant reduction in perivascular cholinergic innervation was only observed in cortical layers II-III by about 16% and IV by about 24% compared to that estimated in layers II-IV of S1BF from 18-month-old wild type mice (Fig. 3.21).

In order to disclose whether the changes in perivascular cholinergic innervation observed in the S1BF region from 18-month-old Tg2576 mice are due to the presence of β-amyloid plaques, the perivascular cholinergic innervation densities were estimated both in areas within the S1BF with no plaque depositions, and in neighboured areas that demonstrated a significant plaque load. As graphed in (Fig.3.22), in the S1BF region from 18-month-old transgenic Tg2576 mice there was no significant difference in the perivascular cholinergic innervation in areas that demonstrated significant plaque load and those with no plaque deposits, regardless of the cortical layer examined (Fig.3.22).

**Fig. 3.22:** Comparison of the perivascular density of cholinergic innervation estimated in areas of cortical layers II-III, IV, V and VI of S1BF region from 18-month-old transgenic Tg2576 mice that demonstrated a significant plaque load (black columns) and in areas with no β-amyloid plaque deposits (grey columns). The data are expressed as ratio of cholinergic terminal endings / vessel contacts (located within a 3-µm-distance range from the vessels) and the total length of microvessels assessed in a particular area of S1BF region and represent the mean ± SD (N=3-5).


CHAPTER 4: DISCUSSION

4.1 β-amyloid and brain vascular system: the vascular hypothesis of Alzheimer’s disease

4.1.1 Evidences of a role of vascular mechanisms in Alzheimer’s disease

In the brain, the cerebral blood flow is tightly regulated to assure adequate and timely blood supply to brain regions that have momentarily high energy demand because of enhanced neural activity, a phenomenon called functional hyperemia (Iadecola et al., 2009). Already at very early stages of AD, changes in the cerebral blood flow, such as reduced blood supply at rest and altered perfusion to activated areas have been observed, which provided evidence for suggesting a causal relationship between vascular mechanisms and the development of sporadic AD. This vascular hypothesis of AD was firstly formulated by de la Torre and Mussivand in 1993 (for reviews, see de la Torre, 2010; Kalaria, 2010; Nicolakakis and Hamel, 2011). Insufficient cerebral blood flow may induce hypoxia-sensitive pathways leading to inflammation with upregulation of pro-inflammatory cytokines, and to oxidative stress with generation of reactive oxygen species which may be detrimental to vascular integrity and function. Indeed, cerebrovascular abnormalities such as thickening of the microvascular basement membranes, decreased luminal diameter, and microvascular degeneration, in particular in the temporal-parietal cortex, have frequently been observed in AD patients (Nicolakakis and Hamel, 2011).

In advanced AD cases β-amyloid (Aβ) deposition occurs also in cerebral vessels (cerebral amyloid angiopathy, CAA) that may result in smooth muscle cell degeneration and weakening of the vascular wall, impairing vasomotor function, and increasing the risk of cerebral hemorrhage (Weller et al., 2009). Indeed, the recently detected brain microbleeds (small dot-like lesions) in AD brains have been suggested to be the missing link between the amyloid cascade hypothesis and the vascular hypothesis (Cordonnier and van der Flier, 2011). The CAA which is observed during normal aging, and in the majority of AD cases, is likely caused by the failure of Aβ elimination from the brain parenchyme, whereas receptor for advanced glycation end products (RAGE), and low density lipoprotein receptor related protein 1 (LRP1) have been suggested to play a role in controlling the Aβ transport through the blood brain barrier (for reviews, see Zlokovic, 2008b, 2011). Pathology of the blood brain barrier (BBB) is an early finding in white matter lesions associated with AD and altered function of BBB transport may also have an impact on the accumulation of Aβ in the brain.
(Kalaria, 1992; Zlokovic, 2008; Farrall and Wardlaw, 2009). Moreover, serum inflammatory proteins may increase BBB permeability, what exacerbate the loss of BBB integrity in an escalating cycle of Aβ protein accumulation and BBB damage (Farrall and Wardlaw, 2009). Recently, Zlokovic (2011) presented a two-hit vascular hypothesis of Alzheimer’s disease. Hit one represents the primary damage of brain microcirculation resulting in neuronal dysfunction and injury mediated by BBB dysfunction associated with leakage and secretion of many neurotoxic molecules and impaired brain capillary flow, while dysfunctional BBB also leads to impaired β-amyloid clearance, and oligaemia to increased β-amyloid generation and accumulation in the brain (hit two).

A recent study in transgenic AD-like mice (Tg2576) further supported the “clearance hypothesis” demonstrating impaired perivascular solute drainage from the brain in aged mice as compared to younger ones (Hawkes et al., 2011). Currently, in a transgenic AD mouse model with strong CAA pathology, a particular role of early perivascular astrocytic dysfunction in impairing cerebrovascular and metabolic pathology has been suggested (Merlini et al., 2011).

Studies in transgenic mouse models of AD suggested that the compromised cerebral hemodynamics observed in AD appears to be associated with inflammation (Paris et al., 2003). The local activation of microglia and reactive astrocytes is accompanied by production and secretion of proinflammatory cytokines such as interleukin-1β (IL-1β), tumour necrosis factor (TNF)-α, and transforming growth factor (TGF)-β1. Endothelial cells are known to respond sensitive to inflammatory stimuli by production of reactive oxygen species that may further exacerbate the vascular damages (Iadecola, 2004).

A number of risk factors are assumed to mediate the cerebrovascular dysfunctions and to trigger AD pathology, such as hypertension, hyperlipidemia, enhanced homocysteine levels, diabetes type 2, metabolic syndrome (obesity, hypertension, cardiovascular disease, atherosclerosis) as well as genetic factors (ε4 allele of ApoE), age, and life style (Rocchi et al., 2009; de la Torre, 2010; Milionis et al., 2008). However, it is still a matter of debate whether neurovascular dysfunction and vascular lesions play a causative role for the neurodegenerative processes as suggested by a number of reports (for review, see Zlokovich, 2008b). However, regardless of that, cerebrovascular diseases appear to play an important role in determining the presence and severity of the clinical symptoms of AD (Snowdon et al., 1997).
4.1.2 Effect of β-amyloid on brain vascular system

The microvascular degenerations observed in AD may also be the consequence of the vasoactive detrimental effects of Aβ. A number of studies provided evidence that the cerebrovascular degenerations are related to Aβ deposition in AD (Attems et al., 2004; Buee et al., 1994, 1997; Fischer et al., 1990; Kalaria et al., 1998; Kalaria, 2002; Mann et al., 1986; Suter et al., 2002). Amyloid deposition occurring in cerebral vessels, results in smooth muscle cell degeneration and leads to weakening of the vascular wall and increasing the chance of lobar hemorrhages (Itoh et al., 1993; Weller et al., 2009).

Observations in transgenic AD-like mice also revealed Aβ-mediated impairments of endothelium-dependent regulation of cortical microcirculation, abnormal vascular autoregulation, reduced cerebral blood flow and attenuated cerebrovascular reactivity to functional hyperemia already before onset of any plaque load, further supporting the link of Aβ to the mechanisms of vascular dysfunction (for comprehensive reviews, see Iadecola et al., 2009; Nicolakakis and Hamel, 2011). However, the view that elevated soluble Aβ levels are sufficient to cause cerebrovascular dysfunction has also been challenged by studies in Tg2576 transgenic mouse model (Shin et al., 2007).

