β-Amyloid-mediated Mitochondria-dependent Cell Death Pathways in Alzheimer´s Disease

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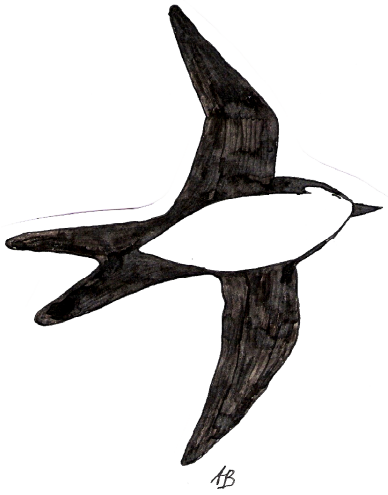

Die Erinnerung ist ein Paradies, aus dem man nicht vertrieben werden kann.

Jean Paul

Für meine Familie

β-Amyloid-vermittelte, mitochondrien-abhängige Zelltodmechanismen in der Alzheimer Demenz

Dissertation

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TABLE OF CONTENTS

1 INTRODUCTION

1 Introduction

1.1 Alzheimer's Disease

1.1.1 Historical Background

The disease was first described by Alois Alzheimer (Fig. 1.1), a neurologist and psychiatrist in Frankfurt am Main. He extensively investigated the pathology of the nervous system together with his colleague Franz Nissl. At the beginning of the last century, he reported that one of his patients, Auguste D., suffered from severe memory impairment and desorientation. After death, he dissected the brain and described histopathological changes which are recognized still today as the typical hallmarks of Alzheimer´s Disease (AD): extracellular senile plaques and intracellular neurofibrillary tangles. Kraepelin, who invited Alzheimer to work with him at the university of Heidelberg in 1902, proposed the term Alzheimer´s disease for this illness. His monumental work on Alzheimer´s disease (Alzheimer 1907) was published In 1907.

Figure 1.1: Alois Alzheimer (1864-1915).

1.1.2 Definition

Alzheimer´s disease (AD), the most frequent form of dementia, is a progressive neurodegenerative disorder characterized by cognitive deterioration, progressive memory loss, impairment of activities of daily living and a variety of neuropsychiatric symptoms and behavioural disturbances evolving over several decades (Braak, H., Braak, E., 1991). The disease first destroys a person's ability to learn and to remember recent experiences, to reason, make judgements, communicate and carry out daily activities. As the illness progresses, patients also experience growing changes in behaviour and personality leading to anxiety, suspiciousness, agitation or hallucinations (www.alzorg.com). In the end-stage of the disease patients need the support of relatives or caregivers to handle basic functions such as eating, personal hygiene and dressing. This underlines the very helpless situation of affected persons

being bedridden, incontinent and dependent on custodial care. Death occurs, on average, about nine years after clinical diagnosis, but the duration can vary from three to twenty years.

1.1.3 Epidemiology

In Germany, 18% of the whole population, approximately 14.8 million people, are 65 years or older. Currently, 1.2 million people in Germany suffer from AD, with about 70% being supported by their relatives. Worldwide, more than 12 million individuals suffer from Alzheimer's disease (AD), and it accounts by far for most cases of dementia that are diagnosed beyond age 60. The disorder currently affects nearly 2% of the entire population in industrialized countries; the risk of AD dramatically increases as a consequence of the longer life expectancy. Most patients are suffering from the so-called late-onset AD (LOAD), whose biggest known risk factor is increasing age and genetic predisposition. The likelihood of developing LOAD approximately doubles every five years, and by the age of 85, the risk increases to nearly 35%. In contrast to LOAD, in early-onset AD (EOAD), symptoms can appear as early as age 40 or even younger. These individuals make up only 10% of all cases of AD. For this form, a hereditary background is believed to be of higher importance than for LOAD. Each year, 100.000 victims die and 360.000 new cases are diagnosed. By 2020, 30 million people will be affected by this devastating disorder worldwide and by 2050, the number could increase to 45 million. Today, the disease causes enormous health care expenses and lost wages of both patients and caregivers which has an enormous impact on society. By 2030, when the entire baby boom generation is over 65, the number of people affected by AD could rise to levels that may exceed the ability of the population to absorb the added cost.

1.1.4 Diagnosis

Nowadays, dementia is a commonly recognized disease diagnosed by the use of the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association 1994) (Watt, D. F., 1994) or ICD 10 (International Classification of Diseases). Diagnosis of AD is most often based on the criteria developed by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS–

ADRDA), according to which it is classified as definite (histological confirmation), probable (typical clinical syndrome without histological confirmation), or possible (atypical clinical features but no alternative diagnosis apparent; no histological confirmation) (Mckhann, G., Drachman, D. et al., 1984). Typical sensitivity and specificity values for the diagnosis of probable Alzheimer's disease with the use of these criteria are 0.65 and 0.75 (Chui, H., Zhang, Q., 1997). Neuro-imaging methods play an increasingly important role in the diagnosis of AD: structural imaging of the brain with computed tomography (CT), magnetic resonance imaging (MRI), positron-emission tomography (PET, Fig. 1.2) or single-photon-emission CT (Silverman, D. H. S., Small, G. W. et al., 2001). Above and beyond the improved diagnostic methods for AD, it should be emphasized that the low rates of recognition of dementia by family members and physicians (Callahan, J. J., 2004; Ross, G. W., Petrovitch, H. et al., 1997) constitute a major barrier to the appropriate care for many patients with AD. Additionally, scientists are facing the problem of clearly differentiate between the multiple forms of dementia, amongst which vascular dementia plays an important role as well as mixed forms of dementia.

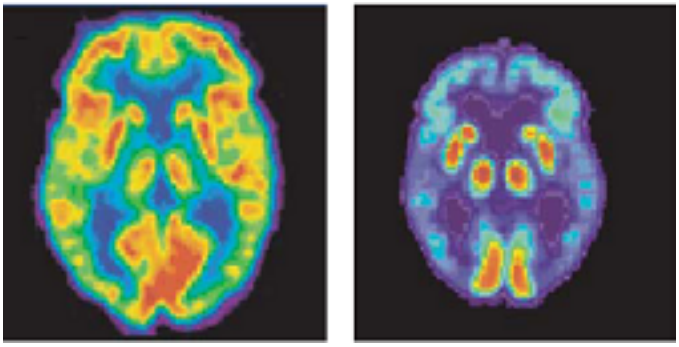

Figure 1.2: Positron emission tomography scans (glucose utilisation).
Red and yellow areas indicate high glucose uptake. AD brain (right) is characterised by impaired glucose metabolism in the frontal cortex and temporal lobes compared to healthy brain (left) (taken form Mattson, 2004). Red and yellow areas indicate high glucose uptake. AD brain (*right*) show a strong decrease in energy metabolism in the frontal cortex and temporal lobes compared to a healthy brain (*left*) (taken from Mattson, 2004).

1.1.5 Risk factors

1.1.5.1 Age

For the late-onset AD (LOAD), the most important risk factor known is increasing age (Mayeux, R., 2003; Braak, H., Braak, E. et al., 1996). Incidences increase about hundredfold between ages 65 and 90, which underlines the importance of the risk factor aging.
LOAD is a progredient illness developing over several decades with first symptoms appearing in an advanced age. Since women posses a higher life expectancy, they are consequently more affected by the disease than men.

1.1.5.2 Genetics

Inherited forms of AD are exceptionally rare, with only a few hundred families being affected throughout the world. In this case, scientists also use the term “Familial Alzheimer’s Disease” (FAD). Naturally, there is a 50% chance to inherit any single gene from either parent, and as half of any generation is affected, a characteristic pattern of inheritance is observed. As mentioned above, early-onset AD (EOAD) make up only 10% of all AD cases, including approximately 1% of FAD. Three genes are known to cause this rare familial form of EOAD: The amyloid precursor protein (APP) on chromosome 21, the presenilin-1 (PS-1) gene on chromosome 14 and the presenilin-2 (PS-2) gene on chromosome 1. Presenilin genes encode polytopic transmembrane proteins, which are processed by proteolytic cleavage. Besides the processing of selected proteins including APP, they have been suggested to be functionally involved in developmental morphogenesis and unfolded protein responses (Suh, Y. H., Checler, F., 2002). Notably, all familial cases are very rare, but the PS-1 gene seems to be the most common and most probably there are other genes causing EOAD remaining to be discovered. Nowadays, more than 120 mutations in the PS-1 gene are identified, all leading to an extremely early onset of the disease before age 40. Most interestingly, all known presenilin mutations increase the production of the neurotoxic $A\beta_{1\text{-}42}$ forming the core of the senile plaques. In contrast to PS, mutations in the APP gene (Fig. 1.3) mainly increase total

Aβ levels (Scheuner, D., Eckman, C. et al., 1996; Citron, M., Oltersdorf, T. et al., 1992; Suzuki, N., Cheung, T. T. et al., 1994). This strongly supports the "amyloid hypothesis" proposing that flaws in processes governing production, accumulation or disposal of Aβ are the primary cause of AD. Patients with Down's syndrome, possessing three copies of chromosome 21, almost always develop AD in the middle age, most probably due to three copies of the APP gene and an elevated production of total Aβ. The first FAD-associated mutation of the APP gene was discovered in 1991. In this mutation, V717 was replaced by isoleucine (APP770 V717) (Goate, A., Chartierharlin, M. C. et al., 1991).

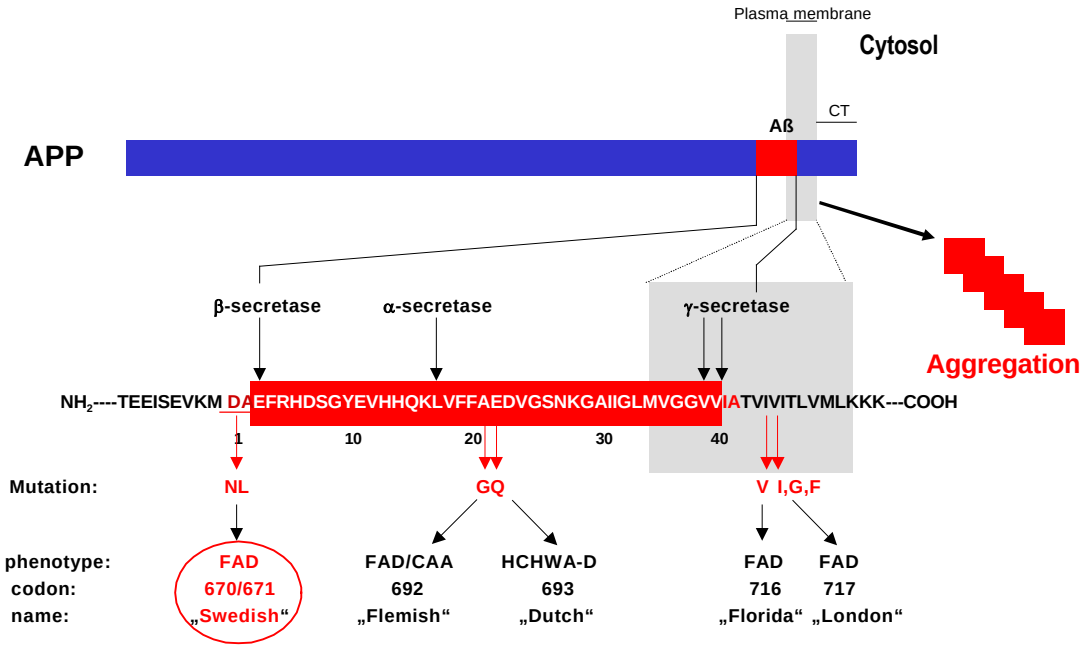

Figure 1.3: APP mutations

α-secretory pathway: The processing of APP by α-secretase leads to the secretion of a large soluble ectodomain of APP (sAPPα) and to the retention of an 83-residue COOH-terminal fragment (C83) in the membrane. β-secretory pathway: cleavage of APP by β-secretase produces a membrane-bound C-terminal fragment (C99) which is further cleaved by a γ-secretase leading to the release and secretion of pathogenic Aβ-peptide and a C-terminal fragment (APP intracellular domain: C57/C59, AICD). Missense mutations in the APP-gene either interfere with α- secretase or enhance β- or γ-secretase cleavage, resulting in an increased production of the toxic Aβ peptide (taken from Eckert, 2003).

In the "Swedish mutation" (APPsw), which results in an average onset of the disease at age 53, two amino acids are replaced (APP770KM670/671NL) next to the N-terminus of the Aβ sequence (Haass, C., Lemere, C. A. et al., 1995b). In contrast to

the mutation at V717, the processing of APPsw does not result in a relative over-production of $A\beta_{1-42}$, but in an elevated production of total Aβ (Cai, X. D., Golde, T. E. et al., 1993; Citron, M., Vigopelfrey, C. et al., 1994). Also, increased cleavage of the β-secretase site was found in distinct subcellular compartments for the APPsw mutation (Haass, C., Lemere, C. A. et al., 1995b): It was found that wild-type APP (APPwt) was re-internalised prior to β-secretase cleavage, while APPsw is cleaved earlier in secretory vesicles, leading to a six- to eightfold increase in Aβ secretion (Cai, X. D., Golde, T. E. et al., 1993).

ApoE4 is the only gene that has been definitely associated with late onset AD (LOAD) (Fig. 1.4). ApoE4 is located on chromosome 19, which has three common variants, E2, E3, E4. The E2 allele probably decreases the risk of developing AD or is protective, in contrast to E4, which is reported to be associated with an increased Aβ metabolism (Saunders, A. M., Strittmatter, W. J. et al., 1993). Consequently, the presence of two copies of the ApoE4 allele leads to an increased risk for developing AD (Corder, E. H., Saunders, A. M. et al., 1993).

Other genes probably enhancing the risk for AD include: A gene encoded on chromosome 10, insulin degrading enzyme (IDE), a metalloendopeptidase implicated in the Aβ degradation (Bertram, L., Blacker, D. et al., 2000). Mutations of IDE are reported to reduce Aβ degradation for 15-30% (Farris, W., Mansourian, S. et al., 2004). Minor changes in the antichymotrypsin gene, various ApoE receptor genes, the serotonin transporter gene and the butyrylcholinesterase E gene are likely to be associated with AD, but to a lower extent than the association of ApoE4 with AD.

	Chromosome	Gene	Mutations	Onset	Feature	Effect on Aß
EO FAD	21	APP	7	45-64	dominant	Aß↑
	14	PS 1	>120	28-55	dominant	Aß↑
	1	PS 2	2	40-75	dominant	Aß↑
EO AD				< 60		
LO FAD	19	Apo E	Poly-morphism ε 2, ε 3, ε 4	61-65	Risk factor ε 4	
LO AD				> 60		

Figure 1.4: Genetic risk factors of AD.

Mutations leading to early-onset familial AD (EOFAD) and to early-onset AD (EOAD) appear on the chromosomes 21, 14 and 1, with APP and PS-1/2 being the affected genes. All mutations are dominantly inherited and lead to an increase in Aβ levels. A risk factor for late-onset AD and late AD (LOAD) is the Apo E 4 allele. Polymorphisms of Apo E are encoded on chromosome 19 (taken from Eckert, 2003).

1.2 Pathological hallmarks

Still today it is unknown which factors trigger LOAD, but scientists believe that the damage of the brain begins years before the clinical symptoms appear. As other forms of dementia also cause memory loss, the definitive diagnosis of AD still requires post-mortem examination of the brain. Brain regions involved in learning and memory processes, including the temporal and frontal lobes and hippocampus, are reduced in size in AD patients as the result of degeneration of synapses and death of neurons leading to a massive reduction of total brain weight and cortex volume at the end-stage of the disease. The affected brain regions also contain significantly high numbers of so-called “plaques” and “tangles” to qualify as affected by AD (Dickson, D. W., 1997; Braak, H., Braak, E. et al., 1996). Plaques are extracellular deposits of fibrils and amorphous aggregates of various Aβ isoforms. Aβ was first isolated from blood vessels of Down’s syndrome and AD patients and then identified as the main constituent of amyloid plaques (Masters, C. L., Simms, G. et al., 1985).

Other proteins are found in the plaques such as ApoE, chymotrypsin, ubiquitin and inflammatory factors. Neurites associated with the plaques are often damaged, which lead to the conclusion that Aβ is the primary toxic species in AD (“amyloid hypothesis”). The neurons to be particularly affected use glutamate or acetyl choline as neurotransmitters together with neurons that produce serotonin and norepinephrine. Neurofibrillary tangles consist of microtubule-associated, hyperphosphorylated tau protein. Plaques and tangles are present mainly in the entorhinal cortex, hippocampus, basal forebrain and amygdala: brain regions involved in learning, memory and emotional behaviours.

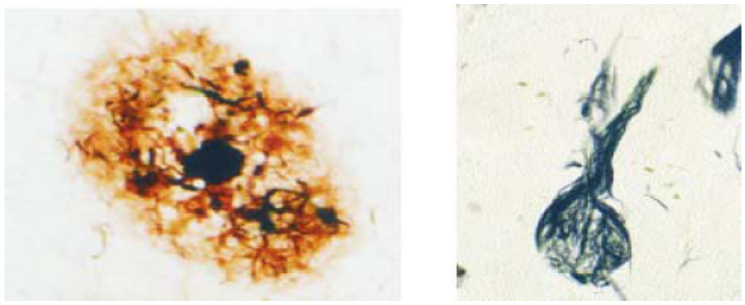

Figure 1.5: Amyloid plaques (*left*) and neurofibrillary tangles (*right*).
Amyloid plaques (left) and neurofibrillary tangles are the pathological hallmarks in AD brain: The dense amyloid core mainly consisting of $A\beta_{1-42}$ is surrounded by an amyloid halo consisting of various Aβ isoforms. Cytoskeletal changes, namely hyperphosphorylation of tau protein, cause neurofibrillary tangles (right) (Bogdanovic, N., 2000).

1.3 Neurofibrillary tangles

Neurofibrillary tangles are found in many neurologic diseases, summarized as tauopathies, indicating that they are not a specific hallmark of AD. Other degenerative disorders associated with hyperphosphorylated tau and the formation of neurofibrillary tangles are, amongst others, amyotrophic lateral sclerosis, corticobasal degeneration and supranuclear progressive paralysis.
Neurofibrillary tangles are intracellular fibrillar aggregates of the microtubule-associated protein tau that exhibit hyperphosphorylation and oxidative modifications. Functionally, microtubules are strongly involved in intracellular transport processes. Under physiological conditions, tau is a microtubule-associated protein (MAP) regulating the formation of axonal microtubules (Drubin, D., Kobayashi, S. et al., 1986), signal transduction (Jenkins, S. M., Johnson, G. V. W., 1998; Jenkins, S. M., Johnson, G. V. W., 1998) and neurite outgrowth (Biernat, J., Mandelkow, E. M., 1999). Hyperphosphorylation of tau can be executed by various proteins: cyclin-dependent kinase 5 (cdk 5), glycogen synthase kinase-3β (GSK-3β), or extracellular signal-regulated kinases (ERKs) (Liu, F., Iqbal, K. et al., 2002; Noble, W., Olm, V. et al., 2003; Moran, C. M., Donnelly, M. et al., 2005). Interestingly, pronounced phosphorylation at select Ser/Thr-Pro motifs but no phosphorylation at Ser262 in the repeat domain of tau protein by recombinant GSK-3β has been described recently (Godemann, R., Biernat, J. et al., 1999). Abnormally hyperphosphorylated tau accumulates and aggregates to paired helical filaments (PHFs) finally leading to

neurodegeneration (Garcia, M. L., Cleveland, D. V., 2001). Interestingly, hyperphosphorylation of tau also blocks APP trafficking, suggesting that this process might be the connecting link between the tau and amyloid theory (Mandelkow, E. M., Biernat, J. et al., 2004).

1.4 APP processing

The Alzheimer's disease APP belongs, together with the amyloid precursor-like protein 1 (APLP-1) and 2 (APLP-2), to a superfamily of proteins that appear functionally related (Coulson, E. J., Paliga, K. et al., 2000), as these transmembrane proteins type I are characterized by their orientation in the plasma membrane: the N terminus is directed to the extracellular space and the C terminus is located in the cytosol. They are also described as integral membrane proteins with a single membrane-spanning domain. Although APLPs are highly homologous to APP in the N and C-terminal domains, they lack Aβ, the main constituent of neuritic plaques in AD. After post-translational modification, mainly N- and O- glycolysation and sulfatation of tyrosine, APP can be processed proteolytically (Georgopoulou, N., McLaughlin, M. et al., 2001). APP is produced in several different isoforms ranging in size from 695 to 770 amino acids with APP695 being the most abundant form in brain produced mainly by neurons. APP695 differs from longer forms of APP in that it lacks a kunitztype protease inhibitor sequence in its ectodomain (Tanaka, S., Nakamura, S. et al., 1988). The normal function of APP is not yet determined, but in the last years many insights about how APP might exert its function became clear. APP is widely expressed in cells throughout the body where the amount produced is influenced by the developmental and physiological state of the cells. Recent findings suggest that APP plays a pivotal role in extracellular signal transduction by interacting with G_0, a GTP-binding protein (Nishimoto, I., Okamoto, T. et al., 1993; Mahlapuu, R., Viht, K. et al., 2003; Karelson, E., Fernaeus, S. et al., 2005). Furthermore, APP is reported to interact with kinesin I corroborating its potential role in axonal transport processes (Kamal, A., Stokin, G. B. et al., 2000). APP is mainly located at the cell surface or on the lumenal side of ER and Golgi membranes, with part of the peptide embedded in the membrane (Fig. 1.6). APP possesses critical components of the mitochondrial-targeting signal, with positively charged residues at 40, 44, and 51, respectively.

Chemical cross-linking together with immunoelectron microscopy show that the mitochondrial APP exists in NH_2-terminal inside trans-membrane orientation and in contact with mitochondrial translocase proteins. Accumulation of full-length APP in the mitochondrial compartment in a transmembrane-arrested form caused mitochondrial dysfunction and impaired energy metabolism (Anandatheerthavarada, H. K., Biswas, G. et al., 2003).

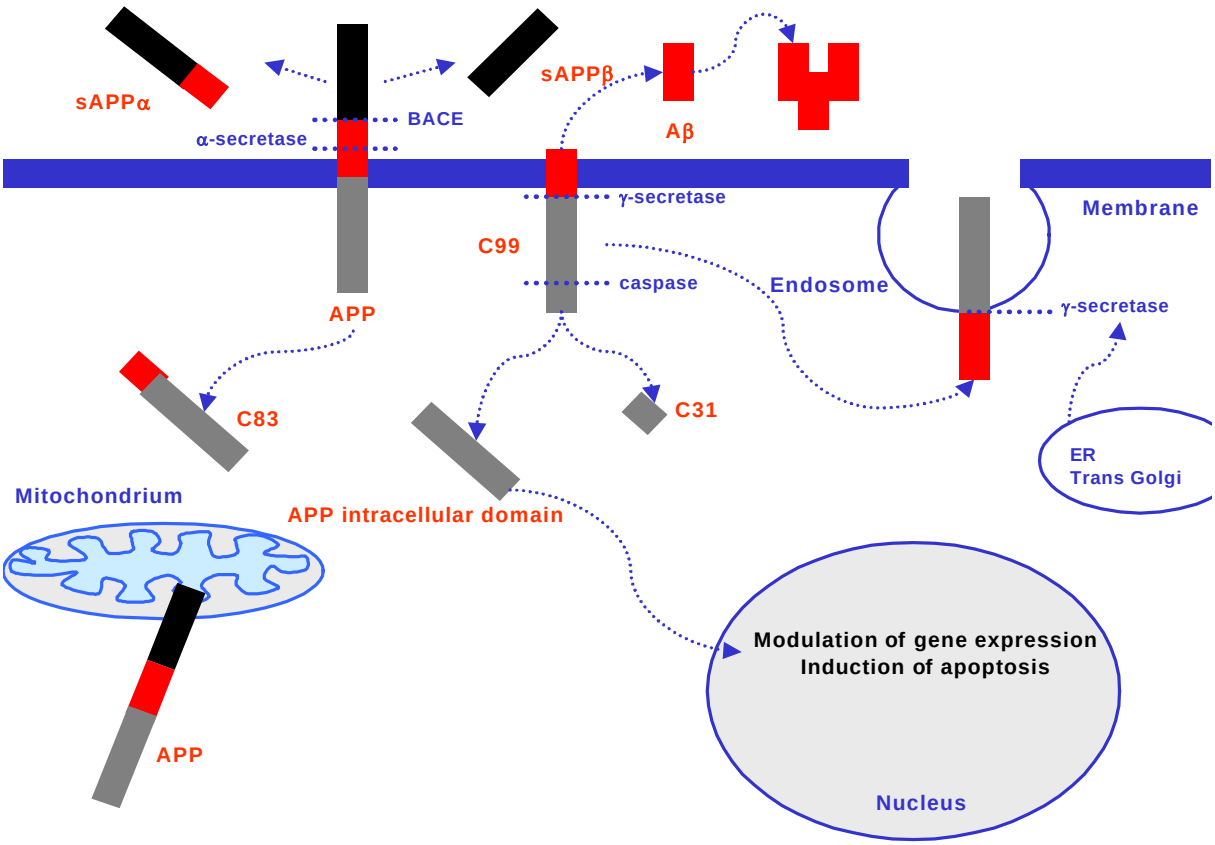

Figure 1.6: APP processing and trafficking.

APP is mainly located at the cell surface or on the lumenal side of ER and Golgi membranes, with part of the peptide embedded in the membrane where it can be proteolytically processed by α- , β- and γ-secretases. Recent findings suggest that altered APP trafficking might contribute to the progress of the disease.

Mature APP (N- and O-glycosylated forms) is phosphorylated at threonine 668 (numbering for APP695 isoform), specifically in neurons (Ando, K., Ohmori, K. et al., 2002). Phosphorylation of mature APP has been described during and after neuronal differentiation in PC12 cells. The phosphorylated form of APP is specifically distributed in neurites as is mostly present in growth cones. This suggests that the

phosphorylated form of APP may play an important role in neurite outgrowth of differentiating neurons (Suzuki, T., Ando, K. et al., 1997; Lee, M. S., Kao, S. C. et al., 2003). Most likely, glycogen synthase kinase-3β (GSK-3β) phosphorylates APP, an enzyme which is also involved in the pathogenic hyperphosphorylation of tau. The phosphorylation site was confirmed by endoproteinase digestion of APP and peptide sequencing (Aplin, A. E., Jacobsen, J. S. et al., 1997). Enzyme activities involved in cleavage of APP at the α-, β- and γ-secretase sites are described in the following.

1.4.1 α-secretase

The identity of α-secretase remains unclear, although TACE (an enzyme cleaving members of the TNF receptor family at the cell surface), and disintegrin and metalloproteases, ADAM-9, -10, -17 respectively, are candidates (Asai, M., Hattori, C. et al., 2003; Buxbaum, J. D., Liu, K. N. et al., 1998; Lammich, S., Kojro, E. et al., 1999). Most of the APP protein is cleaved by α-secretase releasing sAPPα (sAPPα) into the extracellular space while leaving a 83-amino-acid carboxy-terminal APP fragment (C83, CTFα) at the cell surface. C83 is cleaved by γ-secretase resulting in the formation of the fragments p3 and p7. Of note, production of sAPPα increases in response to electrical activity and activation of muscarinic acetyl choline receptors, suggesting that neuronal activity increases α-secretase cleavage of APP (Pascual, J., Fontan, A. et al., 1991). Another approach indicates that the treatment with acetyl cholinesterase inhibitors increases ADAM-10 activity by promoting its trafficking in neuroblastoma cell lines (Zimmermann, M., Gardoni, F. et al., 2004). sAPPα, in turn, is reported to be neuroprotective via its neurotrophic properties (Small, D. H., Nurcombe, V. et al., 1994; Furukawa, K., Sopher, B. L. et al., 1996). Biochemical, epidemiological, and genetic findings demonstrate a link between cholesterol levels, processing of APP and AD. The treatment of various peripheral and neuronal cell lines with either cholesterol-extracting agents or lovastatin resulted in a drastic increase of secreted α-secretase-cleaved sAPPα (Kojro, E., Gimpl, G. et al., 2001).

1.4.2 β-secretase

Altered proteolytic processing of APP by β-secretase resulting in the production and aggregation of neurotoxic forms of Aβ is central to several genetic forms of AD. The membrane-bound aspartyl protease BACE-1, β-site cleaving enzyme, has been identified as β-secretase encoded on chromosome 11 (Yan, R. Q., Bienkowski, M. J. et al., 1999; Vassar, R., Bennett, B. D. et al., 1999; Sinha, S., Anderson, J. P. et al., 1999). Tissue culture and animal studies indicate that β-secretase BACE-1 is expressed in all tissues with highest levels in neurons, and β-secretase cleavage occurs mainly in the plasma membrane, but also at the Golgi apparatus and ER. Three recent publications report increased β-secretase activity in sporadic AD (Holsinger, R. M. D., Mclean, C. A. et al., 2004; Yang, L. B., Lindholm, K. et al., 2003b; Li, R., Lindholm, K. et al., 2004c), but it needs to be confirmed whether BACE-1 over-expression is crucial in the pathogenic cascade of sporadic AD. Cleavage of APP by β-secretase (BACE) liberates sAPPβ into the extracellular space and leaves a C-terminal fragment (C99, CTFβ) bound to the membrane. C99 is internalised and further processed by γ-secretase to produce $A\beta_{1\text{-}40/42}$ in endocytic compartments (Evin, G., Zhu, A. Q. et al., 2003). Another isoform of BACE, BACE-2, shows α-secretase activity and is located on chromosome 21 (Bennett, B. D., Babu-Khan, S. et al., 2000). The enzyme is found in the Golgi apparatus and endosomes being highest expressed in neurons. The physiological role of BACE-2 is unknown, but most probably the enzyme has no key role in APP processing, and it is not considered to be a drug target for AD.

1.4.3 γ-secretase

1.4.3.1 Presenilins

Recent studies strongly suggest the possible pathogenic interactions between APP and presenilins implicating a potential role for presenilins in γ-secretase cleavage. It is supposed that high molecular complexes of presenilins together with anterior pharynx defective (APH), nicastrin and presenilin enhancer 2 (PEN-2) exhibit γ-secretase activity under physiological conditions (Capell, A., Beher, D. et al., 2005). PS-1 and PS-2 are two homologous membrane-spanning proteins with two aspartyl residues in the transmembrane domains 6 and 7 potentially being responsible for the protease-like activity. It seems at least possible that presenilins are involved in γ-secretase cleavage since numerous missense mutations of presenilins are associated with the autosomal dominant form of familial AD (Fraser, P. E., Yang, D. S. et al., 2000; Chen, F. S., Yang, D. S. et al., 2000; Menendez, M., 2004; Finckh, U., Kuschel, C. et al., 2005). Reduced γ-secretase activity was observed in neuronal cultures of PS-1 knockout mice. Of note, knockout of the γ-secretase component PS-1 caused a lethal phenotype indicating that γ-secretase cleavage of Notch-1 must be of fundamental importance during embryonic development (Huppert, S. S., Ilagan, M. X. G. et al., 2005; Doerfler, P., Shearman, M. S. et al., 2001; Selkoe, D., Kopan, R., 2003).

1.4.3.2 γ-secretase cleavage

Expression of the four proteins APH, nicastrin, presenilin and PEN-2 in yeast lead to reconstituted γ-secretase activity (Fig. 1.7) (Edbauer, D., Winkler, E. et al., 2003). The entire biology of the enzyme is yet not fully understood, as the reconstitution from purified components has not yet been accomplished, and the structure of the active site remains unknown. As mentioned above, C83 and C99 are substrates of γ-secretase: C83 cleavage results in the formation of p7 and p3, C99 cleavage results in the formation of various Aβ isoforms and AICD, APP intracellular domain, a fragment that translocates to the nucleus where it may regulate gene expression, including the induction of apoptotic genes (Leissring, M. A., Murphy, M. P. et al., 2002). It has been reported that AICD is neurotoxic by increasing the expression of GSK-3β (Kim, H. S., Kim, E. M. et al., 2003). The identification of the γ-secretase components and the generation of gene-targeted models quickly lead to the identification of γ-secretase substrates other than APP, including the notch receptor 1 (Notch-1), the Notch ligands delta-like protein 1 (Delta-1) and Jagged 2 (Jag-2), v-erb-a erythroblastic leukaemia viral oncogene homologue 4 (ErbB-4) and others (Ni, C. Y., Murphy, M. P. et al., 2001) were rapidly found.

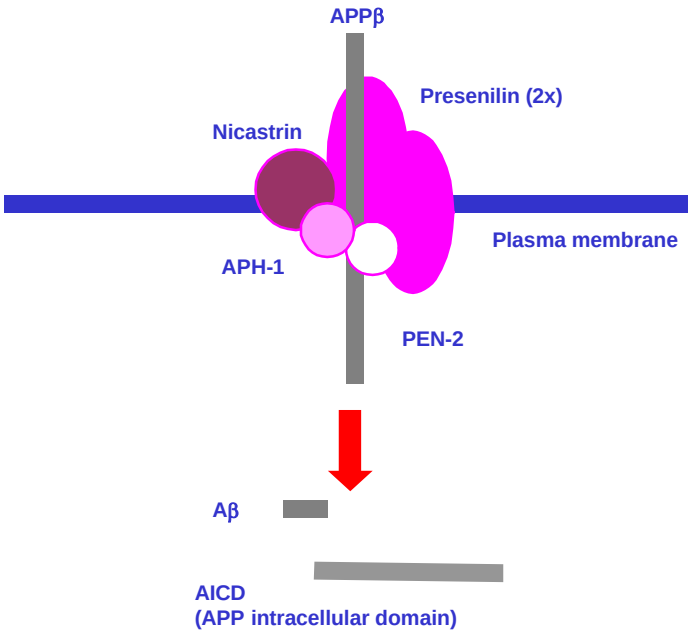

Figure 1.7: γ-secretase complex.

1.4.4 Amyloid β

Recent data suggest that intracellular or extracellular accumulation and oligomerization of Aβ plays a central role in the pathogenesis of the disease, as previous findings suggest that Aβ is a neurotoxic side product of APP processing (Canevari, L., Abramov, A. Y. et al., 2004; Hardy, J., Allsop, D., 1991). Generally, Aβ consists of 39-43 amino acids, whereas the $A\beta_{1-40}$ and $A\beta_{1-42}$ forms are the most prominent ones. Under physiological conditions, $A\beta_{1-42}$ makes up only 10% of the total brain Aβ, but most of the gene mutations that segregate with the inherited forms of AD result in increasing the ratio of $A\beta_{1-42}/A\beta_{1-40}$ production, and $A\beta_{1-42}$ also accumulates in neurons of AD brains (Iwatsubo, T., Odaka, A. et al., 1994). This is very interesting since it has been reported that $A\beta_{1-40}$ and $A\beta_{1-42}$ are produced in distinct subcellular compartments: $A\beta_{1-40}$ is processed in the trans-Golgi, $A\beta_{1-42}$ is from the ER (Hartmann, T., Bieger, S. C. et al., 1997). New data strongly suggest that the production and initial accumulation of Aβ takes place intracellularly in neurons of human brains or of transgenic mice (Oddo, S., Caccamo, A. et al., 2003b). Kienlen-Campard et al. demonstrated that although APP processing leads to production of extracellular $A\beta_{1-40}$ and soluble APP in rat cortical neurons, these extracellular derivatives do not induce neuronal death (Kienlen-Campard, P., Miolet, S. et al., 2002). In their setting, neurons undergo apoptosis as soon as they accumulate intracellular $A\beta_{1-42}$ following the expression of full-length APP or a C-terminally deleted APP isoform. Aβ is a hydrophobic peptide being able to aggregate and to form oligomers. Aβ oligomers evolve over time, whereas spherical oligomer formation precedes the formation of the curvilinear strings or protofibrils (Harper, J. D., Wong, S. S. et al., 1999; Kayed, R., Head, E. et al., 2003). Then, chains of clusters called fibrils together with β-sheeted structures form the end-stage of “plaques”. Recent reports suggest that the toxicity of Aβ does not lie in the insoluble fibrils that accumulate but rather in the soluble oligomeric intermediates, as their amount correlates best with the progress of the disease monitored by cognitive function (Mclean, C. A., Cherny, R. A. et al., 1999; Lue, L. F., Kuo, Y. M. et al., 1999). These soluble oligomers include spherical particles and curvilinear structures called “protofibrils” that appear to represent strings of the spherical particles (Hartley, D. M.,

Walsh, D. M. et al., 1999). These neurotoxic Aβ species comprise small diffusible Aβ oligomers (referred to as ADDLs, for Aβ-derived diffusible ligands), which were found to kill mature neurons in organotypic central nervous system cultures at nanomolar concentrations. At cell surfaces, ADDLs bound to trypsin-sensitive sites and surface-derived tryptic peptides blocked binding and afforded neuroprotection. Remarkably, neurological dysfunction evoked by ADDLs occurred well in advance of cellular degeneration. Without lag, and despite retention of evoked action potentials, ADDLs inhibited hippocampal long-term potentiation, indicating an immediate impact on signal transduction. Impaired synaptic plasticity and associated memory dysfunction during early stage of AD and severe cellular degeneration during the end stage could be caused by the biphasic impact of ADDLs acting upon particular neural signal transduction pathways (Lambert, M. P., Barlow, A. K. et al., 1998).

1.5 Molecular mechanisms of cell death

1.5.1 Necrosis

Necrosis can occur in response to various cytotoxic stimuli, like acute ischemia, exposure to excitotoxins or mechanical injury of the cell membrane. Necrosis is highly unregulated- no active, energy-consuming processes are involved. The histological features of necrotic cell death are mitochondrial and nuclear swelling, dissolution of organelles, and condensation of chromatin around the nucleus (Fig. 1.8). The rupture of nuclear and cytoplasmic membranes follows these events. Degradation of DNA by random enzymatic cuts in the molecule begins.

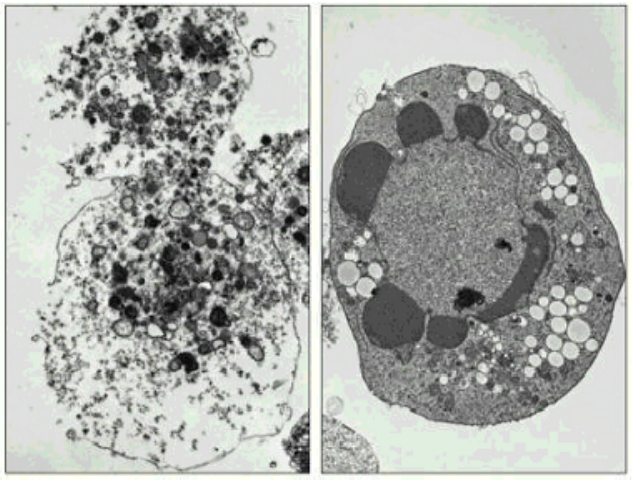

Figure 1.8: Necrotic (*left*) and apoptotic cell (*right*).
(taken from The Cell, 2004).

1.5.2 Apoptosis (Programmed cell death)

The mitochondrial matrix contains numerous enzymes, including those that convert pyruvate and fatty acids to acetyl CoA and those of the citric acid cycle that oxidize this acetyl CoA to CO_2. The energy available from combining molecular oxygen with the reactive electrons carried by NADH and $FADH_2$ is harnessed by an electron-transport chain (ETC) in the inner mitochondrial membrane. Protons are pumped out of the matrix to create a transmembrane electrochemical proton gradient, which includes contributions from both a membrane potential and a pH difference. The large amount of free energy released when protons flow back across the inner membrane provides the basis for ATP production in the matrix by the ATP synthase (F_1F_0 ATPase). The transmembrane electrochemical gradient is also used to drive the active transport of selected metabolites across the mitochondrial inner

membrane, including an ATP-ADP exchange between mitochondria and the cytosol. The resulting high ratio of ATP to its hydrolysis products favours ATP hydrolysis, allowing to drive a large number of the cell's energy-requiring processes. In addition to their function as major energy-providing organelles of the cell, they accomplish a crucial role in apoptosis (Gulbins, E., Dreschers, S. et al., 2003; Gottlieb, R. A., Granville, D. J., 2002). Mitochondria harbour several death factors that are released upon apoptotic stimuli. Alterations in mitochondrial functions, increased oxidative stress and apoptosis have been observed in various diseases. Mitochondria may thus trigger the abnormal onset of cell death by at least three general mechanisms, and most probably their effects are interrelated: (i) disruption of electron transport, oxidative phosphorylation, ATP production, (ii) alteration of cellular redox potential, (iii) release of proteins that trigger activation of caspase family proteases, (Fig. 1.9).

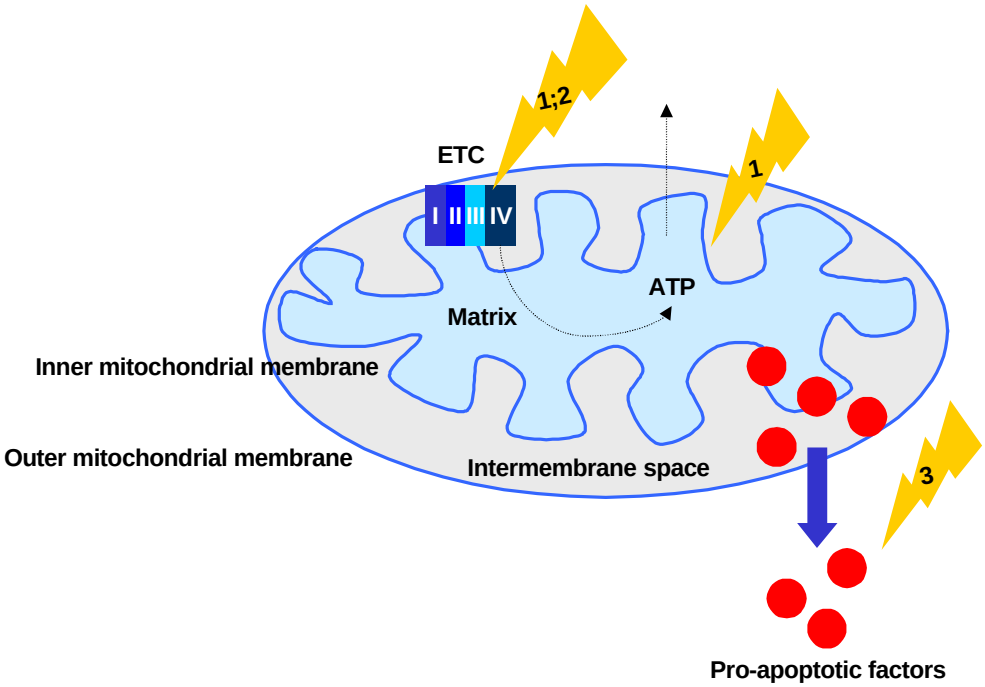

Figure 1.9: Mitochondria- executioners of cell death.

At least three mechanisms lead to mitochondrial disturbances in apoptosis: 1. Disruption of electron transport (ETC= electron transport chain), 2. Alteration of cellular redox-potential, 3. Release of pro-apoptotic factors.

Extrinsic and intrinsic pathways can initiate programmed cell death, apoptosis (Fig. 1.10). The extrinsic pathway plays an important role in immune response and tumour suppression (Reed, J. C., 2000). The intrinsic pathway can be initiated by various stimuli like reactive oxygen species, ER stress, UV radiation, cytotoxic substances, and growth factor withdrawal (Newmeyer, D. D., Ferguson-Miller, S., 2003; Rudner, J., Jendrossek, V. et al., 2002).The mechanism can further be dissected into: (i) the initiation and signalling phase, (ii) the signal amplification phase and (iii), the execution phase. Apoptosis involves changes in the cytoplasm, ER, mitochondria and nucleus as well as the activation of a group of cysteinases called caspases that cleave various protein substrates to sculpt morphological and biochemical aspects of the cell death process. Some groups propose a model for initiation of apoptosis in which mitochondria and caspases engage in a self-amplifying pathway of mutual activation (Green, D., Kroemer, G., 1998; Nicholson, D. W., 2001). In most of the apoptotic scenarios, impairment of mitochondrial function is an early event. In the final phase of apoptosis, executioner caspases (caspase-9 and -3) are activated. More specifically, caspases have a dual function in the apoptotic process: first, as signal transduction molecules that act as facultative inducers of mitochondrial membrane changes (initiator/signaling caspases), and second, as processing enzymes that orchestrate the apoptotic phenotype (executioner caspases). In the last years, investigative work aimed at the inhibition of caspases to prevent apoptosis (Arnoult, D., Karbowski, M. et al., 2003; Riedout, H. J., Stefanis, L., 2001). But recently, it has become clear that inhibition of caspases does not always prevent irreversible loss of cellular function, although it does prevent the acquisition of typical apoptotic morphology.

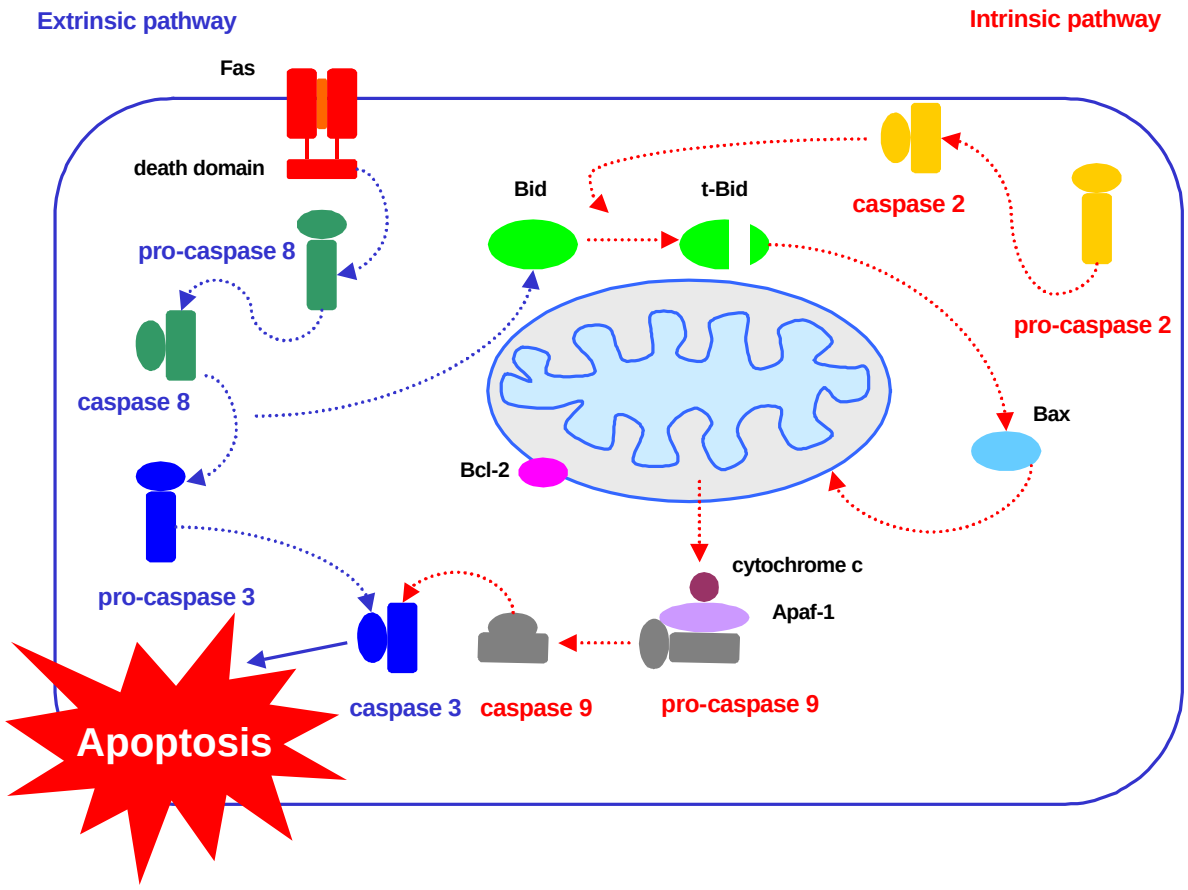

Figure 1.10: Extrinsic and intrinsic signaling pathways of apoptosis.
Caspase-8 is the most important signaling caspase in the extrinsic pathway (*blue arrows*) activating the executioner caspase-3 that finally leads to apoptosis. Caspase-2 is involved in the intrinsic apoptotic pathway (*red arrows*), initiating the cleavage of Bid to t-Bid which in turn promotes the insertion of Bax into the outer mitochondrial membrane. In the next step, pro-apoptotic factors, such as cytochrome c, are released into the cytosol. Cytochrome c forms the apoptosome together with Apaf-1, cleaving procaspase-9. Again, it comes to an activation of caspase-3 by caspase-9.

1.5.2.1 Members of the Bcl-2 family

Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis (Adams, J. M., Cory, S., 1998). Those most similar to Bcl-2 (Bcl-2, Bcl-xL) promote cell survival by inhibiting adapters needed for activation of caspases that dismantle the cell (Fig. 1.11). More distant relatives, like Bax instead promote apoptosis apparently through displacing the adapters from the pro-survival proteins (Reed, J. C., 1997; Borner, C., 2003; Festjens, N., van Gurp, M. et al., 2003).

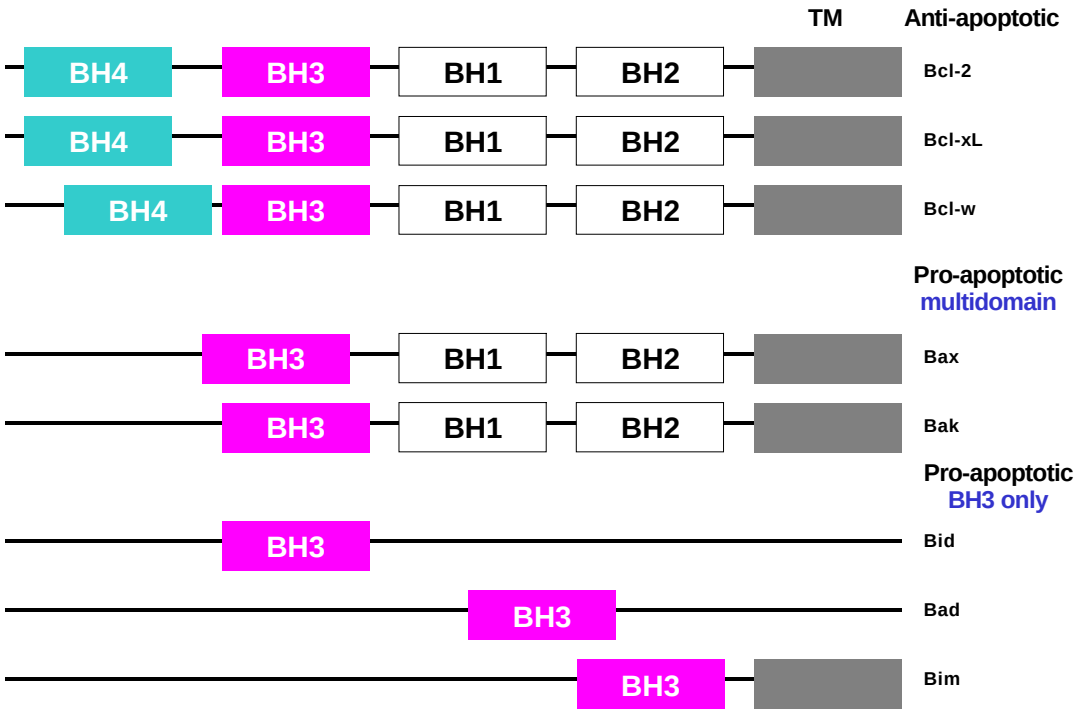

Figure 1.11: Pro- and anti-apoptotic members of the Bcl-2 family.

Pro- and anti-apoptotic members of the family differ in the numbers and orientation of their domains. The anti-apoptotic members (Bcl-2, Bcl-xL, Bcl-w) are characterized by the domains BH1-4 and a trans-membrane domain (TM). Pro-apoptotic members can be sub-divided in multi-domain (Bax, Bak) and BH3-only (Bid, Bad, Bim) proteins (taken from Borner et al., 2003, modified).

Pro-apoptotic members of the Bcl-2 family (Bid, Bax) are originally located in the cytosol. Bid is activated when cleaved by caspase- 2 to truncated Bid (t-Bid) (Li, H. L., Zhu, H. et al., 1998). Following binding to the BH3-domain-only pro-apoptotic protein t-Bid, Bax oligomerizes and then integrates in the outer mitochondrial membrane, where it triggers cytochrome c release (Fig. 1.12). Bax mitochondrial membrane insertion triggered by Bid-cleavage represents a key step in pathways leading to apoptosis (Eskes, R., Desagher, S. et al., 2000; Sharpe, J. C., Arnoult, D. et al., 2004). In many types of apoptosis, the pro-apoptotic protein Bax additionally undergoes a change in conformation at the level of the mitochondria (Wei, M. C., Zong, W. X. et al., 2001). Today it is unclear how Bax promotes the release of cytochrome c. It might be that Bax oligomers induce the opening of the permeability transition pore (PTP) in the outer mitochodrial membrane, a composite proteaceous channel that is involved in the regulation of mitochondrial membrane permeability, by which pro-apoptotic factors might be released: Bax binds to PTP, and PTP opening

induced by Bax results in the relase of cytochrome c into the cytosol (Marzo, I., Brenner, C. et al., 1998).

In contrast to this, it was also shown that Bax alone is sufficient to trigger the release of cytochrome c from isolated mitochondria and that this process cannot be blocked by PTP inhibitors. This is corroborated by the other studies investigating whether Bax promotes cell death directly through its putative function as a channel protein versus indirectly by inhibiting caspases (Jurgensmeier, J. M., Xie, Z. et al., 1998). In this experimental setting, Bax did not induce swelling of mitochondria in vitro unlike Ca^{2+}, a classical inducer of mitochondrial permeability transition. The organelle swelling caused by permeability transition (inducer: Ca^{2+}) causes a non-selective outer membrane rupture. These findings dissociate these two events, implying that Bax uses an alternative mechanism independent from Ca^{2+} for triggering release of cytochrome c from mitochondria. Concluding, these results might speak for the existence of at least two distinct mechanisms leading to cytochrome c release. Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL) are located in the cytosolic compartment and in the outer mitochondrial membrane (Susin, S. A., Zamzami, N. et al., 1996). Their mode of action is very diverse, but Bcl-2 is known to not only inhibit the release of cytochrome c, but also of other mitochondrial pro-apoptotic factors. Bcl-xL in turn blocks the formation of the apoptosome (cytochrome c, Apaf-1 and procaspase-9 complex) and modulates Ca^{2+} levels of the endoplasmatic reticulum (ER). It is possible that Bcl-2-family proteins can influence the levels of releasable Ca^{2+} in the ER, and thus determine whether the released Ca^{2+} is sufficient to overload mitochondria and induce cell death (Kuwana, T., Newmeyer, D. D., 2003).

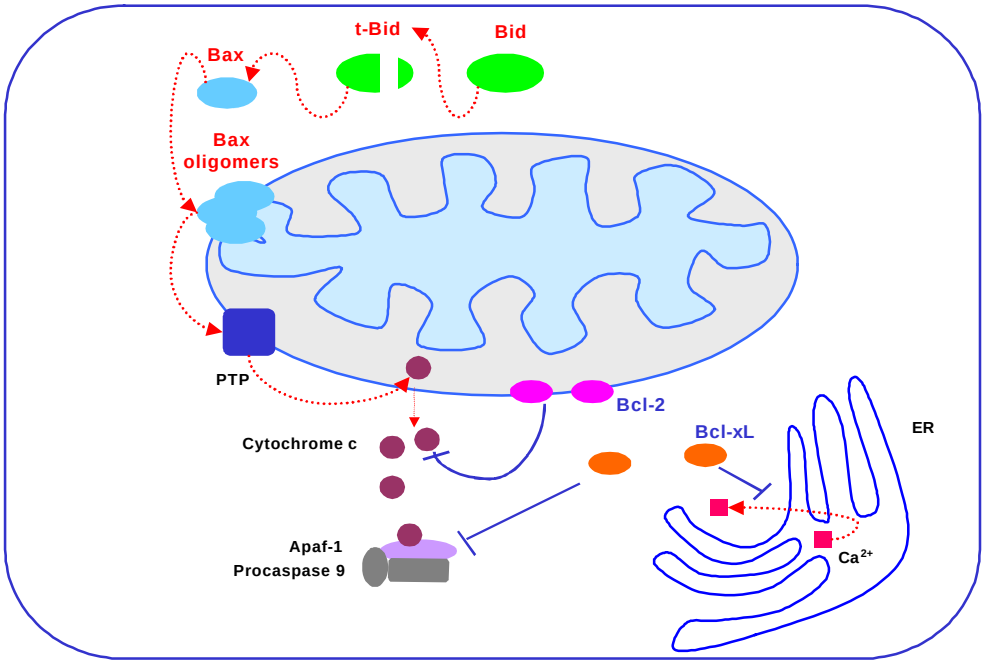

Figure 1.12: Pro- and anti-apoptotic actions of Bcl-2 proteins.

Pro-apoptotic Bcl-2 proteins (*red*) are located downstream mitochondria in the apoptotic pathway. Truncated Bid (t-Bid) induces oligomerization of Bax into the outer mitochondrial membrane. Bax oligomers trigger PTP opening leading to the release of cytochrome c. Anti-apoptotic Bcl-2 proteins (*blue*) can block the release of cytochrome c and the formation of the apoptosome (Apaf-1, procaspase-9 and cytochrome c complex). Additionally, Bcl-xL inhibits the release of Ca^{2+} from the endoplasmatic reticulum (ER).

1.5.2.2 Pro-apoptotic mitochondrial proteins

Mitochondria exert their important role during cell death via the release of pro-apoptotic proteins participating in the degradation phase of apoptosis: Oxidative stress induced by various stimuli results in the release of cytochrome c into the cytoplasm from the mitochondrial intermembrane space where it initiates the caspase-dependent apoptosis cascade (Fig. 1.13) (Newmeyer, D. D., Ferguson-Miller, S., 2003). It is noteworthy that the main part of cytochrome c remains in the mitochondria bound to the electron transport chain (ETC). An essential role of cytosolic cytochrome c in the execution of apoptosis was demonstrated by its direct microinjection into the cytosol, thus bypassing the need for cytochrome c release from the mitochondrial intermembrane space. Microinjection of cytochrome c caused caspase-dependent apoptosis (Takeyama, N., Miki, S. et al., 2002). First, cytochrome c binds to Apaf-1, a mammalian homologue of Ced-4, and procaspase-9 to form the apoptosome which then activates caspase-3 finally triggering apoptosis (Yoshida, H., 2003; Henshall, D. C., Bonislawski, D. P. et al., 2001). The collapse of the mitochondrial membrane along with the release of cytochrome c from mitochondria is followed by the activation of caspases, nuclear fragmentation and cell death (Smaili, S. S., Hsu, Y. T. et al., 2003). The temporal and causal relationship of the release of Smac/DIABLO (in short named Smac), second mitochondrial activator of caspase, compared to cytochrome c was examined by several groups (Rehm, M., Dussmann, H. et al., 2003). The average duration of Smac/DIABLO release is greater than that of cytochrome c. However, there is no significant difference in the time to the onset of release, and both cytochrome c and Smac/DIABLO release coincided with mitochondrial membrane potential depolarization. When Smac/DIABLO is released into the cytosol, it blocks inhibitor of apoptosis proteins (IAPs) (Adrain, C., Creagh, E. M. et al., 2001). IAP, belonging to the anti-apoptotic cytosolic factors, inhibits caspase-9 activation. Over-expression of Smac/DIABLO increases cells sensitivity to apoptotic stimuli (Du, C., Fang, M. et al., 2000). Neutralization of Smac/DIABLO by inhibitors of apoptosis (IAPs) is a caspase-independent mechanism for apoptotic inhibition potentially offering a new therapeutic strategy in apoptosis-mediated diseases (Wilkinson, J. C., Wilkinson, A. S. et al., 2004), while in turn inhibition of IAP's protection by Smac/DIABLO is discussed as a new therapeutic opportunity in

cancer therapy (MacKenzie, A., LaCasse, E., 2000). The third factor being co-released with cytochrome c and Smac/DIABLO is HtrA2/Omi, a caspase-dependent pro-apoptotic protein. Mature HtrA2/Omi is released together with mature Smac/DIABLO from the mitochondria into the cytosol upon disruption of the outer mitochondrial membrane during apoptosis. Mature HtrA2/Omi can induce apoptosis in human cells in a caspase-independent manner through its protease activity and in a caspase-dependent manner (Hegde, R., Srinivasula, S. M. et al., 2002). HtrA2/Omi is largely membrane-associated in healthy cells, with a significant proportion observed within the mitochondria. Once shifted into the cytosol, HtrA2/Omi can interact with IAPs, preventing IAP inhibition of active caspase-3 (Verhagen, A. M., Silke, J. et al., 2002).

The structure of AIF has been identified as a flavoprotein of relative molecular mass 57.000 that shares homology with the bacterial oxidoreductases. The functions of apoptosis inducing factor (AIF), a phylogenetically ancient mitochondrial intermembrane flavoprotein, appear to be much more diverse (Lorenzo, H. K., Susin, S. A. et al., 1999). Under physiological circumstances, AIF is located behind the outer mitochondrial membrane. In response to apoptotic stimuli, AIF translocates to the cytosol and then to the nucleus (Plesnila, N., Zhu, C. L. et al., 2004).

Endowed with the unique capacity to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation when translocated to the nucleus, AIF is essential for programmed cell death during cavitation of embryoid bodies, the very first wave of (caspase-independent) cell death indispensable for morphogenesis (Cande, C., Cohen, I. et al., 2002). This has been confirmed by the finding that a wide-ranging caspase inhibitor prevents none of the AIF effects. Paradoxically, in a mouse mutant with progressive cerebellar and retinal degeneration, the down-regulation of AIF expression lead to apoptosis of neurons in association with an imbalance in free radical metabolism and cell cycle re-entry (Bonni, A., 2003). In addition to its apoptogenic activity on nuclei, AIF participates in the regulation of apoptotic mitochondrial membrane permeabilization and exhibits an NADH oxidase activity. Overexpression of Bcl-2, which controls the opening of mitochondrial permeability transition pores, prevents the release of AIF from the mitochondrion but does not affect its apoptogenic activity (Susin, S. A., Lorenzo, H. K. et al., 1999a).

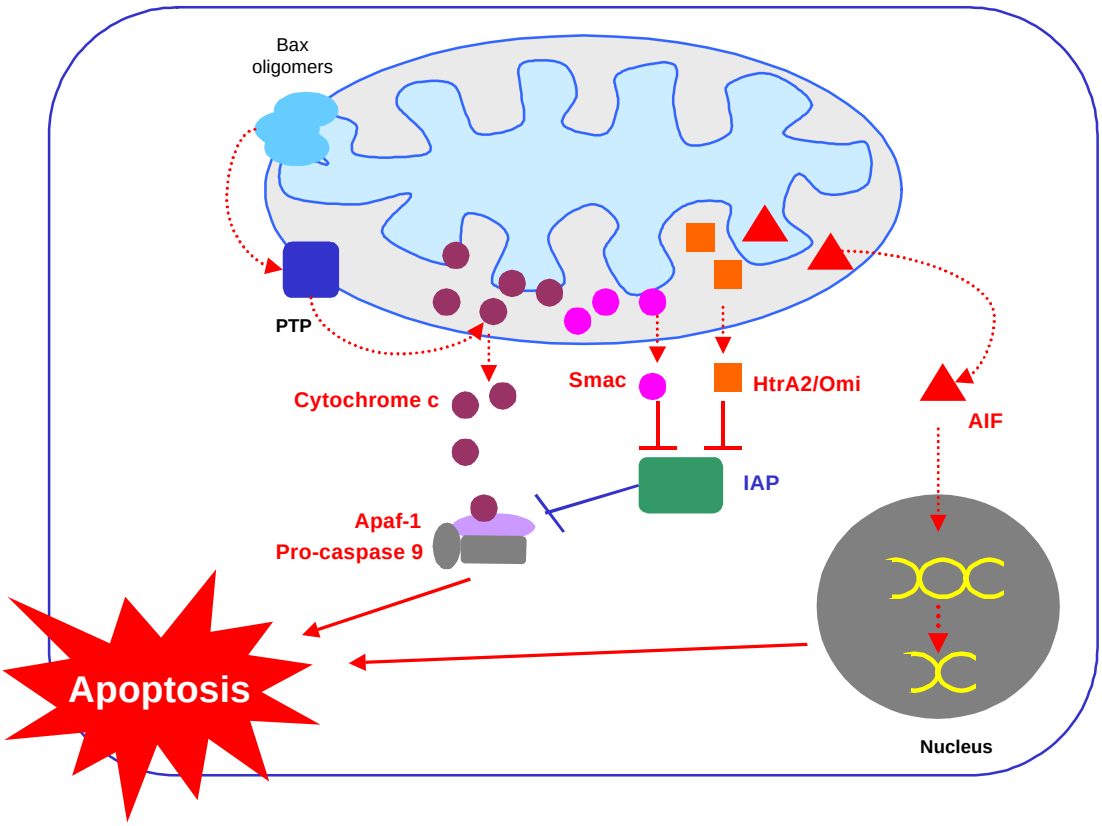

Figure 1.13: Pro-apoptotic mitochondrial proteins.

Apoptosis can be executed in a caspase-dependent or independent way. Cytochrome c, Smac/DIABLO and HtrA2/Omi release directly or indirectly leads to the activation of caspases. In contrast to this, AIF induces nuclear condensation and chromatin fragmentation (*red*: pro-apoptotic factors/actions, *blue*: anti-apoptotic factors/ actions).

1.5.3 Apoptosis and Alzheimer's disease

Over the past 10 years, mitochondrial defects have been implicated in a wide variety of degenerative diseases, aging, and cancer. A growing body of data suggests that apoptosis under mitochondrial control is implicated in neuronal death processes (Ghribi, O., DeWitt, D. A. et al., 2001). The essential serine protease HtrA2/Omi is discussed to be involved in Aβ neurotoxicity in AD as it interacts with Aβ in yeast two-hybrid assays and cell culture experiments (Park, H. J., Seong, Y. M. et al., 2004). FAD mutations in presenilins render neurons vulnerable to apoptosis induced by Aβ, trophic factor deprivation and other stimuli, suggesting that PS-1 in mitochondria is involved by regulating HtrA2/Omi activity (Mattson, M. P., Chan, S. L., 2003;

Popescu, B. O., Ankarcrona, M., 2004; Ankarcrona, M., Hultenby, K., 2002; Gupta, S., Singh, R. et al., 2004). APP mutations are ample to trigger apoptosis in cultured cells (McPhie, D. L., Lee, R. K. K. et al., 1997). It has been shown in our own group that the APPsw mutation increases vulnerability of PC12 cells against oxidative stress (Eckert, A., Steiner, B. et al., 2001; Leutz, S., Steiner, B. et al., 2002; Marques, C. A., Keil, U. et al., 2003; Keil, U., Bonert, A. et al., 2004; Marques, C. A., Keil, U. et al., 2003; Marques, C. A., Keil, U. et al., 2003). Neurotoxic forms of Aβ may be a trigger of apoptosis in AD because pro-apoptotic proteins are associated with Aβ deposits in the brains of AD patients and Aβ can induce apoptosis of cultured neurons. Aβ causes increased oxidative stress in cell culture as could be shown by the induction of NF-κB, a transcription factor thought to be regulated by oxidative stress (Behl, C., Davis, J. B. et al., 1994b). Oxidative stress-mediated neurotoxicity of Aβ finally results in mitochondrial damage (Pike, C. J., RamezanArab, N. et al., 1997). Aβ inhibited cytochrome c oxidase (COX-4) activity in isolated brain mitochondria (Canevari, L., Clark, J. B. et al., 1999). Furthermore, a selective defect in cytochrome c oxidase has been observed in brains of AD patients (Maurer, I., Zierz, S. et al., 2000). Activity changes in mitochondrial enzymes including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase have been described in AD brain (Gibson, G. E., Zhang, H. et al., 1998; Casley, C. S., Canevari, L. et al., 2002b). Aβ-binding alcohol dehydrogenase (ABAD) might be a direct molecular link from Aβ to mitochondrial toxicity (Fig. 1.14). Aβ interacts with ABAD in the mitochondria of AD patients and transgenic mice. The crystal structure of Aβ-bound ABAD shows substantial deformation of the active site that prevents nicotinamide adenine dinucleotide (NAD) binding. An ABAD peptide specifically inhibits ABAD-Aβ interaction and suppresses Aβ-induced apoptosis and free-radical generation in neurons (Lustbader, J. W., Cirilli, M. et al., 2004).

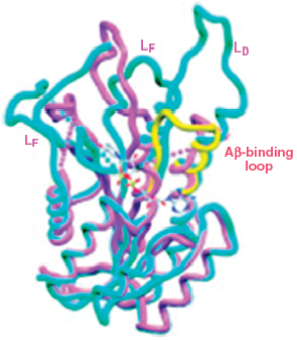

Figure 1.14: Structure of Aβ-bound ABAD.

Superposition of Aβ-bound human ABAD (*magenta*) and rat ABAD in complex with NAD (*cyan*) (taken from Lustbader, 2004).

Endoplasmic reticulum-associated Aβ-binding protein (ERAB), L-3-hydroxyacyl-CoA dehydrogenase type II (HADH II), is expressed at high levels in Alzheimer's disease (AD)-affected brain. It was reported that L-3-hydroxyacyl-coenzyme A dehydrogenase type II (ERAB) activity is implicated in Aβ-induced cytotoxicity (Du Yan, S., Shi, Y. G. et al., 1999).

Studies on patients with these diseases have revealed much about the complexities of mitochondrial genetics, which involves interplay between mutations in the mitochondrial and nuclear genomes. However, the pathophysiology of mitochondrial diseases has remained perplexing (Wallace, D. C., Shoffner, J. M. et al., 1995). Evidence that many neurons undergo apoptosis in AD includes the presence of high levels of activated apoptotic proteins such as caspase-3 and Bax in neurons that exhibit neurofibrillary tangle pathology (Engidawork, E., Gulesserian, T. et al., 2001a). In addition, DNA-damage and up-regulation of the pro-apoptotic proteins p53 and Bax occur in vulnerable neuronal populations at a relatively early stage in the disease process (Gomez-Lazaro, M., Fernandez-Gomez, F. J. et al., 2004; Culmsee, C., Mattson, M. P., 2005). The interaction of Aβ with mitochondrial factors might give rise to new targeting sites for the development of new drugs against neurodegenerative disorders. Not only a wide variety of apoptotic, but also of necrotic stimuli disrupt mitochondrial physiology in Aβ-mediated toxicity (Behl, C., Davis, J. B. et al., 1994a). In coherence with this, data of our own group support the fact that necrosis and apoptosis do not necessarily exclude each other (Marques, C. A., Keil,

U. et al., 2003). In frontal cortex and cerebellum of AD subjects, protein levels of caspase-3, -8, and -9, DFF45 (DNA fragmentation factor 45), and FLIP (Fas associated death domain (FADD)-like interleukin-1β-converting enzyme inhibitory proteins) were decreased, while those of ARC (apoptosis repressor with caspase recruitment domain) and RICK (Receptor interacting protein (RIP)-like interacting CLARP kinase) were increased. In contrast, cytochrome c and Apaf-1 were unchanged (Engidawork, E., Gulesserian, T. et al., 2001b). The current findings showed that dysregulation of apoptotic proteins indeed exist in AD brain and support the notion that it may contribute to neuropathology of AD. Hence, therapeutic strategies that ablate caspase activation may be of some benefit for AD sufferers.

1.5.4 Neurotrophic factors

The neurotrophins are a family of structurally and functionally related small basic proteins including nerve growth factor (NGF). All neurotrophins share a low affinity receptor, p75 ^{NTR}, but they transduct their specific actions mainly by interacting with the tyrosine kinase (Trk) family of transmembrane receptors including three subtypes, Trk A, B, and C. NGF preferably interacts with TrkA (Fig. 1.15) (Grimes, M. L., Zhou, J. et al., 1996). NGF, which is secreted by the target and interacts with receptors on the axon tip, regulates survival, differentiation, and maintenance of responsive neurons. Yet it is uncertain how the NGF signal is communicated retrogradely from distal axons to neuron cell bodies.

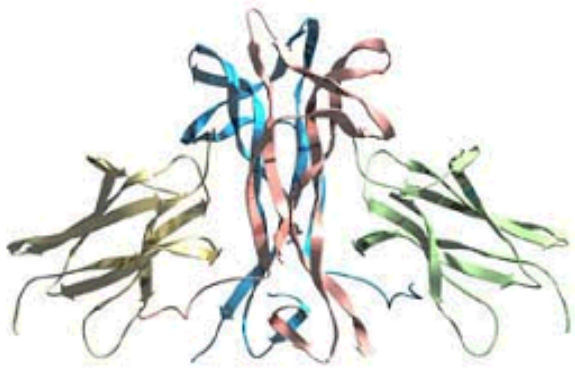

Figure 1.15: Structure of homodimeric form of human NGF in complex with domain 5 of the Trk receptor. (taken from The Cell, 2004)

Most probably, retrograde transport of activated receptors in endocytic vesicles could convey the signal. Some studies raise the possibility that NGF induces formation of signaling endosomes containing activated TrkA. NGF induces transcription-dependent neural differentiation, and the ERK family of MAPKs has been implicated as the dominant signal pathway that mediates this response. Constitutive active forms of c-Raf-1, MEKK1 and MKK6, proximal regulators of the ERKs, JNKs, and p38 MAPKs, respectively, were all stimulated by NGF. These findings indicate that the ERK and JNK pathways collaborate downstream of the NGF receptor. Besides the MAPK pathway the phosphoinositide 3-kinase (PI3K) pathway seems to play an important role (Fig. 1.16) (Jin, L., Hu, X. H. et al., 2005; Jones, D. M., Tucker, B. A. et al., 2003). PI3K activation stimulates Akt, which directly phosphorylates glycogen synthase kinase-3 (GSK-3) to switch off its catalytic activity. GSK-3 genes encode two closely related proteins, GSK-3α and GSK-3β, both of which are widely distributed in the brain. GSK-3β seems to play an important role in regulation of cell

proliferation and neurite outgrowth. Inactivation of GSK-3β by phosphorylation results in the activation of pathways that are normally repressed by GSK-3 (Doble, B. W., Woodgett, J. R., 2003). Very interestingly, GSK-3β also seems to play an important role in AD (Kaytor, M. D., Orr, H. T., 2002), being implicated in amyloid plaque formation associated with AD by influencing APP metabolism (Aplin, A. E., Gibb, G. M. et al., 1996).

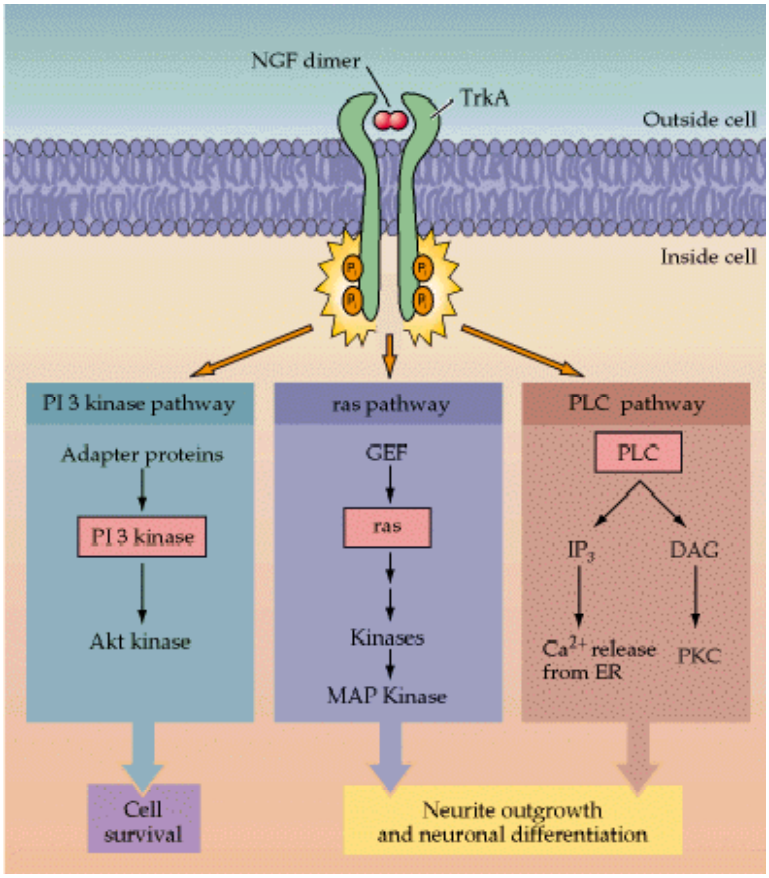

Figure 1.16: Mechanism of action of NGF.

NGF binds to a high-affinity tyrosine kinase receptor, TrkA, on the plasma membrane to induce phosphorylation of TrkA at two different tyrosine residues. These phosphorylated tyrosines serve to bind various adapter proteins or phospholipase C (PLC). The adapter proteins, in turn, activate three major signaling pathways: the PI3 kinase pathway leading to activation of Akt kinase, the ras pathway leading to activation of MAP kinases and the PLC pathway leading to release of intracellular Ca^{2+} and activation of PKC. The ras and PLC pathways primarily stimulate processes responsible for neuronal differentiation, while the PI3 kinase pathway is primarily involved in cell survival (taken from The Cell, 2004).

1.6 Current therapy

Today, there is no known treatment to cure AD, but there are many research programs running pursuing promising studies focused on halting or preventing the disease process (Carreiras, M. C., Marco, J. L., 2004; Breen, K. C., 2004; Cummings, J. L., 2004). The current medications aim on helping to control symptoms of the disease as well as on managing agitation, depression, or psychotic symptoms, such as hallucinations or delusions. In Germany, acetyl cholinesterase inhibitors and memantine are specifically approved for the treatment of AD, and several other anti-dementive drugs are on the market, amongst which standardized Ginkgo biloba extract (EGb 761) and Piracetam are the most important ones.

1.6.1 Acetyl cholinesterase inhibitors

The current standard of care for mild to moderate AD includes treatment with acetyl cholinesterase inhibitors to improve cognitive function. Aricept® (donezepil), Exelon® (rivastigmine) and Reminyl® (galantamine) belong to this group. It has been shown that people suffering from AD have low brain levels of acetyl choline due to a selective loss of cholinergic neurons (Davies, P., Maloney, A. J. F., 1976). Consequently, maintaining higher levels of the neurotransmitter in the postsynaptic area of intact neurons slows the progressive cognitive impairment. For Reminyl® (galantamine), besides its action via nicotinic receptors, neuro-protective effects have been reported after glucose- or oxygen-deprivation of rat hippocampal neurons (Sobrado, M., Roda, J. M. et al., 2004; Chadwick, K. J., Rylett, R. J., 2004).

1.6.2 NMDA antagonists

The NMDA receptor is possibly involved in long term potentiation of disturbed memory processes in dementia (Lipton, S. A., Rosenberg, P. A., 1994; Bading, H., Segal, M. M. et al., 1995). An NMDA (N-methyl-D-aspartate) antagonist, memantine, has been approved for the treatment of advanced AD (Molinuevo, J. L., 2003; Moebius, H. J., Wirth, Y., 2005).

In contrast to cholinesterase inhibitors being indicated for mild to moderate stages, memantine is approved for moderate and severe stages of the disease. The drug protects nerve cells against excess amounts of glutamate, a neurotransmitter whose release is probably increased in AD brain. Memantine is a drug considered to be neuro-protective. However, its benefit has been demonstrated in preclinical studies.

1.6.3 Ginkgo biloba extract

The Gingko biloba extract EGb 761 is a well-tolerated drug approved for the treatment of degenerative, vascular and mixed forms of dementia (Kleijnen, J., Knipschild, P., 1992; Kanowski, S., Herrmann, W. M. et al., 1996; Le Bars, P. L., Velasco, F. M. et al., 2002; Le Bars, P. L., Kastelan, J., 2000). It has been demonstrated by numerous studies and meta-analyses that EGb 761 is a prominent drug to treat AD (Andrieu, S., Gillette, S. et al., 2003). Evidence is provided that the extract EGb 761 owns antioxidant and neuroprotective properties. It was demonstrated by our group that apoptosis was reduced in vitro by the treatment with Ginkgo biloba extract EGb 761. Mice feeded with the extract for two weeks exhibited significantly reduced ROS-induced apoptosis. Notably, this effect is most prominent in old mice (Schindowski, K., Leutner, S. et al., 2001).

Additional data demonstrate that the extract reconstitutes mitochondrial membrane potential of PC12 cells, isolated brain mitochondria and dissociated brain cells after previous mitochondrial damage by sodium nitroprusside (Eckert, A., Keil, U. et al., 2005). Furthermore, the ATP production was reversed by the extract. These findings strongly support that protection of mitochondrial function is a very specific and sensitive property of the Gingko biloba extract EGb 761. Others found that Ginkgo biloba extracts exhibit anti-inflammatory, anti-oxidant and anti-coagulant properties and increase blood flow to the brain. Further studies emphasize that the benefit of the extract in the treatment of AD (Christen, Y., 2004; Kurz, A., Van Baelen, B., 2004). In addition, Ginkgo biloba extract revealed positive effects on neuronal plasticity of adult mice indicating a direct interaction with the glutamatergic system and raise interesting implications with respect to a mechanism explaining its effect on cognitive enhancement in human subjects experiencing dementia (Williams, B., Watanabe, C. M. H. et al., 2004).

1.6.4 Piracetam

Piracetam (2-oxo-1-pyrorolidine acteamide) is applied for the symptomatic therapy of cerebrovascular disturbances. Due to its nootropic properties (Giurgea, C. E., Greindl, M. G. et al., 1983), the substance improves cognitive functions without causing undesirable side effects such as sedation. Extensive investigative work has been performed in our group in order to demonstate that the nootropic substance piracetam recovers mitochondrial function in cell culture models and in dissociated brain cells of mice (Keil, U., Scherping, I. et al., 2006). These important findings further emphasize the importance of the reconstitution of mitochondrial function in the therapy of cerebrovascular disturbances in dementia. Mitochondrial stabilization and protection might be an important mechanism to explan many of piracetam´s beneficial effects in elderly patients.

1.7 Disease-modifying strategies

New drugs are wanted that interfere with the neurodegenerative mechanisms to stop neuronal cell death. As mentioned before, one strategic option could be the stabilization of mitochondrial membrane potential. Furthermore, modulation of APP and Aβ metabolism as well as the promotion of neurotrophic pathways are under investigation.

1.7.1 Statins

ApoE4, playing a regulatory role in the transport of cholesterol, is a known risk factor for developing AD (see chapter 1.1.6.2). Epidemiological studies demonstrated that statin therapy was associated with a decreased risk for the development of AD (Wolozin, B., Kellman, W. et al., 2000). Notably, statins positively regulated α-secretase processing of APP, resulting in elevated amounts of the neuro-protective α- APP (Kojro, E., Gimpl, G. et al., 2001; Fassbender, K., Masters, C. et al., 2001). Since many studies suggest a strong link between cholesterol and Alzheimer´s disease, studies were reviewed by our own group that have examined cholesterol levels in AD patients and control subjects. Of note, elevated cholesterol levels were not identified as a risk factor for AD. Thus, alternative hypotheses have to be

discussed, including cholesterol domains, e.g. (Eckert, G. P., Wood, W. G. et al., 2005). This is underlined by our recent finding that statins have pleiotropic effects in addition to lower cholesterol levels, and that they may act on different pathways involving distinct gene expression patterns (Johnson-Anuna, L. N., Eckert, G. P. et al., 2005). Interestingly, simvastatin had the greatest influence on gene expression as it modified the expression of 23 genes, amongst which some genes were identified of being involved in apoptotic pathways. Currently, prospective clinical trials are underway to investigate the effect of statins on Aβ production.

1.7.2 NSAIDS

Some studies suggested that non-steroidal anti-inflammatory drugs (NSAIDs) may lower the risk of developing AD and slow its progression (Bradbury, J., 2004; Tuppo, E. E., Arias, H. R., 2005). As NSAIDs help by reducing inflammation, it is supposed that they might also slow the inflammatory response occurring during the progression of AD. It is well known that the treatment with NSAIDs is strongly associated with the risk factor liver and kidney damage as well as gastrointestinal bleeding. A trial called the Alzheimer's disease prevention trial (ADAPT) sponsored by the National Institute of Aging (NIA), a part of the National Health Institute (NIH), was designed to assess the potential benefit of long-term use of non-NSAIDs in decreasing the risk of developing AD. The trial was stopped due to an apparent increase in cardiovascular and cerebrovascular events among the participants taking naproxen when compared with those on placebo. Thus, further studies are needed before general recommendations can be made concerning the use of NSAIDs.

1.7.3 β-secretase inhibitors

In the last years, β-secretase inhibitors were designed to block Aβ production and to explore whether β-secretase may be a potential target for new AD therapeutics. The transmembrane domain is a new feature of a mammalian aspartic protease with its active site on the lumenal side of the membrane where β-secretase cleaves APP. Three groups, reporting that β-secretase-knockout mice are deficient in Aβ production, independently provided the in-vivo validation of BACE-1 as β-secretase. This was not unexpected but it additionally demonstrated that there is no compensatory mechanism for β-secretase cleavage in mice (Luo, Y., Bolon, B. et al., 2001; Cai, H. B., Wen, H. J. et al., 2004; Roberds, S. L., Anderson, J. et al., 2001), an important predisposition for inhibitor development. Theoretically, there should be BACE-1 substrates other than APP, and two candidates were identified recently (Kitazume, S., Nakagawa, K. et al., 2004; Lichtenthaler, S. F., Schobel, S. et al., 2004), but the most important and unexpected finding of the knockout studies was the lack of serious vital problems due to β-secretase ablation. A detailed analysis demonstrated that the knockout mice are normal in terms of gross morphology and anatomy, tissue histology, haematology and clinical chemistry and also on gene expression levels and phenotypic assessment of older BACE-1-knockout mice. BACE-1-knockout over-expressing APP were engineered which did not develop any Aβ deposits over life time supporting the fact that there is no alternative mechanism for APP cleavage that could drive plaque formation (Luo, Y., Bolon, B. et al., 2001). Also, no obvious deficits in basal neurological and physiological functions were observed by behavioural analysis of the knockout mice (Roberds, S. L., Anderson, J. et al., 2001). Concluding, the absence of Aβ production and distinct pathology in the BACE-1-knockout mice is encouraging for β-secretase drug development. Today, the therapeutical use of β-secretase inhibitors is still restricted due to the unavoidable toxic side effects. But it seems at least probable that in future times, promising candidates will be developed since potent and specific peptidic inhibitors are identified for aspartic proteases. Nevertheless, the generation of small molecules with the desired pharmacokinetic properties remains problematic (Middendorp, O., Ortler, C. et al., 2004; Polgar, T., Keseru, G. M., 2005; Stachel, S. J., Coburn, C. A. et al., 2004).

1.7.4 γ-secretase inhibitors

The obvious lethality of the presenilin-knockout mice reinforces the serious concerns about mechanism-based toxicity of γ-secretase inhibitors. The effect of γ-secretase inhibitors on thymocyte differentiation (known to be Notch 1-dependent) in organ culture systems was analysed, and indeed, γ-secretase inhibitors reduce thymocyte numbers and block thymocyte differentiation (Hadland, B. K., Manley, N. R. et al., 2001; Doerfler, P., Shearman, M. S. et al., 2001). Currently, it is unclear whether therapeutically useful potent γ-secretase inhibitors can be generated. Regardless the serious concerns about γ-secretase inhibitor therapy, some intriguing findings offer new perspectives: It was shown that, at very high concentrations, certain non-steroidal anti-inflammatory drugs (NSAIDs) can modulate γ-secretase cleavage, such that $A\beta_{1-42}$ is reduced and the production of a smaller isoform, $A\beta_{1-38}$, which is expected to be less prone to aggregation than $A\beta_{1-42}$, is increased. In this setting, Notch cleavage (corresponding to the 49 position of Aβ) was not blocked (Weggen, S., Eriksen, J. L. et al., 2003). These compounds could possibly modulate γ-secretase or its substrate, because the effect is observed in cells that lack the NSAID targets cyclo-oxygenase I (COX-1) and II (COX-2) (Beher, D., Clarke, E. E. et al., 2004). Other known non-COX targets of NSAIDs, such as lipoxygenases, peroxisome proliferative activated receptor-γ (PPAR-γ) and nuclear factor-κB (NF-κB) are not affected. Potent compounds using this $A\beta_{1-42}$ modulator mechanism circumventing the Notch-related side effects of total γ-secretase inhibition, could re-emerge as promising drug targets. Large collections of compounds to identify molecules that reduce Aβ production in the absence of overt toxicity have been screened and it was determined whether β- or γ-secretase activity had been inhibited by secondary assays. This approach only identified γ-secretase pathway inhibitors, and the potent compounds inhibited the production of all Aβ isoforms. Medicinal chemistry programs ultimately lead to drug-like molecules that could reduce plasma and soluble brain Aβ in mice only a few hours after a single administration (Dovey, H. F., John, V. et al., 2001; Lanz, T. A., Himes, C. S. et al., 2003a). Bristol-Myers Squibb announced that the first γ-secretase inhibitor had entered Phase II clinical trials (Molinoff, P. et al., unpublished data) in 2001. Also, most other companies have not

disclosed anything about the development status of their γ-secretase-inhibitor programs. The only exception is Lilly, recently publishing the results of a 6-week Phase II trial of a functional γ-secretase inhibitor which demonstrated a significant decrease in Aβ concentration in plasma, but not in cerebrospinal fluid, at a dose that was tolerated well during the short trial (Siemers, E., Dean, R. A. et al., 2004). The safety, tolerability and decrease of Aβ levels by γ-secretase inhibitor was further investigated in a Phase I trial (Siemers, E., Skinner, M. et al., 2005). Aβ levels decreased dose-dependently after multple doses for 14 days, and the reported adverse events were described as manageable.

1.7.5 Neurotrophins

One promising AD treatment strategy is to protect or repair the neurons affected by the disease process. Nerve growth factor (NGF) and other neurotrophic factors have been studied as potential therapeutic agents and attempts have been made to use NGF itself as a clinical therapy. However, these attempts have not been successful at least partly because NGF is rapidly degraded and lacks the ability to cross the blood brain barrier. While there is widespread interest in the use of neurotrophic factors to prevent and/or reduce the neuronal cell loss and atrophy observed in neurodegenerative disorders, little research has been performed examining the expression and functional role of these factors in the normal and diseased human brain (Connor, B., Dragunow, M., 1998). It has been shown recently that the activation of the high-affinity receptor for nerve growth factor (NGF), tyrosine kinase receptor (TrkA), results in increased secretion of the amyloid precursor protein (sAPPα) into the culture medium. Interestingly, in the parietal cortex of AD patients a significant reduction in TrkA immunoreactivity has been reported suggesting a role of neurotrophins in the process of neurodegeneration in AD (Savaskan, E., Muller-Spahn, F. et al., 2000). Exogenous sAPPα increased neuron viability in both short- and long-term cortical neuron cultures grown in the absence of serum (Rossner, S., Ueberham, U. et al., 1998b). It has been supposed that the activation of mitogen activated protein (MAP) kinase alone is sufficient to promote APP secretion, whereas inhibition of MAP kinase will reduce APP secretion only when phospholipase C gamma or phosphatidylinositol 3-kinase are additionally inhibited (Rossner, S., Ueberham, U. et al., 1999). This suggests that pharmacological manipulations activating the MAP kinase pathway may result in increased secretory APP processing. Furthermore, much attention has been drawn to glycogen synthase kinase 3 (GSK-3) and its implication in amyloid plaque formation associated with AD by influencing APP metabolism. The expression and secretion of βAPP is increased in rat cerebral cortices that have been denervated by subcortical lesions of the nucleus basalis of Meynert. It has been previously shown that sAPPα produced by α-secretase activity potentiates the neuritogenic activity of NGF in vitro. The neurotrophic interactions of NGF and sAPPα have been characterized using differentiated PC12 cells and rat primary cortical neurons. NGF required the

expression of βAPP to maintain a neuronal phenotype (Luo, J. J., Wallace, M. S. et al., 2001).