Aβ may cause degeneration of both the larger perforating arterial vessels as well as cerebral capillaries, presumably mediated through the induction of reactive oxygen species by activation of NADPH oxidase, which may subsequently severely affect regulation of cerebral blood vessels and brain perfusion as well as impair the blood brain barrier (for reviews, see Cole and Vassar, 2009; Iadecola et al., 2009; Smith and Greenberg, 2009; Weller et al., 2009). The enzyme NADPH oxidase has emerged as a major source of vascular oxidative stress and experimental studies demonstrated that NADPH oxidase-derived free radicals may play a role in the cerebrovascular abnormalities associated with Aβ (Iadecola, 2004; Park et al., 2008; Iadecola et al., 2009). Free radicals can trigger inflammation by activating redox sensitive transcription factors, like NFkB and AP1 (Iadecola, 2010). Activation of NMDA receptors by excess glutamate is also known to promote a breakdown of the blood brain barrier and resulting in dysfunction of the affected endothelial barrier integrity (Sharp et al., 2003; Del Zoppo and Mabuchi, 2003; see for review Humpel, 2011). Endothelial dysfunction caused by oxidative stress can initiate release of vascular endothelial growth factor (VEGF) and prostanoids, with promotion of vascular leakage, protein extravasation, and inflammation (Marchesi et al., 2008). Further, extravasated plasma protein triggers perivascular edema and axonal demyelination (Farrall and Wardlaw, 2009). Demyelination, in turn, increases the
oxygen use of axons and enhances the local energy deficit and hypoxia (Trapp and Stys, 2009; Fotuhi et al., 2009; Ihara et al., 2010; Iadecola 2010).

Aβ is also a potent vasoconstrictor in the brain, as has been shown in vivo and in vitro by application of exogenous Aβ to normal blood vessels and to mouse cortex. Moreover, Aβ peptides have been described to inhibit angiogenesis both in vitro and in vivo (Paris et al., 2004a; Paris et al., 2004b), and deregulation of angiogenic factors may contribute to various neurological disorders including neurodegeneration (for review, see Ruiz de Almodovar et al., 2009). One of the key angiogenic factor, the vascular endothelial growth factor (VEGF), a highly conserved heparin-binding protein (Sun and Guo, 2005), was originally found in vascular endothelial cells and is able to induce vascular endothelial cell proliferation, migration and vasopermeability in many types of tissue (Ferrara et al., 2003).

Increased intrathecal levels of VEGF have also been observed in brains of AD patients as compared to age-matched healthy individuals (Kalariya et al., 1998; Tarkowski et al., 2002; Yang et al., 2004) that has been correlated with the clinical severity of the disease (Ryu et al., 2009). However, the functional significance of VEGF up-regulation in the pathogenesis and progression of AD is still a matter of debate. While VEGF and other angiogenic factors were found to be enhanced in AD (Pogue and Lukiw, 2004; Thirumangalakudi et al., 2006; Vagnucci and Li, 2003; Desai et al., 2009), neovascularization has been observed only in the hippocampus of AD patients (Desai et al., 2009).

The brain VEGF is sequestered by amyloid plaques consequently reducing its bioactivity (Yang et al., 2004). Vice versa, Aβ may inhibit vasculogenesis through impairment of VEGF signalling (Patel et al., 2010), and the vascular regeneration is suppressed through induction of endothelial autophagy (Hayashi et al., 2009). Therefore, in AD a loss of neurovascular trophic support by inducing a state of "neurotrophin resistance" via impairment of growth factor signaling through pro-inflammatory cytokines has been suggested (Iadecola 2010; Tong et al., 2008; Venters et al., 2000). Moreover, the transcriptional profiling of the homeobox gene MEOX2, a regulator of vascular differentiation, considerably altered in AD and may mediate reductions in brain capillary density and resting cerebral blood flow, loss of the angiogenic response to hypoxia as revealed by studies with knock-out mice (Wu et al., 2005).
DISCUSSION

4.1.3 Effect of ischemia and hypoperfusion on APP processing

There are reports that ischemia and hypoperfusion may trigger accumulation and cleavage of APP into Aβ, and its deposition in the brain, as well as hyperphosphorylation of tau and PHF formation (for reviews, see Zlokovic, 2008, 2011). The upregulation of VEGF in response to hypoxic, ischemic or hypoglycemic stress (Marti and Risau, 1998; Marti et al., 2000; Stein et al., 1995; Yancopolous et al. 2000) suggests its involvement also in processing of APP. In turn, APP is also highly expressed in the endothelium of neoforming vessels (Paris et al., 2005), and inhibitors of β- and γ-secretases have been reported to inhibit angiogenesis and tumour growth (Paris et al., 2005), suggesting a role of APP metabolism also during angiogenesis. Recently, VEGF has been shown to also be involved in the induction of microglial-mediated inflammation by Aβ deposits via the microglial VEGF receptor subtype Flt-1 serving as a chemotactic receptor to mobilize microglial cells (Ryu et al., 2009).

As vascular endothelial cells are also capable to express and to secrete APP (Ciallella et al., 1999), it has been hypothesized that VEGF may also be involved in formation and deposition of Aβ. This hypothesis has been addressed by examining the effect of VEGF on APP processing in brain slice cultures, as well as in primary neuronal, astroglial and vascular endothelial cells derived from AD-like transgenic Tg2576 mice (Bürger et al., 2009, 2010). Exposure of brain slices by VEGF resulted in an inhibition of the formation of soluble Aβ peptides, which was accompanied by a transient decrease in β-secretase activity, as compared to controls (Bürger et al., 2009). Similar studies in primary neurons, astrocytes, and endothelial cells expressing the Swedish mutation of human APP, further provided evidence that VEGF affects APP processing but differentially acting in cells that form the neuron-glia-vascular unit (Bürger et al., 2010, Schliebs et al., 2011).

4.1.4 Effect of β-amyloid on cholinergic function in brain vascular system

There is a large body of evidence that cerebral blood flow and local glucose delivery is controlled by neuronal activity, known as neurometabolic and neurovascular coupling (Iadecola, 2004; Girouard and Iadecola, 2006; Hamel, 2006; Nicolakakis and Hamel, 2011). Dysfunctions of the regulation of the cerebral blood circulation may affect vital control mechanisms that ensure delivery of adequate amounts of substrate and to maintain the homeostasis of the microenvironment of the neurovascular unit. The neurovascular unit defines the cellular interaction between brain capillary endothelial cells, pericytes, the end feet of perivascular astrocytes, and neuronal axons (Iadecola, 2004; Zlokovic, 2011). Pial arteries at the surface of the brain are densely innervated by perivascular nerves that originate
from autonomic and sensory ganglia, whereas intracerebral arterioles and capillaries receive afferents that originate from subcortical neuronal centers as well as from local cortical interneurons. (for comprehensive review, see Hamel, 2006; Van Beek and Claassen, 2011).

Basing on findings by immunocytochemistry and electron microscopy, the cholinergic axons originating from the basal forebrain project not only to the cortical neuropile but also to arterioles, capillaries and to perivascular astrocytes within the cerebral cortex (Vaucher and Hamel, 1995). Furthermore, there is physiological evidence that the central cholinergic pathways are involved in the regulation of cerebral cortical blood flow. Electrical or chemical stimulation of cholinergic basal forebrain neurons resulted in increased cerebral blood flow (Biesold et al., 1989; Lacombe et al., 1989; Kurosawa M. et al., 1989; Hamel, 2004). The involvement of ACh as a neurotransmitter in the control of regional cerebral blood flow was further demonstrated by administration of cholinergic drugs (Elhusseiny and Hamel, 2000; Farkas and Luiten, 2001). While application mAChR antagonist decreased cerebral blood flow, the inhibition of AChE led to increased cerebral blood flow (Farkas and Luiten, 2001). This response was found to be dependent on nitric oxide (NO) production, and presumably mediated through the M5-mAChR subtype (Elhusseiny and Hamel, 2000). The basal forebrain cholinergic fibers can either directly affect the cerebrocortical microvasculature, or innervate subpopulations of GABAergic interneurons releasing the vasodilators NO and VIP (Vaucher et al., 1997; Cauli et al., 2004). The interneurons appear to serve as a functional relay to adapt perfusion to locally increased neuronal activity (Hamel, 2006).

On the other hand, damages of the neurovascular unit either by oxidative stress, inflammation, or Aβ accumulation may induce degeneration of vascular cholinergic nerve terminals and subsequent retrograde cell death of basal forebrain cholinergic neurons. The loss of cholinergic innervation of components of the neurovascular unit may affect APP processing with enhanced Aβ formation and deposition, microglia activation and inflammation, thus suggesting a link between Aβ production, impairments in cerebrovascular function and basal forebrain cholinergic deficits in AD (cholinergic-vascular hypothesis of AD; Humpel and Marksteiner, 2005; Claasen and Jansen, 2006). Indeed, a semiquantitative immunohistochemical study in aged Tg2576 mice revealed an Aβ-mediated decrease in cholinergic innervation of cortical blood vessels (Kouznetsova and Schliebs, 2006), which has been assumed to contribute to the alterations of the cerebrovascular system observed in transgenic Tg2576 mice (Bürger et al., 2009).
4.2 Aim of study and main results obtained

The present study was undertaken to elucidate whether the age-related Aβ production and deposition may affect the morphological integrity of cerebral microvessels, cholinergic terminals and their intracerebral neurovascular architectonic, and whether size or type of plaques play a role in this process. We wanted to contribute to the question whether changes in cerebral vessels, cortical cholinergic denervation, Aβ formation and deposition may interact with each other.

The availability of transgenic mice that produce human Aβ peptides from birth and progressively develop Alzheimer-like-amyloid deposits in the aged brain appears to be a unique experimental approach to address such questions. To in vivo characterize the developmental relationship of amyloid formation and deposition, cortical cholinergic innervation and cerebrovascular abnormalities, transgenic Tg2576 mice that overexpress the Swedish double mutation of human APP, and demonstrate significant cerebral cortical deposition of Aβ plaques at ages from 9 months onwards (Apelt et al., 2004), were considered as an appropriate in vivo animal model. In this developmental study transgenic Tg2576 mice at ages between 4 and 18 months were investigated in order to reveal any effect of both soluble Aβ and Aβ plaque deposits on maturation and morphological integrity of cerebral cortical capillaries, cholinergic terminals and their cerebral neurovascular relationship with each other.

Using the somatosensory cortex as a representative region, serial cryocut sections, obtained from mice at ages ranging from 4 up to 18 months, were subjected to immunohistochemistry to label vascular endothelial cells, cholinergic nerve terminals and Aβ plaques, followed by a thorough quantitative evaluation of the age-related spatial relationship between cerebral cortical capillaries, amyloid plaques and cholinergic terminals, using computer-assisted imaging analysis.

The main results obtained are as follows:

1. Density of blood vessels estimated in the somatosensory cortex of transgenic mice did not differ to that obtained in wild type mice before onset of plaque deposition (younger than 12 months). However, in aged, 18-month-old Tg2576 mice, demonstrating high plaque loads, decreased blood vessel densities, particularly in layer IV of the somatosensory cortex, were observed.
2. The density of cholinergic terminals estimated in the somatosensory cortex of wild type mice did not change with aging regardless of the cortical layer examined, while in cortical layers II/III and IV of somatosensory cortex of transgenic Tg2576 mice age-related decreases in cholinergic fibre densities were assessed. Quantitative morphometric analysis demonstrated an age-related reduction in the number of varicosities on cholinergic fibres, particularly in layer IV, in both transgenic Tg2576 mice and non-transgenic littermates.

3. Cholinergic innervation of microvessels in the somatosensory cortex decreased with aging in both Tg2576 mice (in cortical layers I to V) and non-transgenic littermates (only in layers I and V), as revealed by estimating the ratio of the number of cholinergic vascular contacts and total length of blood vessel. There was no significant difference in the perivascular cholinergic innervation in areas that demonstrated significant plaque load and those with no plaque deposits regardless of the cortical layer examined.

4.3 Age-related changes in cerebral cortical microvessels in the presence and absence of β-amyloid plaque load

The aim of this part of the study was to elucidate whether the age-related Aβ production and deposition may affect the morphological integrity of cerebral microvessels located in the vicinity of amyloid plaques, and whether size or type of plaques play a role in this process. The developmental profile of blood vessels in the somatosensory cortex of transgenic Tg2576 mouse brain observed between ages of 4 and 10 months did not differ to that of normal mice, indicating that soluble Aβ production does not have any impact on cerebral blood flow. However, following deposition of cortical Aβ plaques from age of 12 months onwards, the density of brain capillaries in cerebral cortical regions decreased with further aging. Indeed, the laser scanning microscopic inspection of brain sections of aged Tg2576 mouse brain demonstrated that the distribution of brain vessels in the vicinity of Aβ deposits differed between senile and diffuse plaques. The lower density of capillaries in areas very close to senile plaques as compared to that located more distal, indicates an impaired blood supply of cortical tissue in close vicinity to senile plaques. Moreover, the density of microvessels estimated in cortical regions with high plaque load was found to be significant lower as compared to areas with low plaque burden, which is in agreement with a previous study (Paris et al., 2004a). This indicates that the deposition of Aβ plaques nearby to cerebral blood
vessels may produce mechanical, displacing actions on the cerebrovasculature. However, accumulation of Aβ around vessels may also exert toxic actions on the integrity of cerebral capillaries by inducing pro-inflammatory events (Apelt and Schliebs, 2001; Patel et al., 2005), including perivascular astrocytosis that may lead to cerebrovascular abnormalities. Interestingly, in double transgenic PS1/APPswe Alzheimer mice a decreased cerebral blood flow compared to PS1 littermates was observed which could be improved by chronic treatment with cyclooxygenase-2 inhibitors, thus suggesting a link between inflammation and compromised cerebral hemodynamics in AD (Paris et al., 2003). Indeed, Aβ has been found to enhance endothelin-induced vasoconstriction in isolated human cerebral arteries which appeared to be mediated through the involvement of a pro-inflammatory pathway (Paris et al., 2000, 2003).

On the other hand, neuropathological studies demonstrated that Aβ itself is associated with structural changes in the vessels including loss of smooth muscle cells, fibrinoid necrosis, and weakening of the vessel wall (Greenberg et al., 1995). Similarly, mice overexpressing human mutant APP have been observed to develop cerebrovascular amyloid angiopathy, morphologically similar to that detectable in the human AD brain (Calhoun et al., 1999; Christie et al., 2001; Van Dorpe et al., 2000), and impairments in vessel function of vasodilatation by disrupting smooth muscle cell organization (Christie et al., 2001). Moreover, in culture Aβ has been shown to be toxic to endothelial cells (Thomas et al., 1996).

Recently it was described that APP is also highly expressed in the endothelium of neoforming vessels which led to the suggestion of a role of APP processing during angiogenesis (Paris et al., 2005). Both in vitro and in vivo studies in Tg2576 mice demonstrated anti-angiogenic actions of Aβ peptides indicating that the amyloidogenic route of APP processing mediated through actions of β- and γ-secretases may play an essential role in affecting angiogenesis (Paris et al., 2004b, 2005).

Otherwise, there are also reports that ischemia and hypoperfusion may trigger accumulation and cleavage of the APP into Aβ, and its deposition in the brain (Bennett SA. et al., 2000; Jendroska et al., 1995). The upregulation of the vascular endothelial growth factor (VEGF) in response to hypoxic, ischemic or hypoglycemic stress (Marti et al., 1998, 2000; Stein et al., 1995; Yancopolous et al. 2000) suggests its involvement also in APP processing.
In a recent study, this hypothesis has been addressed by examining the effect of VEGF on APP processing in brain slice cultures and primary neuronal cells derived from brain tissue of Tg2576 mice (Bürger et al., 2009, 2010). Already six hours of exposure of brain slice cultures by VEGF resulted in an inhibition of the formation of soluble Aβ peptides, which was accompanied by a transient decrease in β-secretase activity, providing evidence of a role of VEGF in β-amyloidogenesis (Bürger et al., 2009).

In conclusion, the data presented in this part of the study add further evidence that Aβ, in addition to its well-described neurotoxic effects, may also contribute to neuronal dysfunction through its actions on the cerebrovasculature.