Moreover, in-vitro evidence is provided that NGF can abolish Aβ-induced apoptosis (Kuner, P., Schubenel, R. et al., 1998). Substituted pyrimidines have modest neurotrophin-like activities (Matsumoto, K., Yamamoto, K. et al., 2003; Fyfe, J. A., Beauchamp, L. M. et al., 2004) and their neuroprotective effect has been studied in models of cerebral ischemia, Parkinson and Huntington's disease. Since it is known that PI3K activation by neurotrophins stimulates Akt which in turn phosphorylates GSK-3, a multifunctional serine/threonine kinase playing a pivotal role not only in the regulation of cell proliferation and neurite outgrowth, but also in APP processing, post-translational modification and transport is considered to be the connecting link between the two main hallmarks of AD, as abnormal tau phosphorylation as well as altered APP processing. In siRNA experiments (Phiel, C. J., Wilson, C. A. et al., 2003), reduced GSK-3β protein expression levels promote $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion. Thus, the reduced phospho-GSK-3β expression in APPsw per se might be at least one regulatory factor leading to increased Aβ levels in these cells.

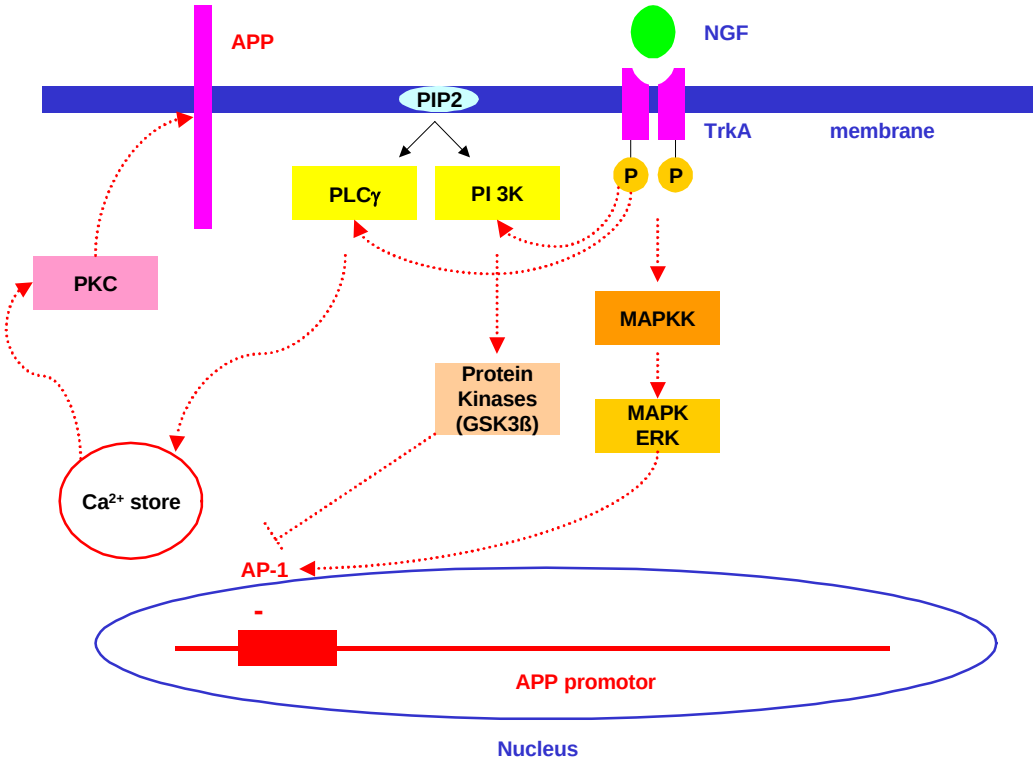

Figure 1.17: NGF signaling pathway and APP processing.

NGF interacts with TrkA in order to convey its signal intracellularly. TrkA receptor binding initiates MAP kinase, PI3K or PLC γ signalling. Besides modulating the phosphorylation status of tau and APP, GSK-3β additionally blocks AP-1 leading to modified APP transcription.

1.8 Aims of the thesis

Although massive investigative work has been accomplished to finally reveal the causes of AD, many pathways being involved in the Alzheimer's disease progress remain obscure, which has fatal consequences for the disease's therapy: The drugs currently used in the therapy of AD are effective, but most of them have only modest symptomatic effects. Actually, no effective causative disease-modifying therapy is available.

Among the approaches towards disease modifying treatment of Alzheimer's disease blocking the initial step of the amyloid cascade, Aβ generation, has received most attention. Aβ generation requires two proteases, β- and γ-secretase, and inhibition of these enzymes is a key focus of AD drug development. Progress in this area has been slow, because these enzymes were not entirely identified. After intracellular generation of Aβ, the peptide is secreted into the extracellular space where it accumulates to form one characteristic hallmark of AD: amyloid plaques. Although the primary toxic species in sporadic AD is still unknown, neurotoxicity of Aβ seems at least partly to be associated oxidative stress, mitochondrial defects, calcium dysregulation, excitatory toxicity and deficiency of survival factors. Missense mutations located within the APP-gene close to the Aβ sequence were the first familial AD-gene to be identified, but yet little is known of how these mutant genes lead to these early onset forms of AD. Interestingly, FAD and sporadic AD have common clinical and neuropathological features. Therefore, using transgenic models expressing FAD-mutations are promising approaches to elucidate the underlying mechanisms of neuronal death in AD. On the basis of many studies in cell cultures, transgenic animal models, and patients, we know that FAD mutations alter APP processing with respect to an enhanced Aβ production. However, the underlying mechanisms responsible for the massive neurodegeneration induced by Aβ in the brain from AD patients are still not completely understood.

The major aim of this thesis was therefore to elucidate neurotoxic biochemical pathways induced by Aβ with special focus on the mitochondria-dependent cell death pathway, since recent data of our group indicate the pivotal role of mitochondria in the pathogenesis of AD.

The first aim of this thesis was to characterize three different APP-expressing cell lines with regard to APP expression and localization, Aβ production, secretion and intracellular distribution as well as β- and γ-secretase activity. As cell models, the neuronal PC12 (rat pheochromocytoma), HEK (human embryonic kidney 293) and SY5Y (human neuroblastoma, APPwt only) cell lines were used, which have been transfected with human wildtype APP or the human APP containing the Swedish double mutation. This mutation results in a three- to sixfold increased Aβ production compared to wild-type APP (APPwt). Compared to experiments using high concentrations of Aβ at micromolar levels applied extracellularly to cells, these cell lines may allow to investigate chronic effects of intracellularly produced and secreted Aβ in a dose-dependent manner. Thus, these cell models could represent a very suitable approach to elucidate the AD-specific cell death pathways mimicking various pathological conditions. Furthermore, the toxicity of different Aβ species should be assessed, since evidence is provided that soluble, intracellular Aβ species already reveal the maximum toxicity and that the insoluble, extracellular Aβ aggregates, even though noxious, do not represent the main toxic species in AD. In particular, the question was addressed whether Aβ dimers represent already the neurotoxic correlate. Therefore, the effects of monomeric, dimeric and oligomeric/fibrillar Aβ on the MTT reduction potential of SY5Y and PC12 cells were investigated. In addition, the effects of intracellularly produced Aβ dimers of human neuroblastoma (SY5Y) cells stably transfected with APPK623C, a mutation causing the intracellular formation of APP and Aβ dimers, on the MTT reduction potential were investigated.

Preceding work of our group revealed that Aβ can induce changes in the mitochondrial activity particularly after oxidative stress. It was shown that initiator- and executioner caspases were activated in a time-dependent pattern while mitochondrial membrane potential and ATP levels decreased significantly (Marques, C. A., Keil, U. et al., 2003; Keil, U., Bonert, A. et al., 2004). Therefore, the second aim of this thesis was to figure out alterations in protein expression of pro-/antiapoptotic factors associated with the mitochondria-dependent cell death pathway under baseline conditions and after oxidative stress. Thus, proteins up- and down-stream acting of mitochondria that are involved in apoptosis were a matter of investigation. In order to assess the interplay of members of the Bcl-2 family during oxidative stress, protein expression levels and subcellular distribution were

investigated. Furthermore, the time-dependent release of pro-apoptotic mitochondrial factors was investigated in order to characterize caspase-dependent and -independent mitochondrial apoptosis. This study should furthermore help to clarify whether Bcl-2 family proteins and other pro-apoptotic mitochondrial factors can serve as potentially new targets for AD disease modification or not.

In order to develop new AD-specific drugs, many efforts aim at the inhibition of secretases or at restoring and maintaining neuronal function. Therefore, in the last part of the thesis, two disease-modifying strategies should be investigated. Following the determination of baseline γ- and β-secretase activity, the time- and concentration-dependent decrease in Aβ levels after treatment with different β- and γ-secretase inhibitors was characterised in cells stably transfected with APPwt and APPsw. In order to additionally examine their beneficial value, the effect of the secretase inhibitors on mitochondrial activity mirroring cellular viability was determined. Furthermore, evidence is provided that substituted pyrimidines have mild growth-promoting activities. In particular, the substituted pyrimidine KP544 seems to be able to potentiate growth-promoting effect of NGF in primary neuronal cultures. In order to clarify the role of substituted pyrimidines as potential neuro-modulatory drugs in AD, we investigated the effects of KP544 on protein expression, APP processing and mitochondrial activity in PC12 cells with special regard to the APP Swedish double mutation.

1.9 Ziele der Arbeit

In den letzten Jahren wurde die Entstehung der Alzheimer Demenz intensiv untersucht, jedoch liegen noch viele Mechanismen, die an der Entstehung der Erkrankung beteiligt sind, im Dunkeln. Dies hat fatale Konsequenzen für die Therapie: Die Arzneimittel, welche zur Zeit zur Behandlung der Alzheimer Demenz eingesetzt werden sind effektiv, aber viele haben nur bescheidene symptomatische Wirkungen. Momentan gibt es keine kausale krankheitsmodulierende Behandlungsoption.

Unter den Therapieansätzen, die zu einer krankheitsmodulierenden Behandlung führen sollen, erhält die Blockade des initialen Schrittes der Amyloidkaskade, die Bildung von Aβ, die meiste Aufmerksamkeit. Zur Bildung von Aβ bedarf es zweier Proteasen, β- und γ-Sekretase, und die Inhibition dieser Enzyme repräsentiert ein Hauptaugenmerk der Alzheimer-Forschung. Die Fortschritte auf diesem Gebiet erfolgten nur langsam, da die Enzyme lange nicht identifiziert werden konnten. Nach der intrazellulären Bildung von Aβ wird das Peptid in den Extrazellularraum sekretiert, wo es zu einem Hauptmerkmal der Alzheimer Demenz akkumuliert: Amyloid-Plaques. Bis heute sind die primären toxischen Spezies der sporadischen Alzheimer Erkrankung noch nicht bekannt. Es scheint jedoch die Neurotoxizität von Aβ, assoziiert mit oxidativem Stress, mitochondrialen Defekten, Kalzium-Dysregulierung, exzitatorischer Toxizität und Mangel von Survivalfaktoren eine wesentliche Rolle zu spielen. Auf dem APP-Gen lokalisierte Mutationen nahe der Aβ-Sequenz waren die ersten familiären Alzheimer-Gene, die identifiziert wurden. Heute ist immer noch wenig darüber bekannt, wie diese Genmutationen zu dem frühen Beginn der Alzheimer Demenz führen. Interessanterweise haben familiäre und sporadische Alzheimer-Formen gemeinsame klinische und neuropathologische Charakteristika. Aus diesem Grund stellen transgene Tier- oder Zellkulturmodelle, die familiäre Mutationen exprimieren, vielversprechende Optionen dar, um die Mechanismen der Alzheimer Erkrankung aufzuklären.

Basierend auf vielen Studien in Zellkultur, an transgenen Tiermodellen und an Patienten weiß man heute, dass familiäre Alzheimer-Mutationen die APP-Prozessierung beeinflussen und zu einer erhöhten Aβ-Produktion führen.

Frühere Ergebnisse unserer Arbeitsgruppe demonstrieren, dass Aβ die neuronale Empfindlichkeit gegenüber oxidativem Stress erhöht und so die Neurodegeneration und den Zelltod fördert.

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Frühere Ergebnisse unserer Arbeitsgruppe demonstrieren, dass Aβ die neuronale Empfindlichkeit gegenüber oxidativem Stress erhöht und so die Neurodegeneration und den Zelltod fördert. Diese Ergebnisse waren die Grundlage des ersten Ziels dieser Arbeit, die zum Ziel hatte Aβ-induzierte, neurotoxische, biochemische Prozesse aufzuklären. Zu diesem Zweck wurde der Effekt der Schwedischen Doppelmutation (APPsw, KM670/671NL) auf oxidativen Stress-vermittelte Zelltodmechanismen untersucht. Die Schwedische Doppelmutation führt zu einer im Vergleich zur Wildtyp-Form (APPwt) drei- bis sechsfach erhöhten Aβ-Produktion. Es wurden PC12- (Rattenpheochromozytom-), HEK- (humane embryonale Nierenkarzinom-) und SY5Y- (humane Neuroblastom-) Zellen als Modell gewählt, die zu einem mit wildtyp APP (APPwt) oder mit der Schwedischen APP-Doppelmutation transfiziert wurden. APPsw-transfizierte PC12-Zellen sekretieren Aβ in picomolaren Konzentrationen, was sehr gut die physiologische Situation in Gehirnen von Alzheimer Patienten wiederspiegelt. Im Gegensatz dazu werden zur Untersuchung der Toxizität von extrazellulärem Aβ unphysiologische hohe Konzentrationen im mikromolaren Bereich appliziert. Dieses Zellkulturmodell ist somit sehr gut geeignet, um Alzheimer-spezifische Zelltodwege zu untersuchen, die unter pathologischen Bedingungen auftreten und zu denen auch intrazelluläres Aβ beiträgt.

Die verschiedenen Zelltypen, mit APPwt bzw. APPsw transfizierte PC12- und HEK-Zellen erlauben die Untersuchung von APP- oder Aβ-vermittelten dosisabhängigen Effekten. APPsw-transfizierte HEK-Zellen zeigen eine dreißigfach erhöhte Aβ-Produktion im Vergleich zu APPsw-transfizierten PC12-Zellen. Somit steht hier ein Modell für die Untersuchung chronisch erhöhter Aβ-Spiegel zur Verfügung.

Humane SY5Y-Zellen sind neuronalen Ursprungs und wurden stabil mit APPwt transfiziert.

Ein weiteres Ziel dieser Arbeit war die Untersuchung der Proteinexpression und Sekretion in humanen Zelllinien (SY5Y, HEK) und der Ratten-Zellline (PC12), sowie der Vergleich beider Parameter in neuronalen (PC12, SY5Y) und nicht-neuronalen Zelllinien (HEK).

Mitochondriales APP trägt möglicherweise zu der Neurozoxizität bei, wie kürzlich von einer Arbeitsgruppe demonstriert wurde. Um die Charakterisierung der APP-Expression und Aβ-Sekretion zu vertiefen, wurde die intrazelluläre Verteilung von APP und Aβ untersucht.

Es wird vermutet, dass lösliche, dimere und oligomere Aβ- Spezies im Gegensatz zu unlöslichen, extrazellulären, fibrilläre Aβ-Aggregaten die primären toxischen Spezies der Alzheimer Demenz darstellen und somit die höchste Toxizität aufweisen. Dehalb war das zweite Ziel dieser Arbeit, die Toxizität unterschiedlicher Aβ- Spezies zu charakterisieren: Die Toxizität monomerer, dimerer und fibrillärer Aβ- Spezies wurde durch Bestimmung des MTT Reduktionspotentials von SY5Y- und PC12-Zellen wurde untersucht. Anhand von SY5Y-Zellen (K623C SY5Y), die mit einem APP-Dimer-bildenden Konstrukt stabil transfiziert wurden, wurden diese Effekte mit denen von intrazellulär gebildeten Aβ-Dimeren auf das MTT Reduktionspotential verglichen.

Frühere Ergebnisse unseres Arbeitskreises beschrieben Caspase-Aktivierung und mitochondriale Veränderungen nach Schädigung durch oxidativen Stress besonders im Zusammenhang mit der APPsw-Mutation in PC12-Zellen. Initiator- und Executioner- Caspasen werden in einer zeitabhängigen Reihenfolge aktiviert, wobei zeitnah das mitochondriale Membranpotential und die ATP-Spiegel signifikant sanken.

Das dritte Ziel dieser Arbeit stellte die Charakterisierung weiterer Proteine dar, die eine wichtige Rolle in der Apoptose und bei oxidativem Stress spielen: Proteine der Bcl-2-Familie und pro-apoptotische, mitochondriale Faktoren. Die Expression und die subzelluläre Verteilung wurden unter Bedingungen untersucht, die zur Charakterisierung der Caspasen analog sind. In diesem Zusammenhang wurde auch die zeitabhängige Freisetzung von pro-apoptotischen mitochondrialen Faktoren im Hinblick auf caspase-abhängige und -unabhängige Apoptose untersucht. Basierend auf den Ergebnissen wurde diskutiert, ob Bcl-2 Proteine oder pro-apoptotische mitochondriale Faktoren potentielle therapeutische Targets in der Alzheimer Demenz darstellen.

Die Etablierung von geeigneten Immunisierungsmethoden zur Heilung der Alzheimer Demenz (Lemere, C. A., Spooner, E. T. et al., 2003; Selkoe, D. J., Schenk, D., 2003) und die Inhibition von Sekretasen stellen zwei Ansätze dar, um die Akkumulation von neurotoxischen Aβ-Formen entscheidend zu hemmen.

Somit bestand das vierte Ziel dieser Arbeit darin, krankheitsmodifizierende Therapiestrategien genauer zu untersuchen: Die basale β- und γ-Sekretase-Aktivität wurde bestimmt um im Anschluß die Effekte der Sekretase-Inhibition in Zellkultur näher zu untersuchen. Es gelang, konzentrationsabhängige Effekte auf die Aβ-Spiegel nach β- und γ-Sekretase- Behandlung darzustellen. Zusätzlich wurde der Einfluss von Sekretase-Inhibitoren auf die mitochondriale Aktivität von APPwt- und APPsw-transfizierten PC12- und HEK-Zellen untersucht.

Neurotrophe Faktoren als therapeutische Agentien zielen darauf ab, die neuronale Funktion bei neurodegenerativen Erkrankungen aufrecht zu erhalten. Zuvor wurde berichtet, dass substituierte Pyrimidine leichte wachstumsfördernde Eigenschaften besitzen.

KP544, ein substituiertes Pyrimidin, soll die wachstumsfördernde Eigenschaft von NGF verstärken. Hier wurde die Rolle von substituierten Pyrimidinen als potentielle neuromodulatorische Wirkstoffe in der Alzheimer Demenz untersucht.

Die Effekte von KP544 auf Protein-Expression, APP-Prozessierung und mitochondriale Aktivität von PC12-Zellen wurden mit besonderem Augenmerk auf die APPsw-Mutation in PC12-Zellen charakterisiert.

2 MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

dB-cyclic AMP	Sigma, Munich, Germany
Bovine Serum Albumin (BSA)	Sigma, Munich, Germany
Complete mini	Sigma, Munich, Germany
DAPT	Calbiochem, Schwalbach, Germany
Digitonin	Sigma, Munich, Germany
Dithiotreitol (DTT)	Sigma, Munich, Germany
Dulbecco´s modified eagle medium (DMEM)	Life Technologies, Karlsruhe, Germany
Dulbecco´s Phosphate buffered saline (PBS)	Life Technologies, Karlsruhe, Germany
ECL chemiluminescence reagent	Amersham, Freiburg, Germany
EDTA	Sigma, Munich, Germany
Fetal Calf Serum, mycoplasma-screened (FCS)	Life Technologies, Karlsruhe, Germany
Geneticine sulfate (G418)	Life Technologies, Karlsruhe, Germany
Glycine	Sigma, Munich, Germany
Horse Serum, mycoplasma-screened (HS)	Life Technologies, Karlsruhe, Germany
Hybond P PVDF membrane	Amersham, Freiburg, Germany
Hydrochloric acid	Sigma, Munich, Germany
Hydrogen peroxide (H_2O_2) 30%	Sigma, Munich, Germany
Hygromycine B	Sigma, Munich, Germany
L-685458	Calbiochem, Schwalbach, Germany
Methanol (MeOH)	Merck, Darmstadt, Germany
N,N-Dimethyformamide	Sigma, Munich, Germany
Nerve growth factor, 7S (NGF)	Calbiochem, Schwalbach, Germany
Nonidet P-40	Sigma, Munich, Germany
NuPAGE 4-20% Gradient gels	Invitrogen, Karlsruhe, Germany
NuPAGE SDS Sample Buffer (4X)	Invitrogen, Karlsruhe, Germany

NuPAGE MES SDS Running Buffer (20X)	Invitrogen, Karlsruhe, Germany
NuPAGE Reducing Agent (10X)	Invitrogen, Karlsruhe, Germany
NuPAGE Transfer Buffer (20X)	Invitrogen, Karlsruhe, Germany
Optimem	Life Technologies, Karlsruhe, Germany
Penicillin /Streptomycine- solution (PenStrep)	Life Technologies, Karlsruhe, Germany
Phenoylmethylsulfonylfluoride (PMSF)	Sigma, Munich, Germany
Poly-L-lysine hydrobromide	Sigma, Munich, Germany
Ponceau Red	Sigma, Munich, Germany
Sodium chloride (NaCl)	Sigma, Munich, Germany
Sodium dodeyl sulfate (SDS)	Sigma, Munich, Germany
β-Secretase Inhibitor II	Calbiochem, Schwalbach, Germany
Tris(hydroxymethyl)-aminomethane (Tris)	Sigma, Munich, Germany
Triton X 100	Sigma, Munich, Germany
Trypsin	Life Technologies, Karlsruhe, Germany
Tween 20	Sigma, Munich, Germany
Ultraglutamine	Life Technologies, Karlsruhe, Germany
Western Breeze Blocker Diluent A	Invitrogen, Karlsruhe, Germany
Western Breeze Blocker Diluent B	Invitrogen, Karlsruhe, Germany

2.1.2 Kits

$A\beta_{1-40}$ ELISA	Biosource, Solingen, Germany
$A\beta_{1-42}$ ELISA	Biosource, Solingen, Germany
β-secretase activity assay	Calbiochem, Schwalbach, Germany
Cell Proliferation Kit I (MTT Assay)	Roche Diagnostics, Mannheim, Germany
Clontech cell fractionation kit	BD Biosciences, Heidelberg, Germany
Cytotoxicity Detection Kit (LDH Assay)	Roche Diagnostics, Mannheim, Germany
DC Protein Assay	BioRad, Munich, Germany
γ-secretase activity assay	RnD, Wiesbaden, Germany
Quantikine M Cytochrome c ELISA	RnD, Wiesbaden, Germany

2.1.3 Antibodies

2.1.3.1 Primary antibodies

Goat anti-Actin	Santa Cruz, Heidelberg, Germany
Mouse anti-APP/ Aβ/CTF (W-02)	Abeta, Heidelberg, Germany
Mouse anti-Bcl-2	BD Pharmingen, Heidelberg, Germany
Mouse anti-Calreticulin	Chemicon, Wiesbaden, Germany
Mouse anti-COX-4 (Cell Fractionation Kit)	BD Biosciences, Heidelberg, Germany
Mouse anti-Nitrotyrosine	Chemicon, Wiesbaden, Germany
Rabbit anti-AIF	Chemicon, Wiesbaden, Germany
Rabbit anti-Bax	CellSignaling, Frankfurt, Germany
Rabbit anti-Bcl-xL	CellSignaling, Frankfurt, Germany
Rabbit anti-Cytochrome c (Cell Fractionation Kit)	BD Biosciences, Heidelberg, Germany
Rabbit anti-ERAB	Chemicon, Wiesbaden, Germany
Rabbit anti-GSK-3β	CellSignaling, Frankfurt, Germany
Rabbit anti-MEK 1	Abcam, Cambridge, UK
Rabbit anti-Na-K-ATPase	BD Transduction Labs, Heidelberg, Germany
Rabbit anti-phospho-GSK-3β	CellSignaling, Frankfurt, Germany
Rabbit anti-Smac/DIABLO	Chemicon, Wiesbaden, Germany

Antibodies were diluted in a total volume of 14 ml TBST containing 0.5% BSA and 100 µl sodium azide (100x). Appropriate dilutions ranged from 1:100 to 1:10000, depending on the antibody. Optimal conditions were determined by dilution series according to manufacturer's instructions. These dilutions were stored at 4-8°C and were used several times, 5-10 times on the average. Before the application of W-02 on PVDF membranes, the membrane was boiled for 5 min in ddH_2O to achieve optimal results.

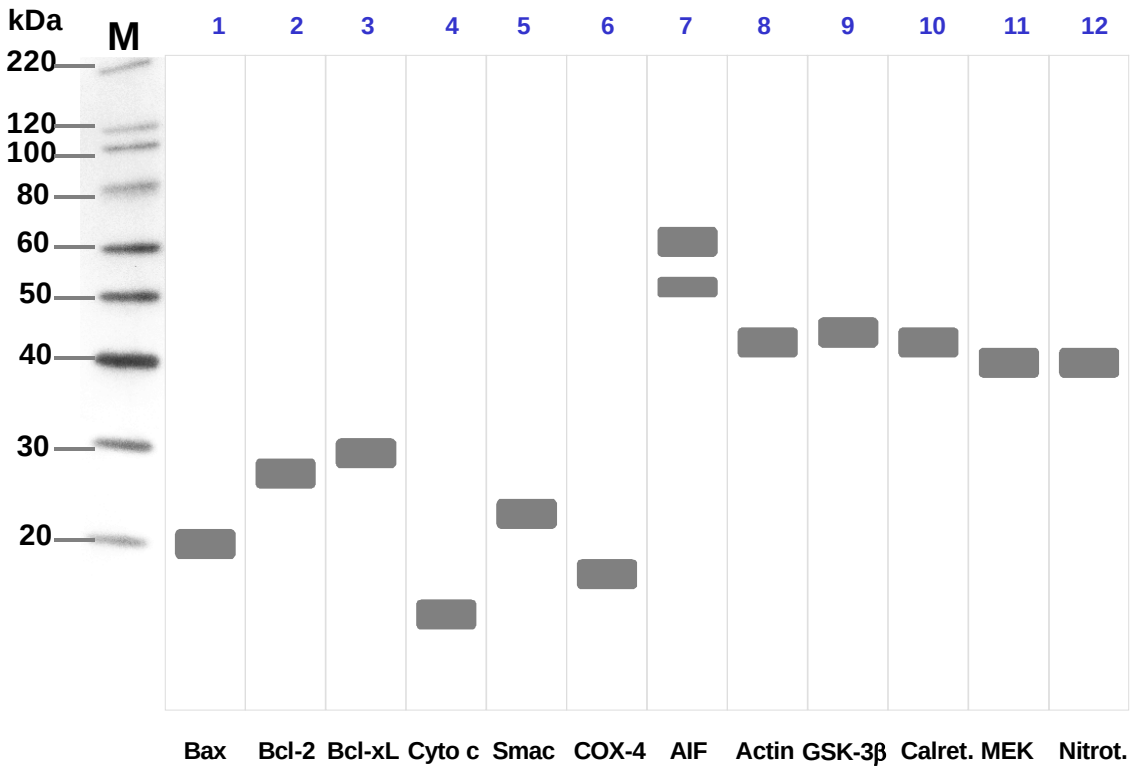

Figure 2.1: Schematic protein bands for the antibodies in use.

On the left side, typical bands for the Marker MagicMark® (M, Invitrogen, Germany) are shown. The marker is horseradish peroxidase-coupled. Via the chemiluminescent reaction with ECL, the bands of the marker appear on the film. The following lanes 1-12 represent schematic protein bands for the antibodies used in this work. Schematic bands for APP, sAPPα, CTF and Aβ are shown in Fig. 2.8.

2.1.3.2 Secondary antibodies

Anti-goat IgG secondary antibody	Calbiochem, Schwalbach, Germany
Anti-mouse IgG secondary antibody	Calbiochem, Schwalbach, Germany
Anti-rabbit IgG secondary antibody	Calbiochem, Schwalbach, Germany

Generally, secondary antibodies were freshly diluted 1: 1000 with TBST prior to use.

2.1.4 Buffers and Solutions

Glycine stripping solution:

Glycine	8.0 g
ddH_2O	1.0 l
HCl conc.	2.5 ml

MTT solubilization reagent (20% SDS/ 50% DMFA):

N,N-Dimethyformamide	90 ml
Solubilization Solution (kit)	90 ml
SDS	27 g

LDH reaction buffer:

1. Reconstitution of the catalyst (Diaphorase/NAD^+) with 1ml ddH_2O
   1 aliquot
2. Catalyst + dye (Iodo-tetrazolium chloride/ sodium lactate; kit)
   45 aliquots

The reaction buffer was prepared freshly prior to usage.

PBS buffer:

Dulbecco´s phosphate buffered saline (10X) was diluted with ddH_2O and stored at 4°C until usage.

STEN lysis buffer:

Nonidet P-40	1%
Tris pH 7.6	50 mM
NaCl	150 mM
EDTA	2 mM

TBST (10X):

Tris	24.2 g
NaCl	80 g
HCl conc.	13 ml
Tween 20	5 ml

The solution was adjusted to pH 7.8. 100 ml were diluted with 900 ml ddH_2O.

Tris Buffer 5 mM:

1.21 g/ml Tris was dissolved in 2 l ddH_2O and pH was adjusted to 7.4.

Digitonin lysis buffer:

NaCl	75 mM
NaH_2PO_4	1 mM
Sucrose	250 mM
PMSF	1 mM
Digitonin	0.05 %

Formic acid lysis buffer:

Concentrated formic acid was diluted to 40% with ddH_2O.

Mitochondria lysis buffer:

Triton X was diluted to 0.1% with ddH_2O.

γ-secretase inhibitor stock solution:

γ-secretase inhibitors (DAPT, L-685458) were dissolved in DMSO resulting in a 1 mM stock solution. The stock solution was aliquoted and kept at -20°C until usage.

β-secretase inhibitor stock solution:

β-secretase inhibitor II was dissolved in DMSO resulting in a 1 mM stock solution. The stock solution was aliquoted and kept at -20°C until usage.

Caspase and JNK inhibitors:

The inhibitor were dissolved in DMSO resulting in 1 mM stock solutions. The stock solutions were aliquoted and kept at -20°C until usage.

Reconstitution of monomeric, dimeric and oligomeric/fibrillar Aβ isoforms:

Aβ stock solutions were adjusted to a final concentration of 0.5 mM with 25% NH_3 (pH 9.8). Complete dissolution of Aβ was achieved by sonification for 15 min and aliquoted to be stored unti usage. Aβ stocks were diluted with 5 mM Tris (pH 7.4) to 2.5 µM and kept in a waterbath (37°C) for 24h to aggregate before assay start.

NGF stock solution:

NGF was dissolved in PBS containing 2% serum (0.5% HS, 1.5% FCS) at a final concentration of 50 ng/ml. The stock solution was aliquoted and kept at -20°C until further usage.

Freezing medium:

FCS	40%
Medium (without supplements)	50%
DMSO	10%

NuPage Running buffer (Gradient gels):

MES SDS Running Buffer (20X)	50 ml
ddH_2O	950 ml

NuPage Transfer buffer (Gradient gels):

Transfer buffer (20X)	50 ml
Methanol	100 ml
Antioxidant	1 ml
ddH_2O	849 ml

Schaegger Running Buffer (Kathode, 5x):

Tris	0.5 M
Tricine	0.5 M
SDS	0.5%

Schaegger Running Buffer (Anode, 10x):

Tris (pH 6.8)	2 M

Schaegger Buffer for the preparation of Tricine gels:

Tris (pH 8.5)	3 M
SDS	0.3%

Sample Buffer (Reducing conditions):

Tris/Hcl (pH 6.8)	1.2 g
Gycerol	24 ml
SDS	8.0 g
DTT	3.1 g
Trypan blue	200 ml

Tris-Acrylamide gels (18%):

Acrylamide (40%)	8.75 ml
Bis-acrylamide	4.82 ml
Tris (pH 8.8)	5.00 ml
SDS (10%)	0.20 ml
ddH2O	1.13 ml
TEMED	0.01 ml

Schaegger Tricine Gels (15%):

Acryl-/ Bisacrylamide solution (29:1)	7.50 ml
Schaegger Buffer (pH 8.5)	5.00 ml
ddH_2O	0.75 ml
Gycerol (87%)	1.75 ml
APS (10%)	0.10 ml
TEMED	0.01 ml

2.1.5 Apparatus

Arpege 70 tank for liquid nitrogen	Air liquide, Paris, France
Asys Hightech Digiscan plate reader	Asys, Eugendorf, Germany
Biorad PowerPac 300	BioRad, Munich, Germany
BioRad Sub Cell Model 96	BioRad, Munich, Germany
Centrifuge GS-6R	Beckman, Munich, Germany
Combitips plus 0.1, 0.5, 1, 2.5, 5 ml	VWR International, Darmstadt, Germany
Coverslides for Neubauer chamber	VWR International, Darmstadt, Germany
Cryo S	Greiner, Frickenhausen, Germany
Falcon 2050 Polystyrene tubes	Becton-Dickinson, Heidelberg, Germany
Freezer (-20°C)	Liebherr GmbH, Ochsenhausen, Germany
Freezer (-80°C)	Heraeus Instruments, Hanau, Germany
Hamilton syringe, 25+50 µl	Hamilton, Bonaduz, Switzerland
IKA HS 250 basic Shaker	IKA Labortechnik, Staufen, Germany
Incubator Heraeus Type BB 6220	Heraeus Instruments, Hanau, Germany
Laminar flow hood	Heraeus Instruments, Hanau, Germany
Microfuge R centrifuge	Beckman, Munich, Germany
Microscope Modell TMS type 104	Nikon, Japan
Microtiter plates, 6, 96 wells flat bottom, sterile	Greiner, Frickenhausen, Germany
Milli Q Plus PF Millipore system	Millipore, Eschborn, Germany
Multical pH 526 pH meter	WTW, Weilheim, Germany
Multipette plus	Eppendorf, Wesseling-Berzdorf, Germany

Neubauer chamber superior	VWR International, Darmstadt, Germany
Pipette tips 10- 1000 µl and 5 ml	Greiner, Frickenhausen, Germany
Pipettes 5, 10, 25 ml sterile	Greiner, Frickenhausen, Germany
Pipettus akku	Hirschmann, VWR, Germany
Polypropylene tubes 15, 50 ml	Greiner, Frickenhausen, Germany
Promax 1020 Shaker	Heidolph, Kelkheim, Germany
Transferpette 1, 10, 100, 5000 µl	Eppendorf, Wesseling-Berzdorf, Germany
Unitek HB-130 Heating Block	CLF, Emershacker, Germany
Variomag mono Magnetic agitator	H+P Labortechnik, VWR, Darmstadt, Germany
Vortex model REAX 2000	Heidolph, Kelkheim, Germany
Wallac 1420 Victor2 plate reader	Perkin Elmer, Rodgau-Jürgesheim, Germany
X-Cell gel electrophoresis system	Invitrogen, Karlsruhe, Germany
Ziegra ice machine	Bader, Frankfurt, Germany

2.1.6 Computer software

Graph Pad Prism 3.0, GraphPad Software
Microsoft Excel software 2000
Umax Vistascan software
ScionImage Software
Kodak ds1D software v. 2.03
Wallac 1420 Workstation version 2.09

2.2 Methods

2.2.1 Cell culture and transfection

2.2.1.1 Thawing cells

All cell clones were kept in liquid nitrogen until usage. Before thawing cells, medium was brought to room temperature. Then, a total volume of 10 ml was given into a falcon tube and a culture dish. After gently thawing cells in the water bath, they were added to the falcon tube and centrifuged at 1000 x g for 5 min to remove toxic DMSO. Medium was removed, the pellet consisting of cells was resuspended in 1 ml fresh medium. The suspension was pipetted into the culture dish medium while gently shaking. Culture dishes were stored in the incubator for 24h. Then, depending on confluency of the cells, the first splitting step was undertaken or the medium was changed. First experiments were performed at passage 3, since at that time, growth rate of the clones was synchronized. In this way, false results due to differing growth rates were avoided. Depending on incubation periods, growth rates were independently determined by counting the cells before assay start.

2.2.1.2 Freezing cells

Before starting the freezing procedure, all media in use were brought to 4-8°C. Freezing tubes were kept on ice. Then, cells were collected with cool and fresh culture medium and centrifuged at 1000 x g for 5 min. In the next step, they were resuspended in 1 mL of freezing medium and transferred to the tubes. After overnight storage at -80°C in a freezing box containing isopropanol, cells were moved to the liquid nitrogen.

2.2.1.3 PC12 cells

PC12 cells were transfected with DNA constructs harbouring human mutant APP (APPsw, K670M/N671L) gene, wild-type APP (APPwt) gene, inserted downstream of a CMV promotor, using FUGENE technique (Roche Diagnostics). The transfected cells APPwt PC12, APPsw PC12 and control PC12 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and 5% heat-inactivated horse serum, 50 units/ml penicillin, 50 µg/ml streptomycin, and 400 µg/ml G418 at 37°C in a humidified incubator containing 5% CO_2.

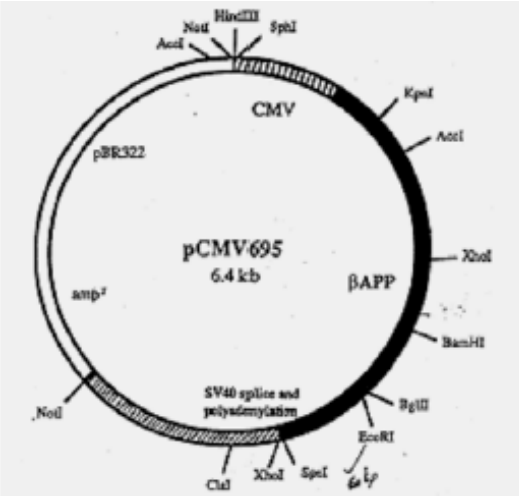

Figure 2.2: APP-pCMV695 vector
(taken from Marques C., 2004)

2.2.1.4 HEK cells

HEK cells were equally transfected with DNA constructs harbouring human mutant APP (APPsw, K670M/N671L) gene, wild-type APP (APPwt) gene, inserted downstream of a CMV promotor, using FUGENE technique (Roche Diagnostics). The transfected cells APPwt HEK, APPsw HEK and control HEK were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and 50 units/ml penicillin, 50 µg/ml streptomycin, and 400 µg/ml G418 at 37°C in a humidified incubator containing 5% CO_2.

2.2.1.5 SH-SY5Y cells

APP mutations were introduced by polymerase chain reaction amplification using the megaprimer method. Two outside primers spanned the *Bgl*II (forward, TCG GCC TCG TCA CGT GTT C) and *Xmn*I sites (reverse, CAA CTG GCT AAG GGG CTA TGT G) in APP while the reverse mutagenic primers introduced mutations at position 623 of APP695 (K623C, CCA ATG ATT GCA CCA CAA TTC GAA CCC). For cloning of the mutated DNA fragment the restriction enzymes *Xho*I and *Cla*I were used. The resulting cDNAs were cloned into the mammalian expression vector pCEP4 at *Sma*I and *Sal*I sites. Stably expressing cell lines have been obtained by transfecting the human neuroblastoma SH-SY5Y with the plasmid K623C containing the entire coding region of APP. Human neuroblastoma SY5Y cells were grown at 37°C in RPMI medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, minimal essential medium nonessential amino acid mix, 50 units/ml pencillin, 50 µg/ml streptomycin. For stable expression, SY5Y cells were transfected with receptor cDNAs individually or in combination, selected with hygromycin.

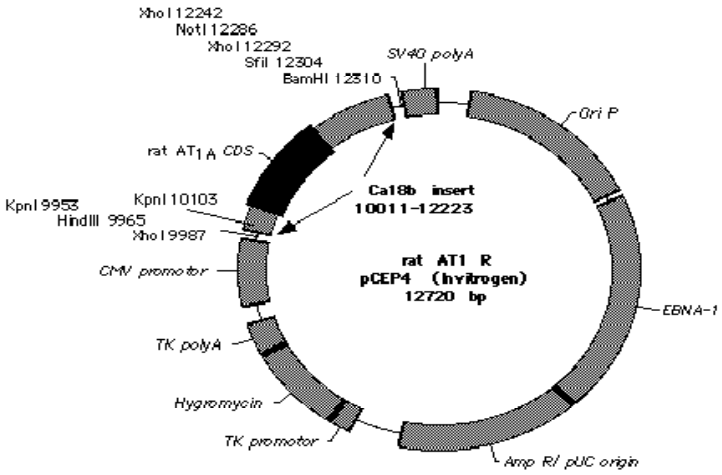

Figure 2.3: pCEP4 vector
(taken from Invitrogen, Karlsruhe, Germany)

2.2.1.6 Cell Differentiation

For the determination of MTT reduction potential, PC12 cells were plated on Poly-L-lysine (Sigma, Germany) coated 96 well plates at a density of 10.000 cells per well in DMEM supplemented with 5% fetal calf serum, 10% heat-inactivated horse serum and 50 µg/ml penicillin/streptomycin. Stably transfected clones expressing human mutant APP (APPsw) were additionally maintained with G418 (Gibco, Germany) at a

concentration of 400 µg/ml. Differentiation of PC12 cells was induced within 12h after addition of DMEM. Depending on the assay design, PC12 cells were treated with varying concentrations of nerve growth factor (NGF, Sigma) and 2´-O-dibutyryladenosine 3´: 5´-cyclicmonophosphate (db-cyclic AMP, Sigma) Under standard conditions, full differentiation was achieved by the treatment with NGF (50 ng/ml) and db-cyclic AMP (500 µM) for 5d. This procedure is according to the protocol used by most workers in this field, and in this time, PC12 cells posses the maximal number of neurites.

For western blot experiments, cells were plated in culture dishes (IWAKI, Germany) at a density of 5 x 10^6 cells. After incubation, cells were washed with ice-cold PBS and centrifuged at 800 x g for 5 min at 4°C. After centrifugation, PBS was removed, and cells were resuspended in lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40) supplemented with the protease inhibitor cocktail Complete mini® (Roche Diagnostics, Germany) and incubated at 4°C for 10 min. After removal of cell debris by centrifugation at 800 x g at 4°C for 5 min, the total protein content of the supernatant was determined by the method of Lowry. For the following western blot procedure, see chapter 2.2.8.

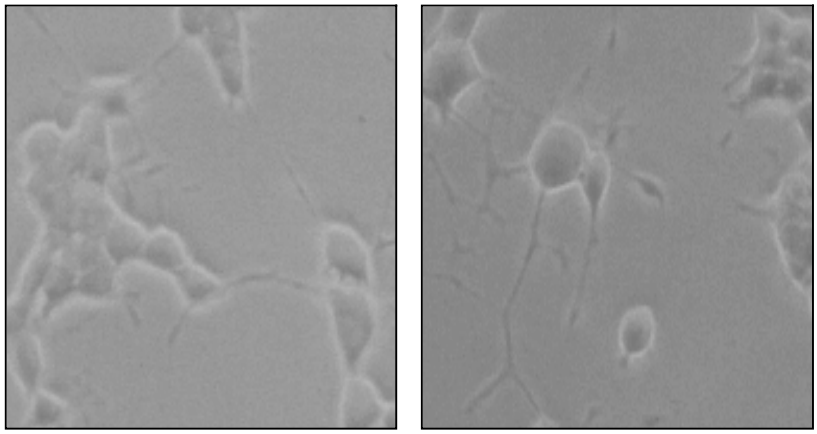

Figure 2.4: Terminally differentiated PC12 cells.

PC12 cells were treated with NGF (50 ng/ml, *left*) or with a combination of NGF and db-cyclic AMP (50 ng/ml, 500 µM, *right*) in order to achieve full differentiation. For microscopic analysis, cells were plated at a density of 50.000 cells/ chamber slide and treated with the above-mentioned concentrations of NGF and db-cyclic AMP.

2.2.2 Determination of cell number

Cells were collected from culture dishes and centrifuged at 1000 x g for 5 min. The supernatant consisting of medium was removed and 1 ml of fresh medium was added to gently resuspend the cells. 10 µl of cell suspension was given into a tube with 90 µl Trypan blue solution. After mixing, 10 µl were applied on a Neubauer counting chamber. The chamber consists of 9 equally large squares, with 1 mm^2 area each square. The depth of a square is 0.1 mm resulting in a total volume of 0.1 µl. Viable cells appear white, as broken or damaged cells are stained blue by Trypan blue entering intracellular compartments. The cell number of 4 squares were determined and the mean was formed indicating the total cell number in 1 ml cell suspension. Then, cell number was adjusted at 1 x 10^6 cells/ml by the addition of fresh medium. After mixing, the adequate volume of cell suspension was pipetted into the corresponding wells or plates.