4.4 Age-related changes of cholinergic terminals in cholinceptive target regions in the presence and absence of β-amyloid plaque load

Based on investigations demonstrating that already soluble Aβ, before any amyloid plaque formation, may induce changes in a number of cholinergic function (Klingner et al., 2003), the study was designed as semiquantitative immunohistochemistry to determine distribution and density of VACHT as specific cholinergic marker in brain sections from both young adult 4-month-old transgenic Tg2576 mice that display enhanced levels of cortical human Aβ peptides but not yet any plaque deposition, and from aged 18-month-old transgenic animals with high load of both soluble and plaque-forming fibrillary Aβ peptides.

4.4.1 VACHT – a reliable marker for detection of cholinergic terminals in cerebral cortex

The VACHT is a 12-transmembrane domain protein with N- and C-terminal regions directed to the cytosol. VACHT is predominantly present in synaptic vesicles in cholinergic nerve endings (Gilmor et al., 1996; Weihe et al., 1996; Mundigl et al., 1993; Nakata et al., 1998). Endogenous VACHT-immunoreactivity observed in varicosities is co-localized with synaptic vesicle marker such as synaptophysin, synaptotagmin I and FM4-64 (Barbosa et al., 1999; Santos et al., 2000; for review, see Prado, 2002).

Therefore, VACHT has been used as a specific marker for immunohistochemical visualisation of cholinergic neuronal fibres and nerve terminals (Weihe et al. 1996). The rabbit polyclonal anti-VACHT serum (Phoenix Pharmaceuticals, Inc., Belmont, USA) used in this study, was raised against a synthetic peptide representing the C-terminus from cloned rat VACHT, which was used to identify the presence of VACHT in the mouse cerebral cortex.
In preliminary studies, the immunohistochemical detection of VAChT in cholinergic nerve terminals in mouse brain was compared to that for ChAT, the mostly used marker for detection of cholinergic cell bodies and partly terminals. In the present study double immunofluorescence using polyclonal rabbit anti-VAChT serum and the polyclonal goat anti-ChAT antibody demonstrated a complete overlap of immunoreactive cholinergic fibres in the parietal cortex (see 3.2.1). Microscopic inspection of VAChT immunohistochemistry revealed the presence of lattice-like fibre network with fine varicose pervading all VAChT-immunopositive fibres in cortical tissue. However, VAChT staining was much more intense than that for ChAT, allowing a higher quality of staining and more reliable images to be used for semiquantitative evaluation. Moreover, in situ hybridization histochemistry demonstrated similar distributions of VAChT mRNA and ChAT mRNA as compared to that of the corresponding proteins (Ichikawa et al., 1997), and VAChT binding studies also correlated with ChAT activities (Kish et al., 1990; Efange et al., 1997), thus providing strong evidence that VAChT immunohistochemistry represents a useful and reliable marker to detect cholinergic terminals in mouse cerebral cortex.

4.4.2 The barrel field of the somatosensory cortex 1 (S1BF) as a model region to reveal age-related changes in cholinergic innervation

The mouse S1BF was chosen as a representative model region to investigate age-related and cortical layer-specific changes in the density of cholinergic terminals both in the presence and absence of amyloid plaque load.

The mean density of VAChT-immunoreactivity determined in S1BF of wild type and of transgenic Tg2576 mice but assessed in areas distal from any plaque (> 100 µM), was comparable regardless of the cortical layer studied. The immunohistochemistry demonstrated a normal anatomical distribution and morphology of cholinergic intracortical network in S1BF, while the fibre density was highest in cortical layers I-III, followed by layers V-VI, and lowest in layer IV. This distribution was comparable to previous findings in rat brain using AChE and ChAT-histochemistry as cholinergic marker proteins (Wainer and Mesulam, 1990).

The young 4-month-old transgenic mice displayed no transgene-related changes despite of higher soluble Aβ production, and showed a similar cholinergic laminar distribution of VAChT density in S1BF, as compared to non-transgenic littermates. Developmentally, in wild type mice no significant differences in the global density of VAChT-immunoreactivity between young-adult and aged animals were found. However, in the cortex of 18-month-old
transgenic Tg2576 mice that demonstrated considerable Aβ burden and high levels of soluble Aβ peptides, the quantitative analysis of total VACHT-immunoreactivity revealed significant age-related regionally degradation and loss of cholinergic innervation in layers II/III and IV, as compared to corresponding values obtained in S1BF of younger transgenic mice. Our results compare well with a previous study in aged mice carrying familial AD-linked mutations, also demonstrating selective cholinergic denervation and decrease in number of ChAT-positive varicosities detected in hippocampus at 12/14 month-old and in the parietal cortex across different layers, at 18 months of age (Aucoin et al., 2005).

4.4.3 VACHT expression: morphological and morphometric studies

To reveal whether the transgene-mediated changes in VACHT-immunoreactivity are accompanied by morphological changes, morphometric studies of cholinergic afferents in two select cortical layers (II-III and IV) in S1BF were performed by determining fibre length and counting the number of boutons present in a particular fibre. For morphological characterization of cholinergic fibres high-power resolution of digital photomicrographs obtained from LSM microscope were used, which facilitated determination of varicosities present at cholinergic fibres.

The detailed fine structural analysis of images showed that cholinergic fibres in tissue of aged transgenic and wild type mice have many swollen varicosities with larger in size compared to young animals, which was also observed in other studies on aging and in AD brains (see e.g., Nyakas et al., 2011). Moreover, cholinergic dystrophic neurites surrounding the plaque observed in S1BF of aged transgenic mice were endowed with numerous boutons being larger in size as normal, which is in agreement with a number of other studies (Onorato et al., 1989; Hu et al., 2003; Shi et al., 2009). Thus it has been proposed that abnormal accumulations of proteins and organelles within the swollen structures of cholinergic terminals point to the importance of axonal transport in AD (De Vos et al., 2008). It was also observed that the majority of cholinergic axon terminals in transgenic S1BF appeared to display a lower density of varicosities, which were distributed not homogeneously throughout the axonal profile, as compared to young animals. In general, the cholinergic terminals in S1BF of aged transgenic mice looked different to that observed in wild type tissue, presumably due to the action of Aβ.

There is much debate and and support of the idea that major aspects of AD neuropathology are the result of failures in axonal transport. It has been demonstrated that the fast axonal
transport is inhibited by Aβ peptide oligomers, which further results in bidirectional axonal transport inhibition (Pigino et al., 2009). In particular, the axonal transport of vesicular ACh transporter (VACHT) was found to be inhibited by Aβ (Nyakas et al., 2011). Moreover, Aβ (1-42) promotes cholinergic dysfunction by triggering aberrant neuronal fibres sprouting, what may lead to reorganisation of morphological integrity of neuronal composition and might cause formation of fiber swelling forming grape-like structures (Masliah et al., 2003; Gaykema et al., 1992; see e.g., Nyakas et al., 2011). On the other hand, the aberrant neuritic sprouting may also be interpreted as an adaptive restorative response which subsequently turns into neuritic degeneration (Nyakas et al., 2011). Additionally, the chronic deterioration of intra-axonal transport capacity of cholinergic neurons and their cortical projections could promote the retrograde degeneration of the basal forebrain cholinergic fibres in case of AD (Nyakas et al., 2011). On the other hand, the reduction of microtubule-dependent axonal transport may stimulate pathological cleavage of APP resulting in Aβ deposition and plaque formation (Slotkin et al., 2005; see e.g., Nyakas et al., 2011).

Our finding of a relative increase in the density of varicosities in aged transgenic Tg2576 mouse brain compared to non-transgenic littermates suggests a role of Aβ in mediating neurodegenerative effects on cholinergic cerebral cortical fibre network, which is in agreement with many other studies (Wong et al., 1999; Hu et al., 2003; Klingner et al., 2003). Thus, in a number of other transgenic AD-like mouse models at early stages of amyloid pathology a significant plasticity of cortical cholinergic pre-synaptic VACHT-positive boutons by means of reorganization of the cholinergic network has also been observed. However, when comparing different transgenic AD-like mouse models a rather complex interaction between number and size of cholinergic synapses and AD-related mutated transgenes and amyloid burden has been found (Wong et al., 1999). E.g., transgenic mice expressing mutated human presenilin-1 (M146L) alone (with normal APP and no human amyloid generation) did not demonstrate any changes in the density of cholinergic synapses (Wong et al., 1999).