2.2.3 Determination of protein content

The content of total protein was determined following the method of Lowry. The assay principle is based on the reaction of an alkaline copper-tartrate solution with the protein, resulting in the formation of a copper-protein complex. In the following, the complex reduces a reagent consisting of phospho-molybdate and phospho-wolfram leading to the formation of a blue-coloured adduct. The absorption at 620 nm can be read with an ELISA microplate reader. In all assays, a standard curve using BSA and the appropriate lysis buffer is generated by which the total protein content of each pobe was calculated. For general purpose, the standard dilutions ranged from 0.6 mg/ml to 2 mg/ml. were freshly prepared from BSA stocks (100 mg/ml) prior to each determination

2.2.4 Detection of Aβ levels

For the detection of $A\beta_{1-40}$ and $A\beta_{1-42}$, a specific sandwich enzyme-linked immunosorbent assay employing monoclonal antibodies was used. The ELISA was performed according to the instructions given in the Aβ ELISA Kit by Biosource. The assay principle is that of a standard sandwich ELISA, which utilizes a monoclonal

mouse anti-human $A\beta_{1-16}$ capture antibody and a cleavage-site-specific rabbit anti-human $A\beta_{1-40}$ or $A\beta_{1-42}$. According to the manufacturer´s instructions, the ELISA specifically recognizes human Aβ, thus rat Aβ from PC12 control cells is barely detected. The minimum detection level for $A\beta_{1-40}$ is at 7.8 pg/ml, that of $A\beta_{1-42}$ is at 15.6 pg/ml. In brief, 2 to 5 x 10^6 cells were plated on culture dishes the day before assay start. Then, medium was changed and the treatment (secretase inhibitors) was started. At the end of the incubation, cells were visually checked for potential contamination and confluency. The culture supernatant was collected by centrifugation at 800 x g for 5 min. 990 µl of culture supernatant was transferred to adequate freezing tubes, 10 µl of a 1mM PMSF stock solution was added and probes were gently mixed before immediate freezing at -80°C. For the determination of Aβ in cell lysates, the same treatment conditions were chosen. Here, the cell lysates were collected and washed with ice-cold PBS. Then, the appropriate lysis buffer (Tris, formic acid, 400 µl, chapter 2.1.4) was added to the probes and left to incubate for 15 min. Cell debris was removed by centrifugation at 800 x g for 5 min, and cell lysates were stored until assay performance. Before performing the Aβ ELISA, standard dilutions from the reconstituted Aβ solution given in the kit were freshly prepared and applied on the 96 well ELISA plate. Excess standard solutions could generally be stored at -80°C for several weeks, but a mix of Lot numbers of ELISA kits is not recommendable. After thawing the probes, they were added to the 96 well ELISA plate and incubated overnight at 4-8°C without shaking. The next day, after washing the plates three times with the appropriate wash buffer provided in the kit, the primary antibody dilution was added to the plate and the incubation was continued for another 2h. It is noteworthy that in 2004, Biosource changed the assay protocol for the $A\beta_{1-40}$ ELISA: Here, the primary antibody was directly added to the probes and the mixture was incubated overnight as mentioned above. The rest of the protocol is identical to that of the $A\beta_{1-42}$ ELISA currently available. Then, the plate was again intensively washed three times before the addition of the secondary antibody. The incubation was continued for further 2h followed by four washing steps. Colour development is initiated by the addition of tetramethylbenzidine (TMB) forming a blue chromophore during the reaction. When 1 N HCl is added, the chromophore turns yellow, resulting in an absorbance at 450 nm. Absorbance was measured in a ELISA plate reader shortly after the addition of 1 N HCl. Sample concentrations were

calculated from the standard curve and Aβ contents were calculated by the formula given for the standard regression in the Excel graph (Fig. 2.5).

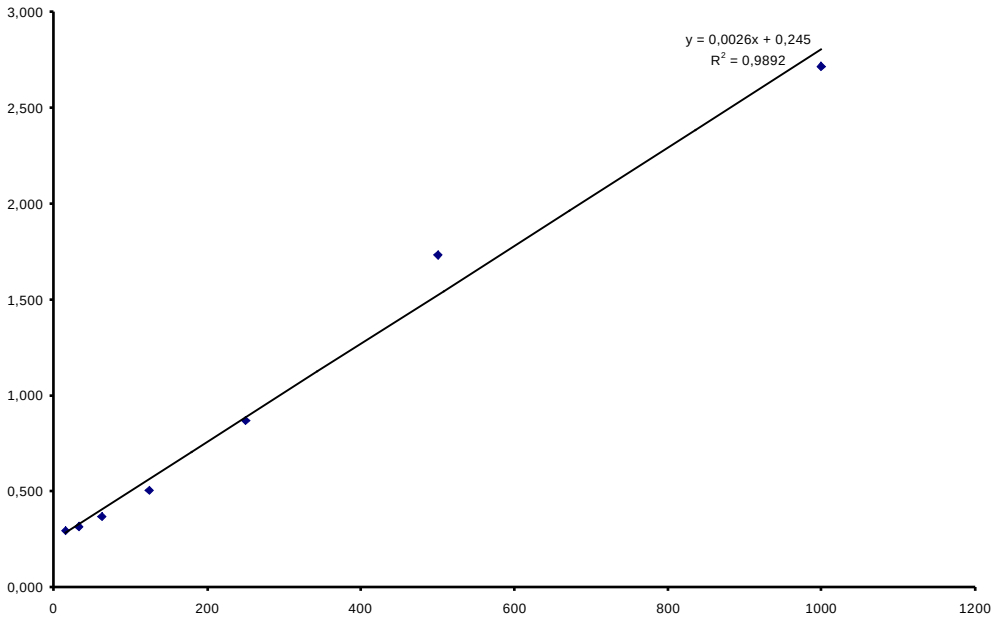

Figure 2.5: Aβ standard curve (example).

A standard dilution curve was performed in each experiment to exactly determine Aβ contents of the probes by the resulting regression formula.

2.2.5 Isolation of cytosolic and mitochondrial fractions

Cytosolic and mitochondrial fractions were isolated by digitonin permeabilization. Digitonin depletes cholesterol from the plasma membrane, thus causing pore formation. Mitochondria generally posses less cholesterol in the outer membrane. Consequently, mitochondria remain intact during digitonin treatment. After the establishment of optimal lysis conditions (Fig. 2.6), 5 x 10^6 cells were washed with ice cold phosphate-buffered saline (PBS) and were resuspended for 15 min on ice in permeabilization buffer containing 75 mM NaCl, 1 mM NaH_2PO_4, 250 mM sucrose, 1 mM PMSF, additional protease inhibitors (Complete® mini, Roche Diagnostics, Germany), and 0.05% digitonin. To confirm the results, we checked successful lysis by trypan blue staining. Alternatively, we used the Clontech cell fractionation kit (BD Biosciences, Germany). Here, cells were treated with 200 µl lysis buffer provided in the kit. Following a centrifugation step at 800 x g at 4°C for 10 min, the supernatant was separated from the pellet consisting of cellular debris. The crude mitochondrial pellet was collected by centrifugation at 13.000 x g at 4°C for 60 min. The supernatant containing cytoplasm proteins was stored at –20°C for further investigation.

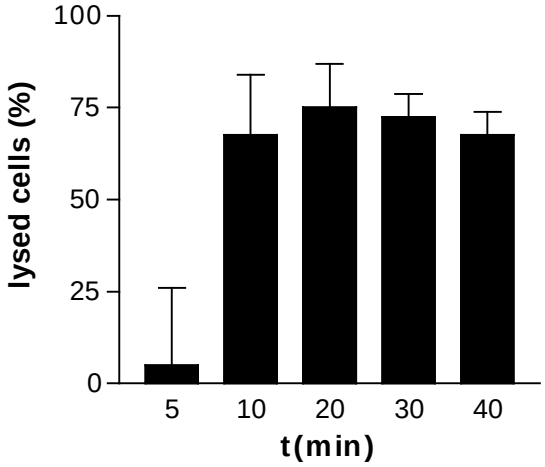

Figure 2.6: Digitonin lysis of PC12 cells.

PC12 cells were treated with 0.05% digitonin for various periods of time. Membrane permeabilization was assessed by trypan blue staining. Blue cells bodies indicate successful permeabilization.

The pellet consisting of mitochondria was resuspended in 0.1% Triton X and mechanically lysed. Succesful separation of mitochondrial and cytosolic fractions was checked by western blot for COX-4. COX-4 (cytochrome c oxidase 4) is part of the electron transport chain located in the inner mitochondrial membrane. Cytosolic fractions should be free of any mitochodrial components, like COX-4, namely. Before the performance of western blot experiments, the total protein content was determined by the method of Lowry (chapter 2.2.3) and equal amounts of protein were loaded on the gel.

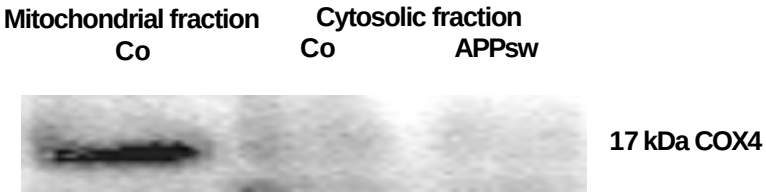

Figure 2.7: COX-4 western blot of cytosolic and mitochondrial fraction.
In the western blot of the mitochondrial fraction, COX-4 migrates at 17 kDa. Cytosolic fractions of APPsw and control cells are free from COX-4 protein.

2.2.6 Sample preparation of cell lysates

Equal amounts of cells were collected an resuspended in 4X NuPAGE SDS Sample lysis buffer (Invitrogen, Germany). After sonification for 30 min, the probes were boiled at 95°C for 10 min, centrifuged at 10.000 x g for 5 min and then assayed by western blot. Alternatively, cells were lysed in STEN lysis buffer (chapter 2.1.4) for 15 min on ice and crashed by exposure to liquid nitrogen followed by determination of protein content. The advantage of STEN lysis is the reduced viscosity of the lysate, which faciliates gel loading. Furthermore, protein levels could be detected by the method of Lowry (chapter 2.2.3) to assure correct and equal protein loading.

2.2.7 Sample preparation of medium probes

As mentioned above (chapter 2.2.4), for Aβ ELISA and western blot experiments, 2-5 x 10^6 cells (2 or 5 ml total volume of cell suspension) were seeded into culture dishes and left to settle overnight. The next day, depending on the assay design, treatment was started. After incubation, 1 ml of the medium was collected and

centrifuged at 1000 x g for 5 min to remove cells and debris. Then, the protease inhibitor PMSF was added at a concentration of 1 µM to the medium of treated cells. Probes were frozen at –80°C until further usage. For western blot, samples were diluted with 4X NuPAGE SDS Sample buffer, boiled at 95°C for 10 min and centrifuged at 10.0000 x g for 5 min.

2.2.8 Western Blot

2.2.8.1 Self-made tricine and glycine western blots

After determination of the total protein content by the method of Lowry (chapter 2.2.3), depending on the western blot to be performed, cell extracts, medium probes, cytosolic and mitochondrial fractions were loaded on acrylamide gels and examined by SDS-PAGE. Since the usage of gradient gels was first reduced to the application of the antibody W-02 detecting APP and Aβ, we produced self-made glycine and tricine western blots depending on the protein to be detected. Generally, we performed glycine or tricine western blot with acrylamid amounts ranging form 10 to 18% at 90 V for 2-3h. For proteins with a molecular weight less than 30 kDa, tricine western blots (chapter 2.1.4) were more appropriate. The gels were prepared a day before SDS Page performance. High molecular weight proteins were separated on glycine western blots with acrylamide amounts ranging from 8-12%. Because of the intesnive stripping procedure, proteins were then transferred on Hybond P PVDF membranes (Amersham Biosciences, Germany) at 25 V for 90 min, being more resistant to the treatment than nitrocellulose membranes. Membranes were saturated with 5% nonfat dry milk in TBST for one hour before antibody exposure. Then, the PVDF membranes were exposed to the corresponding antibodies overnight.

2.2.8.2 Gradient gels

To detect APP and Aβ on the same western blot, we used NuPAGE 4-12% gradient gels (Invitrogen, Germany). The proteins were transferred on Hybond P PVDF membranes as described above and blocked for one hour with Western Breeze blocking solution, a comparable alternative to 5% nonfat dry milk. PVDF membranes were incubated with mouse anti-human APP W-02 (Abeta, Germany). The W0-2

antibody is directed against amino acids 4-10 of the human Aβ sequence, therefore detecting the mature and immature APP, sAPPα, C83, C99 and Aβ (Fig. 2.8). After treatment for 1h with the corresponding, horseradish peroxidase-coupled secondary antibodies (Calbiochem, Germany), the protein bands were detected by ECL chemiluminescence reagent (Amersham, Germany). After detection, the membranes were treated with stripping buffer for 2h before reprobing with distinct antibodies (chapter 2.1.4).

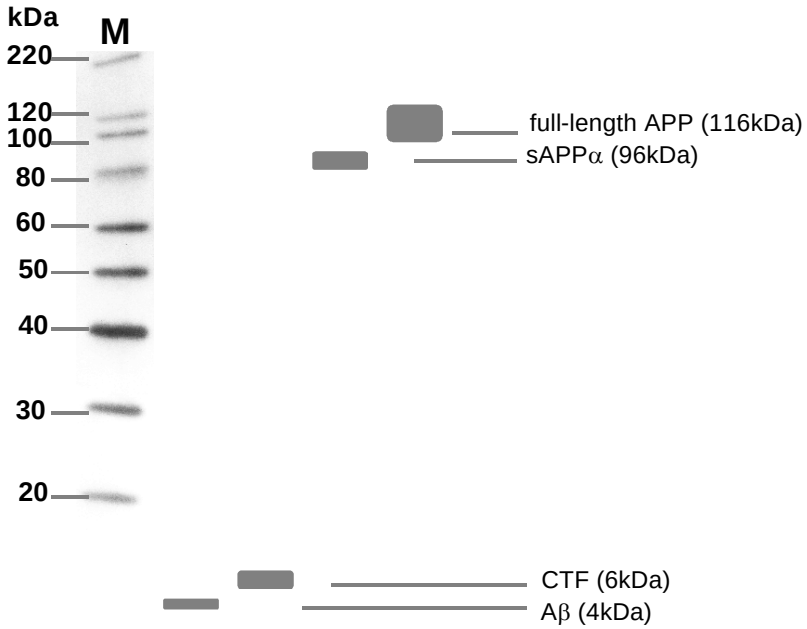

Figure 2.8: Scheme of a gradient gel.

On the left side, typical bands for the Marker MagicMark® (Invitrogen, Germany) are shown. The marker is horseradish peroxidase-coupled. Via the chemiluminecent reaction with ECL, the bands of the marker appear on the film. On the right side, bands of Aβ (4 kDa), CTF (C83, C99, approx. 6 kDa), sAPPα (glycosylated forms, 96 kDa) and full-length APP (glycosylated forms, 116 kDa). It is not possible to definitely differentiate between CTFs and Aβ, since the protein bands are too close to each other on the gradient gel.

2.2.9 Quantikine M Cytochrome c ELISA

For the sample preparation, 5 x 10^6 cells were plated on culture dishes the day before assay start. The next day, H_2O_2 (500 µM) was added to the cells to start incubation. At the end of the incubation, cells were collected by centrifugation at 800 x g for 5 min. The resulting pellet was washed with ice-cold PBS and again centrifuged at 800 x g for 5 min to remove the PBS wash buffer. In the following, cells

were lysed with the lysis buffer provided in the kit for 15 min while gently resuspending the pellet. Cell debris was removed by centrifugation at 800 x g for 5 min and the samples were stored at –80°C for further investigation. The ELISA was performed according to the instructions given in the Cytochrome c ELISA kit by RnD Systems, Germany. The assay employs the quantitative sandwich enzyme immunoassay technique and was designed to replace cytochrome c western blot. A monoclonal antibody specific for cytochrome c has been pre-coated onto a microplate. Samples are pipetted into the wells and any cytochrome c present is bound by the immobilized antibody (Fig. 2.9). After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for cytochrome c is added to the wells. Following four wash steps to remove any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and colour (blue) develops in proportion to the amount of cytochrome c bound in the initial step. The colour development is stopped by the addition of 1 N HCl and the intensity is measured by a ELISA microplate reader.

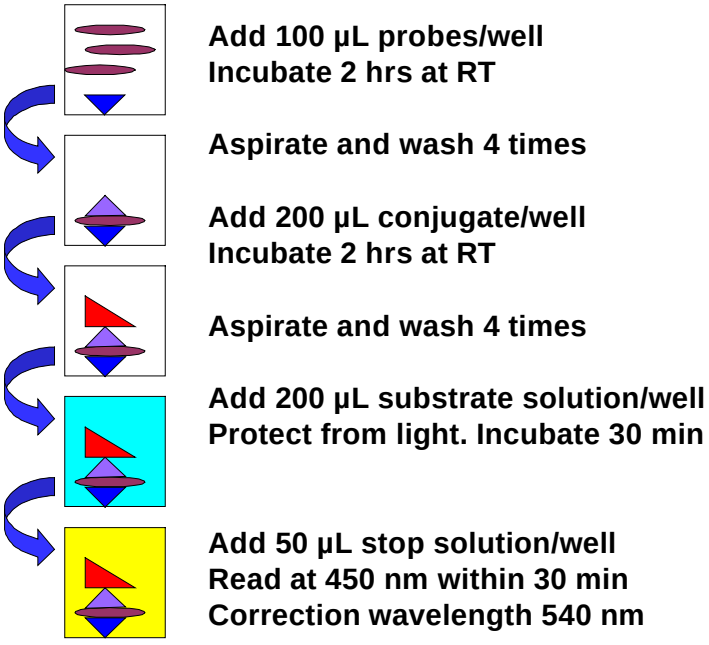

Figure 2.9: Assay procedure Quantikine M Cytochrome c ELISA.

2.2.10 MTT-Assay

The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells (Vistica, D. T., Skehan, P. et al., 1991; SLATER, T. F., 1963). This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH (Fig. 2.10). The formazan crystals formed are solubilized and the resulting coloured solution is quantified using a scanning multiwell spectrophotometer (ELISA reader).

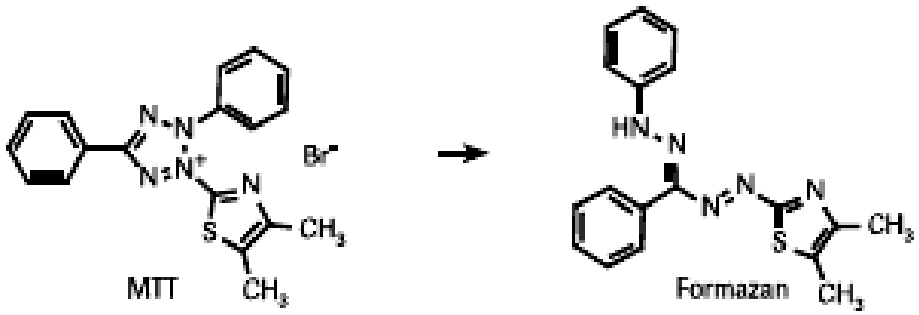

Figure 2.10: Metabolization of MTT to a formazan salt.

5-10 x 10^5 (PC12, HEK, SY5Y) cells were seeded per well the day before treatment (Fig. 2.11). Two hours before the end of incubation, MTT reagent was added at a final concentration of 1.0 mg/ml. Incubation was continued for another 2h, and the formazan crystals were then solubilized by 100 µl of a 20% SDS/50% N,N-dimethyl-formamide solution. After complete 12h of solubilization, the absorption was measured at 550 nm with a correction wavelength of 620 nm using an ELISA microplate reader.

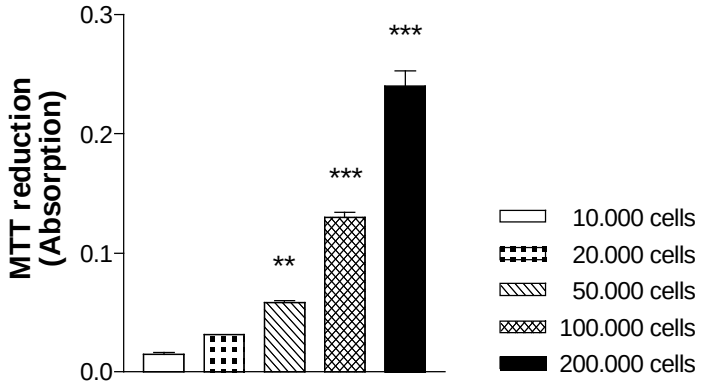

Figure 2.11: MTT assay of SY5Y cells.

In order to establish the optimal conditions of the MTT assay for SY5Y cells, increasing cell numbers of SY5Y cells were plated on 96 well plates the day before assay start. Then, the assay was started by the addition of the MTT reagent. After solubilization, the absorption was measured at 450 nm with a correction wavelength at 620 nm. Considering the total absorption values, 50.000- 100.000 cells/well were considered as optimal. One-way ANOVA $p<0.0001$; posthoc *t* test: ** $p<0.01$, ***$p<0.001$ vs 10.000 cells.

2.2.11 LDH assay

The assay principle relies on the fact that lactate dehydrogenase can pass the plasma membrane of damaged (necrotic or apoptotic) cells. Outside the cell, lactate dehydrogenase catalyses the oxidation of lactate to pyruvate and the reduction of NAD^+ to $NADH+H^+$ in the same time. $NADH+H^+$ reduces a yellowish tetrazolium salt resulting in a deep red formazan salt (Fig. 2.12). Consequently, the absoption of the formazan correlates with lactate dehydrogenase activity. Cells were plated at a density of 1 x 10^5, according to manufacturer´s instructions (Roche Diagnostics, Germany) and the assay was started. After incubation, cells were centrifuged at 1000 x g, the supernatant was pipetted in a fresh 96 well plate, and LDH reagent was added. After half an hour, stop solution (1 N HCl) was added and absorption was measured at 420 nm with a correction wavelength of 620 nm using an ELISA microplate reader.

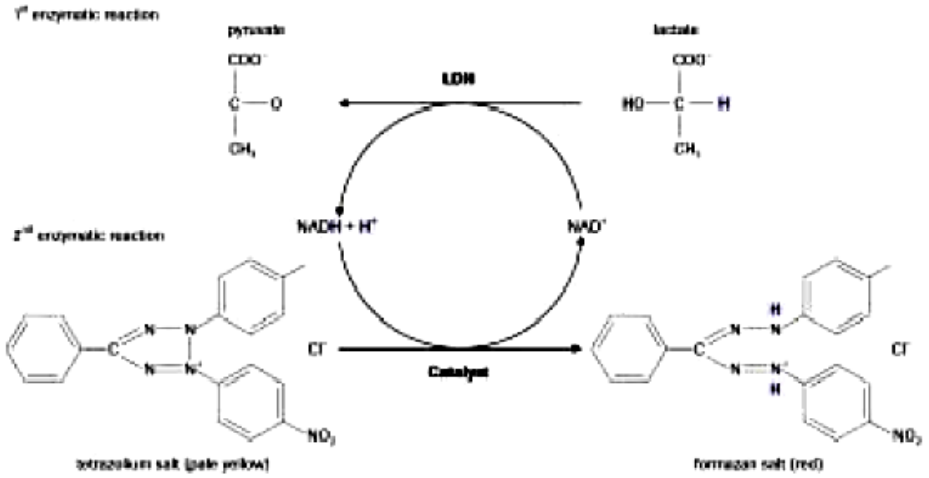

Figure 2.12: Test principle LDH assay.

2.2.12 Determination of ATP levels

SY5Y cells were plated the day before at a density of 2 x 10^4 cells/well in a white 96 well plate. SY5Y cells were incubated with varying concentrations of H_2O_2 for different periods of time. The kit is based upon the bioluminescent measurement of ATP. The bioluminescent method utilises an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin. The emitted light is linearly related to the ATP concentration and is measured using a luminometer. The bioluminescent method utilises an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction:

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{++}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT} \quad (\text{Emission at } 565 \text{ nm})$$

Figure 2.13: Test priciple ATP assay.

2.2.13 γ-secretase activity assay

The assay detects the enzymatic activity of the γ-secretase class of proteases associated with the cleavage of amyloid precursor protein (APP) from cell lysates using a fluorometric reaction. First, cells of interest are lysed to collect their intracellular contents. Principally, protein content is determined by the method of Lowry (chapter 2.2.3) allowing to always apply the same amount of protein. Generally, the applied volume of lysis buffer should be kept as low as possible to achieve maximum protein contents. The cell lysate is then tested for secretase activity by the addition of a secretase-specific peptide conjugated to the reporter molecules EDANS and DABCYL (Fig. 2.14).

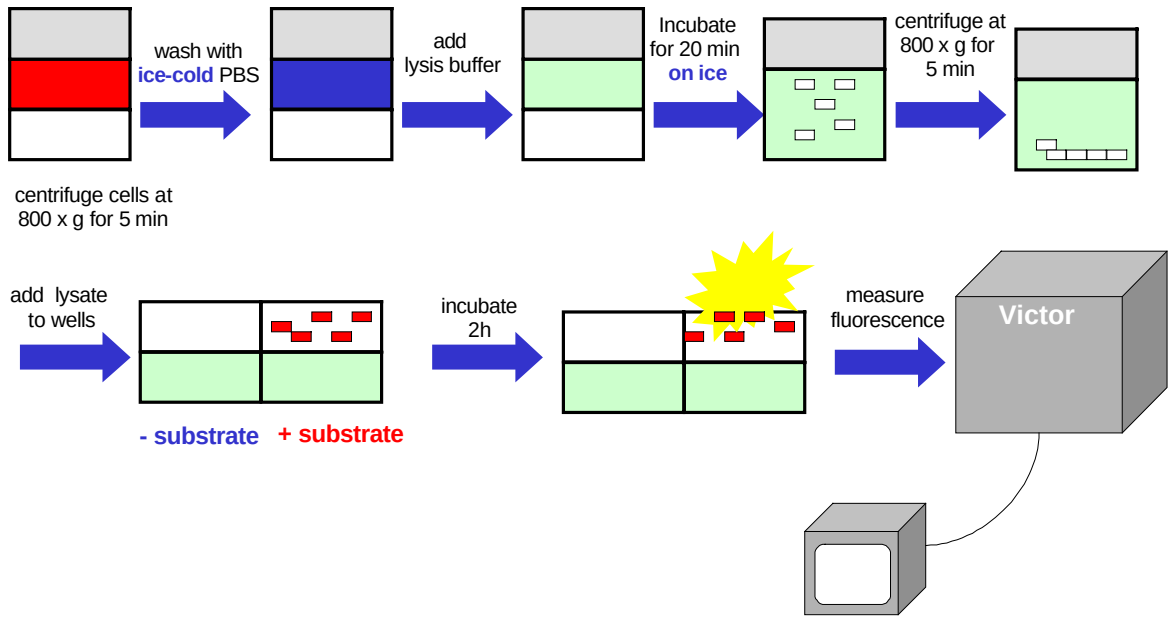

Figure 2.14: Assay protocol for the γ-secretase activity assay.

In the uncleaved form, the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety which exhibits maximal absorption at the same wavelength (495 nm) (Fig. 2.15). Cleavage of the peptide by the secretase physically separates EDANS and DABCYL allowing the release of a fluorescent signal. The substrate sequence provided with the kit corresponds to the amino acid sequence associated with γ-secretase cleavage of amyloid precursor protein (amino

acids 710-718) and includes the London mutation. The level of secretase enzymatic activity in the cell lysate is proportional to the fluorometric reaction. Background controls are performed by reactions without addition of the substrate. Then, these values are subtracted from the experimental results prior to calculating the x-fold increase.

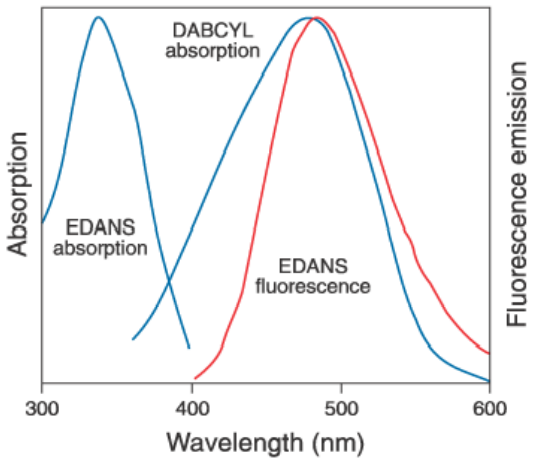

Figure 2.15: Test principle of the γ-secretase activity assay.
In the uncleaved form, the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety which exhibits maximal absorption at the same wavelength (495 nm). Cleavage of the peptide by the secretase physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal.

2.2.14 β-secretase activity assay

First, cells of interest are lysed to collect their intracellular contents. Principally, protein content is determined by the method of Lowry (chapter 2.2.3) allowing to always apply the same amounts of protein. The assay principle is that of the γ-secretase activity assay. Generally, the applied volume of lysis buffer should be kept as low as possible to achieve maximum protein contents. The cell lysate is then tested for secretase activity by the addition of a secretase-specific peptide conjugated to the reporter molecules EDANS and DABCYL. In the uncleaved form, the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety which exhibits maximal absorption at the same wavelength (495 nm). Cleavage of the peptide by the secretase physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal. A reconstituted β-secretase

was provided within the kit as a positive control to assure the proper working of the test (Fig. 2.16).

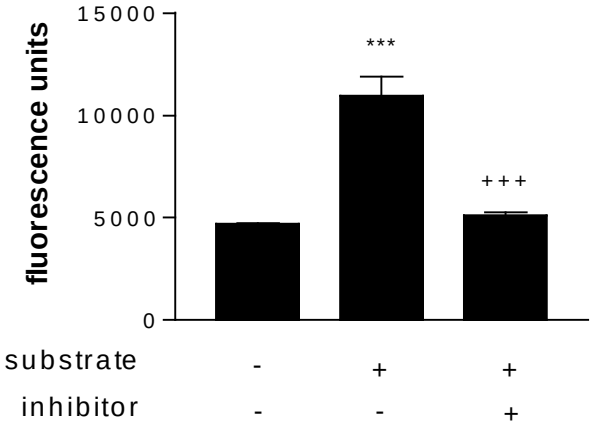

Figure 2.16: Enzyme activity of reconstituted β-secretase.

First, activity of reconstituted β-secretase without substrate was determined. After the addition of substrate, β-secretase activity increases to the twofold. Addition of β-secretase inhibitor completely blocks enzyme activity. One-way ANOVA $p<0.001$; posthoc *t* test: *** $p<0.001$ vs. β-secretase (minus substrate/inhibitor); $^{+++}$ $p<0.001$ vs. β-secretase (plus substrate/inhibitor).

2.2.15 Statistical Analysis

Data are given as mean ± SEM. For statistical comparison, Students *t*-test, One-way ANOVA followed by Tukeys posthoc test, Repeated Measures ANOVA or Two-way ANOVA were used. p values less than 0.05 were considered statistically significant. For the statistical analysis, GraphPad Prism 3.0 software was used.

3 RESULTS

3 Results

3.1 Characterization of APP processing and Aβ secretion

3.1.1 Secretion of Aβ in APPwt and APPsw PC12, HEK and SY5Y cells

In order to investigate the effect of chronic APP expression and Aβ production on oxidative stress-induced apoptosis, APP expression and Aβ secretion levels of three different cell lines were determined. PC12 cells, rat pheochromocytoma cells, are of neuronal origin and represent a convenient model for the investigation of neuro-degenerative diseases. Stably APP-transfected PC12 cells suffering from chronic exposure to APP and Aβ, with secretion levels in the picomolar range, mimic physiological conditions since comparable $A\beta_{1-40}$ levels have been determined in post-mortem dissected brains from patients suffering from sporadic AD. We could show that $A\beta_{1-40}$ levels of stably transfected PC12 cells bearing the Swedish double mutation are elevated to the twofold compared to control cells ($p<0.05$ vs. control), whith APPwt PC12 cells secreting 20 pg/ml less $A\beta_{1-40}$ into the culture medium compared to APPsw PC12 cells (Fig. 3.1). Furthermore, APPwt and APPsw clones express similar levels of APP (Eckert, A., Steiner, B. et al., 2001). Culture supernatants of PC12 cells were assayed for secreted $A\beta_{1-42}$, but due to the extremely low secretion levels (approx. < 15pg/ml), the obtained values were below the detection limit of the $A\beta_{1-42}$ ELISA kit.

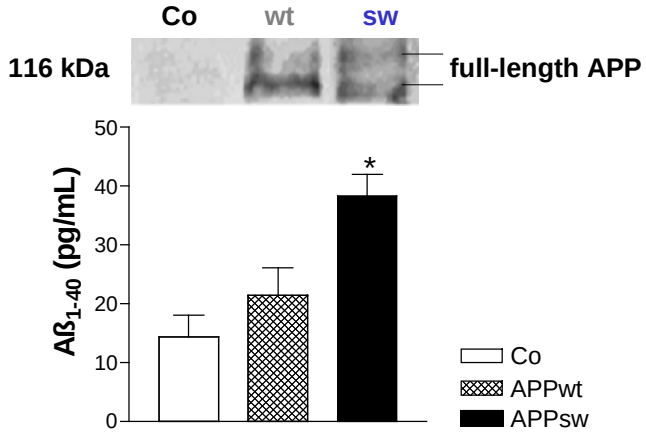

Figure 3.1: Secreted $A\beta_{1-40}$ levels of PC12 cells.

Culture supernatants of 5 x 10^6 PC12 cells (APPwt, APPsw, Co) were collected after 24h incubation and assayed for $A\beta_{1-40}$ by ELISA. One-way ANOVA: ***p<0.0001, posthoc *t* test: *p<0.05 vs. control (Co). Data are shown as means ± SEM of 3-4 independent experiments. Inlay: APP expression levels of PC12 cells.

HEK cells, human embryonic kidney cells, are of non-neuronal origin. They were stably transfected with APPwt and APPsw and represent convenient models for the investigation of APP processing, Aβ formation and secretion (Forman, M. S., Cook, D. G. et al., 1997). HEK control cells express low amounts of endogenous human APP (Fig. 3.2, Inlay) that is proteolytically processed resulting in measurable levels of secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ (Fig. 3.2 A+B). In comparison to APPsw PC12 cells, APPsw HEK cells secrete 30-fold higher levels of $A\beta_{1-40}$ and eightfold higher levels of $A\beta_{1-42}$, thus reaching the micromolar range. $A\beta_{1-42}$ levels of APPsw HEK cells were twentyfold lower than $A\beta_{1-40}$ levels (Fig. 3.2 A+B). APPsw HEK cells secrete tenfold more $A\beta_{1-40}$ ($p<0.01$ vs. APPwt control) and sevenfold more $A\beta_{1-42}$ ($p<0.05$ vs. APPwt control) than APPwt and control HEK cells ($p<0.05$, $p<0.001$ vs. control) (Fig. 3.2 A). APP expression, which does not alter between APPsw and APPwt PC12 cells, differs between HEK clones since APPsw HEK cells express more APP than APPwt HEK cells (Fig. 3.2, Inlay). Due to the characteristically high APP and Aβ levels in APPsw HEK cells, they serve as a comparative model for massive, chronic Aβ overproduction in contrast to chronically low exposure levels of Aβ in PC12 cells.

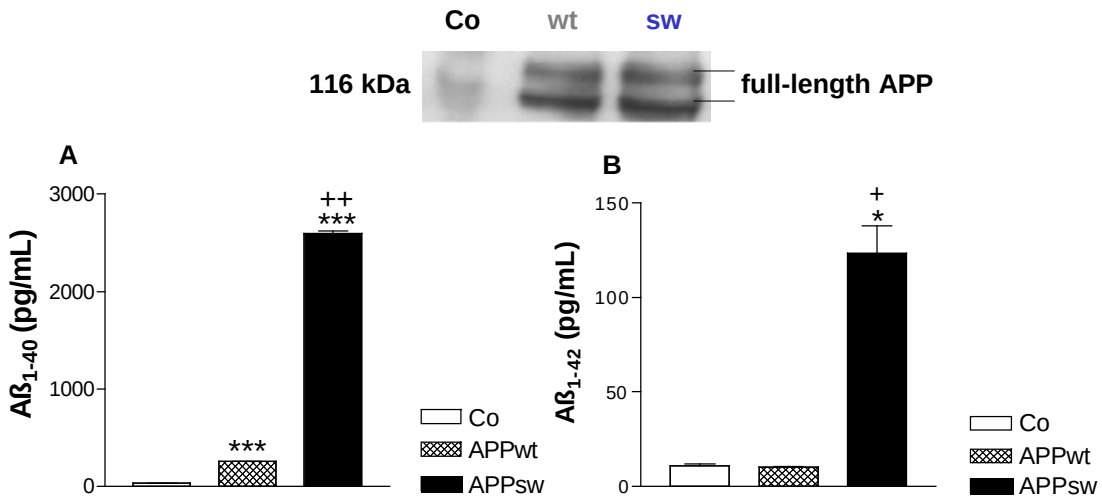

Figure 3.2: Secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ levels of HEK cells.

Culture supernatants of 5 x 10^6 HEK cells (APPwt, APPsw, Co) were collected and assayed for $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ (B) by ELISA. A) Secreted $A\beta_{1-40}$. B) Secreted $A\beta_{1-42}$. One-way ANOVA: ***$p<0.001$, posthoc *t* test: *$p<0.05$, *** $p<0.001$ vs. control (Co), $^{+}p<0.05$, $^{++}p<0.01$ vs. APPwt control. Data are shown as means ± SEM of 3-4 independent experiments. Inlay: APP expression levels of HEK cells.

SY5Y cells, human neuroblastoma cells, represent a neuronal cell line of human origin. The cell model of stably expressing APPwt clones closely mimics conditions in brains of patients suffering from sporadic AD. APPwt SY5Y cells secrete significantly ($p<0.001$) higher levels of $A\beta_{1-40}$ compared to control cells (Co).

Secretion levels of endogenous $A\beta_{1-40}$ control (Co) SY5Y cells are roughly in the range of control HEK cells (Fig. 3.3). Secreted $A\beta_{1-40}$ levels of APPwt SY5Y cells (approx. 210 pg/ml) are comparable to the levels of APPwt HEK (approx. 280 pg/ml) and. When compared to PC12 cells, APPwt SY5Y cells secrete sevenfold higher $A\beta_{1-40}$ levels than APPsw PC12 and tenfold higher $A\beta_{1-40}$ than APPwt PC12 cells.

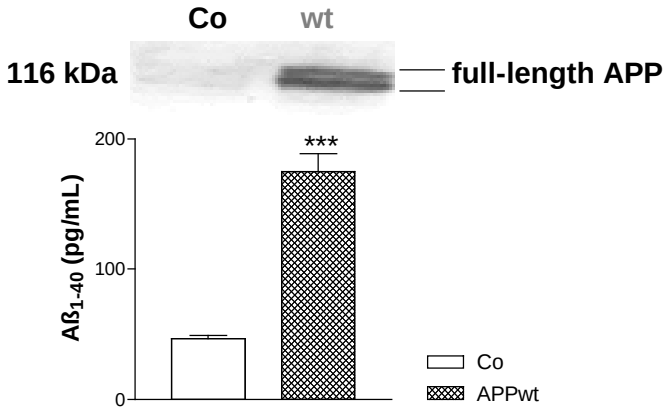

Figure 3.3: Secreted $A\beta_{1-40}$ levels of SY5Y cells.

Culture supernatants of 5 x 10^6 SY5Y cells (APPwt, Co) were collected and assayed for $A\beta_{1-40}$ by ELISA. One-way ANOVA: ***p<0.001, posthoc *t* test: ***p<0.001 vs. control (Co). Data are shown as means ± SEM of 3 independent experiments. Inlay: APP expression levels of SY5Y cells.

3.1.2 Intracellular Aβ levels of cell lysates

Recently, it has been shown that in a model of rat cortical neurons intracellular, dimeric and oligomeric Aβ forms are the key toxic species of neuronal cell death (Kienlen-Campard, P., Miolet, S. et al., 2002). There is only little known about intracellular Aβ levels in stably transfected cell lines. We determined intracellular soluble and insoluble Aβ levels in HEK cells in order to further characterize the situation in stably transfected cells and to compare the results with those obtained from primary cultures. In order to discriminate between soluble and insoluble Aβ species, we performed particular extraction methods.

Tris buffer lysis was performed for the extraction of soluble Aβ and insoluble Aβ species were extracted by formic acid (FA) lysis.

The experiments were performed with HEK cells, because they secrete Aβ in large quantities which lead us to the speculation that intracellular Aβ might correlate with extracellular Aβ reaching detectable levels.

HEK control cells secrete low amounts of endogenous human Aβ detectable by the ELISA kit. Nevertheless, intracellular, soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels of HEK control cells were at the detection limit given in the ELISA kit (<15 pg/ml) (Fig. 3.4 A+B). Compared to secreted extracellular $A\beta_{1-40}$, intracellular $A\beta_{1-40}$ levels are approximately more than hundredfold lower in APPsw, twentyfivefold lower in APPwt and tenfold lower in control HEK cells (Co). Secreted $A\beta_{1-40}$ levels are twentyfold higher than $A\beta_{1-42}$ levels. Intracellular $A\beta_{1-42}$ levels are in the same range like intracellular $A\beta_{1-40}$ levels (Fig. 3.4 B). Nevertheless, we can conclude that intracellular, soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels are of similar quantity (Fig. 3.4 A+B), with a tendency to higher soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPsw HEK cells, although not significant.

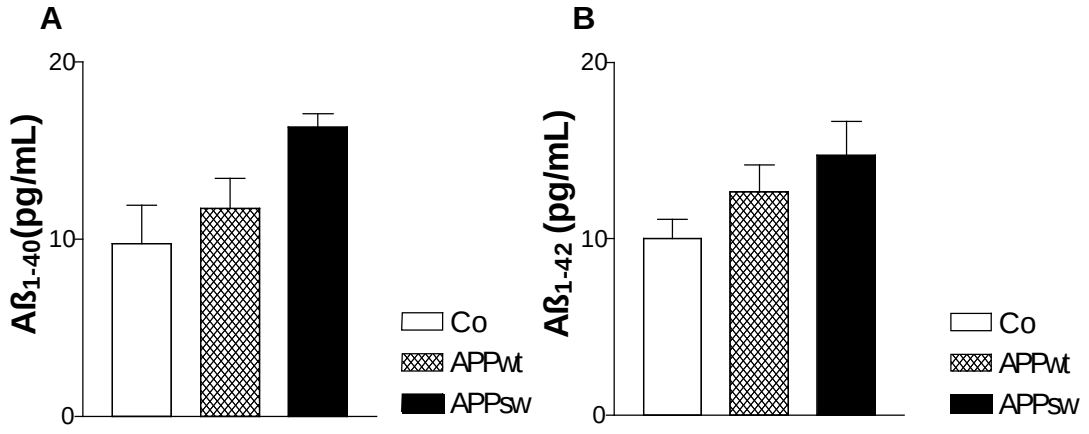

Figure 3.4: Intracellular soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels of HEK cells.

Tris lysis was performed on 10 x 10^6 HEK cells (APPwt, APPsw, Co) and lysates were then assayed for $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ (B) by ELISA. Data are shown as means ± SEM of 3 independent experiments.

For the detection of insoluble intracellular Aβ species, a formic acid (FA) extraction was performed on HEK cells (Fig. 3.5 A+B). We observed a slight, but not significant increase in insoluble $A\beta_{1-40}$ levels of APPsw and APPwt HEK cells compared to control HEK cells (Fig. 3.5 A).

Intracellular, insoluble $A\beta_{1-42}$ was not detectable in APPwt and APPsw HEK cells. The FA extract of APPsw HEK cells contained approximately 60 ng/ml insoluble $A\beta_{1-42}$ (Fig. 3.5 B). Concluding we may state that in APPsw HEK cells, the ratio of $A\beta_{1-40}$ and $A\beta_{1-42}$ is shifted in favour of intracellular, insoluble $A\beta_{1-42}$ and that the accumulation of insoluble Aβ species is a specific feature of APPsw HEK cells.

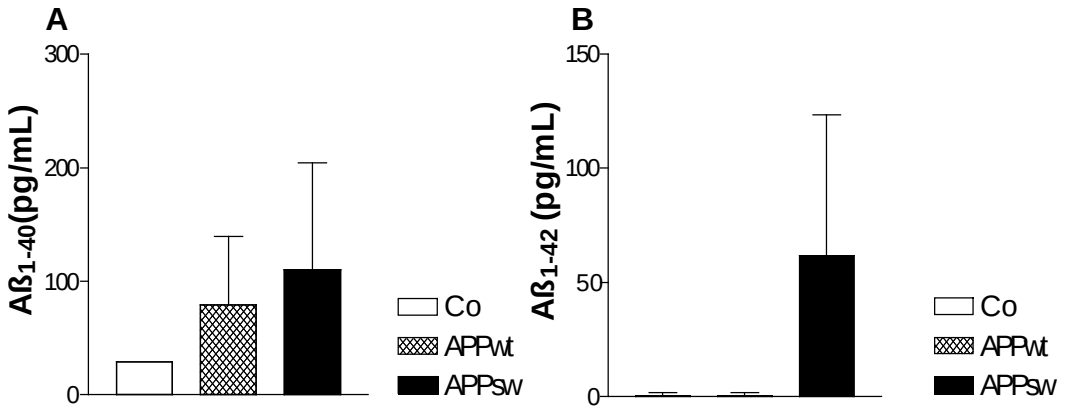

Figure 3.5: Intracellular insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels of HEK cells.

Formic acid lysis was performed on 10 x 10^6 HEK cells and lysates were then assayed for $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ (B) by ELISA. One-way ANOVA: ns., posthoc *t* test: ns. Data are shown as means ± SEM of 3 independent experiments.

3.1.3 APP and mitochondria

In the recent years, there has been intensive discussion about APP processing and Aβ generation and their impact on neuronal cell death. APP, besides its ER targeting domain being responsible for the correct post-translational transport of APP, additionally owns a mitochondrial-targeting domain. It has been demonstrated recently that the accumulation of full-length APP in the mitochondrial compartment in a transmembrane-arrested form causes mitochondrial dysfunction and impaired energy metabolism in rat cortical neurons (Anandatheerthavarada, H. K., Biswas, G. et al., 2003). Using confocal laser scanning microscopy, we could show that mitochondria and APP are partly co-located in the cell (Keil, U., Bonert, A. et al., 2004). Full-length APP (116 kDa) was detected in the mitochondrial fraction of PC12 cells by western blot (Fig. 3.6). In contrast to this, the purified cytosolic fraction is free from APP, indicating that in this fraction, possible impurities from the plasma membrane are underneath the detection limit. The cytosolic fraction is free from

cytochrome c oxidase-4 (COX-4, 14 kDa), a marker for the inner mitochondrial membrane, demonstrating the absence of mitochondrial debris. As expected, Actin (45 kDa), the major intracellular protein component, is found in the cytosolic and mitochondrial fraction.