It is interesting to note, that the significant elevation of density of VACHT-immunoreactive cholinergic synapses in Tg2576 mice occurred prior to plaque formation (Wong et al., 1999) and in aged mice with high plaque load (Klinger et al., 2003). The double mutant transgenic mice (hAPP/PS1), which have extensive amyloidosis, demonstrated a prominent diminution in the density of VACHT-immunoreactive synapses and reduction in the size these cholinergic synapses (Wong et al., 1999), presumably due to the synergistic effect of AD-related transgenes. Similarly, a negative correlation of plaque size and VACHT-positive cholinergic
density was observed in close proximity of plaques in doubly APP/PS1 transgenic mouse line (Hu et al., 2003).

The quantitative morphometric analysis further demonstrated an age-related loss of varicosities on VACHT-immunoreactive fibres in layers II-IV of S1BF regardless of the transgeneity of the mice studied, with more severe reduction of VACHT-positive boutons in cortical layer IV. In previous neuropathological studies layers II and III are described to be more severe involved with AD pathological features than the deeper layers (Pearson et al., 1985; Lippa et al., 1992; Gomez-Isla et al., 1996; see e.g., Romito-DiGiacomo et al., 2007). The neurons of the superficial layers are mainly involved in cortico-cortical connections whereas those in deeper layers project preferentially to non-cortical regions. In vitro studies have shown that the neurons in deeper cortical layers are more resistant to the toxic influence of Aβ(1–42), as compared to the neurons of the more superficial layers (Romito-DiGiacomo et al., 2007), hypothesizing that the differential regional vulnerability in AD depends on the extent of connectivity rather than on single neurons.

Interestingly to note, that AChE is postulated to play also a non-enzymatic role in the development of neuritic projections (Slotkin et al., 2009). During brain development neurotransmitters serve important trophic functions that control neuronal cell replication and differentiation, synaptic organization, and morphologic assembly of brain nuclei (see e.g., Slotkin et al., 2009). There is ample evidence that ACh plays a role in structural and functional remodeling of cortical circuits (see review, Schliebs and Arendt, 2006). Also synaptic physiology and behavioral effects depend at least on molecular and/or structural changes in neuronal connections, as demonstrated previously for the somatosensory and visual cortex (see for review Hohmann, 2003). The cortico-cortical connections within rodent primary somatosensory cortex have been implicated as a major component involved in the rapid reorganization of cortical barrel maps. However, other investigators have suggested a role for subcortical structures in reorganization (see e.g., Arnold et al., 2001). Thus, layer IV receives a strong afferent input from ventroposterior thalamic projection neurons and any divergence in the thalamocortical projection to multiple cortical barrels could also provide an anatomical substrate for cortical plasticity and must be considered in any mechanism of rapid cortical reorganization (Arnold et al., 2001). During ontogenesis there is a great refinement of thalamo-cortical input to layer IV that underlies the maturation of its topographical map. Neonatal synaptic plasticity for NMDA- or kainite receptors and a rapid maturation in
GABAergic inhibition exhibit functional properties necessary for mature sensory processing (Daw et al., 2007).

In conclusion, the data presented in this study provide further evidence, that age-related Aβ deposition alters the network of cholinergic connectivity, at least in the S1BF of transgenic Tg2576 mice. The alteration in neuronal synaptic plasticity in the aged transgenic mice under Aβ peptide overproduction may most likely be considered as compensatory mechanism in support of cholinergic neurotransmission.

4.5 Age-related changes in cholinergic innervation of cerebral cortical microvessels in the presence and absence of β-amyloid plaque load

In the present study, the mouse somatosensory cortex was representatively used to study the effect of β-amyloid on the cholinergic innervation of intracerebral microvessels in transgenic mice and non-transgenic littermates by applying semiquantitative dual immunohistochemistry of cholinergic terminals and blood vessels.

4.5.1 STL – a mono-marker for detection of cortical vessels, senile amyloid plaques and activated microglia in cerebral cortex

To differentiate between cholinergic terminals, intracerebral microvessels, and Aβ deposits in one and the same brain section, a lectin isolated from potatoes, the Solanum Tuberosum lectin (STL) has been tested for its usefulness in labeling blood vessels and Aβ. STL is homotypic endolectin that has carbohydrate-binding specificities to oligosaccharides, recognizing N-acetyllactosamine-containing glycosphingolipids, may adhere to lactosylceramide (Gallagher 1984; Ciopraga et al. 2000) and consists of two nearly identical chitin-binding modules (Van Damme et al., 2004).

The STL-staining allowed the detection of cortical brain vessels in variable size, diameter and branches, from pial vessels covering the surface of the brain till intracortical capillaries, being in agreement with a previous study (Härtig et al., 2009). The comparison of Aβ staining pattern with other Aβ staining procedures by means of Thioflavin S dye and immunohistochemistry with the monoclonal antibody 4G8 revealed that STL labels both diffuse and fibrillar Aβ in brain sections that were not pretreated with formic acid. Moreover, STL may also be used to label glial cells and macrophages (Härtig at al., 2009). In order to reveal which kind of glia (micro-or astroglial cells) is stained by STL, brain sections were subjected to either immunostaining with anti-CD11b antibody, that labels microglial cells, or
Discussion

Glial fibrillary acidic protein (GFAP, that labels astroglial cells), and in each case counterstained with STL. While STL fluorescence was not co-localized with immunoreactivity for astroglial cells, STL was shown to label activated microglial cells.

In conclusion, STL can be used to label brain cortical vessels, senile amyloid plaques and activated microglia. Moreover, it represents an adequate dye to be used in combination with other immunohistological markers.

4.5.2 Cholinergic perivascular innervation of cerebral cortical microvessels in transgenic Tg2576 mice

For a more detailed determination of cholinergic perivascular nerve terminals in the cerebral cortex of 8-month-old transgenic mice, high power Laser scanning microscopy by three-dimensional (3D)-scanning (series of the multi-slice 2D-imaging along the z-axis) of double-stained sections for VACHT and STL was used. This technique allows a more detailed description of the distribution of perivascular cholinergic terminals around blood vessels, its association with surrounding blood vessels of varying sizes in different cortical layers (see Fig. 3.19), and to spatially reconstruct perivascular cholinergic innervations (see Fig. 3.20). The high power Laser scanning microscopy revealed the presence of VACHT-positive nerve fibres closely associated with and partly attached on the wall of large blood vessel. The cholinergic nerve fibres appeared to overlay the vessel wall, or being apposed directly to the wall, and located within the immediate vicinity of small microvessels, being in agreement with a number of other histochemical and ultrastructural studies. Already few decades ago cholinergic nerve terminals have been reported to be present in direct vicinity of cortical microvessels (Eckenstein and Baughman, 1984; Armstrong, 1986; Arnerić et al., 1988). Perivascular AChE-positive nerve fibers at cerebral blood vessels of different mammalian species as well as connections of ChAT-immunoreactive terminals with pial arteries and capillaries have been visualized by many investigators (Arnerić et al., 1988; Suzuki and Hardebo, 1993; Chédotal et al., 1994, Vaucher and Hamel, 1995; for overview, see El-Assouad and Tayebati, 2002). Revealed by co-staining of rat cortical brain section for Phaseolus vulgaris leucoagglutinin (PHA-L, anterogradely transported from cholinergic cells in the substantia innominata), and for ChAT-immunoreactivity, Vaucher and Hamel (1995) showed that cortical arterioles and capillaries receive a cholinergic input from cholinergic nuclei of the basal forebrain. Pharmacological and receptor autoradiographic studies provided evidence that endothelial and smooth muscle cells of cerebral vessels are endowed with a
number of transmitter receptors including different subtypes of the mAChRs (for review, see Hamel, 2004), suggesting a role of neurovascular projections originating from local interneurons, or from distant nuclei, to modulate cerebral blood flow (for review, see Girouard and Iadecola, 2006).

In conclusion, the high power Laser scanning microscopy of brain sections co-stained for VACHT and STL as introduced in this study, represents a useful tool to study various aspects of the perivascular cholinergic network in mouse brain.

4.5.3 Quantitation of cholinergic input on cerebral microvessels of mouse brain

The evaluation of age-related immunohistochemical detection of blood vessels and cholinergic nerve terminals in the somatosensory cortex of Tg2576 mice and non-transgenic littermates provided evidence of Aβ plaque-mediated changes in densities of blood vessels and cholinergic fibres. Therefore the question arose whether the age-related Aβ production and deposition also have consequences on the morphological integrity of the neurovascular unit, in particular on the specific cholinergic innervation of blood vessels.

Brain sections of the somatosensory cortex of 6- and 8-month-old transgenic Tg2576 mice, displaying enhanced level of soluble Aβ but not yet any plaque deposition, and of 18-month-old transgenic animals with high plaque load were subjected to dual immunofluorescent histochemistry for both cholinergic fibres and blood vessels followed by quantitation of cholinergic contacts on microvessels using high power Laser scanning microscopy.

Evaluation of cholinergic contacts/endings on microvessels within a length of 100 μm in somatosensory cortical sections of 6- and 8-month-old mice did not show any transgene-mediated changes in cholinergic innervation of microvessels. However, further postnatal development of Tg2576 mice up to the age of 18 months resulted in a significant loss of cholinergic innervation of blood vessels in almost all cortical layers (except layer VI). Similar reductions of cholinergic contacts with microvessels were also observed in the somatosensory cortex of 18-month-old wild type mice, but restricted to cortical layers I and V, and being less severe as compared to that found in Tg2576 mice.

In 6- and 8-month-old mice the number of varicosities (boutons) and length of microvessels did not differ between transgenic and wild type mice, while an impaired vascular autoregulation has been observed in transgenic APP mice (Kalaria, 2009). Moreover, the
earliest abnormality observed in these mice is a profound alteration in the cerebro-vascular regulation at 2–3 months of age (Iadecola, 2004), which correlates with behavioral abnormalities (Hsiao et al., 1996). Other studies in Tg2576 mice revealed cognitive impairments at the age of 6 months, before significant Aβ deposition (Westerman et al., 2002). Presumably, the beginning of disease-induced cerebral vascular alterations lead to mild disturbancies in regulation of cerebral blood flow, which are still not so severe to be detectable at the morphological level.

The changes we have observed in the cerebral neurovascular cholinergic pathway in layers I to V of somatosensory cortex of 18-months old transgenic mice in comparison to younger transgenic animals, are in agreement with data obtained from other animal studies. Hamel and co-workers (2005) showed in 12 to 18-month old mice carrying familial AD-linked mutations, a selective cholinergic denervation of ChAT-positive varicosities, which initially started in the hippocampus by the age of 12-14 months and then reached the parietal cortex at the age of 18 months (Aucoin et al., 2005). However, despite decreased cholinergic cortical innervation there was no evidence of dystrophic cholinergic varicosities or significant loss in the perivascular area (Aucoin, et al., 2005), which may partly be explained by the different method of detection and quantification of neurovascular connections. However, the data obtained from studies in AD patients also support the results we have observed in Tg2576 mice in the present study (Scheibel et al., 1987 and 1988; Tong and Hamel, 1999). Thus, structural analysis of the capillary plexus in brains of AD patients demonstrated perivascular denervation, and microangiopathy, which may be due to changes in capillary wall structure, with the consequence of leading to profound alterations in blood-brain barrier function (Scheibel et al., 1987 and 1988). Tong and Hamel (1999) showed regional denervation of intracortical microvessels and reduced nitric oxide synthase (NOS)-containing neurons with paralleled loss of total cholinergic nerve terminals in the corresponding areas of the cerebral cortex in neuropathologically confirmed cases of AD in comparison to age-matched control individuals (Tong and Hamel, 1999). The authors showed also dysfunctional interactions between basalo-cortical and intracortical NOS-containing neurons in regulation of cortical perfusion following basal forebrain activation in AD (Tong and Hamel, 2000). Interestingly to note, selective destruction of the cholinergic nucleus basalis magnocellularis in the rabbit leads to the deposition of Aβ in and around cerebral blood vessels (Roher et al., 2000). The data suggest that cortical cholinergic deprivation may result in the loss of vasodilation
mediated by ACh and may lead to cerebral hypoperfusion and a disruption of blood-brain barrier.

Surprisingly, the changes in densities of cholinergic varicosities on cortical microvessels detected in the somatosensory cortex of 18-month-old Tg2576 mice did not correlate with plaque-load, which was also found in another animal study (Aucoin et al., 2005). The authors also did not find a direct relationship between the age-dependent increase in the number of plaques and the decrease in the density of ChAT-positive axonal varicosities in hippocampus and parietal cortex (Aucoin, et al. 2005). Therefore, they proposed non-selective and focal neurotoxic effects on cholinergic axons, assuming that plaque- and oxidative stress-independent diffuse cholinotoxicity, most likely caused by soluble Aβ assemblies, is responsible for the hippocampal and cortical cholinergic denervation (Aucoin et al., 2005). While senile plaques in the AD brain have been believed for a long time to be the most toxic forms responsible for cholinergic neurodegeneration, there is now strong evidence that soluble oligomers of Aβ, such as low-molecular weight monomers, oligomers and amyloid-derived diffusible ligands, as well as protofibrils ensure neurotoxic species that mediate early neuronal dysfunction and initiate neurodegeneration at cholinergic terminals (Lue et al., 1999; McLean et al., 1999; Klein et al., 2001; Hardy and Selkoe, 2002; Auld et al., 2002; Yan and Feng, 2004; for reviews, see Walsh and Selkoe, 2007; Schliebs and Arendt, 2011). These findings fit with observations that the severity of neurodegeneration in AD correlates best with the pool of soluble Aβ rather than with the number of insoluble Aβ plaques (McLean et al., 1999). As soluble Aβ(1-42) have the capacity to bind with high affinity to alpha7 nicotinic acetylcholine receptor (α7nAChR; Wang et al., 2000), and thereby suppressing the neuroprotective properties of α7nAChR, the blockade of α7nAChR by Aβ(1-42) may represent a potential mechanism to explain the cholinergic denervation of microvessels in the absence of any plaques in Tg2576 mouse brain. Moreover, there are other reports that Aβ(1-42) may trigger cholinergic dysfunction by promoting aberrant neuritic sprouting (Masliah et al., 2003; Masliah et al., 1991).

Changes in the cerebrovasculature and the related cholinergic input have also been observed in normal non-transgenic mice even though to a lesser extent as compared to Tg2576 animals, indicating that also normal physiological aging is accompanied by functional and structural decline of cholinergic transmission. The functional cholinergic decline associated with normal
aging may be the result of decrements in “cholinergic” gene expression, impairments in intracellular signaling, and cytoskeletal transport (for review, see Schliebs and Arendt, 2011). The aging-related alterations in densities of microvessels and cholinergic fibres found in the somatosensory cortex of 18-month-old wild type mice compare well with previous morphological studies. Kalaria (1996) described variable age-related changes in the perivascular nerve plexuses of both extraparenchymal and intracerebral vessels, but aging process is regionally selective and does not equally affect the perivascular innervation of CNS vessels (Kalaria, 1996). Burnstock and co-workers reported on age-related changes of perivascular cerebrovascular nerves with reduced expression of vasodilator nerves and an increased expression of vasoconstrictor nerves in the brain of old rats (Mione et al., 1988), whereas marked reductions in the noradrenergic and ACh-positive innervation of the middle cerebral arteries in aged rabbits have been shown (Saba et al., 1984). Another report demonstrated alterations in perivascular cholinergic nerve fibres with slight decrease in choline accumulation and increase in ChAT activity in the major cerebral arteries of the 22-month-old rats (Hamel et al., 1990). A microanatomical study demonstrated age-related decrease of cholinergic ACh-immunoreactive nerve fibres in pial and intracerebral arteries of adult or senescent rats (El-Assouad and Tayebati, 2002). Interestingly, Ypsilanti and co-workers (2008) detected reduced lengths of hippocampal cholinergic ChAT-positive fibers in the aged rat brain with no change in number and size of ChAT-positive neurons (Ypsilanti et al., 2008).