ER-associated amyloid-binding protein (ERAB, 27 kDa), which we supposed to be located in the ER (Du Yan, S., Shi, Y. G. et al., 1999), was detected in the cytosolic fraction. Therefore, it seems possible that ERAB is associated with the outer ER membrane, being easily removed by the fractionation procedure. From the outer ER membrane, ERAB might be transported to other subcellular compartments under conditions of oxidative stress.

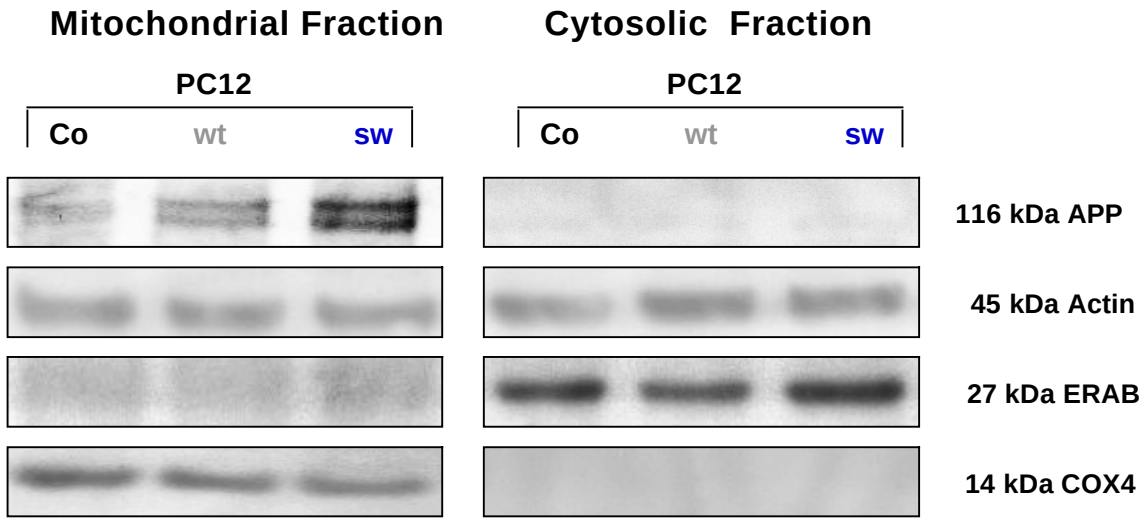

Figure 3.6: APP is present in the mitochondrial fraction of PC12 cells.

Mitochondrial and cytosolic fractions of 5 x 10^6 were subjected to western blot and analysed for the following proteins: full-length APP (116 kDa), Actin (45 kDa), ERAB (27 kDa) and COX-4 (14 kDa). Full-length APP is located in the purified mitochondrial fraction. Actin is found in both, cytosolic and mitochondrial fraction. ERAB is mainly located in the cytosolic compartment. COX-4, a crucial marker for the mitochodrial fraction, is found there exclusively.

In order to assess whether the presence of APP in mitochondria is a more generally observed phenomenon in cell culture, we submitted mitochondrial and cytosolic fractions of APPwt, APPsw and control HEK cells to western blot analysis (Fig. 3.7). Since HEK cells are of non-neuronal origin, they are prone to show an intracellular full-length APP localization distinct from PC12 cells. We may also speculate that this effect is probably linked to the extremely high APPsw- and APPwt-load of HEK cells.

Indeed, full-length APP (116 kDa) was not only present in the mitochondrial but also in the cytosolic compartment of APPwt and APPsw HEK cells. It might be that the APP found in the cytosolic fraction has been associated with the endoplasmatic reticulum and was separated by the fractionation procedure. C-terminal fragments (CTFs, 6 kDa), namely C99 and C83, are equally detected in the cytosolic fraction.

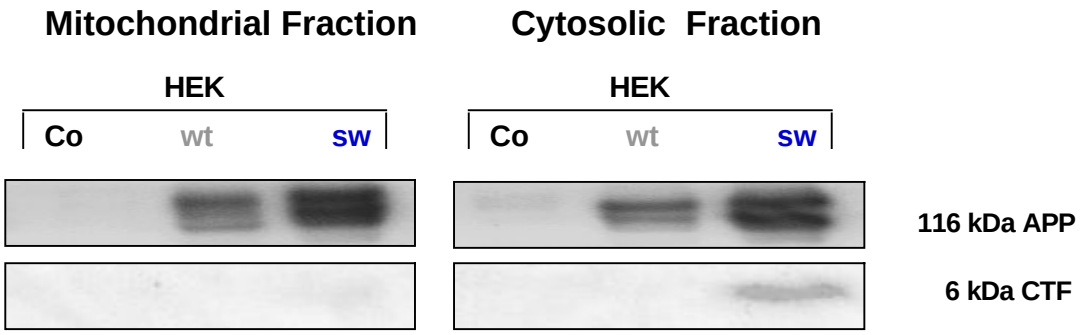

Figure 3.7: APP is present in the mitochondrial fraction of HEK cells.
Mitochondrial and cytosolic fractions of 5 x 10^6 HEK cells were subjected to western blot and analysed for the following proteins: full-length APP (116 kDa) and CTF (6 kDa). Full-length APP is located in the purified mitochondrial fraction. CTFs are exclusively found in the cytosolic compartment.

3.1.4 Aβ and mitochondria

In order to further characterize the intracellular localization of Aβ in our cell models, we determined Aβ levels of purified cytosolic and mitochondrial fractions of PC12, HEK and SY5Y cells by ELISA technique (Fig. 3.8 A-C). PC12 and SY5Y cells show very low cytosolic and mitochondrial $A\beta_{1-40}$ within the detection limit (Fig. 3.8 A+C). Hence, mitochondrial $A\beta_{1-40}$ of APPsw HEK cells (38 pg/ml, $p<0.05$ vs. control, $p<0.05$ vs. APPwt control) was found in significantly elevated amounts compared to APPwt (< 10 pg/ml) and control cells (<10 pg/ml) (Fig. 3.8 B). APPsw HEK cells contain tenfold higher mitochondrial $A\beta_{1-40}$ levels than APPwt and control HEK cells. Due to fact that proteins are generally concentrated in the course of the fractionation procedure, $A\beta_{1-40}$ detected in mitochondrial fraction of APPsw HEK cells (approx. 38 pg/ml) is higher than in Tris lysates (15pg/ml).

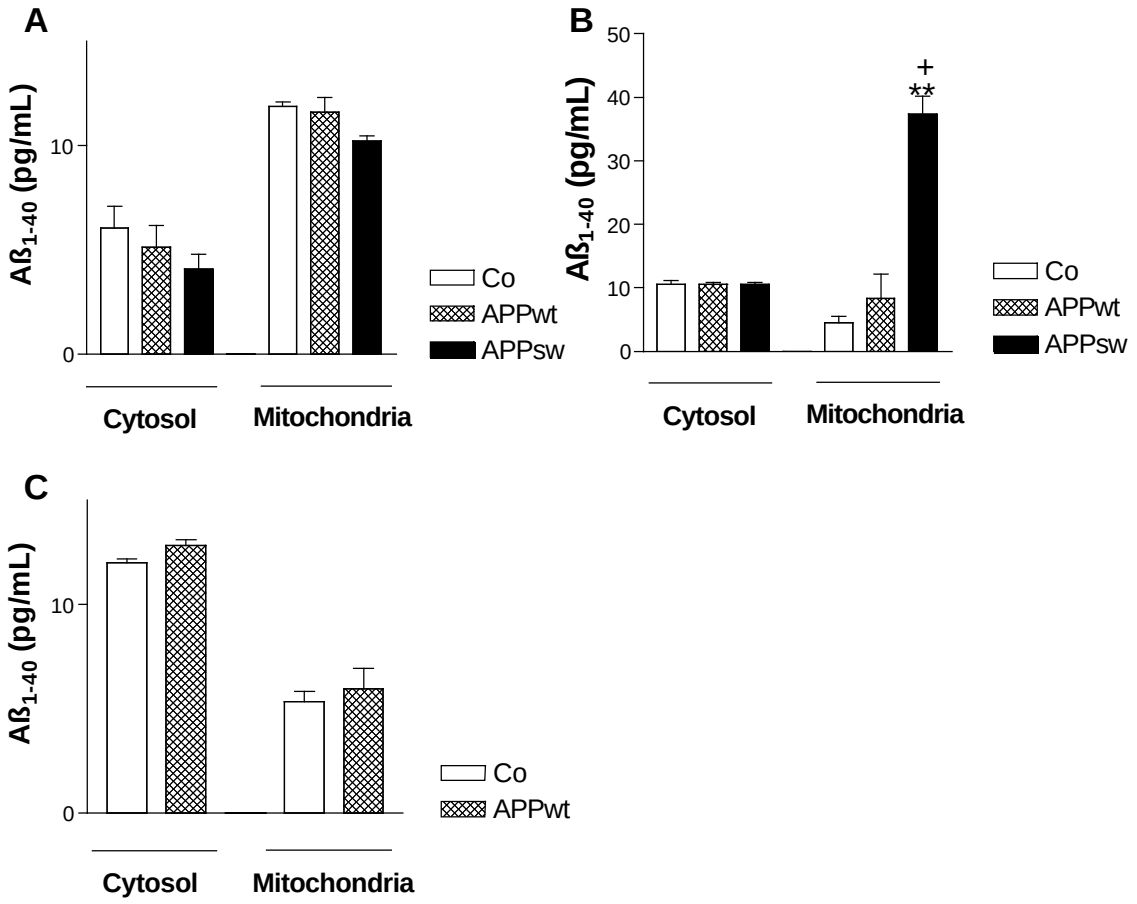

Figure 3.8: $A\beta_{1-40}$ is located in mitochondria of HEK cells.

A) $A\beta_{1-40}$ levels in mitochondrial and cytosolic fractions of 5 x 10^6 PC12 cells. B) $A\beta_{1-40}$ levels in mitochondrial and cytosolic fractions of 5 x 10^6 HEK cells. C) $A\beta_{1-40}$ levels in mitochondrial and cytosolic fractions of 5 x 10^6 SY5Y cells. One-way ANOVA: ***p<0.001, posthoc *t* test: **p<0.01 vs. control (Co), $^{+}$p<0.05 vs. APPwt. Data are shown as means ± SEM of 2-4 independent experiments.

We suggest that mitochondrial fractions were enriched in protein and free of proteases potentially being able to degrade Aβ. In contrast, whole cell lysates contain multiple Aβ-degrading proteases, and most probably, it is a question of protease inhibitor concentration that should be higher than the inhibitor concentration used in culture supernatant probes. Notably, cellular debris consisting of DNA fragments, organelles and plasma membrane could potentially quench the Aβ signal or interact with the detection and capture antibody.

3.1.5 Secretase activity in PC12 and HEK cells

In the preceding chapters, we could demonstrate that PC12, HEK and SY5Y cells revealed individually distinct APP expression and Aβ secretion levels. It was found that β-secretase expression is significantly increased in brains of AD patients (Shen, Y., Yang, L. B. et al., 2002; Fukumoto, H., Cheung, B. S. et al., 2002; Holsinger, R. M. D., Mclean, C. A. et al., 2004; Yang, L. B., Lindholm, K. et al., 2003c; Li, R., Lindholm, K. et al., 2004b) and that β-secretase activity increases with aging in human, monkey and mouse brain (Fukumoto, H., Rosene, D. L. et al., 2004). These findings indicate that β-secretase expression and activity is of considerable in vivo relevance. Moreover, numerous evidence is provided that the APPsw mutation promotes β-secretase activity in APP transgenic mice. In order to characterize APPwt and APPsw-transfected PC12 and HEK cells as suitable models for the investigation of AD-related alterations we determined the activity of the enzyme.
Since secretases are ubiquitously expressed, we questioned whether HEK cells show β- and γ-secretase activity comparable to those of neuronal cells. The studies intended to characterize β– and γ-secretase activity in the cell culture models of AD with special regard to the impact of the enzymes on Aβ production.

3.1.5.1 β-secretase activity

The APPsw mutation is associated with increased β-secretase-mediated cleavage leading to an early onset of Alzheimer's Disease (Haass, C., Lemere, C. A. et al., 1995b). The subsequent γ-secretase cleavage results an enhanced generation of $A\beta_{1-40}$ and $A\beta_{1-42}$. We determined basal β-secretase activity of PC12 and HEK cells (Fig. 3.9 A+B). In APPsw PC12 cells, baseline β-secretase activity is increased to the twofold compared to APPwt ($p<0.05$ vs. APPwt) and control cells ($p<0.05$ vs. control) (Fig. 3.9 A). The same effect, even though less pronounced, was observed in APPsw HEK cells showing a significantly ($p<0.05$ vs. control) enhanced β-secretase activity compared to control HEK cells (see Fig. 3.9 B). The demonstrated data are consistent with previously reported findings of other groups, since β-secretase of APPsw transfected cells is significantly increased.

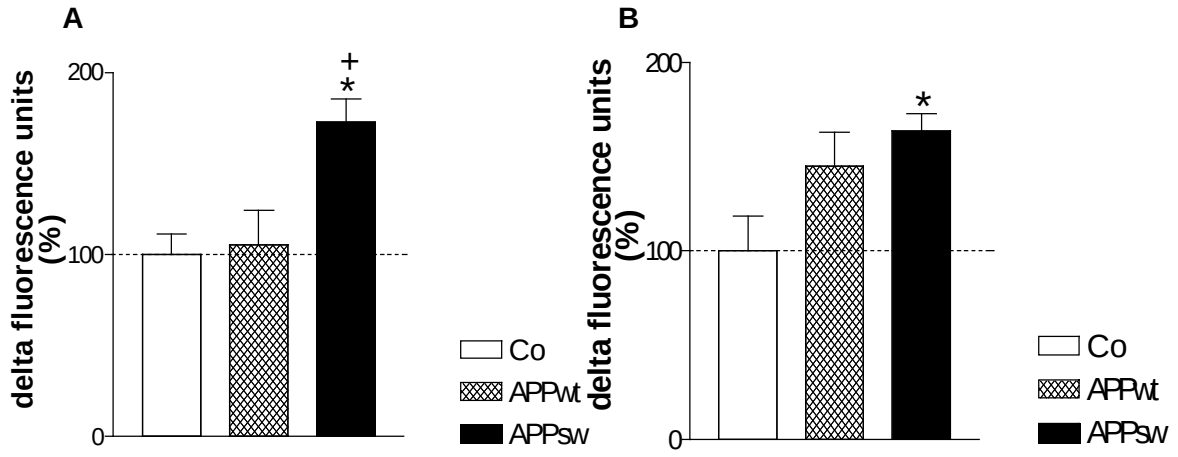

Figure 3.9: Increased β-secretase activity in APPsw PC12 and HEK cells.

Cell lysates of 5 x 10^6 cells were collected and assayed for β-secretase activity. A) β-secretase activity in PC12 cells. B) β-secretase activity in HEK cells. One-way ANOVA: ***p<0.001, posthoc *t* test: *p<0.05 vs. control (Co), $^{+}$p<0.05 vs. APPwt. Data are shown as means ± SEM of 4 independent experiments. Dotted line represents 100% control.

3.1.5.2 γ-secretase activity

In contrast to the increasing evidence for the implication of enhanced β-secretase activity in AD, little is known about AD-dependent alterations in γ-secretase activity. This might be due to the structure of γ-secretase representing a multimeric enzyme complex. It was reported that the isolation and reconstitution from human brain tissue homogenates was related to numerous methodical difficulties (Fraering, P. C., Ye, W. J. et al., 2004; Farmery, M. R., Tjernberg, L. O. et al., 2003; Edbauer, D., Winkler, E. et al., 2003). So far, six different γ-secretase complexes have been identified in the cell (Shirotani, K., Edbauer, D. et al., 2004), and evidence is provided that intracellular localization and environment have a strong impact on its activity (Pasternak, S. H., Callahan, J. W. et al., 2004; Urano, Y., Hayashi, I. et al., 2005; Yu, W. H., Kumar, A. et al., 2004). We assessed whether APPwt and the APPsw mutation have an effect on baseline γ-secretase activity (Fig. 3.10 A+B). γ-secretase activity was significantly (p<0.01) increased in APPsw PC12 cells compared to control cells (Fig. 3.10 A). In APPsw, APPwt and control HEK cells, comparable γ-secretase activity levels were determined. Considering the results obtained from β-

secretase activity measurements with similar results in both cell culture models, we may conclude that γ-secretase activity is not simultaneously elevated in APPwt and APPsw HEK cells compared to APPwt and APPsw PC12 cells. On the one hand we speculate that γ-secretase activity may be at maximum levels in control HEK cells. HEK control cells secrete Aβ levels comparable to those of APPsw PC12 cells (approx. 38 pg/ml), thus already leading to a maximum γ-secretase activity in control HEK cells. This implicates that the Swedish mutation does not prime for an increased γ-secretase activity per se. On the other hand, we may deduct that the APPwt and APPsw transfections do not have an impact on γ-secretase activity in non-neuronal cells. This would lead to the conclusion that the effect of the APPsw mutation on γ-secretase activity is a typical hallmark of neuronal cell lines. In a more abstract manner, we may rise the question whether alterations in composition and localization of the γ-secretase complex, rather than APP-related changes, modulate γ-secretase activity.

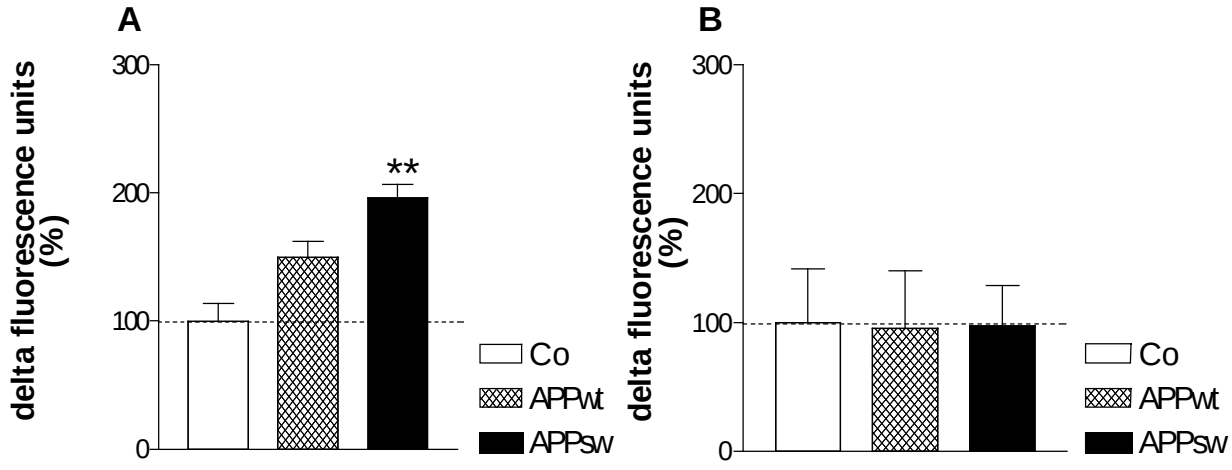

Figure 3.10: Increased γ-secretase activity in APPsw PC12 cells, but not in APPsw HEK cells.

Cell lysates of 5 x 10^6 cells were collected and assayed for γ-secretase activity. A) Delta values (%) of γ-secretase activity in PC12 cells. B) Delta values (%) of γ-secretase activity in HEK cells. One-way ANOVA: ***p<0.001, posthoc *t* test: **p<0.01 vs. control cells (Co). Data are shown as means ± SEM of 4 independent experiments. Dotted line represents 100% control under baseline conditions.

3.1.5.3 Comparison of secretase activity

APPwt and APPsw HEK cells do not show significantly increased γ-secretase activity compared to control cells. Without specific substrate addition, fluorescence levels of HEK cells are comparable those of PC12 cells, indicating that increased, unspecific baseline fluorescence is not the reason for the astonishing results obtained for γ-secretase obtained in HEK cells.

One consistent observation in association with AD is that β-secretase activity is significantly elevated in cells (Haass, C., Lemere, C. A. et al., 1995a) and transgenic mice bearing the Swedish double mutation (Rossner, S., Apelt, J. et al., 2001) as well as in brains of patients suffering from sporadic AD (Tyler, S. J., Dawbarn, D. et al., 2002; Li, R., Lindholm, K. et al., 2004a; Yang, L. B., Lindholm, K. et al., 2003a). In our cell model the APPsw mutation results in a markedly increased $A\beta_{1-40}$ secretion of APPsw HEK cells (2.6 pg/ml) (Keil, U., Bonert, A. et al., 2004) and $A\beta_{1-42}$ (0.2 pg/ml) compared to control HEK cells ($A\beta_{1-40}$: 0.35 pg/ml; $A\beta_{1-42}$: 14 pg/ml) . We could already demonstrate that these high, chronically secreted Aβ levels initiated early apoptosis as monitored by propidium iodide staining under baseline conditions (Keil, U., Bonert, A. et al., 2004). Here, we could demonstrate that the APPsw mutation leads to a significantly increased β-secretase activity in HEK cells (APPsw: 164 ± 15 % vs. control: 100 ± 32 %; $p<0.05$) under baseline conditions (Fig. 3.11). The increase in β-secretase activity might finally result in an increased formation of βCTFs (C99, C-terminal fragments) and consequently to an increased Aβ secretion.

In our model, we did not observe significant alterations of γ-secretase activity in APPsw and control HEK cells under baseline conditions compared to β-secretase activity. Thus, we may conclude that β- and γ-secretase activity are not directly interrelated. The most probable explanation might be that the APPwt and APPsw transfection does not have an impact on γ-secretase activity in non-neuronal cells, while β-secretase activity is equally elevated in neuronal (PC12) and non-neuronal (HEK) cells.

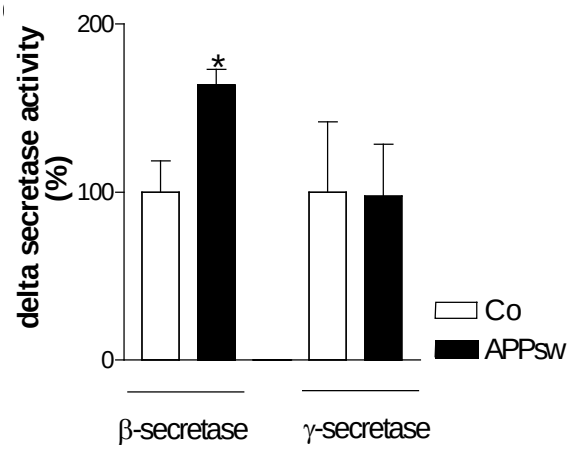

Figure 3.11: Comparison of β- and γ-secretase activity in APPsw and control HEK cells.

β- secretase and γ–secretase activity of APPsw and control HEK cells were compared. One-way ANOVA: $p<0.05$. posthoc *t* test revealed a significant increase in β–secretase activity in APPsw (*$p<0.05$) vs. control HEK cells. All values represent the means ± SEM from 3-4 experiments.

3.2 Toxicity of different Aβ species

It is still under investigation which kind of Aβ species play the most prominent role in the course of AD. There is growing evidence that intracellular, dimeric or oligomeric Aβ mainly contributes to the neuro-degenerative process (Kayed, R., Head, E. et al., 2003). The aim of the following study was to assess whether exposure to extracellular or intracellular Aβ leads to alterations in mitochondrial activity. To do so, various settings were investigated. First, we exposed PC12 and SY5Y cells to different Aβ species (monomeric, dimeric, fibrillar) in order to characterize their effect on MTT reduction potential. In the next step, SY5Y K623C cells bearing a mutation in the APP gene which leads to the formation of intracellular dimeric APP and Aβ were used to investigate the effect of intracelularly produced Aβ on the MTT reduction potential (Schmechel, A., Zentgraf, H. et al., 2003; Scheuermann, S., Hambsch, B. et al., 2001).

SY5Y cells producing dimeric Aβ (SY5Y K623C) were compared to APPwt-transfected and control SY5Y cells under baseline conditions and under H_2O_2-mediated oxidative stress by the MTT assay and the determination of ATP levels. The measurement of the MTT reduction potential his a widely used and well-

established method for the investigation of viability reflecting metabolic activity, function of mitochondria and cell death.

3.2.1 Extracellular Aβ: monomers vs. dimers and fibrils

We exposed PC12 and SY5Y control cells to various monomeric, dimeric and oligomeric/fibrillar Aβ forms. Monomeric, dimeric and Aβ was synthesized and reconstituted as previously described (Schmechel, A., Zentgraf, H. et al., 2003). Commercially available wild-type $A\beta_{1-42}$ (Bachem) was aggregated to form oligomers and/or fibrills. Previous results showed that aggregated wild-type $A\beta_{1-42}$ exhibits maximum toxicity at concentrations ranging from 10-100 nM (Leutz, S., Steiner, B. et al., 2002; Keil, U., Bonert, A. et al., 2004). Generally, PC12 and SY5Y cells were exposed to Aβ isoforms (50, 100 nM) for 24h previous to the measurement of MTT reduction potential. PC12 and SY5Y control cells were first treated with aggregated wild-type $A\beta_{1-42}$.(Fig. 3.12 A+B). The treatment with oligomeric/fibrillar $A\beta_{1-42}$ (50,100 nM) lead to a dose-dependent decrease in mitochondrial viability. In PC12 (50 nM: $p<0.01$; 100 nM: $p<0.001$ vs. control, Fig. 3.12 A) as well as in SY5Y cells (50,100 nM: $p<0.001$ vs. control, Fig. 3.12 B) MTT reduction potential decreased for 40-50%, respectively. Briefly, the results confirmed former findings corroborating the theory that oligomeric/fibrillar aggregates basically contribute to $A\beta_{1-42}$-mediated toxicity.

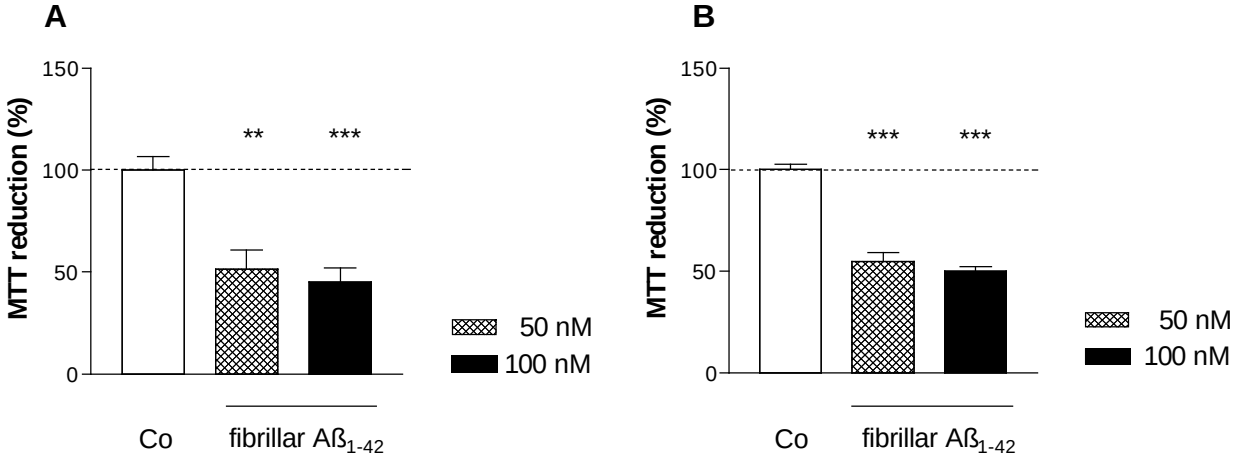

Figure 3.12: Fibrillar wild-type $A\beta_{1-42}$ is toxic to PC12 and SY5Y cells.

The day before assay start, cells were plated on a 96 well plate at a density of 5 x 10^4 cells. A) Treatment of PC12 control cells with oligomeric/fibrillar wild-type $A\beta_{1-42}$ for 24h. B) Treatment of SY5Y control cells with monomeric wild-type $A\beta_{1-42}$ for 24h. One-way ANOVA: ***$p<0.001$, posthoc *t* test: **$p<0.01$, ***$p<0.001$ vs. untreated control. Data are shown as means ± SEM of 5-6 independent experiments. Dotted line represents 100% baseline control.

Exposure of PC12 and SY5Y cells to monomeric wild-type $A\beta_{1-42}$ (50,100 nM) for 24h did not result in a decrease in MTT reduction potential (Fig. 3.13 A+B). These data are consistent with earlier findings of other groups demonstrating that monomeric Aβ does not exhibit toxic effects in cell culture (Kayed, R., Head, E. et al., 2003).

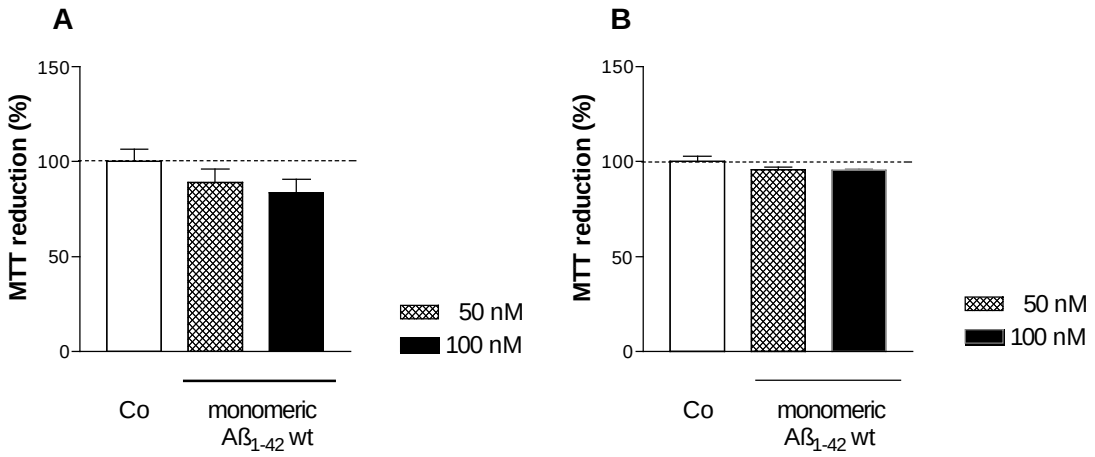

Figure 3.13: Extracellular monomeric wild-type $A\beta_{1-42}$ exhibits no toxicity to PC12 and SY5Y cells.
The day before assay start, cells were plated on a 96 well plate at a density of 5 x 10^4 cells. A) Treatment of PC12 control cells with monomeric wild-type $A\beta_{1-42}$ ($A\beta_{1-42}$ wt) for 24h. B) Treatment of SY5Y control cells with monomeric wild-type $A\beta_{1-42}$ for 24h. One-way ANOVA: ***p<0.001, posthoc *t* test: n.s. Data are shown as means ± SEM of 5-6 independent experiments. Dotted line represents 100% control.

Exposure of PC12 cells and SY5Y to dimeric $A\beta_{1-40}$ (50,100 nM) for 24h lead to a decrease in MTT reduction potential (Fig. 3.14 A+B). In PC12 control cells, the decrease in MTT reduction almost reached significancy at an application 100 nM dimeric $A\beta_{1-40}$ (p=0.06 vs. control). In SY5Y control cells, treatment with 50 nM dimeric $A\beta_{1-40}$ nearly lead to a significant decrease in MTT reduction potential, while by the treatment with 100 nM dimeric $A\beta_{1-40}$, MTT reduction potential decreased significantly (p<0.05 vs. control). Concluding, dimeric $A\beta_{1-40}$ exhibited less toxic effects on MTT reduction potential of PC12 (100 nM: 32%) and on SY5Y cells (100 nM: 10%) compared to fibrillar/oligomeric $A\beta_{1-42}$ (40-50%). This indicates that dimeric $A\beta_{1-40}$, although leading to a modest reduction of MTT reduction potential compared to monomeric wild-type $A\beta_{1-42}$, is less toxic than higher aggregated Aβ species.

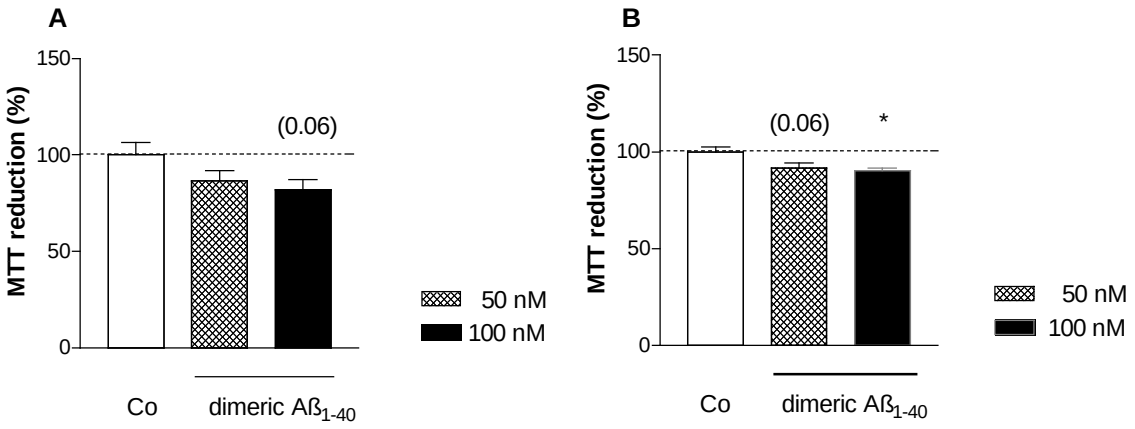

Figure 3.14: Extracellular dimeric $A\beta_{1-40}$ is only slightly toxic to PC12 and SY5Y cells.

The day before assay start, cells were plated on a 96 well plate at a density of 5 x 10^4 cells. A) Treatment of PC12 control cells with dimeric $A\beta_{1-40}$ for 24h. B) Treatment of SY5Y control cells with dimeric $A\beta_{1-40}$ for 24h. One-way ANOVA: ***p<0.001, posthoc *t* test: *p<0.05 vs. control (Co). Data are shown as means ± SEM of 5-6 independent experiments. Dotted line represents 100% control.

The exposure of PC12 and SY5Y cells to dimeric $A\beta_{1-42}$ (50,100 nM) resulted in a significant decrease of MTT reduction of both cell lines (Fig. 3.15 A+B). The extent in decrease is approximately 30% higher than the decrease in MTT reduction potential achieved by exposure to dimeric $A\beta_{1-40}$. MTT reduction level is significantly decreased in PC12 (50 nM: 46%, p<0.001; 100 nM: 50%, p<0.001 vs. control, Fig. 3.15 A) and SY5Y cells (50 nM: 29%, p<0.001; 100 nM: 31%, p<0.001 vs. control, Fig. 3.15 B). Dimeric $A\beta$ ($A\beta_{1-40}$, $A\beta_{1-42}$) species are more toxic than monomeric $A\beta$ ($A\beta_{1-42}$): the MTT reduction achieved by the treatment with dimeric $A\beta_{1-42}$ is comparable to MTT reduction levels resulting from the exposure to fibrillar/oligomeric $A\beta_{1-42}$ (50-55%). Finally, we can conclude that neurotoxic effects, here monitored by MTT, are not exclusively dependent on the $A\beta$ species ($A\beta_{1-40}$, $A\beta_{1-42}$), but also depend on the aggregation status of $A\beta$ species.

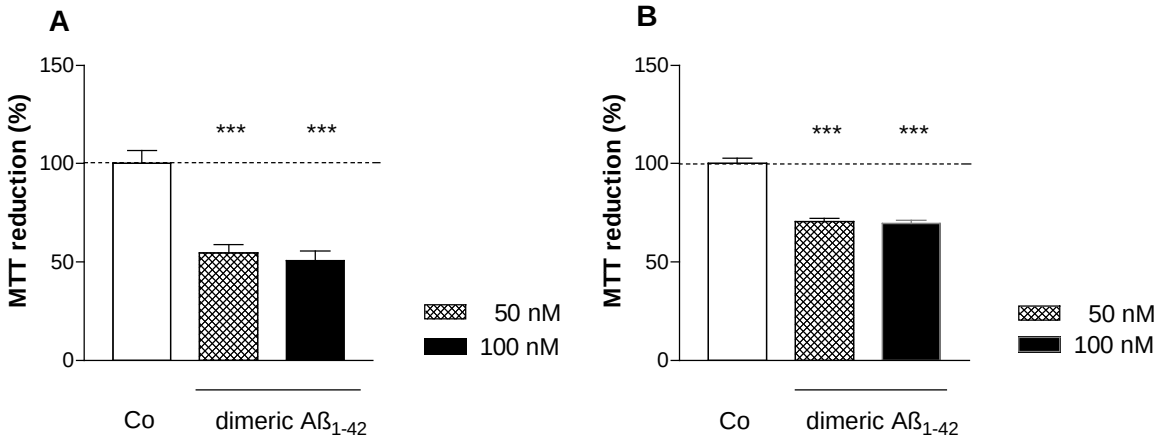

Figure 3.15: Extracellular dimeric $A\beta_{1-42}$ is toxic to PC12 and SY5Y cells.

The day before assay start, cells were plated on a 96 well plate at a density of 5 x 10^4 cells. A) Treatment of PC12 control cells with dimeric $A\beta_{1-42}$ for 24h. B) Treatment of SY5Y control cells with dimeric $A\beta_{1-42}$ for 24h. One-way ANOVA: ***p<0.001, posthoc *t* test: ***p<0.001 vs. control (Co). Data are shown as means ± SEM of 5-6 independent experiments. Dotted line represents 100% control.

3.2.2 Intracellular Aβ: dimers

We investigated the MTT reduction potential and ATP levels of SY5Y cells bearing a dimer-forming APP mutation (APPK623C) in comparison to APPwt and control SY5Y cells. The formation of intracellular APP and Aβ species has been demonstrated before (Scheuermann, S., Hambsch, B. et al., 2001). Here, we focused on the characterization of functional viability parameters: APPwt and control SY5Y cells did show similar MTT reduction potential under baseline conditions, during exposure to 0.1 mM H_2O_2 for 2, 4 and 6h and after exposure to H_2O_2 (0.001- 1.0 mM) for 6h (Fig. 3.16 A+B). MTT reduction was significantly reduced in APPK623C SY5Y cells compared to control SY5Y cells (control: 100%; APPK63C: 74%: p<0.001 vs. control) and compared to APPwt SY5Y cells (APPwt: 92%; APPKC: 74%) under baseline conditions (Fig. 3.16 A+B). The exposure to H_2O_2 (0.1 mM) for 2, 4 and 6h did result in a similar decrease in MTT reduction potential of all clones (Fig. 3.16 A). After 4 and 6h exposure, the decreased MTT reduction potential of APPK623C cells was not significantly different from the values obtained for APPwt and control SYY cells

compared to 2h treatment and baseline conditions (APPK623C: p<0.001 vs. control SY5Y, p<0.001 vs. APPwt SY5Y).

MTT reduction levels decreased significantly already after exposure to 0.01 mM H_2O_2 for 6h in all clones (Fig. 3.16 B). After exposure to 0.001 and 0.01 mM H_2O_2 for 6h and under baseline conditions, the MTT reduction potential of APPK623C cells was significantly decreased compared to APPwt and control SY5Y cells (APPK623C: p<0.01 (baseline), p<0.05 (0.001 mM) vs. control SY5Y, p<0.01 (baseline), p<0.05 (0.001 mM) vs. APPwt SY5Y). At higher concentrations (0.1, 1.0 mM), the MTT reduction potential of APPK623C was not significantly different from that of APPwt and control SY5Y.

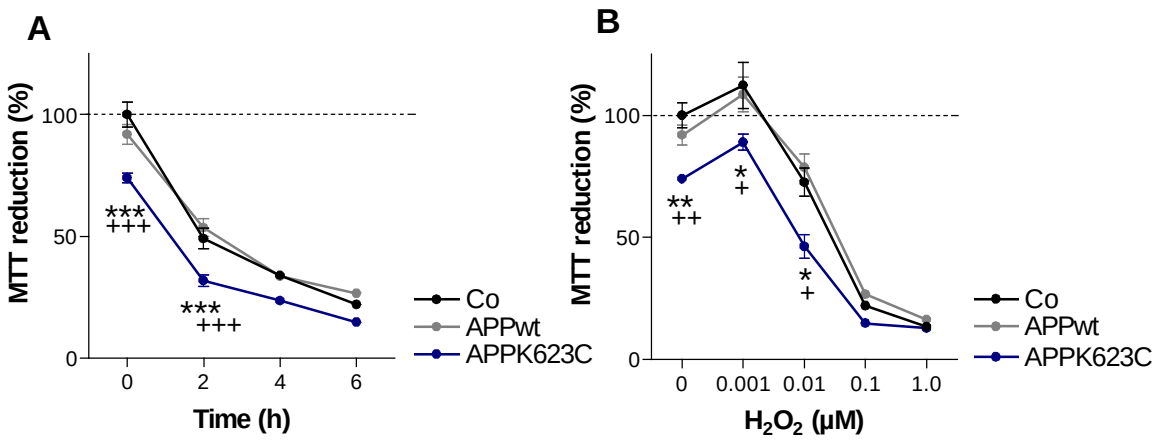

Figure 3.16: Reduced mitochondrial viability in APPK623C SY5Y cells.

A) APPK623C (APPKC), APPwt and control (Co) SY5Y cells were exposed to H_2O_2 (0.1 mM) for 2, 4 and 6 h and MTT reduction potential was assessed by an ELISA reader. One-way ANOVA: ***p<0.001, posthoc *t* test: ***p<0.001 vs. control SY5Y cells (Co); $^{+++}$p<0.001 vs. APPwt SY5Y cells. B) APPK623C, APPwt and control SY5Y cells were exposed to H_2O_2 (0.001-1 mM) for 6h. One-way ANOVA: ***p<0.001, posthoc *t* test: *p<0.051, **p<0.01 vs. SY5Y cells (Co); $^{+}$p<0.05, $^{++}$p<0.01 vs. APPwt SY5Y cells. Data are shown as means ± SEM of 4-6 independent experiments. Dotted line represents 100% baseline control.

We have previously reported that PC12 cells bearing the Swedish double mutation show reduced ATP levels (Keil, U., Bonert, A. et al., 2004). Considering the fact that APPK623C SY5Y cells showed a significantly reduced MTT reduction potential under baseline conditions and during oxidative stress, we determined the relative ATP levels (%) compared to APPwt and control SY5Y cells under baseline conditions and expousre to H_2O_2 (0.1 mM) for 6h and to 0.001-1.0 M H_2O_2 for 6h (Fig. 3.17 A+B). Under baseline conditions, APPK623C show significantly decreased ATP levels

compared to APPwt and control cells (Control: 100%; APPK623C: 53%; p<0.001 vs. control) and compared to APPwt SY5Y cells (APPwt: 93%; APPK623C: 53%; p<0.001) (Fig 3.17 A+B). The exposure to H_2O_2 (0.1 mM, 2, 4, 6h) resulted in a time- and concentration-dependent decrease in MTT reduction potential in all clones. After incubation with H_2O_2, the decrease of ATP-levels in APPK623C cells was similar to the decrease of APPwt and control SY5Y cells after 2, 4 and 6h. Additionally, SY5Y cells were incubated with various concentrations of H_2O_2 (0.001-1 mM) for 6h (Fig. 3.17 A). The exposure to 0.01 and 1.0 mM H_2O_2 for 6h lead to drastically reduced ATP levels in all clones (Fig. 3.17 B). At the lower concentrations (0.001, 0.01 mM), APPK623C SY5Y cells show significantly decreased ATP levels compared to APPwt SY5Y (p<0.001 vs. APPK623C) and control SY5Y cells (p<0.001 vs. APPK623C).

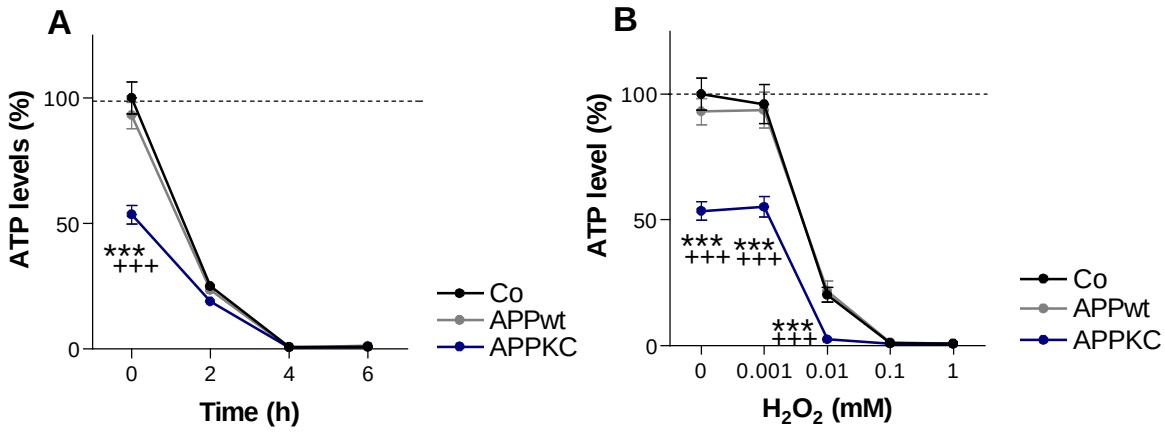

Figure 3.17: Reduced ATP levels in APPK623C SY5Y cells.

A) APPK623C (APPKC), APPwt and control (Co) SY5Y cells were exposed to H_2O_2 (0.1 mM) for 2, 4 and 6 h. One-way ANOVA: ***p<0.001, posthoc *t* test: ***p<0.001 vs. control SY5Y cells (Co); $^{+++}$p<0.001 vs. APPwt SY5Y cells. B) APPK623C (APPKC), APPwt and control (Co) SY5Y cells were exposed to H_2O_2. (0.001-1 mM) for 6h. One-way ANOVA: ***p<0.001, posthoc *t* test: ***p<0.001 vs. control SY5Y cells (Co); $^{+++}$p<0.001 vs. APPwt SY5Y cells. Data are shown as means ± SEM of 4-6 independent experiments. Dotted line represents 100% baseline control.