As cholinergic cells steadily require support by the cholinotrophic nerve growth factor (NGF), the maintenance of functional NGF production and signaling in the aged nervous system is of particular importance. There are reports of sprouting responses of perivascular axons associated with intradural blood vessels after intraventricular infusion of NGF in the adult rat brain (Isaaacson et al., 1990). To maintain the sprouting perivascular axons a continuous supply of NGF is required, while the level of endogenous NGF, or other factors produced by the vascular target tissue, are not sufficient to maintain the newly formed axons (Isaaacson et al., 1995).

In conclusion, the detailed morphological and morphometric analysis of cortical perivascular cholinergic innervation in the somatosensory cortex of both transgenic Tg2576 and normal wild type mice revealed age-dependent alterations that are more severe in cortical tissue suffering of Aβ plaque pathology.
4.6 Summary and conclusions

The present study was undertaken to elucidate whether the age-related Aβ production and deposition may affect the morphological integrity of cerebral microvessels, cholinergic terminals and their intracerebral neurovascular architectonics. We wanted to contribute to the question whether changes in cerebral vessels, cortical cholinergic denervation, Aβ formation and deposition are interrelated.

To *in vivo* characterize the developmental relationship of amyloid formation and deposition, cortical cholinergic innervation and cerebrovascular abnormalities, transgenic Tg2576 mice that overexpress the Swedish double mutation of human APP, and demonstrate significant cerebral cortical deposition of Aβ plaques at ages from 9 months onwards, were considered as an appropriate animal model. Thus, transgenic Tg2576 mice at ages between 4 and 18 months were investigated in order to reveal any effect of both soluble Aβ and Aβ plaque deposits on maturation and morphological integrity of cerebral cortical capillaries, cholinergic terminals and their cerebral neurovascular relationship with each other.

Using the somatosensory cortex as a representative region, serial cryocut sections, obtained from mice at ages ranging from 4 up to 18 months, were subjected to immunohistochemistry to label vascular endothelial cells (GLUT1 immunostaining), cholinergic nerve terminals (VACHT immunostaining) and Aβ plaques (Thioflavin S, and/or *Solanum Tuberosum* lectin staining), followed by a thorough quantitative evaluation of the age-related spatial relationship between cerebral cortical capillaries, amyloid plaques and cholinergic terminals, using computer-assisted image analysis. Moreover, high power Laser scanning microscopy of brain sections co-stained for VACHT (cholinergic nerve terminals), and *Solanum Tuberosum* lectin (STL; dye for cerebral vasculature) was used as tool to study various aspects of the perivascular cholinergic network in mouse brain.

The density of blood vessels estimated in the somatosensory cortex of transgenic mice by anti-GLUT-1 immunohistochemistry did not differ to that obtained in wild type mice before onset of plaque deposition (younger than 12 months). However, in aged, 18-month-old Tg2576 mice, demonstrating high plaque loads, decreased blood vessel densities, particularly in layer IV of the somatosensory cortex, were observed.

The density of cholinergic terminals estimated by evaluation of VACHT immunohistochemistry in somatosensory cortical sections of wild type mice did not change with aging regardless of the cortical layer examined, while in cortical layers II/III and IV of
somatosensory cortex of transgenic Tg2576 mice age-related decreases in cholinergic fibre densities were assessed. However, quantitative morphometric analysis demonstrated an age-related reduction in the number of varicosities on cholinergic fibres, particularly in layer IV, in both transgenic Tg2576 mice and non-transgenic littermates.

Cholinergic innervation of microvessels in the somatosensory cortex decreased with aging in both Tg2576 mice and non-transgenic littermates, as revealed by estimating the ratio of the number of cholinergic vascular contacts and total length of blood vessel. There was no significant difference in the perivascular cholinergic innervation in areas that demonstrated significant plaque load and those with no plaque deposits regardless of the cortical layer examined.

The data presented in this animal study add further evidence that Aβ, in addition to its well-described neurotoxic effects, may also contribute to neuronal dysfunction through its actions on the cerebrovasculature.

The detailed morphological and morphometric analysis of cortical perivascular cholinergic innervation in the somatosensory cortex of both transgenic Tg2576 and normal wild type mice revealed age-dependent alterations in the network of cortical cholinergic connectivity that are more severe in cortical tissue suffering of Aβ plaque pathology.

The changes in densities of cholinergic varicosities on cortical microvessels detected in the somatosensory cortex of 18-month-old Tg2576 mice did not correlate with plaque-load, which was also found in another animal study (Aucoin et al., 2005). These findings fit with observations that the severity of neurodegeneration in AD correlates best with the pool of soluble Aβ rather than with the number of insoluble Aβ plaques.

Taken together, the data obtained in this in vivo study strongly support an age-related interplay of Aβ accumulation, cholinergic dysfunction, and vascular impairments, while it still remains to be elucidated which processes play a causative role and which events are secondary. A potential mechanism is provided by the vascular hypothesis of AD: Aging-, and life-style-associated damages of the brain microvasculature may affect Aβ clearance and perivascular drainage, promoting cerebrovascular Aβ deposition, inducing partial loss of cholinergic vascular innervation and changes in vascular function, angiogenesis and VEGF upregulation with consequences on APP processing and Aβ accumulation.
REFERENCES


REFERENCES


Deutsche Alzheimer Gesellschaft e.V., 2011; http://www.deutsche-alzheimer.de/


REFERENCES


Soffer D. Cerebral amyloid angiopathy—a disease or age-related condition. Isr Med Assoc J. 2006, 8:803-806.


Introduction

The majority of patients suffering of Alzheimer’s disease (AD) demonstrates cerebral vascular changes and impaired regulation of cerebral blood flow, that have been assumed to play an important role in AD pathogenesis (vascular hypothesis of AD). There is strong evidence that both β-amyloid oligomers and plaques contribute to vascular injuries and to functional impairments of the neurovascular unit. *Vice versa*, amyloid lesions can be triggered by hypertension and ischemic brain injury, while β-amyloid aggregates appear to have anti-angiogenic properties. Damaged vascular endothelial cells produce and secrete the Vascular Endothelial Growth Factor (VEGF) which has been shown to favour β-amyloidogenesis by cells forming the neurovascular unit. Moreover, the physiological functions of cerebral microvasculature (vasodilation/-constriction) are controlled by basal forebrain cholinergic neurons. Injured blood brain capillaries may result in cholinergic denervation and retrograde cell loss. Cholinergic dysfunction may result in impaired cerebral blood flow with consequences on normal function of the neurovascular unit including processing of the amyloid precursor protein (APP).
Aim of study

The present study was undertaken to elucidate whether the age-related β-amyloid production and deposition may affect the morphological integrity of cerebral microvessels, cholinergic terminals and their intracerebral neurovascular architectonics, and whether size or type of plaques play a role in this process. We wanted to contribute to the question whether changes in cerebral vessels, cortical cholinergic denervation, β-amyloid formation and deposition are interrelated.