Resuming, we may state that the stably expressed K623C mutation is toxic to SY5Y cells under baseline conditions, as monitored by the decrease in MTT reduction potential (Fig. 3.16 A+B) and strongly decreased baseline ATP levels under baseline conditions (Fig. 3.17 A+B). This implicates that intracellularly produced dimeric Aβ is sufficient to cause impaired energy metabolism and reduced cellular viability in cell culture. The effect was so strong that there was no further increase by additional exposure to H_2O_2 – mediated oxidative stress.

3.3 Characterization of mitochondria-dependent cell death pathways in APP-expressing cells

Proteins of the Bcl-2 superfamily are important players in the regulation of the mitochondrial apoptotic pathway, modulating the release of numerous pro-apoptotic factors from mitochondria which in turn activate executioner caspases. In order to characterize the cell-type specific expression levels and subcellular localization, we submitted cell lysates of PC12, HEK and SY5Y cells to western blot analysis.

3.3.1 Bcl-2 family proteins and mitochondrial pro-apoptotic factors in PC12 cells

The analysis of cell lysates, mitochondrial and cytosolic fractions of PC12 cells (Fig. 3.18) lead to the following result: The anti-apoptotic protein of Bcl-xL was expressed in moderate quantities in PC12 cells. Intracellularly, Bcl-xL is mainly located in the mitochondrial fraction. Bcl-xL is decreased in the cytosolic fraction of APPsw and APPwt PC12 cells, which might contribute the increased vulnerability of APPwt and APPsw cells against oxidative injury. The expression of the pro-apoptotic protein Bax was unaffected in all clones. The protein is located in the mitochondrial and cytosolic compartment, with no difference in the distribution pattern of the clones. Bcl-2 is not expressed in PC12 cells. The result was confirmed by Celio Marques in our lab and by another working group analysing mRNA levels of Bcl-2 (Maroto, R., Perez-Polo, J. R., 1997; Marques, C. A., Keil, U. et al., 2003). Cytochrome c was expressed at comparable levels in all cell types, and the protein was mainly located in the mitochondrial compartment under baseline conditions. Barely no cytochrome c was detected in the cytosolic compartment. Similar expression and distribution was observed by the analysis of the pro-apoptotic mitochondrial factors Smac/DIABLO and AIF. Smac/DIABLO was found to be co-located with cytochrome c in the mitochondrial intermembrane space (Adrain, C., Creagh, E. M. et al., 2001; Du, C., Fang, M. et al., 2000) and AIF was found to be mainly located in the mitochondrial compartment (Cande, C., Cohen, I. et al., 2002; Daugas, E., Susin, S. A. et al., 2000).

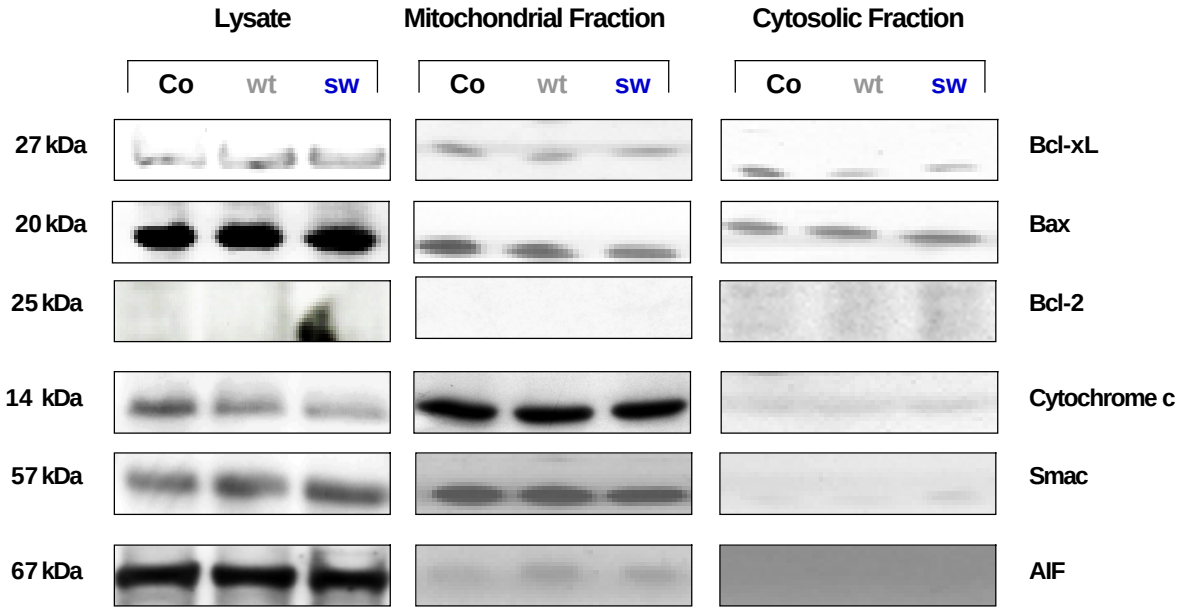

Figure 3.18: Bcl-2 protein family members and mitochondrial pro-apoptotic factors in PC12 cells.
Subcellular fractions (mitochondria, cytosol) were isolated and subjected to western blot analysis as well as cell lysates. The resulting membranes were tested for the following proteins: Bcl-xL (27 kDa), Bax (20 kDa), Bcl-2 (25 kDa), cytochrome c (14 kDa), Smac/DIABLO (57 kDa) and AIF (67 kDa).

3.3.2 Bcl-2 family proteins and mitochondrial pro-apoptotic factors in HEK cells

In the following, we investigated the expression and subcellular localization of Bcl-2 family proteins and mitochondrial pro-apoptotic factors of HEK cells (Fig. 3.19). The anti-apoptotic protein Bcl-xL was expressed in HEK cells, and slightly reduced expression levels were observed in APPwt and APPsw cells. Bcl-xL was mainly found in the mitochondrial fraction of APPwt and APPsw HEK cells, but at lower levels than in control HEK cells. Only a minor part of Bcl-xL is located in the cytosolic fraction of APPsw, APPwt and control HEK cells. Bax was present in cell lysates of all clones, and the protein is equally located in the mitochondrial and cytosolic compartment. In contrast to the situation in PC12 cells, Bcl-2 was highly expressed in HEK cells. APPsw HEK cells express lower levels of Bcl-2 compared to APPwt and control HEK cells. This was confirmed by the analysis of the cytosolic fraction, as APPsw showed decreased Bcl-2 levels compared to APPwt and control cells. Also, we could demonstrate that Bcl-2 is mainly located in the cytosol. The mitochondrial fraction is free from the protein under baseline conditions. Cytochrome c was

expressed at comparable levels in all HEK clones, and the protein was mainly located in the mitochondrial compartment under baseline conditions, whith only insignificant levels of cytochrome c in the cytosolic compartment. The pro-apoptotic mitochondrial factor Smac/DIABLO was abundantly present in the cytosol indicating its potential involvement in the increased apoptosis levels of HEK cells compared to PC12 cells under baseline conditions. AIF expression levels were unaltered in APPwt and APPsw compared to control cells. Similar to PC12 cells, AIF was mainly located in mitochondria of HEK cells.

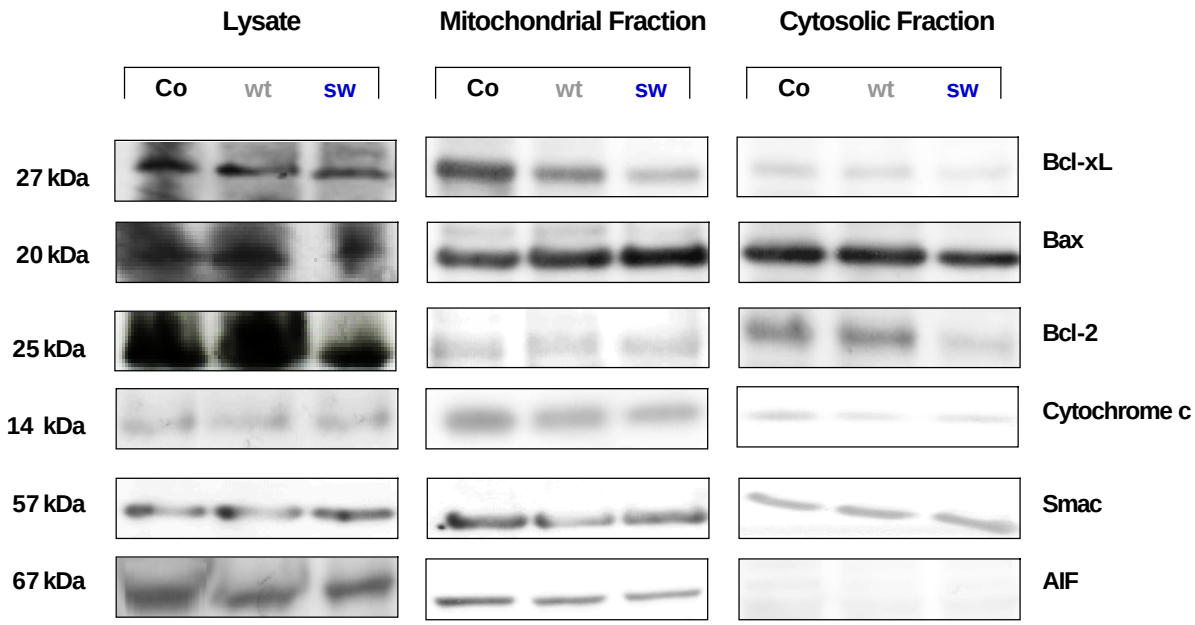

Figure 3.19: Bcl-2 protein family members and mitochondrial pro-apoptotic factors in HEK cells.
Cell lysates and subcellular fractions were isolated as described above and subjected to western blot analysis. The resulting membranes were probed for the following proteins: Bcl-xL (27 kDa), Bax (20 kDa), Bcl-2 (25 kDa), cytochrome c (14 kDa), Smac/DIABLO (57 kDa) and AIF (67 kDa).

3.3.3 Bcl-2 family proteins and mitochondrial pro-apoptotic factors in SY5Y cells

The anti-apoptotic protein Bcl-xL was equally expressed in APPwt and control SY5Y cells (Fig. 3.20). In cell lysates, mitochondrial and cytosolic fractions, the expression levels of Bcl-xL were slightly decreased in APPwt compared to control SY5Y cells. In contrast to HEK cells, Bcl-xL was mainly found in the cytosolic fraction. Only a minor part of Bcl-xL is located in mitochondria of APPwt and control SY5Y cells. According

to the previous findings in PC12 and HEK cells, Bax was found in cell lysates, and the protein is located in both mitochondrial and cytosolic compartments. Bcl-2 was highly expressed in SY5Y cells, with APPwt cells showing decreased Bcl-2 levels compared to control cells. In contrast to HEK cells, Bcl-2 was not present in the cytosolic fraction under baseline conditions, but notably present in the mitochondrial compartment. Cytochrome c was expressed at comparable levels in both APPwt and control SY5Y cells, with equivalent protein levels in the cytosolic and mitochondrial fraction. Smac/DIABLO was found in the cytosol, which is in consistent with the findings in HEK cells. APPwt and control SY5Y cells expressed equal amounts of AIF, and similar to PC12 and HEK cells, AIF was mainly located in mitochondrial fraction.

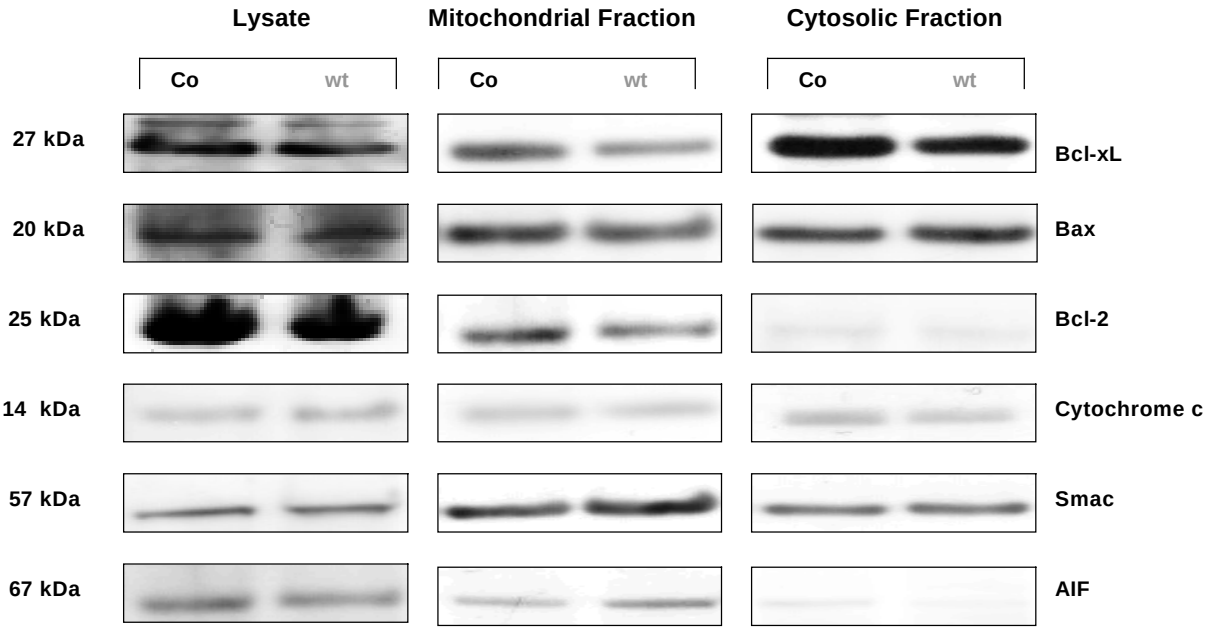

Figure 3.20: Bcl-2 protein family members and mitochondrial pro-apoptotic factors in SY5Y cells.
Cell lysates and subcellular fractions were isolated as described above and subjected to western blot analysis. The resulting membranes were probed for the following proteins: Bcl-xL (27 kDa), Bax (20 kDa), Bcl-2 (25 kDa), cytochrome c (14 kDa), Smac/DIABLO (57 kDa) and AIF (67 kDa).

3.3.4 Bcl-2 family proteins in APP transgenic mice

Our previous findings indicate that decreased Bcl-2 and Bcl-xL expression might be implicated in the increased neurotoxicity mediated by chronic exposure to high levels of Aβ. The next assays aimed at the demonstration of the in vivo relevance of the previous findings in cell culture. Cell lysates of dissociated brain neurons of 3 months-old APP transgenic mice were subjected to western blot analysis for APP (116 kDa), Aβ (4 kDa), Bcl-xL (27 kDa), Bcl-2 (25 kDa) and Bax (20 kDa) (Fig. 3.21). 3 months-old APP transgenic mice exhibit significantly increased APP expression levels compared to non-transgenic littermates. Furthermore, Aβ was scarcely detectable in both APP transgenic and non-transgenic mice. This is in accordance with other findings showing that extracellular Aβ plaque formation is significantly increased in 6 months-old APP transgenic mice. Bcl-xL expression levels of APP transgenic mice were decreased in comparison to non-transgenic littermates. Bcl-2 and Bax expression was analogous in both groups.

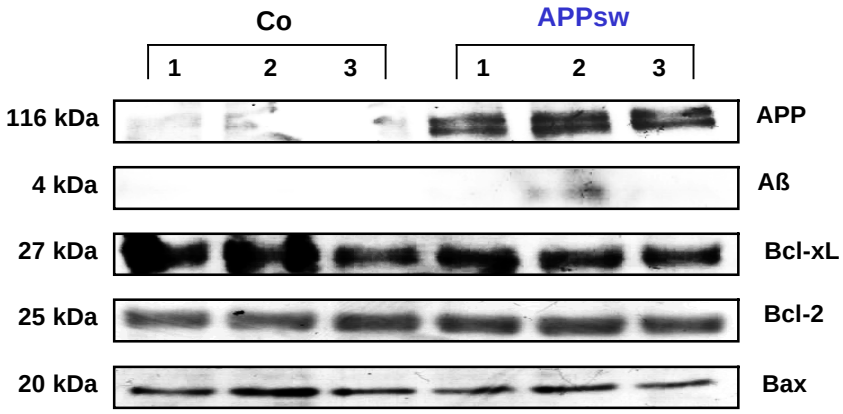

Figure 3.21: Decreased Bcl-xL expression in APPtg mice.

Cell lysates of brain tissue of 3 months-old APP transgenic mice (n=3) and littermates (n=3) were generated by Tris lysis (5 mM). Probes were subjected to western blot for APP (116 kDa), Aβ (4 kDa), Bcl-xL (27 kDa), Bcl-2 (25 kDa) and Bax (20 kDa).

3.3.5 Ratios of pro-and anti-apoptotic proteins of the Bcl-2 family

Altered ratios of pro-(Bax) and anti-(Bcl-2) apoptotic protein levels are generally considered to render cells more vulnerable to apoptotic cell death (Cheng, E. H. Y. A., Wei, M. C. et al., 2001). In order to investigate Bcl-2 family proteins and mitochondrial pro-apoptotic factors in more detail, we compared expression levels of Bcl-2, Bax and Bcl-xL in APPwt, APPsw, control HEK and APPwt, control SY5Y cells by densiometric analysis (Fig. 3.22 A-C). Bcl-2 expression is significantly higher in SY5Y control compared to HEK control cells ($p<0.001$ vs. HEK control) (Fig. 3.22 A). Furthermore, Bcl-2 expression is significantly decreased in APPwt compared to control cells ($p<0.001$ vs. SY5Y control). Although SY5Y control cells exhibited a higher Bcl-xL expression, the increase was not significant (Fig. 3.22 B). Thus, Bcl-xL and Bax expression levels of HEK and SY5Y cells were at the same size (Fig. 3.22 B+C), indicating that a shift in the ratio of pro- and anti-apoptotic Bcl-2 family protein levels might primarily be linked to altered expression levels of the anti-apoptotic Bcl-2.

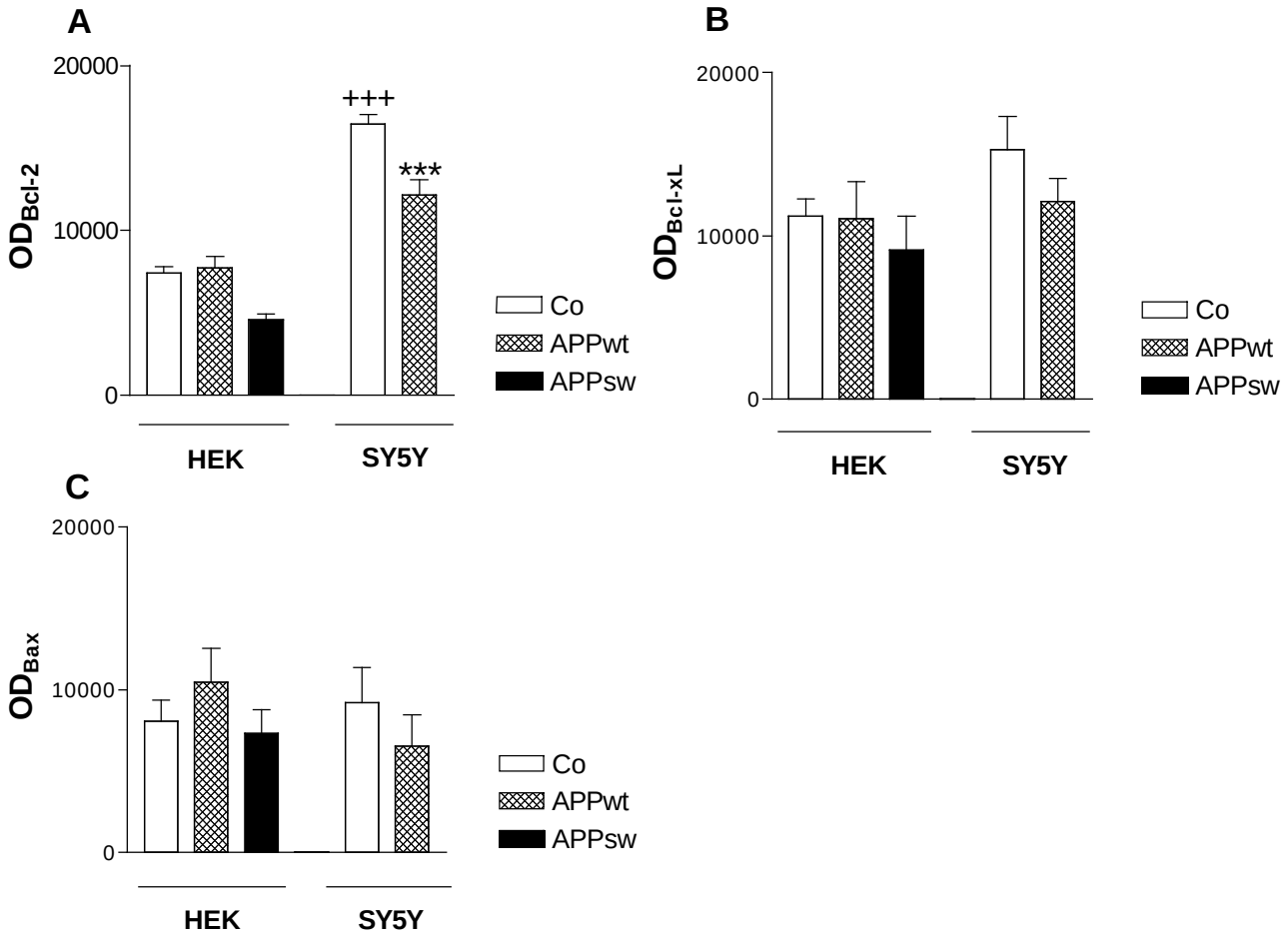

Figure 3.22: Significantly decreased Bcl-2 expression levels in APPwt SY5Y cells.

Western blots of cell lysates of HEK and SY5Y cells for Bcl-2, Bcl-xL and Bax were densiometrically analysed. One-way ANOVA: $p<0.0001$; posthoc *t* test: ***$p<0.001$ vs. SY5Y control; $^{+++}p<0.001$ vs. HEK control. Data are shown as means ± SEM of 3 independent experiments.

As mentioned above, the Bcl-2/Bax ratio is a generally accepted indicator for the vulnerability against apoptotic cell death. Consequently, Bcl-2/Bax and Bcl-xL/Bax ratios were calculated to relate them to baseline apoptosis process of HEK and SY5Y cells (Fig. 3.23 A+B). Although no significant shift of the Bcl-2/Bax ratio was monitored by the densiometric analysis of Bcl-2 in APPsw HEK cells compared to control cells, the Bcl-2/Bax ratio is significantly decreased in APPsw compared to control HEK cells ($p<0.05$ vs. control) (Fig. 3.23 A). The significantly elevated Bcl-2/Bax ratio of control SY5Y cells (100% increase; $p<0.001$ vs. HEK control) clearly reflects increased Bcl-2 expression levels in control SY5Y cells. Notably, the Bcl-2/Bax ratio of APPwt SY5Y cells is significantly decreased compared to control SY5Y cells (APPwt: 0.9; control: 2.0; $p<0.001$).

In turn, the Bcl-xL/Bax ratio of HEK cells was unaltered, while the Bcl-xL/Bax ratio of APPwt SY5Y cells was significantly decreased compared to control SY5Y cells ($p<0.05$ vs. control) (Fig. 3.23 B). Our findings are consistent with current studies pointing out that the Bcl-2/Bax ratio is a valuable indicator for apoptotic processes.

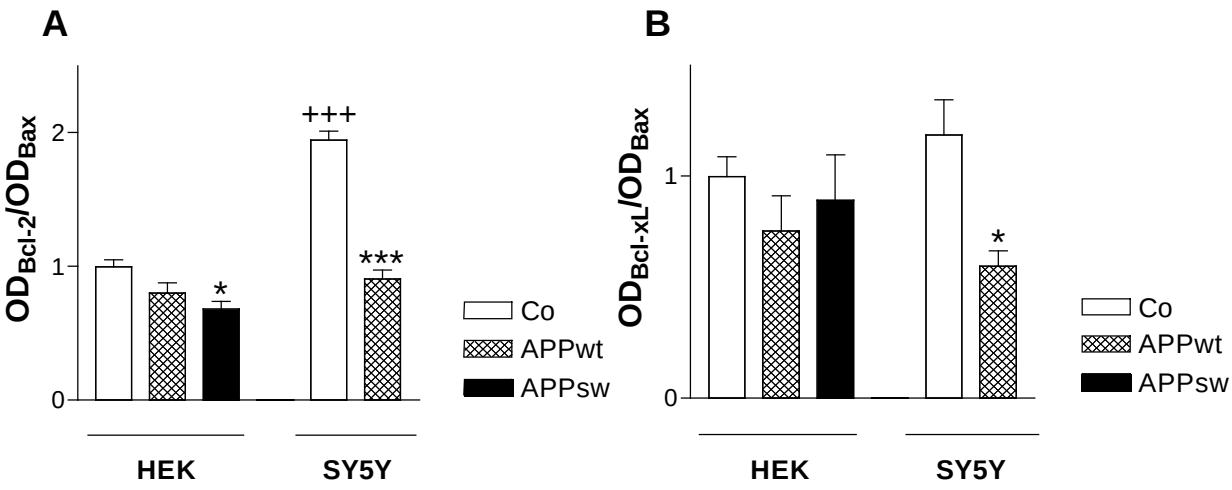

Figure 3.23: Significantly decreased Bcl-2/Bax and Bcl-xL/Bax ratios in APPwt SY5Y and APPsw HEK cells.

Western blots of cell lysates of HEK and SY5Y cells for Bcl-2, Bcl-xL and Bax were densiometrically analysed to calculate Bcl-2/Bax and Bcl-xL/Bax ratios. One-way ANOVA: $p<0.0001$; posthoc *t* test: *$p<0.05$,***$p<0.001$ vs. corresponding control; $^{+++}p<0.001$ vs. HEK control. Data are shown as means ± SEM of 3 independent experiments.

3.3.6 In-vivo relevance of altered Bcl-2, Bcl-xL/ Bax ratios

Western blot analysis indicated that only Bcl-xL expression was altered in APP transgenic mice compared to non-transgenic littermates (Fig. 3.21). In the following, we subjected western blots for Bcl-xL and Bax to densiometric analysis to calculate Bcl-xL/ Bax ratios for APP transgenic mice and non-transgenic littermates (Fig. 3.24). Bcl-xL/Bax ratio were significantly ($p<0.05$ vs. littermate control) decreased in APP transgenic mice. Finally, we speculate that down-regulation of Bcl-xL levels is an early event in chronic exposure to increased APP and Aβ levels.

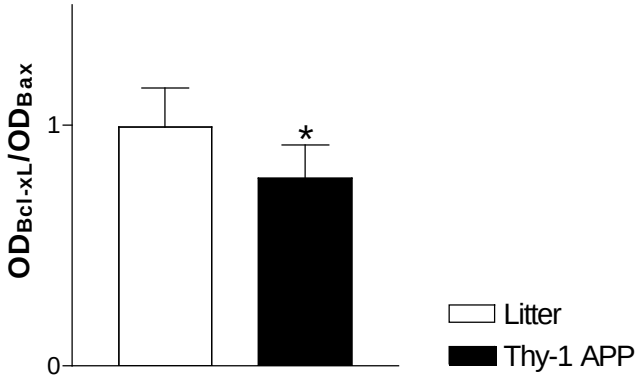

Figure 3.24: Significantly decreased Bcl-xL/ Bax ratio in APPtg mice.

Cell lysates of brain tissue of 3 month-old APP transgenic mice (n=3) and littermates (n=3) were generated by Tris lysis (5 mM). Probes were subjected to western blot for Bcl-xL (27 kDa) and Bax (20 kDa). Bands were evaluated densiometrically and Bcl-xL/Bax ratios were calculated in the following. One-way ANOVA: $p<0.0001$; posthoc *t* test: *$p<0.05$ vs. age-matched littermate control.

3.3.7 Mitochondrial activity during oxidative injury in PC12, HEK and SY5Y cells

Preceding work of our group aimed at the characterization of caspase activation and mitochondrial alterations after oxidative injury with special regard to the APPsw mutation (Marques, C. A., Keil, U. et al., 2003; Keil, U., Bonert, A. et al., 2004). It was shown that initiator- and executioner caspases were activated in a time-dependent pattern while mitochondrial membrane potential and ATP levels decreased significantly during exposure to H_2O_2-mediated oxidative stress. In this setting, PC12 cells were exposed to 500 µM H_2O_2 to investigate caspase-activation. Furthermore, it has been demonstrated by Celio Marques that apoptosis is increased to the maximum after exposure to H_2O_2 at a concentration of 500 µM. Since the aim of this thesis was to investigate alterations in Bcl-2 family members and pro-apoptotic mitochondrial factors to obtain a deeper knowledge of signal transduction during the course of apoptosis, we have chosen the same experimental conditions. First of all, we determined the MTT reduction potential during H_2O_2-mediated oxidative stress in order to compare the results to the time-dependent release of pro-apoptotic factors. This would enable us to determine whether changes in Bcl-2 family members, pro-

apoptotic mitochondrial factors or MTT reduction potential are the most sensitive indicator of impaired cellular functions during oxidative stress-mediated apoptosis.

In order to investigate whether wild-type APP and the APPsw mutation modulate cellular viability and mitochondrial activity during oxidative injury and under baseline conditions, we monitored the MTT reduction potential of APPwt and APPsw PC12 cells after exposure to H_2O_2 (500 µM) for 0, 2, and 4h (Fig. 3.25). Under baseline conditions, APPwt and APPsw cells revealed a reduced MTT reduction potential compared to control cells (APPwt: 87%; APPsw: 69% vs. 100% baseline control). Exposure to H_2O_2 (500 µM) resulted in a significant reduction in MTT reduction potential after 2 and 4h (APPwt: $p<0.001$ vs. APPwt control; APPsw: $p<0.001$ vs. APPsw control). Furthermore, MTT reduction potential was decreased to similar levels in APPwt (2h: 13%; 4h: 3%) and in APPsw cells (2h: 10%; 4h: 3%). Concluding, the APPsw mutation lead to a more pronounced decrease in MTT reduction potential under baseline conditions compared to wild-type APP in PC12 cells, while the massive exposure to H_2O_2 resulted in a drastic reduction of cellular viability in both cell types. These data were coherent with previous findings of our group (Marques, C. A., Keil, U. et al., 2003).

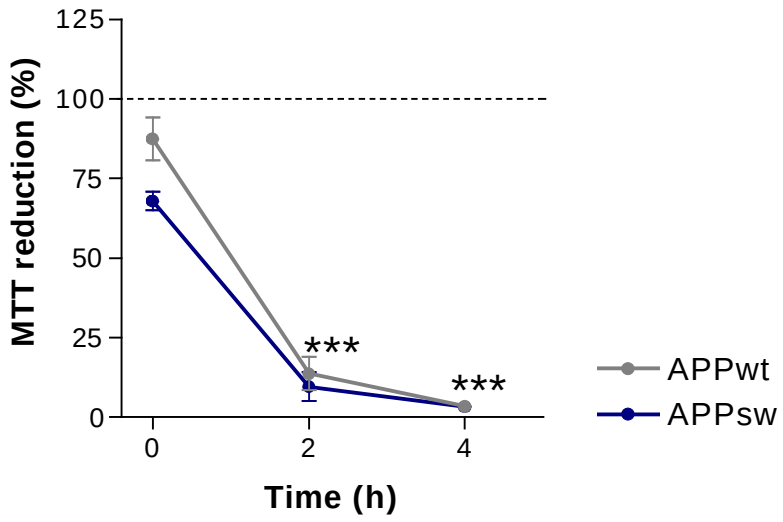

Figure 3.25: Decreased MTT reduction potential in APPwt and APPsw PC12 cells after oxidative injury.
APPsw and APPwt PC12 cells were exposed to H_2O_2 (500 µM) fo 2 and 4h. MTT reduction potential was assessed in the following. One-way ANOVA: $p<0.0001$; posthoc *t* test: ***$p<0.001$ vs. corresponding untreated baseline control (APPwt, APPsw). Data are shown as means ± SEM of 3 independent experiments. Dotted line represents 100% baseline control.

In the following, we exposed APPwt and APPsw HEK cells to H_2O_2 (500 µM) for 2 and 4h (Fig. 3.26). Under baseline conditions, APPsw cells revealed significantly reduced MTT reduction potential compared to APPwt HEK cells (APPwt: 102%; APPsw: 79% vs. 100% baseline control; $p<0.05$ vs. APPwt control). Exposure to H_2O_2 (500 µM) resulted in a significant reduction in MTT reduction potential after 2 and 4h (APPwt: $p<0.001$ vs. APPwt control; APPsw: $p<0.001$ vs. APPsw control). After secondary insult, MTT reduction potential of APPsw cells was significantly decreased compared to APPwt HEK cells ($p<0.01$ vs. corresponding APPwt probe). The APPsw mutation not only lead to a highly pronounced decrease in MTT reduction potential under baseline conditions, but also lead to a significantly decreased MTT reduction after oxidative injury compared to wild-type APP.

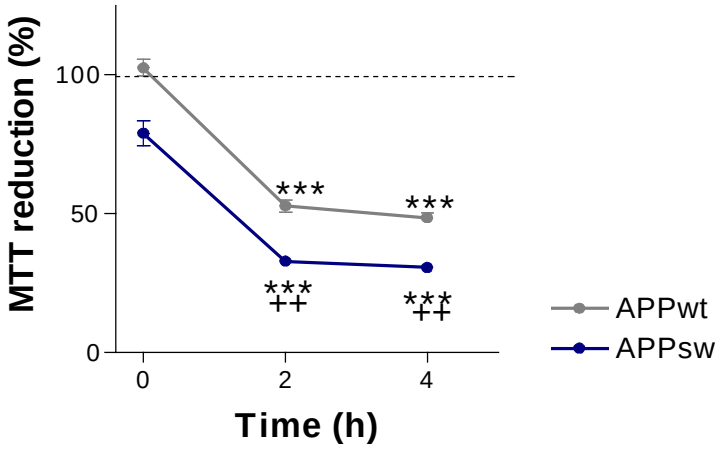

Figure 3.26: Decreased MTT reduction potential in APPwt and APPsw HEK cells after oxidative injury.
APPsw and APPwt HEK cells were exposed to H_2O_2 (500 µM) fo 2 and 4h. MTT reduction potential was assessed in the following. One-way ANOVA: $p<0.0001$; posthoc *t* test: ***$p<0.001$ vs. corresponding untreated control; $^{++}p<0.01$ vs. APPwt. Data are shown as means ± SEM of 3 independent experiments. Dotted line represents 100% baseline control.

We exposed APPwt and control SY5Y cells to varying concentrations of H_2O_2 (0.001-1.0 mM) for 6h and to H_2O_2 (0.1 mM) for 2, 4 and 6h (Fig. 3.27 A+B). Under baseline conditions, APPwt cells revealed a slightly reduced MTT reduction potential compared to control SY5Y cells (Fig. 3.27 A). Exposure to H_2O_2 (0.1 mM) resulted in a significant reduction in MTT reduction potential after 2 and 4h in both cell types ($p<0.001$ vs. untreated control). After secondary insult for 2 and 4h (H_2O_2, 0.1 mM) MTT reduction of APPwt and control SY5Y cells was at the same level.

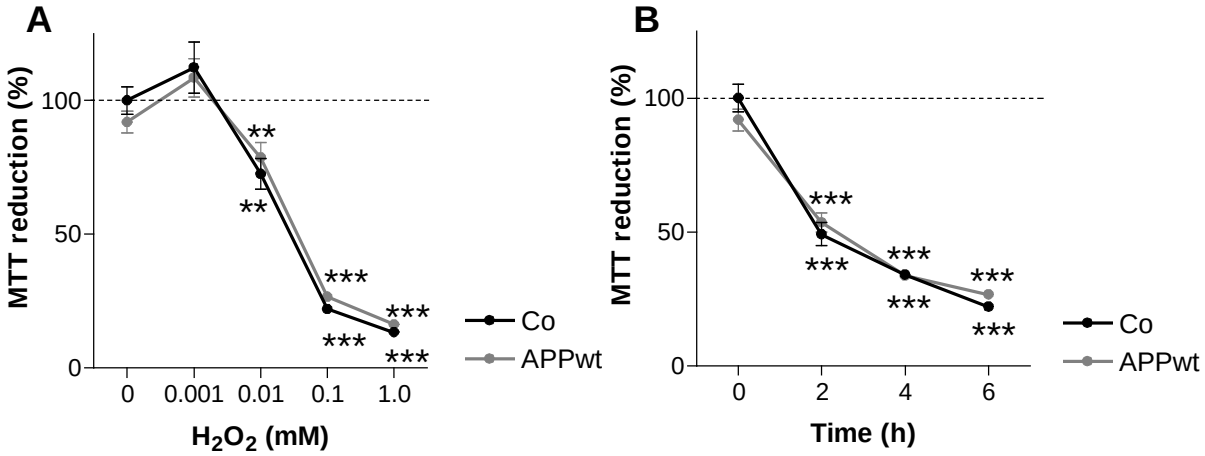

Figure 3.27: Decreased MTT reduction potential in APPwt SY5Y cells after oxidative injury.

A) APPwt and control SY5Y cells were exposed to H_2O_2 (0.001-1.0 mM) for 6h. B) APPwt and control SY5Y cells were exposed to H_2O_2 (0.1 mM) for 2, 4 and 6h. MTT reduction potential was assessed in the following. One-way ANOVA: $p<0.0001$; posthoc *t* test: **$p<0.01$, ***$p<0.001$ vs. corresponding untreated baseline control. Data are shown as means ± SEM of 3 independent experiments. Dotted line represents 100% baseline control.

3.3.8 Subcellular localization of Bcl-2 family members and pro-apoptotic mitochondrial factors during oxidative injury

3.3.8.1 Bcl-xL

Bcl-xL is a Bcl-2 family protein bearing anti-apoptotic functions in the cell (Fig. 3.28 A+B). Here, we investigated the intracellular distribution of Bcl-xL during exposure to oxidative stress. PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. Principally, basal levels of Bcl-xL in the mitochondrial fraction are unchanged in APPwt and APPsw compared to control cells (Fig 3.28 A). During H_2O_2 treatment, mitochondrial Bcl-xL levels did not change. In contrast to this, cytosolic Bcl-xL is decreased in APPsw and APPwt cells compared to control cells under baseline conditions and during H_2O_2 treatment. This was confirmed by the densiometric western blot analysis of Bcl-xL (Two-way ANOVA: $p<0.001$ for cell type) (Fig. 3.28 B), indicating that after oxidative injury, cytosolic Bcl-xL did not translocate to mitochondria.

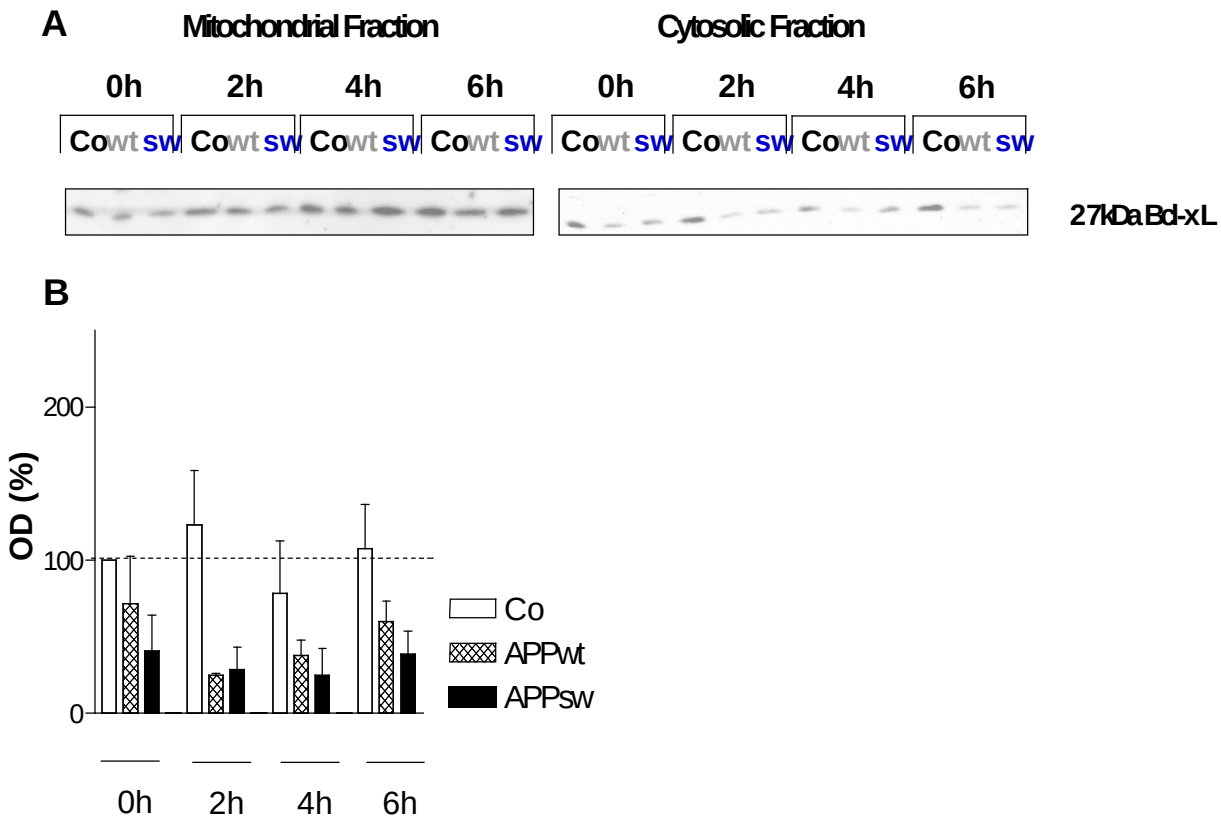

Figure 3.28: Bcl-xL levels under baseline conditions and during exposure to H_2O_2 in PC12 cells.

A) PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. Mitochondrial and cytosolic fractions were separated and subjected to western blot analysis. B) Densiometric analysis of Bcl-xL bands of the cytosolic fraction. Data represent means ± SEM of 3-6 independent experiments. Two-way ANOVA: p=0.0008 (cell type). Dotted line represents 100% control.

Increasing evidence is provided that Bcl-2 family proteins dimerize in order to exert their pro- or anti-apoptotic function. To investigate the dimerization potential of Bcl-xL, we exposed PC12 cells to H_2O_2 (500 µM) for 2, 4 and 6h and treated cell lysates with BMH (5 mM), a commercially available chemical cross-linker. Cell lysates assayed for Bcl-xL did not show any difference in total Bcl-xL content in APPsw, APPwt and control cells (Fig. 3.29). When subjected to cross-linking, heteromeric and dimeric Bcl-xL bands (42, 54 kDa) were detected. Nevertheless, there was no change in heteromer/dimer content during H_2O_2 exposure nor were there any differences between the PC12 clones. Resuming, heteromerization or dimerization of Bcl-xL seems not to be a crucial step in oxidative injury-caused apoptosis.

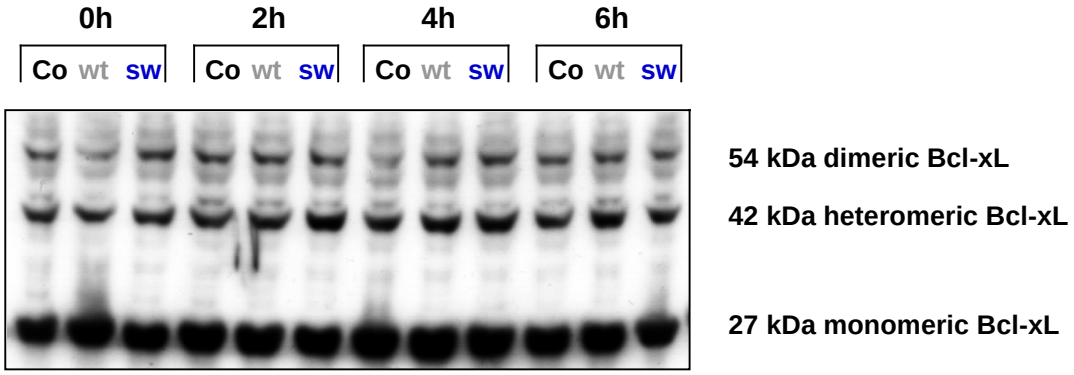

Figure 3.29: Bcl-xL forms dimers and heteromers.

PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. After treatment with the chemical cross-linker BMH (5 mM) for 1h, whole cell lysates were subjected to western blot analysis and assayed for Bcl-xL.

3.3.8.2 Bax

Bax belongs to the pro-apoptotic members of the Bcl-2 family of proteins. Evidence is provided that Bax is a pivotal player in the apoptotic process. It is still under discussion whether Bax insertion and oligomerization in the outer mitochondrial membrane forms pores itself by which mitochondrial factors are released into the cytosol or whether it induces PTP opening thus being the preliminary step to the release of pro-apoptotic, mitochondrial factors. First, we assessed the intracellular distribution of Bax under baseline conditions and after exposure to H_2O_2 (500 µM) for 2, 4 and 6h. We could demonstrate that mitochondrial and cytosolic fractions contain equal amounts of Bax under baseline conditions and during oxidative injury (Fig. 3.30 A+B). Densiometric analysis of the cytosolic fraction revealed a slight, but not significant increase in Bax protein content after 4h followed by a slight reduction in cytosolic Bax contents to baseline levels after 6h exposure (Fig. 3.30 B).