Experimental approach

The availability of transgenic mice that produce human β-amyloid peptides from birth and progressively develop Alzheimer-like -amyloid deposits in the aged brain appears to be a unique experimental approach to address such questions. To in vivo characterize the developmental relationship of amyloid formation and deposition, cortical cholinergic innervation and cerebrovascular abnormalities, transgenic Tg2576 mice that overexpress the Swedish double mutation of human APP, and demonstrate significant cerebral cortical deposition of β-amyloid plaques at ages from 10 months onwards, were considered as an appropriate in vivo animal model. In this developmental study transgenic Tg2576 mice at ages between 4 and 18 months were investigated in order to reveal any effect of both soluble β-amyloid and β-amyloid plaque deposits on maturation and morphological integrity of cerebral cortical capillaries, cholinergic terminals and their cerebral neurovascular relationship with each other.

Using the somatosensory cortex as a representative region, serial cryocut sections, obtained from mice at ages ranging from 4 up to 18 months, were subjected to immunohistochemistry to label vascular endothelial cells (anti-glucose transporter 1 (GLUT1) immunostaining), cholinergic nerve terminals (anti-vesicular acetylcholine transporter (VACht) immunostaining) and β-amyloid plaques (Thioflavin S, and/or Solanum Tuberosum lectin staining), followed by a thorough quantitative evaluation of the age-related spatial relationship between cerebral cortical capillaries, amyloid plaques and cholinergic terminals, using computer-assisted image analysis. Moreover, high power Laser scanning microscopy of brain sections co-stained for VACht (cholinergic nerve terminals), and Solanum Tuberosum lectin (STL; dye for cerebral vasculature) was used as tool to study various aspects of the perivascular cholinergic network in mouse brain.
Results obtained

The main results obtained in the study presented were as follows:

1. Density of blood vessels estimated in the somatosensory cortex of transgenic mice by anti-GLUT-1 immunohistochemistry did not differ to that obtained in wild type mice before onset of plaque deposition (younger than 12 months). However, in aged, 18-month-old Tg2576 mice, demonstrating high plaque loads, decreased blood vessel densities, particularly in layer IV of the somatosensory cortex, were observed.

2. The density of cholinergic terminals estimated by evaluation of VACHT immunohistochemistry in somatosensory cortical sections of wild type mice did not change with aging regardless of the cortical layer examined, while in cortical layers II/III and IV of somatosensory cortex of transgenic Tg2576 mice age-related decreases in cholinergic fibre densities were assessed. However, quantitative morphometric analysis demonstrated an age-related reduction in the number of varicosities on cholinergic fibres, particularly in layer IV, in both transgenic Tg2576 mice and non-transgenic littermates.

3. Cholinergic innervation of microvessels in the somatosensory cortex decreased with aging in both Tg2576 mice and non-transgenic littermates, as revealed by estimating the ratio of the number of cholinergic vascular contacts and total length of blood vessel. There was no significant difference in the perivascular cholinergic innervation in areas that demonstrated significant plaque load and those with no plaque deposits regardless of the cortical layer examined.

4. The high power Laser scanning microscopy of brain sections co-stained for VACHT, and STL represents a useful tool to study various aspects of the perivascular cholinergic network in mouse brain.

Conclusions

1. The data presented in this animal study add further evidence that β-amyloid, in addition to its well-described neurotoxic effects, may also contribute to neuronal dysfunction through its actions on the cerebrovasculature.
2. The detailed morphological and morphometric analysis of cortical perivascular cholinergic innervation in the somatosensory cortex of both transgenic Tg2576 and normal wild type mice revealed age-dependent alterations in the network of cortical cholinergic connectivity that are more severe in cortical tissue suffering of β-amyloid plaque pathology.

3. The changes in densities of cholinergic varicosities on cortical microvessels detected in the somatosensory cortex of 18-month-old Tg2576 mice did not correlate with plaque-load, which was also found in another animal study (Aucoin et al., 2005). These findings fit with observations that the severity of neurodegeneration in Alzheimer’s disease correlates best with the pool of soluble β-amyloid rather than with the number of insoluble β-amyloid plaques.

4. Taken together, the data obtained in this in vivo study strongly support an age-related interplay of Aβ accumulation, cholinergic dysfunction, and vascular impairments, while it still remains to be elucidated which processes play a causative role and which events are secondary. A potential mechanism is provided by the vascular hypothesis of AD: Aging-, and life-style-associated damages of the brain microvasculature may affect β-amyloid clearance and perivascular drainage, promoting cerebrovascular β-amyloid deposition, inducing partial loss of cholinergic vascular innervation and changes in vascular function, angiogenesis and VEGF upregulation with consequences on APP processing and β-amyloid accumulation.

Datum ........................................ Unterschrift ........................................
CURRICULUM VITAE

(Persönlicher Lebenslauf)

Name, Vorname: Kuznetsova, Elena
Geburtsdatum: 06.11.1974
Geburtsort: Smela, UdSSR
Familienstand: verheiratet, 2 Kinder
Privatanschrift: Stephanstraße 8, 04103 Leipzig
Telefon (privat): 0341 9625809; Mobil: 01797073665
E-mail (privat): ekuz74@yahoo.de

Aktuelle Dienstadresse: HELIOS Klinikum Borna (Leipziger Land)
Klinik für Anästhesie, Intensivmedizin, Schmerztherapie und Palliativmedizin
Rudolf-Virchow-Straße 2, 04552 Borna
Tel.(Sekretariat): (0343) 3211681

Schulausbildung:
1982 – 1985 Grundschule in Kuschka und Aschhabad, UdSSR
1985 – 1990 Mittelschule in Termez und Taschkent, UdSSR
1990 – 1992 Mittelschule in Taschkent, UdSSR
06/1992 Allgemeine Hochschulreife (Abitur), Note: sehr gut

Praktische Ausbildung und Hochschulstudium:
12/1992 – 08/1993 Krankenpflegerin im Militärkrankenhaus, Kandalakscha,
Russische Förderung
09/1993 – 06/1999 Studium an der St. Petersburger Staatlichen pädiatrischen
medizinischen Akademie, Russische Förderung;
Abschluss als Diplom-Mediziner, Fachrichtung Pädiatrie
Gesamtnote der Staatlichen Prüfungen: sehr gut

Beruflicher Werdegang:
09/1999 – 12/2003 Assistenzärztin in der Weiterbildung an der St. Petersburger
Staatlichen pädiatrischen medizinischen Akademie, Russische
Förderung, am Lehrstuhl "Geburthilfe und Frauenheilkunde"
08/2001 – 12/2003 Mutterschutz und Erziehungsurlaub mit dem ersten Kind
01/2004 – 06/2007 Wissenschaftliche Mitarbeiterin und Doktorandin am
Paul-Flechsig-Institut für Hirnforschung, Leipzig
07/2007 – 06/2008 Ärztliche Tätigkeit im Rahmen der Anpassungsmaßnahmen
(Anpassungszeit) am HELIOS Klinikum Leipziger Land
seit 07/2008 Ärztin in Weiterbildung, Fachgebiet Anästhesiologie,
am HELIOS Klinikum Borna (Leipziger Land)
seit 08/2011 Mutterschutz und Erziehungsurlaub mit dem zweiten Kind
Sprachkenntnisse:

- Russisch (Muttersprache)
- Deutsch (sehr gute Kenntnisse)
- Englisch (gute Kenntnisse)
- Latein

Datum: ................................. Unterschrift: .................................
Completing my MD degree I have spent four wonderful years at the Department of Neurochemistry of Paul Flechsig Institute for Brain Research at the University of Leipzig. The best moments of my doctoral thesis I have been shared with many people. My first debt of gratitude must go to my supervisor Professor Dr. rer. nat. habil. Reinhard Schliebs who helped me in all sorts of difficulties and problems during my entire working stay at the Paul Flechsig Institute. I would like to express my deep and sincere gratitude to him. I am thankful to Renate Jendrek for perfect technical assistance and Dr. rer. nat. Jens Grosche for the help with Laser Scanning imaging. I am also grateful to Professor Dr. Wolfgang Härtig for his assistance in immunochemistry. I wish to thank my husband Dr. med. Yousef Yafai for his patiency and encouragement. Finally, I wish to express my warm and sincere thanks to all members of the Department of Neurochemistry of the Paul Flechsig Institute.