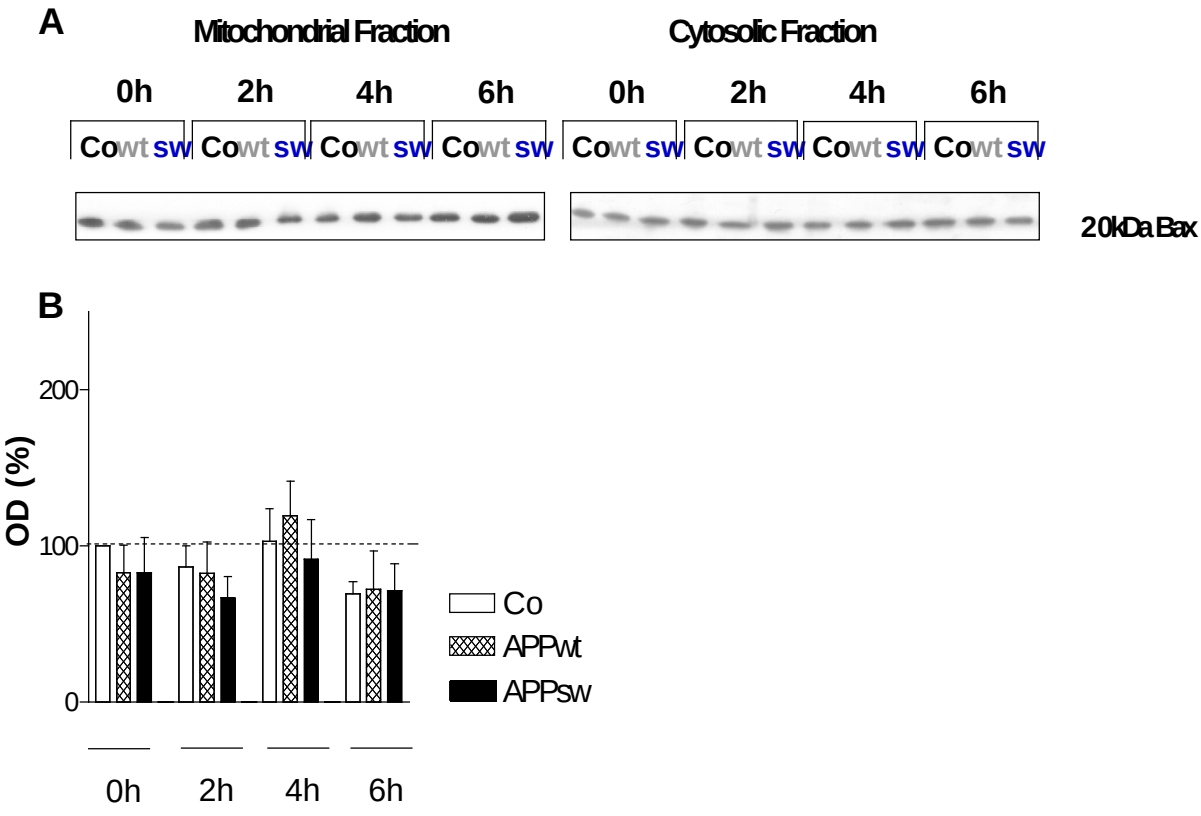

Figure 3.30: Bax slightly increases in the cytosolic compartment after 4h exposure to H_2O_2.

A) PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. Mitochondrial and cytosolic fractions were separated and subjected to western blot analysis. B) Densiometric analysis of Bax bands of the cytosolic fraction. Data represent means ± SEM of 5-8 independent experiments. Dotted line represents 100% control.

It has been described before that Bax oligomerizes and inserts into the outer mitochondrial membrane during oxidative stress (Luetjens, C. M., Kogel, D. et al., 2001). Here, we added BMH (5 mM) to cell lysates after exposure of PC12 cells to H_2O_2 (500 µM) in order to investigate dimerization (Fig. 3.31). We could detect dimeric Bax under baseline conditions. After 4h exposure to H_2O_2, dimeric Bax levels increased. After 6h treatment, levels of dimeric Bax decreased slightly to baseline levels. This is consistent with previous data, as we could asses a slight increase in total cytosolic Bax after 4h treatment (Fig. 3.31 A+B). We suggest that increased dimer formation is initiated after 4h, potentially leading to transient pore formation or the opening of the membrane permeability transition pore in the outer mitochondrial membrane.

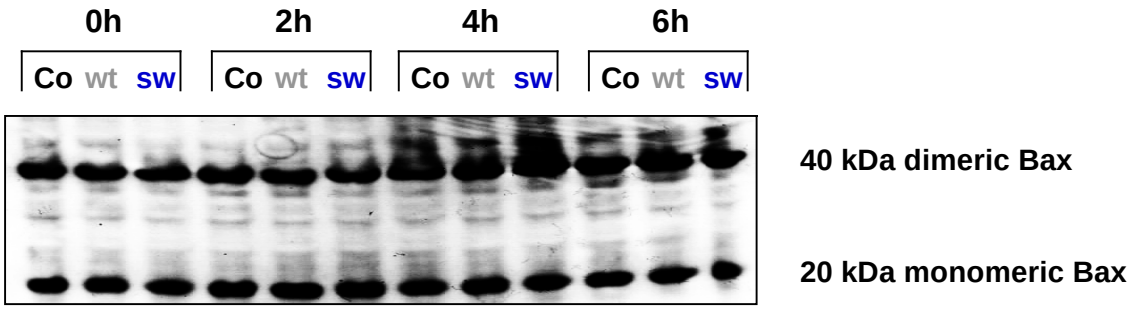

Figure 3.31: Enhanced Bax dimerization after 4h H_2O_2 treatment.

PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. After incubation of cell lysates with the chemical cross-linker BMH (5mM) for 1h, probes were subjected to western blot analysis and assayed for Bax.

3.3.8.3 Cytochrome c

The release of cytochrome c from the mitochondrial inter-membrane space into the cytosol is known to be one crucial step of caspase-dependent apoptosis. It has been described before that only a minor part of cytochrome c is released into the cytosol while the main part, notably cytochrome c bound to the electron transport chain, remains in the mitochondria.

As it has been shown in our group before that exposure to H_2O_2 leads to a time-dependent activation of caspases, namely caspase-9 and caspase-3, as well as to a drop down of mitochondrial membrane potential. In order to further characterize mitochondrial changes during apoptosis, we again exposed PC12 cells to H_2O_2 (500 µM) for 2, 4 and 6h (Fig. 3.32 A+B). Isolated cytosolic and mitochondrial fractions were assayed for cytochrome c. Exposure to H_2O_2 lead to a release of cytochrome c into the cytosol in a time-dependent manner (Two-way ANOVA: $p<0.001$ for time), with a maximum release at 6h of incubation. Under baseline conditions, only very low amount of cytosolic cytochrome c was detected. After 6h incubation, APPwt and APPsw cells revealed a slightly increased release of cytochrome c compared to control cells (Fig. 3.32 A). This result was confirmed by densiometric analysis of the cytochrome c western blot bands (Fig. 3.32 B). The cytosolic probes were subjected to a cytochrome c ELISA confirming the results achieved by densiometric analysis. Interestingly, after 6h, higher values were achieved by ELISA, indicating that

saturation was reached in band intensity by densiometric analysis, resulting in lower, non-proportional increase in measured values.

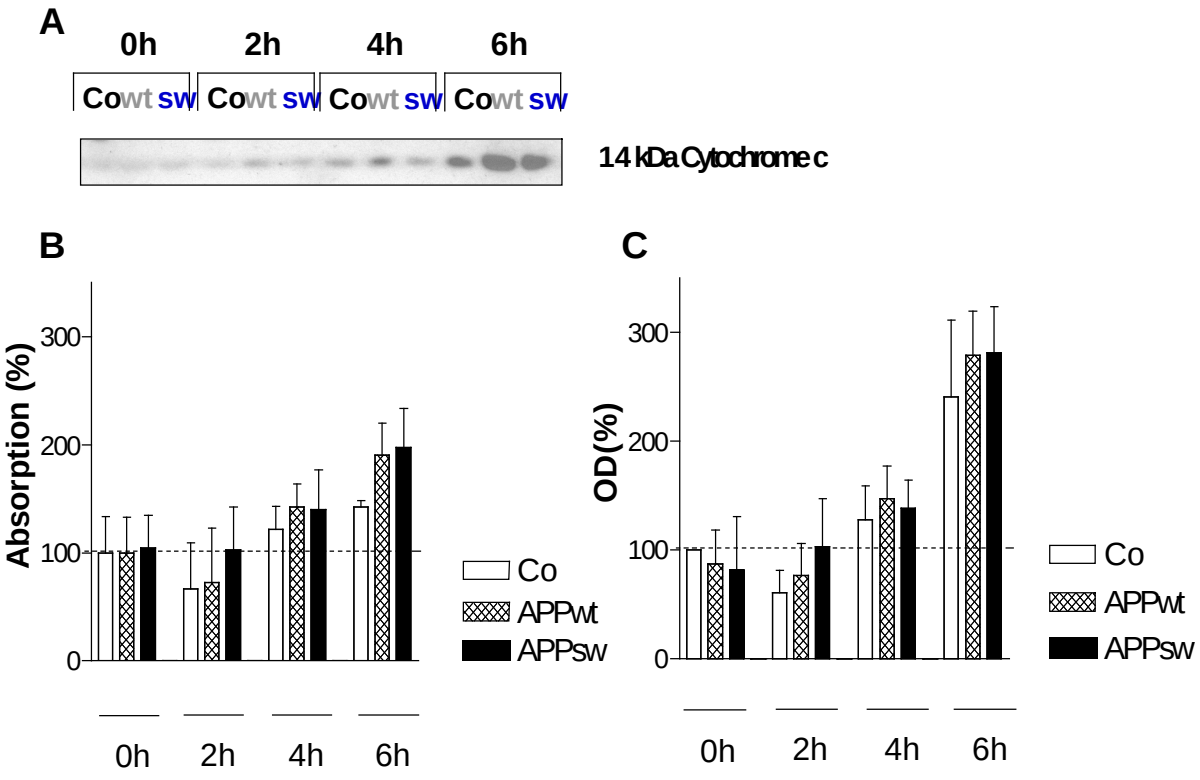

Figure 3.32: Cytochrome c is released in a time-dependent manner after H_2O_2 exposure.

A) PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. Cytosolic fractions were isolated and subjected to western blot analysis. B) Densiometric analysis of cytochrome c bands of the cytosolic fraction. Data represent means ± SEM of 5-10 independent experiments. Two-way ANOVA $p<0.0001$ (time). C) Cytochrome c ELISA of the cytosolic fractions. Data represent means ± SEM of 3-4 independent experiments. Two-way ANOVA: $p=0.0113$ (time). Dotted line represents 100% control.

3.3.8.4 Caspase/ JNK inhibition and cytochrome c release

Previously, it was demonstrated in our group that apoptosis is prevented by caspase and JNK inhibitors (Marques, C. A., Keil, U. et al., 2003). The release of cytochrome c in response to apoptogenic signals is downstream caspase-2 and -8, respectively. The interaction of JNK with the mitochondrial apoptosis signaling cascade through interaction with Bcl-2 family proteins. In order to investigate whether caspase-2 and -8 or JNK inhibitors could prevent mitochondria-mediated apoptosis, we pre-incubated

control PC12 cells with specific enzyme inhibitors after induction of oxidative stress-mediated apoptosis by H_2O_2 (500 µM) (Fig. 3.45). Indeed, as shown by western blot, caspase and JNK inhibition inhibited the release of cytochrome c into the cytosol. Moreover, cytochrome c detected in the cytosolic fraction of probes treated with the inhibitors, almost reached baseline levels.

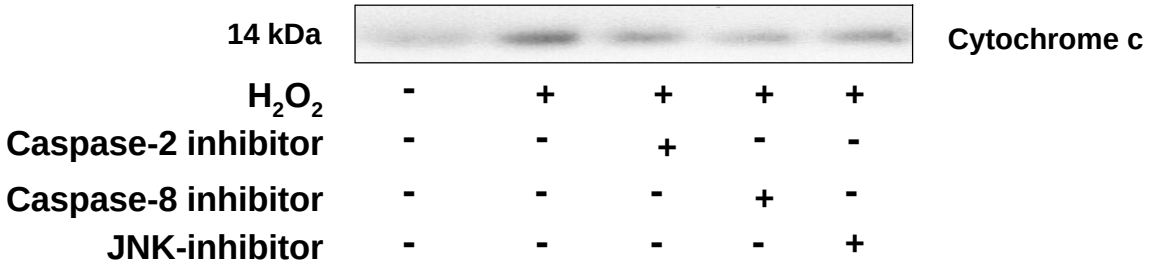

Figure 3.33: Caspase- and JNK inhibition results in a decreased cytochrome c release
Control PC12 cells were exposed to H_2O_2 (500 µM) for 6h alone or in combination with the following inhibitors: Caspase-2 inhibitor (5 µM), caspase-8 inhibitor (5 mM) and JNK inhibitor (500 nM). Cytosolic fractions were collected and analysed by western blot (n=1).

3.3.8.5 Smac/DIABLO

Second mitochondrial activator of caspases (Smac/DIABLO), is originally located in the mitochondrial inter-membrane space and released in response to oxidative stress. It has been described before that Smac/DIABLO is co-released with cytochrome c, blocking IAPs (inhibitor of apoptosis proteins), thus promoting apoptosis. PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h and mitochondrial fractions were analysed by western blot. Indeed, a time-dependent release of Smac/DIABLO was observed at various incubation time points H_2O_2 (Two-way ANOVA: $p<0.001$ (time); $p<0.05$ (cell type) (Fig. 3.34 A+B)). Total amounts of mitochondrial Smac/DIABLO did not decrease during H_2O_2 treatment, indicating that Smac/DIABLO is not completely released into the cytosol as the major part remained in the mitochondrial inter-membrane space. This effect has also been observed for cytochrome c, and the previously postulated co-release of both pro-apoptotic factors could be confirmed (Rehm, M., Dussmann, H. et al., 2003). We suggest that

Smac/DIABLO is released slightly earlier into the cytosol by APPwt and APPsw PC12 cells compared to control cells. These results were confirmed by densiometric analysis of cytosolic Smac/DIABLO bands (Fig. 3.34 B).

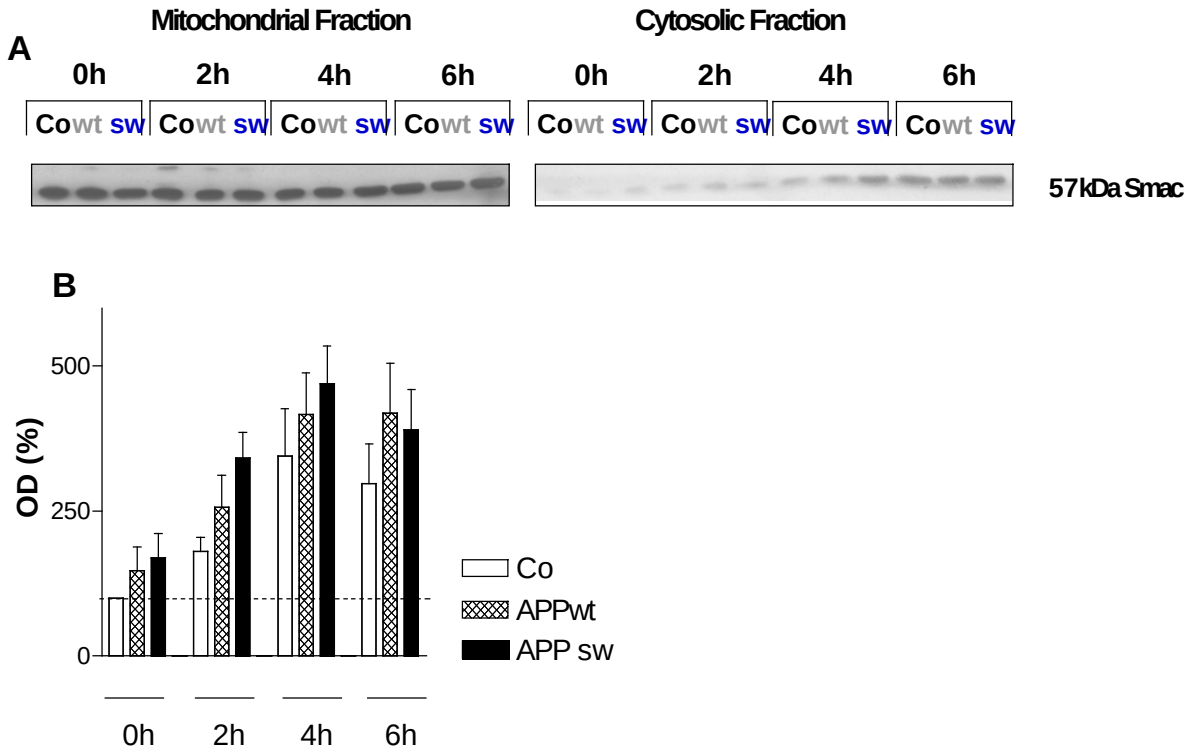

Figure 3.34: Smac/DIABLO is co-released with cytochrome c during H_2O_2 exposure.

A) PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. Cytosolic and mitochondrial fractions were isolated and subjected to western blot analysis. B) Densiometric analysis of Smac bands of the cytosolic fraction. Data represent means ± SEM of 5-9 independent experiments. Two-way ANOVA $p<0.0001$ (time), $p=0.0243$ (cell type). Dotted line represents 100% control.

3.3.8.6 AIF

Under baseline conditions, apoptosis inducing factor (AIF) is located in the mitochondrial matrix. In response to apoptotic stimuli, AIF is released into the cytosol from where it translocates to the nucleus (Arnoult, D., Gaume, B. et al., 2003). AIF induces chromatin condensation and nuclear fragmentation finally leading to apoptosis (Daugas, E., Susin, S. A. et al., 2000). AIF is part of the caspase-independent apoptosis thus caspase-activation is not required. Here, we wanted to asses the subcellular localization and the following translocation of AIF after oxidative injury. Hence, we treated PC12 cells with H_2O_2 (500 µM) for 2, 4 and 6h. After 6h exposure to H_2O_2, AIF accumulated in the mitochondrial compartment. Notably, the accumulation is highest in APPsw PC12 cells (Fig. 3.35), while AIF was not released into the cytosol at that time. Concluding we can say that the initial step of caspase-independent apoptosis is the accumulation of AIF in the mitochondrial compartment.

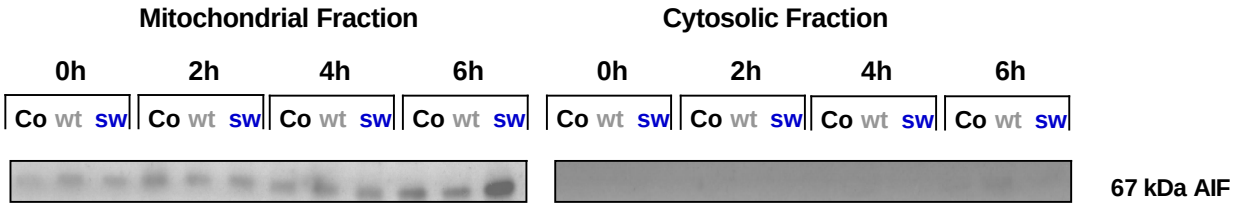

Figure 3.35: Increased accumulation of AIF in mitochondria of APPsw PC12 cells after 6h H_2O_2 exposure. PC12 cells were exposed to H_2O_2 (500 µM) for 2, 4 and 6h. Cytosolic and mitochondrial fractions were isolated and subjected to western blot analysis. Data represent means ± SEM of 4-5 independent experiments.

We incubated the PC12 for 24h with H_2O_2 (500 µM) and assayed mitochondrial and cytosolic fractions for AIF. We observed an accumulation of AIF in APPsw, APPwt and control cells in the mitochondrial fraction after 24h treatment. In contrast to the 6h incubation, no differences in mitochondrial AIF content were observed, indicating that AIF accumulated earlier in APPsw PC12 cells compared to APPwt and control cells (Fig. 3.36). Most interestingly, a cleaved fragment of AIF (57 kDa) was released into the cytosol after 24h H_2O_2 (500 µM) incubation (Fig. 3.36). It has been described before that the cleaved fragment of AIF translocates to the nucleus initiating nuclear

fragmentation and chromatin condensation. Our data corroborate these previous findings leading to the conclusion that apoptosis could be executed in a two-step, caspase-dependent and –independent manner.

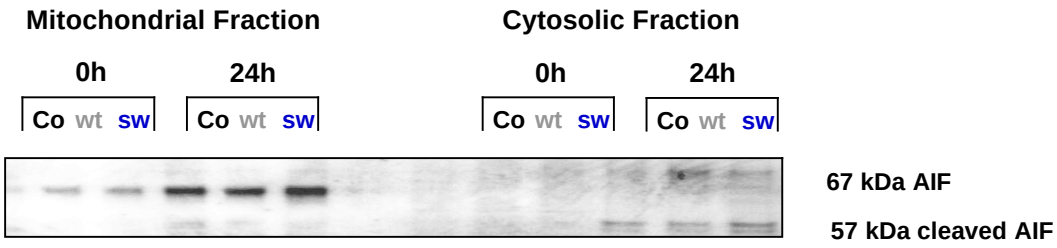

Figure 3.36: H_2O_2 induces AIF fragmentation and the release of a cleaved fragment into the cytosol.
PC12 cells were incubated with H_2O_2 (500 µM) for 24h. Cytosolic and mitochondrial fractions were submitted to western blot analysis. After 24h incubation, AIF accumulates in the mitochondrial fraction, and a cleaved fragment is released into the cytosol. Data represent means ± SEM of 3 independent experiments.

3.4 Evaluation of disease-modifying strategies

3.4.1 γ-Secretase inhibitors

In the past, large collections of compounds to identify molecules that reduce Aβ production in the absence of overt toxicity have been screened and it was determined whether β- or γ-secretase activity had been inhibited by secondary assays. This underlines the very importance of β- and γ-secretase as potential targets in AD. Here, we investigated the effects of two γ- and one β -secretase inhibitors on mitochondrial viability, APP expression and Aβ secretion.

3.4.1.1 Mitochondrial activity

We could previously show that the functional γ-secretase inhibitor DAPT reconstitutes ATP levels in APPsw PC12 cells after 48h treatment. We additionally investigated the effect of γ-secretase inhibition by DAPT on MTT reduction potential of APPwt and APPsw PC12 and HEK cells (Fig. 3.37 A-B). Generally, APPsw and APPwt HEK and PC12 cells revealed a slightly reduced MTT reduction potential under baseline conditions. The exposure of PC12 cells to DAPT (250-1000 nM) for 24h did not result in a significant increase of mitochondrial activity (Fig. 3.37 A). When the treatment of APPsw and APPwt PC12 cells to DAPT (0.25-1.0 µM) was continued for another 24h, this resulted in a significant increase in MTT reduction levels of APPwt (0.5, 1.0 µM; $p<0.05$ vs. untreated APPwt control) and APPsw (1000 nM; $p<0.05$ vs. untreated APPwt control) (Fig. 3.37 B). It should be explained that DAPT did not influence the MTT reduction potential of control cells. In order to prove that the results were not due to increased proliferation rates of APPwt and APPsw PC12 cells, cell numbers were counted after incubation (data not shown). The proliferation rates in the first three days were low, with similar results for all clones. But at an incubation for 5d, poliferation rates had to be taken into account for the calculation of the results.

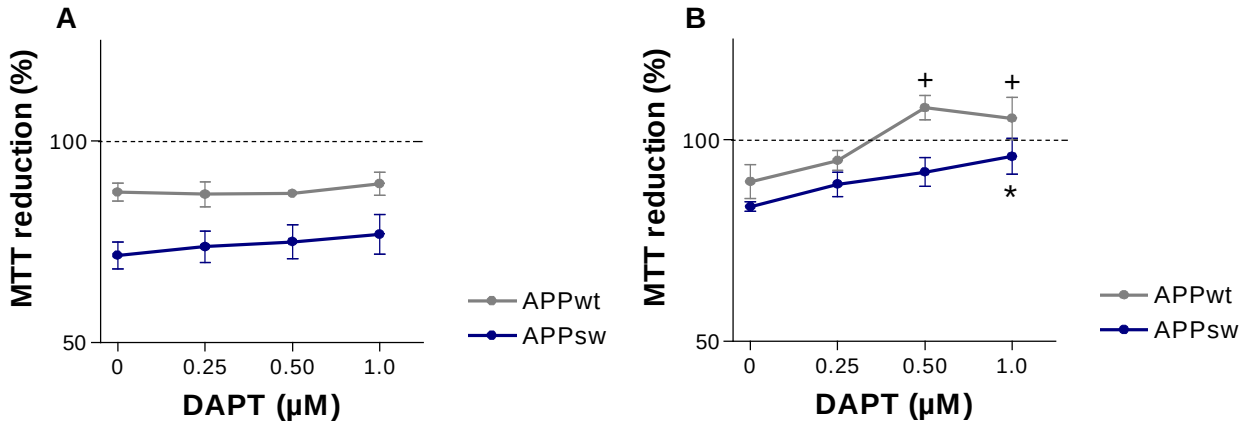

Figure 3.37: The γ-secretase inhibitor DAPT increases mitochondrial viability in PC12 cells.

A) PC12 cells were treated with DAPT (0.25-1.0 µM) for **24h**. B) PC12 cells were treated with DAPT (0.25-1.0 µM) for **48h**. Mitochondrial viability increases significantly in APPsw cells (1.0 µM), and in APPwt PC12 cells (0.5, 1.0 µM) after 48h exposure to DAPT. One-way ANOVA: ***p<0.001, posthoc *t* test: *p<0.001 vs. APPsw baseline control, $^{+}$p<0.001 vs. APPwt baseline control. Data are shown as means ± SEM of 3-5 independent experiments. Dotted line represents 100% baseline of control cells.

In order to exclude that the increase in MTT reduction potential is a cell-type specific process and not directly linked to DAPT, we treated APPwt, APPsw and control HEK cells with concentrations ranging from 0.25 to 1.0 µM for 48h (Fig. 3.38). Simultaneously, MTT reduction potential increased significantly in APPsw HEK cells (p<0.05 vs. untreated APPsw baseline control) after 48h treatment.

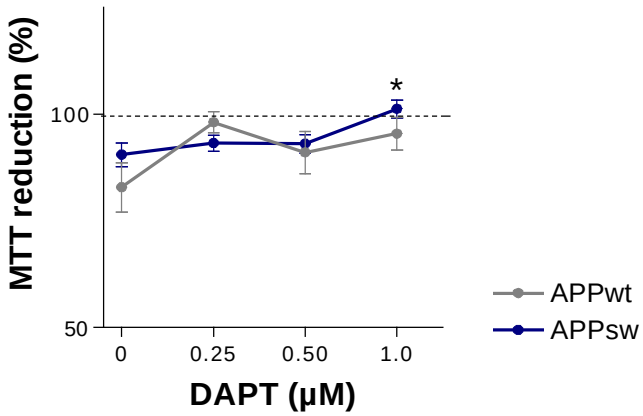

Figure 3.38: The γ-secretase inhibitor DAPT increases mitochondrial viability in APPsw HEK cells.

HEK cells were treated with DAPT (0.25- 1.0 µM) for 48h and MTT reduction potential was measured. Mitochondrial viability increased significantly in APPsw cells (1.0 µM). One-way ANOVA: ***p<0.001, posthoc *t* test: *p<0.001 vs. APPsw baseline control. Data are shown as means ± SEM of 5 independent experiments. Dotted line represents 100% baseline of control cells.

The increase in MTT reduction potential could be directly linked to the Aβ-reducing properties of the γ-secretase inhibitor DAPT, which indicates tha the inhibition of enzymes involved in the processing of APP are promising targets for AD. The development of γ-secretase inhibitors lead to structurally distinct substances, all of which possess Aβ-reducing properties, but differ in the degree of their toxic side effects. Thus, a structurally distinct γ-secretase inhibitor, L-685458, was characterized by its effect on the MTT reduction potential (Fig. 3.39). L-685458 is widely described in literature as a transition-state analogue of γ-secretase, supposed to bind to γ-secretase in a highly specific manner (Funamoto, S., Morshima-Kawashima, M. et al., 2004; Kornilova, A. Y., Das, C. et al., 2003; Lewis, H. D., Revuelta, B. I. P. et al., 2003). In contrast to DAPT, we found a decrease in MTT reduction after L-685458 treatment.

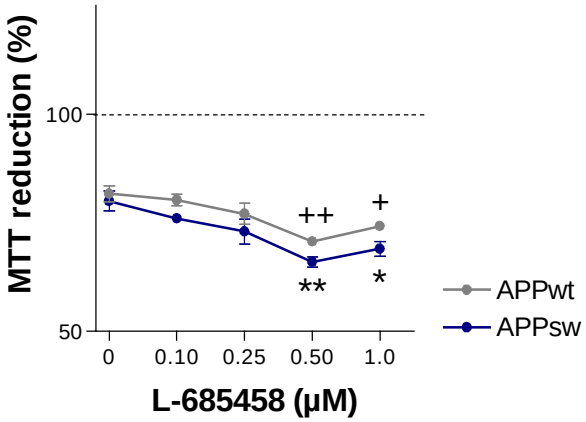

Figure 3.39: The γ-secretase inhibitor L-685458 significantly decreases MTT reduction potential in HEK cells.

Treatment with L-685458 (0.1-1.0 µM) for 48h. One way ANOVA: p<0.0001, posthoc *t* test revealed a significant decrease in MTT reduction in APPsw at 0.5 µM (**p<0.01 vs. APPsw baseline control) and 1.0 µM (*p<0.05 vs. APPsw baseline control) and in APPwt HEK cells at 0.5 µM ($^{++}$p<0.01) and 1000 nM ($^{+}$p<0.05) vs. untreated baseline control. Data are shown as means ± SEM of 5 independent experiments. Dotted line represents 100% baseline of control cells.

This result demonstrates that in the case of the γ-secretase inhibitor L-685458, the toxic side effects monitored by a decrease of the MTT reduction potential beat beneficial effects mediated by the reduction of Aβ levels.

3.4.1.2 Secreted Aβ levels

The functional γ-secretase inhibitor DAPT dose-dependently reduces Aβ levels in plasma, CSF and brains of Tg2576 mice (Lanz, T. A., Himes, C. S. et al., 2003b) and in rat cortical neurons (Kienlen-Campard, P., Miolet, S. et al., 2002). In order to determine Aβ secretion levels, we exposed APPsw and APPwt PC12 cells to increasing concentrations of DAPT (0.1-1.0 µM) for 24h (Fig. 3.40 A+B). Under baseline conditions, APPsw PC12 cells secrete significantly higher $A\beta_{1-40}$ levels than control cells ($p<0.05$ vs. control, Fig. 3.1) and APPwt PC12 cells ($p<0.05$ vs. APPwt control, Fig. 3.1). $A\beta_{1-40}$ secretion of APPsw PC12 cells decreased significantly in a dose-dependent pattern ($p<0.01$, $p<0.001$ vs. untreated APPsw baseline control) (Fig. 3.40 B), while secreted $A\beta_{1-40}$ levels of APPwt PC12 cells remained unaffected. We speculate that this is due to the very low $A\beta_{1-40}$ of APPwt PC12 cells, thus rendering it impossible to achieve any further decrease in secretion levels by DAPT treatment (Fig. 3.40 A).

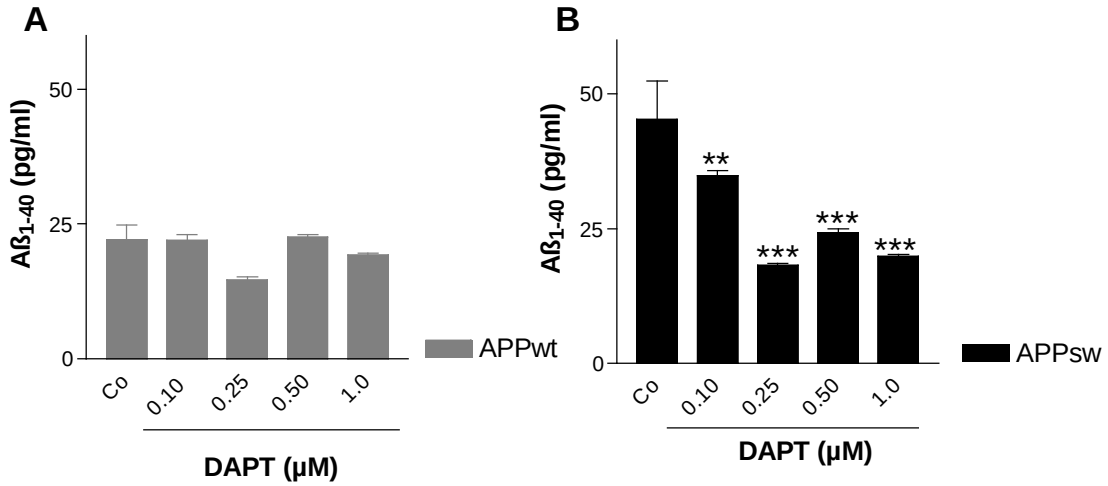

Figure 3.40: DAPT decreases secreted $A\beta_{1-40}$ levels in APPsw PC12 cells in a concentration-dependent manner.

PC12 cells were treated with increasing concentrations of DAPT (0.1-1.0 µM) for 24h. Culture supernatants were collected and assayed for $A\beta_{1-40}$ by ELISA technique. A) $A\beta_{1-40}$ levels of APPwt PC12 cells after exposure to DAPT (0.1-1.0 µM, 24h). B) $A\beta_{1-40}$ levels of APPsw PC12 cells after exposure to DAPT (0.1-1.0 µM, 24h). One-way ANOVA: ***p<0.001, posthoc *t* test: **p<0.01, ***p<0.001 vs. APPsw baseline control. Data are shown as means ± SEM of 3 independent experiments.

Therefore, a similar experimental setting was used for APPwt and APPsw HEK cells. DAPT significantly decreased both secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPwt and APPsw HEK cells (Fig. 3.41 A+B, Fig. 3.42 A-D). 4h treatment with 0.25 µM DAPT did not lead to a decrease in $A\beta_{1-40}$ levels. The treatment with 1.0 µM DAPT significantly reduced $A\beta_{1-40}$ in both cell lines ($p<0.001$ vs. corresponding untreated baseline control) (Fig. 3.41 A+B). Secreted $A\beta_{1-42}$ was not detectable after 4h in culture supernatants of APPwt and APPsw HEK cells. In more detail, we oberved a tenfold reduction in APPwt (Co: 112pg/ml vs. 15 pg/ml) and a 24fold reduction (Co: 486 pg/ml vs. 22pg/ml) of secreted $A\beta_{1-40}$ in APPsw HEK cells after DAPT treatment (1.0 µM).

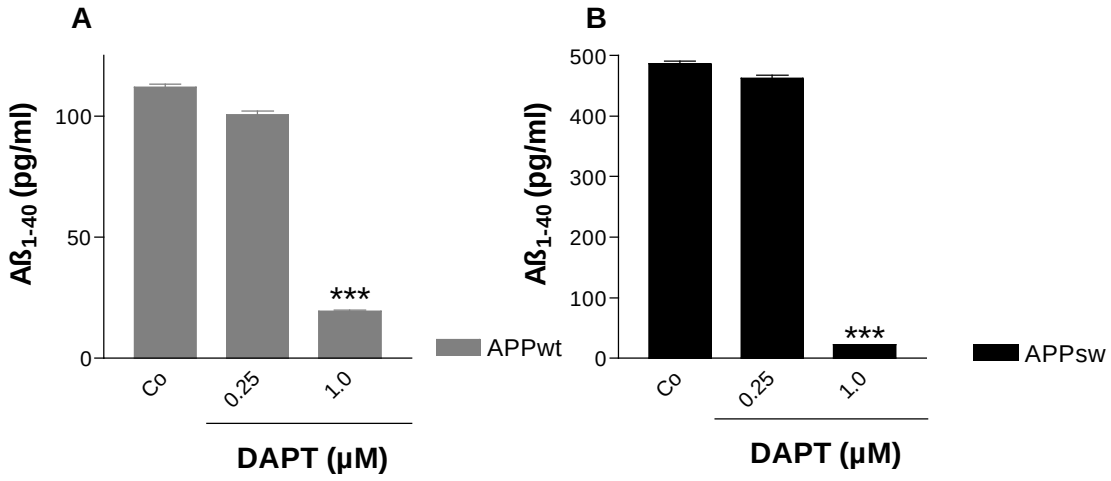

Figure 3.41: DAPT treatment (4h) decreases secreted $A\beta_{1-40}$ levels in a concentration-dependent manner.
A) Treatment of APPwt HEK with DAPT (0.25, 1.0 µM) for 4h. B) Treatment of APPsw HEK with DAPT (0.25, 1.0 µM) for 4h. Culture supernatants were collected after 4h incubation and assayed for $A\beta_{1-40}$ by ELISA technique. One-way ANOVA: ***p<0.001, posthoc *t* test: ***p<0.001 vs. control (Co), Data are shown as means ± SEM of 3 independent experiments.

In the following, we determined secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPwt and APPsw HEK cells after 24h exposure to DAPT (Fig. 3.42 A-D). Treatment of APPwt HEK cells with 0.25 µM DAPT for 24h lead to a significant ($p<0.01$ vs. untreated baseline control) decrease in secreted $A\beta_{1-40}$ levels, while $A\beta_{1-42}$ levels remained unaltered (Fig 3.42 A+B). APPwt HEK cells showed a significant ($p<0.001$ vs. untreated baseline control) reduction of both $A\beta_{1-40}$ and $A\beta_{1-42}$ at 1.0 µM DAPT. Generally, secretion of $A\beta_{1-40}$ was inhibited to a higher extent than the secretion of $A\beta_{1-42}$ in APPwt HEK cells. The

inhibition of γ-secretase by DAPT (0.25, 1.0 µM) in APPsw HEK cells completely abolished both $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion. By DAPT treatment for another 24h, secreted Aβ levels remained equally low in APPsw and APPwt HEK cells (data not shown). Concluding, the γ-secretase inhibitor DAPT blocks $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion to a higher extent in APPsw than APPwt HEK cells. We may assume that the reduction of $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion as an early event achieved by γ-secretase inhibition explains the increase in MTT reduction potential (Fig. 3.38).

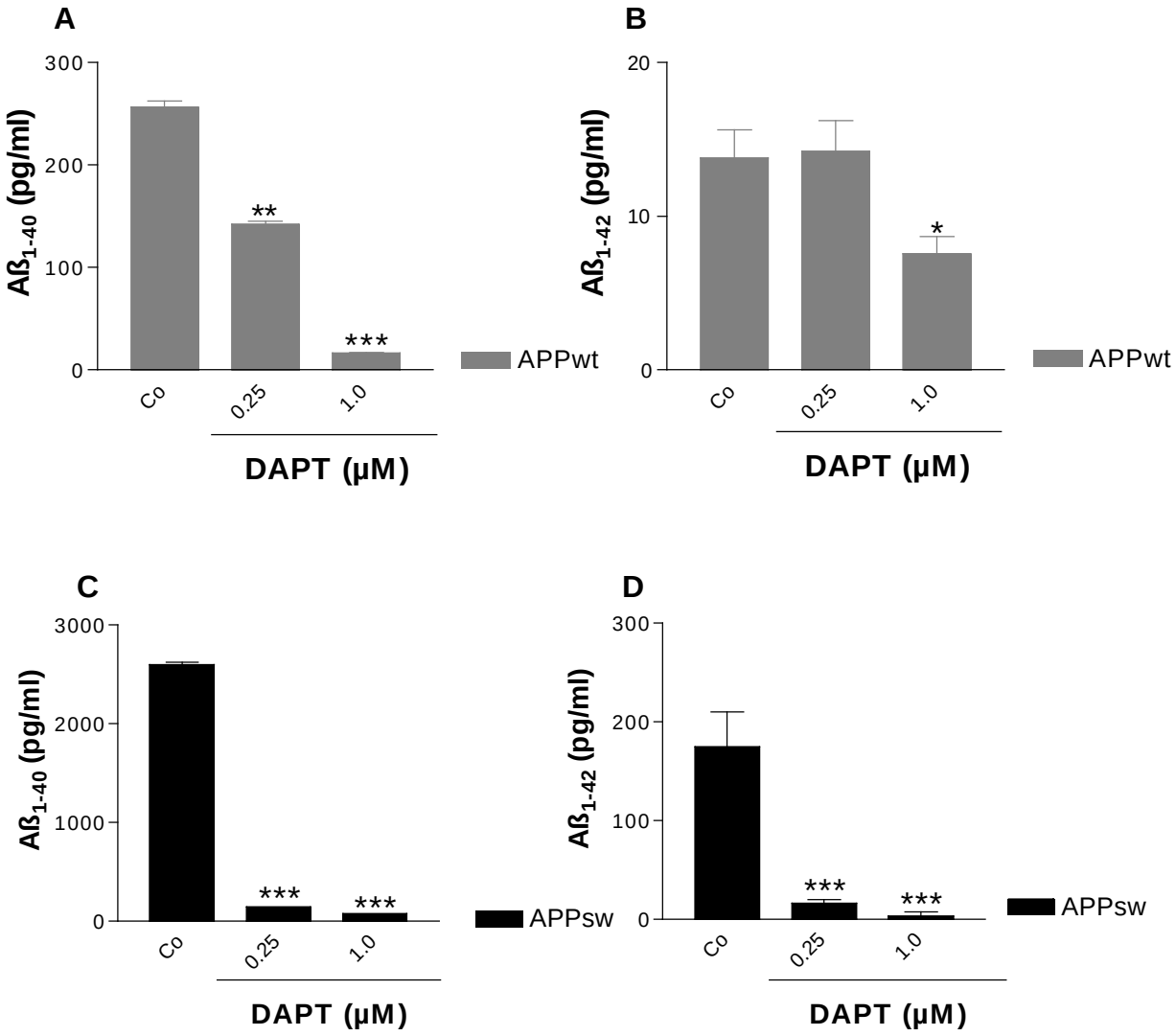

Figure 3.42: DAPT treatment (24h) strongly decreases secreted Aβ$_{1-40}$ and Aβ$_{1-42}$ levels in APPsw HEK cells.

A) Secreted Aβ$_{1-40}$ levels after treatment of APPwt HEK with DAPT (0.25, 1.0 µM) for 24h. B) Secreted Aβ$_{1-42}$ levels after treatment of APPwt HEK with DAPT (0.25, 1.0 µM) for 24h. C) Secreted Aβ$_{1-40}$ levels after treatment of APPsw HEK with DAPT (0.25, 1.0 µM) for 24h. D) Secreted Aβ$_{1-42}$ levels after treatment of APPsw HEK with DAPT (0.25, 1.0 µM) for 24h. Culture supernatants were collected after 24h incubation and assayed for Aβ$_{1-40}$ and Aβ$_{1-42}$. One-way ANOVA: ***p<0.001, posthoc *t* test: *p<0.05, **p<0.01,***p<0.001 vs. untreated baseline control (Co), Data are shown as means ± SEM of 3 independent experiments.

In order to further characterize the decrease in secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ caused by γ-secretase inhibtion, the effects of the structurally distinct inhibitor L-685458 on $A\beta$ secretion, APPwt and APPsw HEK cells were investigated. HEK cells were exposed to varying concentrations of L-685458 for 24h (Fig. 3.43 A-D). Most interestingly, L-685458 induced a significant ($p<0.05$ vs. untreated baseline control) increase in $A\beta_{1-40}$ and $A\beta_{1-42}$ at low concentrations (Fig. 3.43 A+B); $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion was reduced significantly at higher concentrations (0.25 µM: $p<0.05$, $p<0.01$;0.5 µM: $p<0.001$ vs. untreated baseline control) in APPwt HEK cells. Consequently, the concentration-dependent increase in secretion was more pronounced for $A\beta_{1-42}$. Interestingly, the significant reduction of secreted $A\beta_{1-42}$ required equal concentrations as for $A\beta_{1-40}$ reduction. In contrast to the situation in APPwt HEK cells, L-685458 did not induce enhanced secretion of both $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPsw HEK cells (Fig. 3.43 C+D). $A\beta_{1-40}$ levels were significantly lowered at 0.25 µM ($p<0.01$ vs. untreated baseline control) and 0.5 µM ($p<0.001$ vs. untreated baseline control) (Fig. 3.43 C). $A\beta_{1-42}$ secretion was more sensitive to γ-secretase inhibition by L-685458, as 0.02 µM significantly reduced $A\beta_{1-42}$ secretion levels ($p<0.001$ vs. untreated baseline control) (Fig. 3.43 D). It was a very intriguing finding that the γ-secretase inhibitor L-685458 differently modulates secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in APPwt and APPsw HEK cells. This would speak for distinct ways of APPsw and APPwt processing and $A\beta$ production thereof, a phenomenon which will need further discussion.

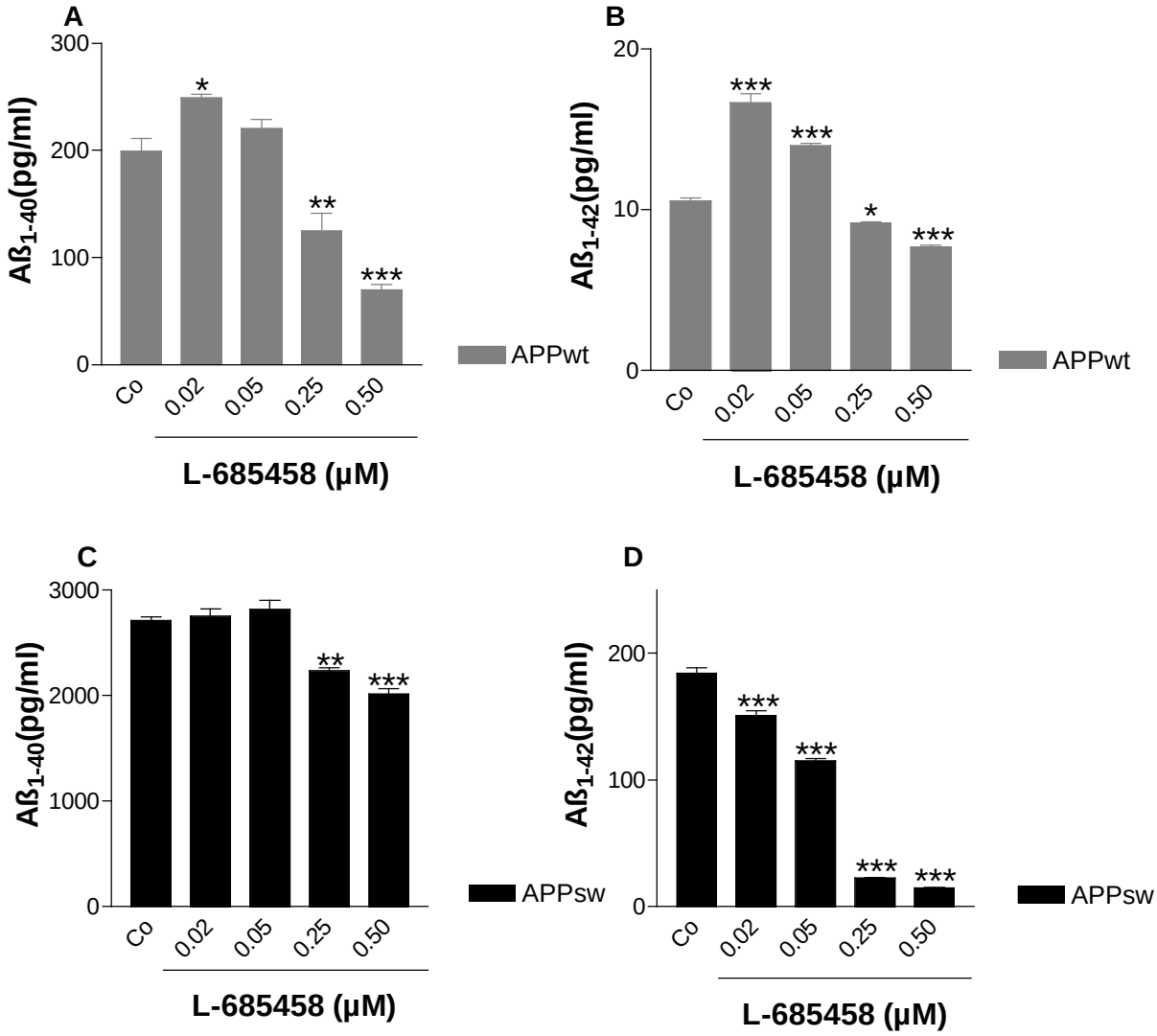

Figure 3.43: L-685458 time- and dose-dependently decreases secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in APPsw and APPwt HEK cells.

A) Secreted $A\beta_{1-40}$ levels after treatment of APPwt HEK with L-685458 (0.02-0.5 µM) for 24h. B) Secreted $A\beta_{1-42}$ levels after treatment of APPwt HEK with L-685458 (0.02-0.5 µM) for 24h. C) Secreted $A\beta_{1-40}$ levels after treatment of APPsw HEK with L-685458 (0.02-0.5 µM) for 24h. D) Secreted $A\beta_{1-42}$ levels after treatment of APPsw HEK with L-685458 (0.02-0.5 µM) for 24h. Culture supernatants were collected after incubation and assayed by ELISA. One-way ANOVA: ***p<0.001, posthoc *t* test: **p<0.01,***p<0.001 vs. untreated baseline control (Co). Data are shown as means ± SEM of 3 independent experiments.

3.4.1.3 Intracellular APP, secreted APP, CTFs and Aβ

It is generally stated that the γ-secretase inhibitor DAPT does not influence APP expression and secretion in rat cortical neurons (Dovey, H. F., John, V. et al., 2001). Here, we show that high concentrations (1.0 µM, Fig. 3.26 A+B), in contrast to lower concentrations (0.25 µM, Fig. 3.44), modulate APP processing in stably transfected HEK cells. Cell lysates and culture supernatants of APPwt, APPsw and control cells were collected after treatment with DAPT and subjected to western blot analysis. Generally, intracellular CTFs (namely C83, 6 kDa) increased during DAPT treatment. Due to the sensitivity of the antibody W-02, secreted Aβ (4 kDa) was detectable in the culture supernatants of APPsw HEK cells, but not in supernatants of APPwt and control HEK cells (Fig. 3.44, 3.45 A+B). Treatment of APPwt, APPsw and control cells with 0.25 µM DAPT for 24 and 48h did not affect the secretion of sAPPα (96 kDa). In contrast to this, Aβ secretion was lowered markedly as no extracellular Aβ was detected in the culture supernatant of APPsw HEK cells. Expression of full-length APP (116 kDa) increased slightly in a time-dependent manner after 24 and 48h DAPT treatment.

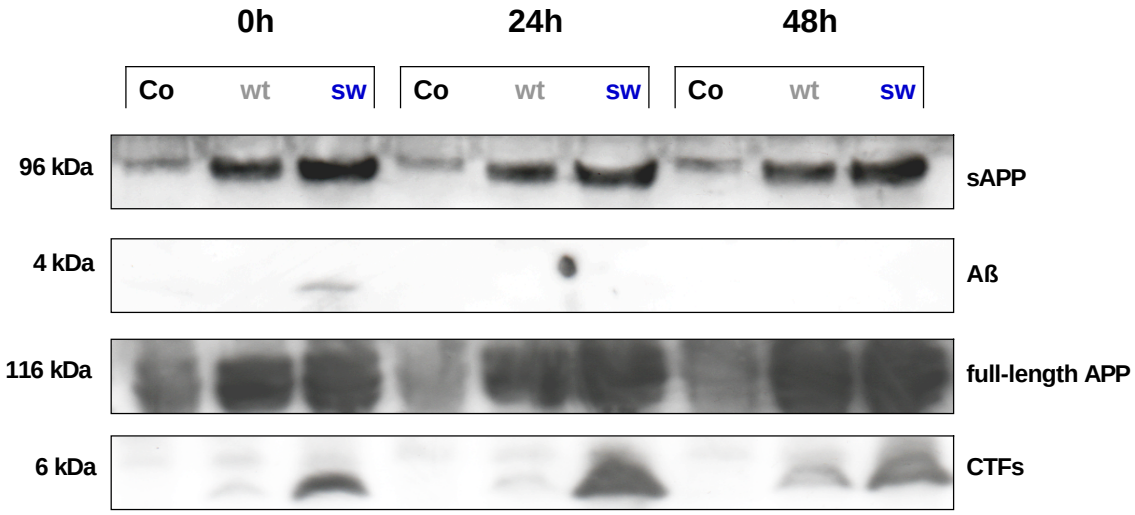

Figure 3.44: DAPT treatment (0.25 µM) time-dependently increases intracellular APP and CTF levels.
APPwt, APPsw and control HEK cells were treated with DAPT (0.25 µM) for 24 and 48h. Culture supernatants and whole cell lysates were collected after incubation and subjected to western blot analysis. Intracellular full-length APP (116 kDa) and CTFs increased, while secreted Aβ levels decreased after 24 and 48h.

We could show before that DAPT treatment (1.0 µM) significantly increased MTT reduction potential of both PC12 and HEK cells. It was of interest whether other effects on APP processing, besides the inhibition of Aβ formation and secretion, could be related to the increase in MTT reduction by DAPT treatment. After 4h exposure to DAPT (1.0 µM), secreted sAPPα levels (96 kDa) slightly decreased in APPwt and APPsw HEK cells. But again, it came to a slight accumulation of intracellular, full-length APP (116 kDa) (Fig. 3.45 A). Prolonged DAPT treatment for 48h lead to reduction of secreted Aβ and intracellular accumulation of CTFs. Of note, a drastic reduction not only of secreted sAPPα (96 kDa), but also of intracellular full-length APP (116 kDa) was observed (Fig. 3.45 B). These findings indicate that altered APP processing, in a more generalized manner, might be modulated by DAPT. Eventually, altered APP trafficking could result in alterations of full-length as well as secreted APP.

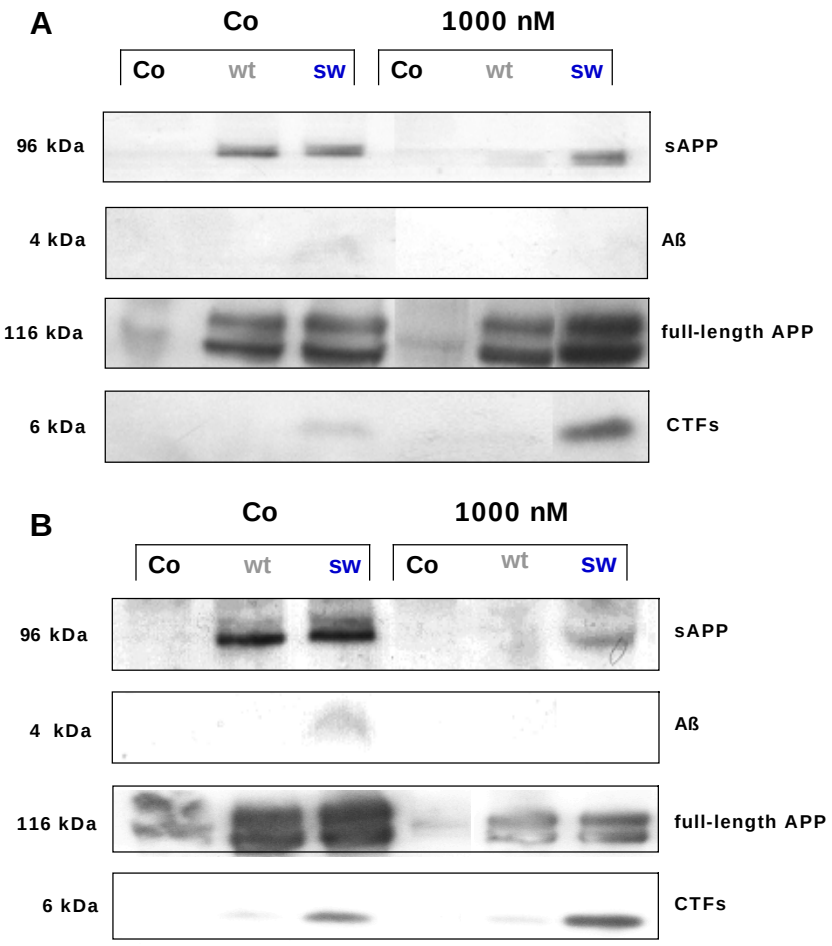

Figure 3.45: DAPT modulates intracellular APP levels.

APPwt, APPsw and control HEK cells were treated with DAPT (1.0 µM) for 4h (A) and 48h (B). Culture supernatants and whole cell lysates were subjected to western blot analysis.

3.4.2 β-secretase inhibitors

3.4.2.1 Mitochondrial activity

It is widely known that peptidic β-secretase inhibitors are toxic due to their unspecific side effects (Citron, M., 2002). As an example for the general characteristics of peptidic β-secretase inhibitors, we chose the β-secretase inhibitor II in order to demonstrate that the MTT reduction potential mirrors the toxicity of the substance in cell culture. HEK cells were exposed to β-secretase inhibitor II for 48h (Fig. 3.46). Concentrations of 2.5 µM and higher were exceedingly toxic to APPsw ($p<0.001$ vs. untreated baseline control) and APPwt ($p<0.001$ vs. untreated baseline control) HEK cells. Furthermore, we examined lower concentrations of the β-secretase inhibitor II as well as shorter incubation periods without observing significantly increased MTT reduction levels in APPwt and APPsw HEK cells (data not shown).

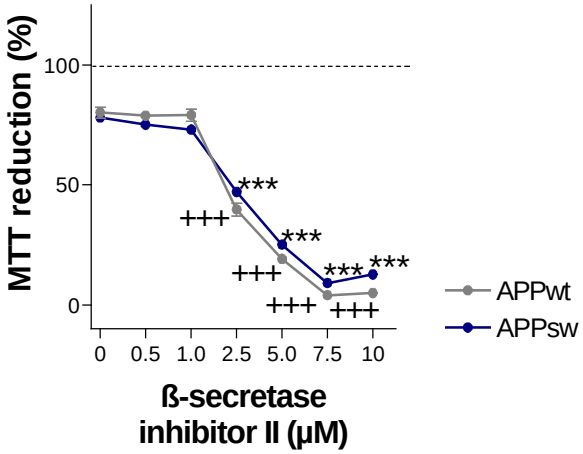

Figure 3.46: The β-secretase inhibitor II significantly reduces MTT reduction potential of APPwt, APPsw and control HEK cells.

Treatment of APPwt and APPsw and control HEK cells with β-secretase inhibitor II (0.5-10 µM) for 48h. One-way ANOVA: $p<0.001$, posthoc *t* test revealed a significant decrease in MTT reduction at concentrations above 2.5 µM in APPsw (***$p<0.001$) and APPwt ($^{+++}p<0.001$) HEK cells vs. corresponding untreated baseline controls. All values represent the means ± SEM from 3-5 experiments, each experiment performed in triplicates. Dotted line represents 100% baseline of control.

3.4.2.2 Aβ secretion levels

HEK cells were exposed to β-secretase inhibitor II for 24h and culture supernatants were assayed for secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ (Fig. 3.47 A-C). Treatment of APPwt HEK cells exposed to β-secretase inhibitor II (0.5-10 µM) revealed no effect on $A\beta_{1-40}$ secretion. Paradoxically, $A\beta_{1-42}$ levels significantly ($p<0.001$ vs. untreated baseline control) increased in a concentration-dependent pattern under the same treatment conditions (Fig. 3.47 B). In APPsw HEK cells, exposure to β-secretase inhibitor II results in an unimportant increase in secreted $A\beta_{1-40}$ levels at concentrations ranging from 0.5-5 µM (Fig. 3.47 C). A similar effect was observed on $A\beta_{1-42}$ secretion levels at 0.5 µM (Fig. 3.47 D). But also, secreted $A\beta_{1-40}$ was significantly ($p<0.001$ vs. untreated baseline control) reduced after 24h treatment with 10 µM β-secretase inhibitor II.

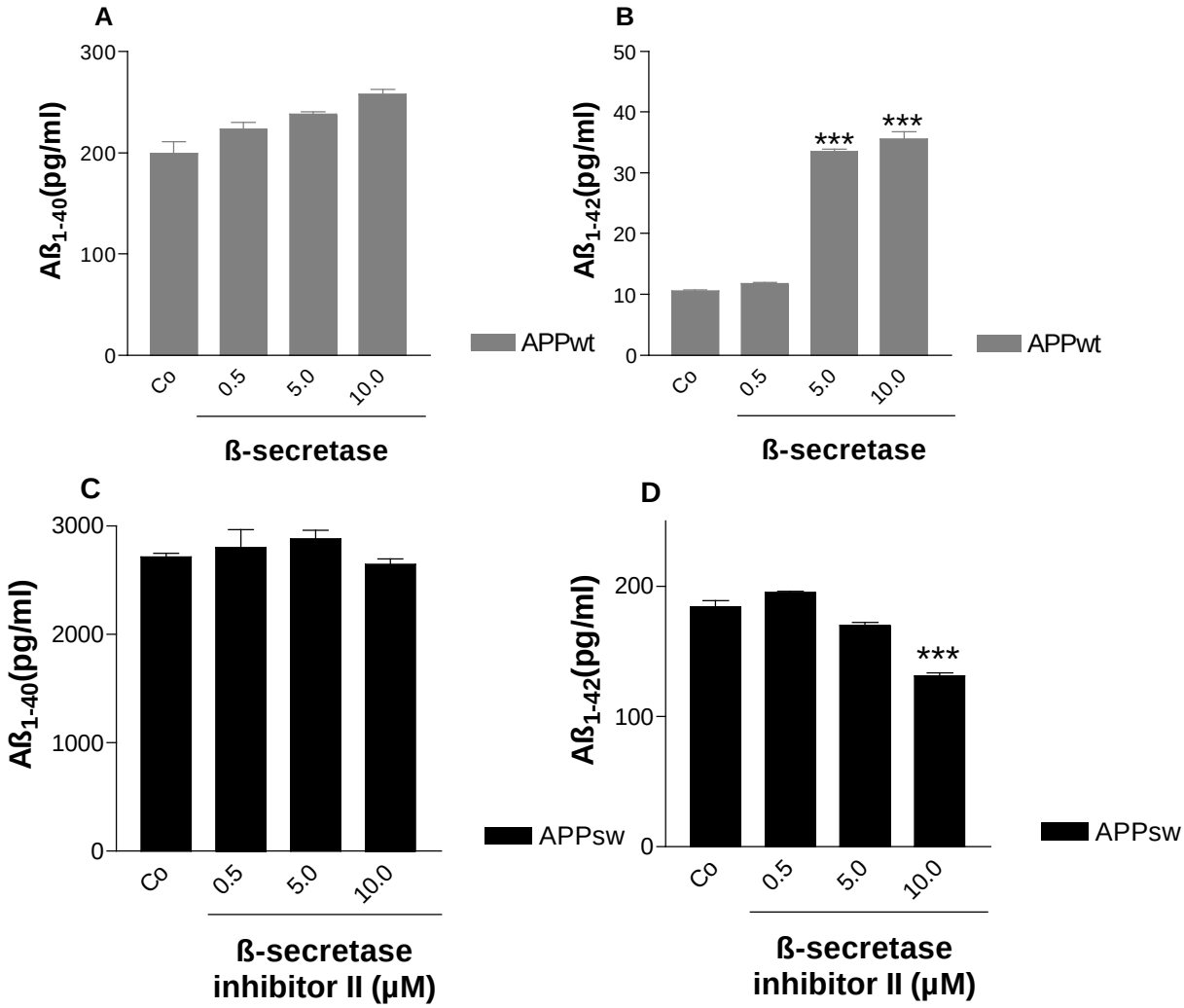

Figure 3.47: Treatment of APPsw HEK cells with the β-secretase inhibitor II (10 µM) leads to decreased secreted $A\beta_{1-42}$.

APPwt and APPsw HEK cells were treated with various concentrations of the β -secretase inhibitor II (0.5-10 µM) for 24h. Culture supernatants were collected and assayed by ELISA. A) Determination of secreted $A\beta_{1-40}$ levels in APPwt HEK cells. B) Determination of secreted $A\beta_{1-42}$ levels in APPwt HEK cells. C) Determination of secreted $A\beta_{1-40}$ levels in APPsw HEK cells. D) Determination of secreted $A\beta_{1-42}$ levels in APPsw HEK cells. One-way ANOVA: $p<0.0001$, posthoc *t* test: $p<0.001$ vs. Co.All values represent the means ± SEM from 3-6 experiments, each experiment performed in duplicates.

3.4.3 Growth factor modulators

3.4.3.1 KP544 protects against oxidative injury

In order to investigate the effect of KP544 on cellular viability during oxidative insult, we treated PC12 cells with hydrogen peroxide (H_2O_2). Interestingly, already under baseline conditions, KP544 showed protective effects on APPsw PC12 cells by significantly increasing the cellular ability to reduce MTT (Fig. 3.48) suggesting that the drug is able to protect against the toxicity of chronic Aβ exposure. After 6h treatment with H_2O_2 (500 µM), mitochondrial activity was very strongly decreased. Even under this condition of extreme disruption of MTT reduction, KP544 (0.01 µM) exhibited a significant protective effect on viability of control ($p<0.05$) as well as on APPsw ($p<0.001$) PC12 cells.

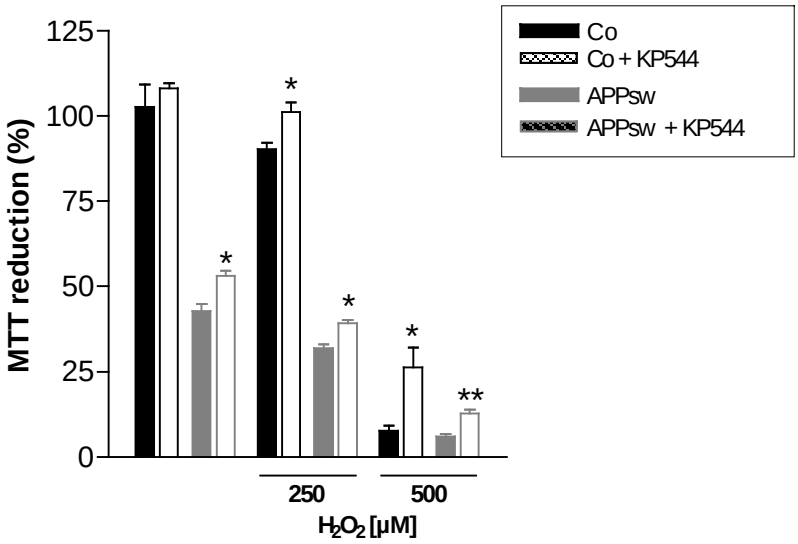

Figure 3.48: KP544 protects undifferentiated PC12 cells against H_2O_2 insult.

APPsw and control PC12 cells were treated with H_2O_2 (250 or 500 µM) in the presence or absence of KP544 (0.01 µM) for 6 hours. Two-way ANOVA revealed a significant difference between cell types ($p<0.001$) and a significant effect of the KP455 treatment ($p<0.001$), posthoc *t* test: *$p<0.05$, **$p<0.01$ vs. corresponding control without KP544. All values represent the means ± SEM from 3-4 experiments, each experiment performed in triplicates.

3.4.3.2 KP544 protects differentiated cells against growth factor withdrawal

In order to investigate the effect of KP544 on cellular viability during trophic factor withdrawal, we treated PC12 cells with increasing concentrations of KP544 (0.001 µM, 0.01 µM, 1.0 µM) for 3 days (Fig. 3.49 A-C). Notably, already under conditions in the presence of NGF (50ng/ml), KP544 did promote cellular survival of both, control and APPsw cells. This effect is strongest pronounced at a concentration of 1.0 µM, but is already present at a concentration as low as 0.001 µM indicating that KP544 can enhance NGF activity on differentiated PC12 cells. Moreover, KP544 was able to enhance mitochondrial activity under conditions of NGF withdrawal in control as well as in APPsw cells (Fig. 3.49 A-C). Again, the strongest increase in the ability to reduce MTT was at a concentration of 1.0 µM KP544. In this experimental design, KP544 exhibited an 1.7fold increase of MTT reduction in control and a 2.5 fold increase in APPsw cells thereby enhancing mitochondrial activity of these cells that suffer from NGF withrawal up to the level of control PC12 with NGF in the medium (Fig. 3.49 C). Again, APPsw cells seem to specifically benefit of the effects of KP544.

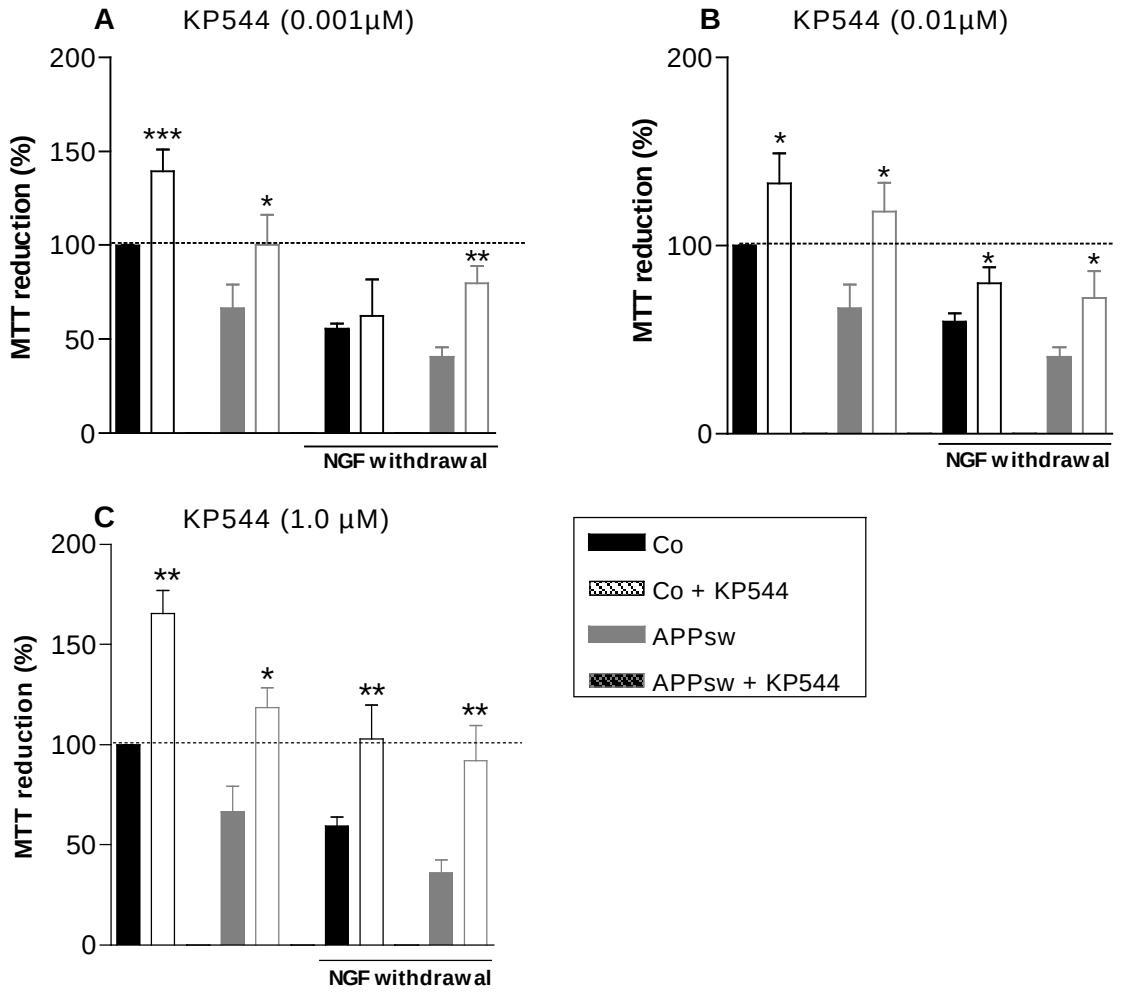

Figure 3.49: KP544 protects fully differentiated PC12 cells against trophic factor withdrawal in a concentration-dependent manner.

Terminally differentiated PC12 cells were treated with increasing concentrations of KP544 - 0.001µM (A), 0.01 µM (B), 1.0 µM (C) - for 3 days in the presence or absence of NGF. A) Treatment with 0.001 µM KP544. Two-way ANOVA revealed a significant difference between cell types ($p<0.05$) and a significant KP544 treatment effect ($p<0.001$). B) Treatment with 0.01 µM KP544. Two-way ANOVA revealed a significant difference between cell types ($p<0.01$) and a significant KP544 treatment effect ($p<0.001$) C) Treatment with 1.0 µM KP544. Two-way ANOVA revealed a significant difference between cell types ($p<0.001$) and a significant KP544 treatment effect ($p<0.001$). A-C) posthoc *t* test: *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. corresponding control without KP544. All values represent the means ± SEM from 4-7 experiments, each experiment performed in triplicates.

3.4.3.3 KP544 modulates the phosphorylation status of GSK-3

Previously, it has been shown that NGF-induced neurite outgrowth of PC12 cells is about twofold enhanced with KP544 treatment (Fyfe, J. A., Beauchamp, L. M. et al., 2004). Moreover, this study suggested that KP544 functions within the cell at a site that is downstream from or independent of mitogen-activated protein kinases (MAPK), since expression levels of ERK1/2 did change after NGF treatment for 30 min or 48h, but no additional effect of KP544 could be detected. In agreement with this observation, we did not find a specific effect of KP544 on expression levels of MEK1/2 (MAP-kinase-kinase), which acts upstream of ERK1/2 (data not shown). Since evidence is provided that the PI 3-K-Akt-GSK-3 pathway is involved in and seems to be even more important for neurite extension than the MAPK pathway (Baki, L., Shioi, J. et al., 2004), we investigated GSK-3β expression and phosphorylation. Therefore, we treated control and APPsw PC12 cells with NGF (50 ng/ml) or with a combination of NGF and KP544 (1.0 µM) for 1 hour and for 48 hours (Fig. 3.50 A+B). In general, the expression and the phosphorylation status of GSK-3β were increased after 48 hours of incubation (Fig. 3.50 A). The expression levels of unphosphorylated GSK-3β did not alter between APPsw and control PC12 nor between treatment groups. The phosphorylation at Ser9 of GSK-3β leads to an inactivation of the enzyme, resulting in the activation of pathways that are normally repressed by GSK-3β. GSK-3β activation is involved in neurodegenerative processes, an inactivation of this enzyme might be beneficial to mitochondrial acitivity, cellular viability and neurite growth. Of note, when cells were exposed to NGF for 1h, there was a slight increase in phospho- GSK-3β levels. Treatment with a combination of KP544 (1.0 µM) and NGF (50 ng/ml) for 1 hour seems to have synergistic effects, specifically in APPsw cells phosphorylation at Ser9 increased significantly ($p<0.05$) compared to untreated APPsw control cells (Fig. 3.50 B). In addition, we observed an increased phosphorylation status ($p<0.001$) of GSK-3β of control cells and APPsw PC12 cells in all treatment groups after 48 hours compared to 1 hour exposure (Fig. 3.50 B), but no additional effects of KP544 to the action of NGF alone possibly due to the fact that maximum expression levels were reached. Interestingly, in all conditions, GSK-3β phosphorylation was significantly decreased in

APPsw cells compared to control PC12 cells (Fig. 3.50 B) (Two-way ANOVA: $p<0.05$ between cell types).

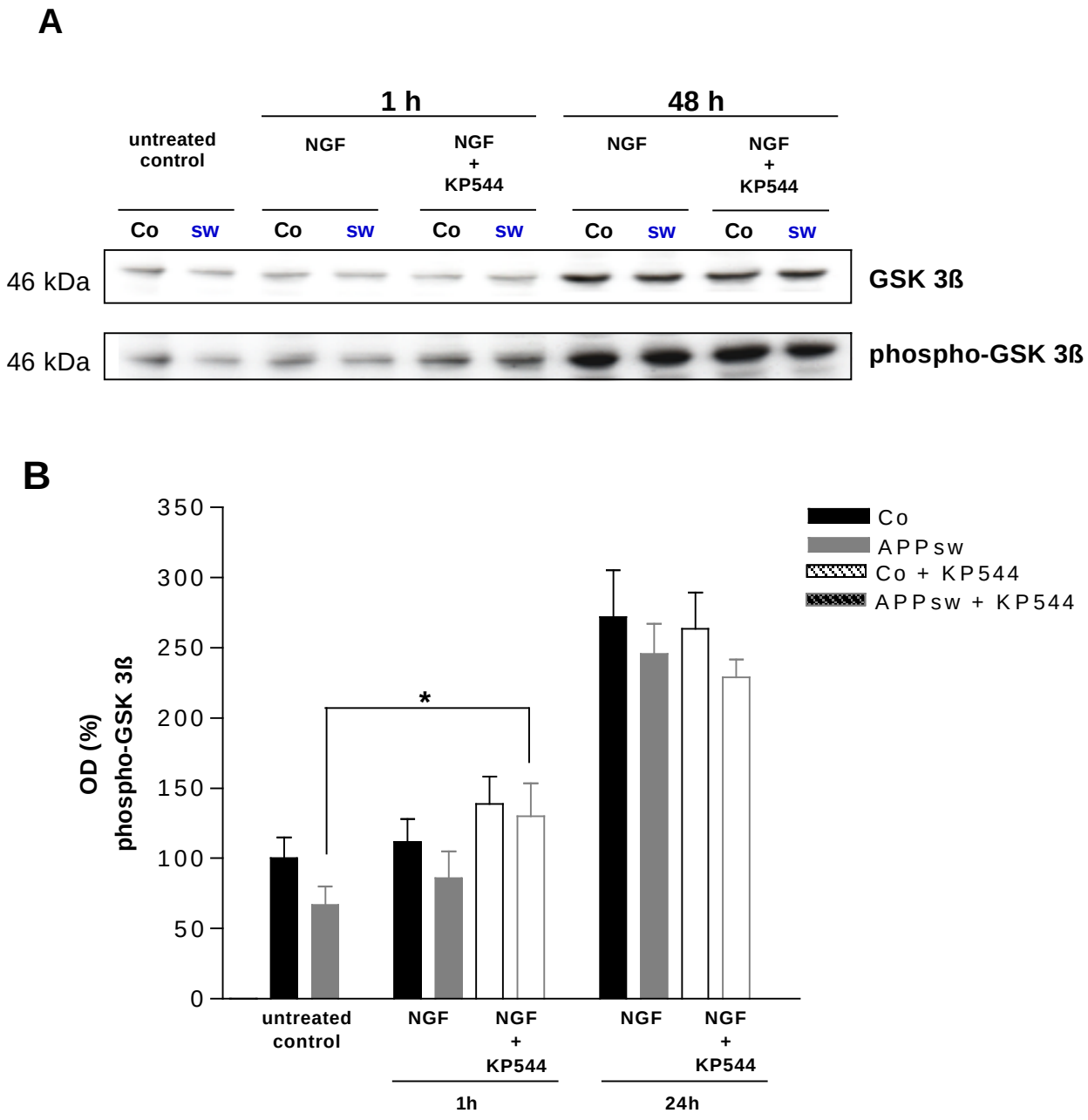

Figure 3.50: Expression levels and phosphorylation status of GSK-3β.

APPsw and control PC12 cells were treated with NGF (50 ng/ml) or NGF (50 nM) in combination with KP544 (1 µM) for one hour and for 48 hours. A) Western blot analysis of GSK-3β and phospho-GSK-3β expression. B) Densiometric analysis of bands of phosphorylated GSK-3β. Two-way ANOVA revealed a significant difference between cell types ($p<0.05$), posthoc *t* test: *$p<0.05$ vs. untreated APPsw control cells. All values represent the means ± SEM. from 4-6 experiments.

3.4.3.4 KP544 decreases the secretion of $A\beta_{1-40}$ in APPsw PC12 cells

Recent studies have shown that that GSK-3β interacts with and binds presenilin, a component of the high molecular weight γ-secretase complex that cleaves APP, in normal human brains and cells (De Ferrari, G. V., Inestrosa, N. C., 2000). Although GSK-3β is known to be involved in tau phosphorylation, its role in APP processing is poorly understood. Interestingly, GSK-3 inhibitors, like lithium, abolished GSK-3β-mediated Aβ increase and reduced plaque burden in APP transgenic mice (Su, Y., Ryder, J. et al., 2004). In our cell model, the Swedish double mutation leads to a significantly increased secretion of $Aß_{1-40}$ when compared to control cells (Keil, U., Bonert, A. et al., 2004). We treated APPsw PC12 cells with NGF (50 ng/ml), KP544 (1.0 µM) and a combination of both for 48 hours (Fig. 3.51). $A\beta_{1-40}$ levels were significantly reduced by NGF treatment ($p<0.05$). Of note, KP544 treatment even reduced $A\beta_{1-40}$ levels to a higher extent ($p<0.01$) (NGF: 43% reduction; KP544: 57% reduction). The combined treatment with KP544 and NGF showed no further reduction of $A\beta_{1-40}$ suggesting that NGF and KP544 act synergistically but not additively on APP processing pathway in this experimental setting.

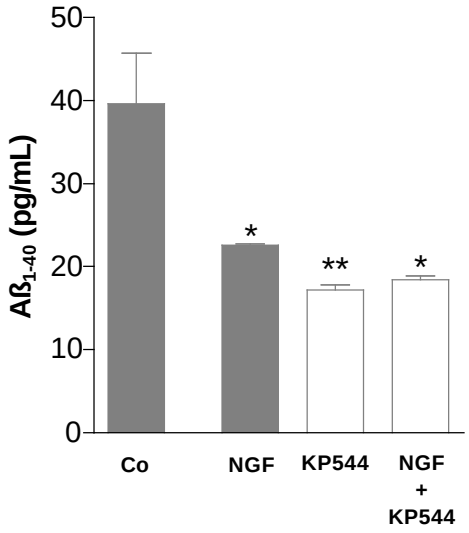

Figure 3.51: KP544 reduces levels of secreted $A\beta_{1-40}$ in APPsw PC12 cells.

APPsw PC12 were treated with KP544 (1.0 µM), NGF (50 nM) or a combination of both for two days. Medium was collected and analysed by $A\beta_{1-40}$ ELISA. One-way ANOVA: $p<0.01$, posthoc test: $^*p<0.05$, $^{**}p<0.01$ vs. untreated APPsw control. All values represent the means ± SEM from 4-7 experiments.

4 DISCUSSION

4 Discussion

4.1 APP over-expression, intracellular Aβ and APP localization, Aβ secretion and secretase activity

4.1.1 Aβ secretion

Extracellular addition of Aβ to cultured neurons or injection into the brain causes neurotoxic effects (Yankner, B. A., Dawes, L. R. et al., 1989; Geula, C., Wu, C. K. et al., 1998). However, it is difficult to discern cause-effect relationships from such data, especially in view of the nonspecific actions of the applied high-dosage Aβ on cells or isolated mitochondria (Pereira, C., Santos, M. S. et al., 1998; Canevari, L., Clark, J. B. et al., 1999; Casley, C. S., Canevari, L. et al., 2002b). Concerning experiments on isolated mitochondria, one should not leave out of sight that interactions with other organelles, which might be substantial for the investigated processes, are lacking (cross talk of mitochondria and ER, e.g.). Hypothesizing that at low levels, Aβ would seek out specific cellular targets such as the Receptor for Advanced Glycation Endproducts (RAGE) as a multiligand receptor (Yan, S. D., Chen, X. et al., 1996b; Schmidt, A. M., Hori, O. et al., 1996; Yan, S. D., Chen, X. et al., 1996a), these specific interactions would not be detectable in isolated mitochondria.

Therefore, we used transfected cell lines expressing APPwt and APPsw as a model of investigation, offering the advantage to study chronic, dose-dependent effects of APP and Aβ from the picomolar to the nanomolar range. These cell lines, first characterized by their $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion levels, are a valuable tool to study AD-related alterations reflecting different stages of the disease. PC12 cells secrete low Aβ levels reflecting the physiological situation in brains of patients suffering from sporadic AD (Scheuner, D., Eckman, C. et al., 1996; Selkoe, D. J., 2002). In contrast to this, APPsw HEK cells secrete extremely high, pathological levels of Aβ which might reflect the situation of patients suffering from familial AD. Furthermore it has been stated by others that the transfection of APPsw in non-neuronal cells matches best with the expression of APPwt in neurons (Forman, M. S., Cook, D. G. et al., 1997). Therefore, we additionally characterized SY5Y cells stably expressing APPwt as a model of reference. Since it is postulated that Aβ can be re-internalized from the

extracellular space into the cell body, it was surprising to find a strong variation in secreted Aβ levels between the cell lines. Re-internalization of Aβ might be partly dependent on the amounts secreted and might thus cause intracellular alterations in protein expression and energy metabolism (for further discussion, see 4.1.2). Consequently, the cell lines allow us to study cellular alterations linked to the presence of secreted Aβ. Considering the fact that the biggest part of the AD patients are suffering from the sporadic form, most experiments were performed with this cell model.

4.1.2 Intracellular Aβ

Most of the gene mutations that segregate with the inherited forms of AD result in increasing the ratio of $A\beta_{1-42}$/ $A\beta_{1-40}$ production (Suzuki, N., Cheung, T. T. et al., 1994). $A\beta_{1-42}$ also accumulates in neurons of AD patients (Wirths, O., Multhaup, G. et al., 2004). First evidence for intracellular Aβ was reported by Wertkin et al. who observed intracellular Aβ in a differentiated neuronal cell line (Wertkin, A. M., Turner, R. S. et al., 1993). A more detailed analysis of NT2N neurons showed substantial amounts of $A\beta_{1-40}$ and $A\beta_{1-42}$ at a ratio of 3 : 1 in cell lysates, whereas analysis of secreted forms yielded a ratio of 20 : 1 for $A\beta_{1-40}$: $A\beta_{1-42}$ (Turner, R. S., Suzuki, N. et al., 1996).

Intraneuronal Aβ deposition in the hippocampus was described in transgenic mice expressing APP with the Swedish mutation (Shie, F. S., LeBoeur, R. C. et al., 2003). Takahashi et al. used immunoelectron microscopy to determine the subcellular localization of Aβ. They could show that intraneuronal $A\beta_{1-42}$ in APP-transgenic mice accumulated predominantly in multivesicular bodies (MVBs) within presynaptic and postsynaptic compartments, as well as in human AD brains. This accumulation was associated with an altered synaptic morphology, which preceded extracellular amyloid plaque deposition (Takahashi, R. H., Nam, E. E. et al., 2002). In accordance with these findings it has been previously shown that intraneuronal Aβ accumulation precedes plaque formation in transgenic mice expressing mutant APP695 with the Swedish, Dutch and London mutations in combination with mutant presenilin-1. These mice displayed abundant intraneuronal Aβ immunoreactivity in hippocampal and cortical pyramidal neurons (Wirths, O., Multhaup, G. et al., 2001). This was even more pronounced in a transgenic mouse model expressing the Swedish and London

mutant APP751 together with mutant PS-1 (Blanchard, V., Moussaoui, S. et al., 2003). The intraneuronal immunoreactivity declined with increased plaque accumulation (Wirths, O., Bayer, T. A. et al., 2002). Moreover, the neuronal loss did not correlate with the amount of extracellularly deposited Aβ, suggesting that high levels of intraneuronal Aβ are linked to the neurotoxic effects (Schmitz, C., Rutten, B. P. F. et al., 2004).

A recent report described a new triple-transgenic mouse model expressing mutant APP in combination with mutant PS-1 and mutant tau-protein. These mice displayed early synaptic dysfunction before plaque or tangle deposition was evident, together with early intraneuronal Aβ immunoreactivity preceding plaque deposition. Interestingly, tau and Aβ immunoreactivity colocalized in hippocampal neurons, implying that early intraneuronal Aβ accumulation affects tau pathology (Oddo, S., Caccamo, A. et al., 2003a; Oddo, S., Caccamo, A. et al., 2003b).

Aβ peptides are generated at different subcellular sites. Whereas $A\beta_{1-40}$ is generated solely in the trans-Golgi network (TGN), $A\beta_{1-42}$ is generated in the endoplasmic reticulum (ER) as well as Golgi compartments (Hartmann, T., Bieger, S. C. et al., 1997; Xu, H. X., Sweeney, D. et al., 1997; Greenfield, J. P., Tsai, J. et al., 1999).

This has been shown by the retention of APP in the endoplasmic reticulum/intermediate compartment (ER/IC), which leads to an elimination of intracellular $A\beta_{1-40}$, whereas the synthesis of intracellular $A\beta_{1-42}$ was not affected (Cook, D. G., Forman, M. S. et al., 1997).

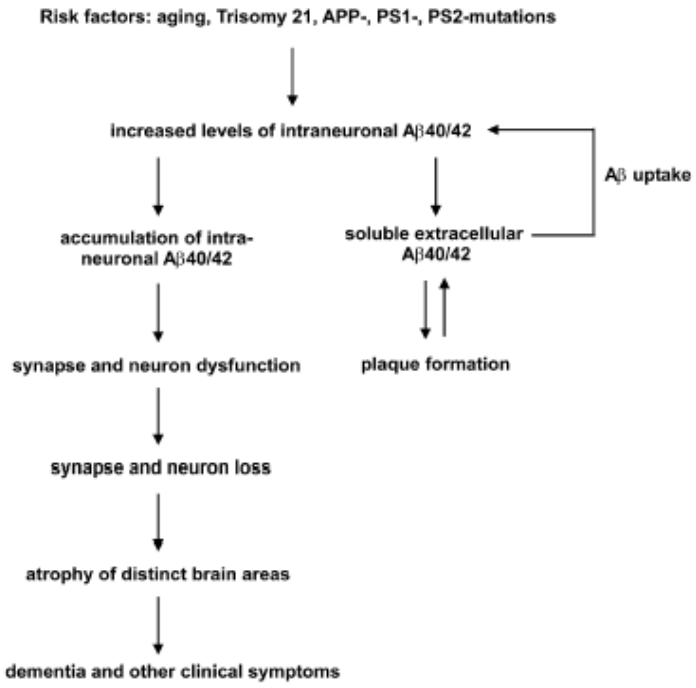

Figure 4.1: The modified β-amyoid cascade.

Scheme showing the current β-amyloid hypothesis, which is based on biochemical, neuropathological, molecular-biological and genetic evidence (taken from Wirths, 2004).

Altogether, these data strongly suggest that the neuronal production of $A\beta_{1-42}$ is a critical event in AD, and it has been speculated that intraneuronal $A\beta_{1-42}$ is toxic, but the toxicity has not been demonstrated. Kienlen-Campard et al. report that the long term expression of human APP in rat cortical neurons induces apoptosis. They observed that although APP processing leads to production of extracellular $A\beta_{1-40}$ and soluble APP, these extracellular derivatives did not induce neuronal death. On the contrary, neurons underwent apoptosis as soon as they accumulated intracellular $A\beta_{1-42}$ following the expression of full-length APP or a C-terminal deleted APP isoform (Kienlen-Campard, P., Miolet, S. et al., 2002). Therefore they postulate that the accumulation of intraneuronal $A\beta_{1-42}$ is the key event in the neurodegenerative process. In order to investigate the role of intracellular Aβ forms, we determined intracellular soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in HEK cells. Generally, we found that intracellular Aβ levels were extremely low in HEK cells, barely not detectable by the Aβ ELISA kits used in our group (Fig. 3.4, 3.5). This fact should not be left out of

sight during the interpretation of the following data. We observed that the amounts of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were in the same range for all HEK clones (APPwt, APPsw, control), which could indicate a shift in the $A\beta_{1-42}$: $A\beta_{1-40}$ ratio in favour of $A\beta_{1-42}$ for intracellular, soluble Aβ. Intracellular, insoluble $A\beta_{1-40}$ was detectable in APPwt, APPsw and control HEK cells, whereas insoluble $A\beta_{1-42}$ was only detectable in APPsw HEK cells. Thus, insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ was elevated in comparison to soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPsw HEK cells. Here, the $A\beta_{1-42}$: $A\beta_{1-40}$ ratio was clearly shifted in favour of $A\beta_{1-42}$, which lead us to the conclusion that $A\beta_{1-42}$ partly accumulates intracellularly and forms insoluble aggegrates. Furthermore, we may deduct that accumulation of intracellular, insoluble $A\beta_{1-42}$ is a pathogenic event, as it only occurs in APPsw HEK cells. Under baseline conditions, only APPsw HEK cells show decreased MTT reduction potential and decreased ATP levels (Keil, U., Bonert, A. et al., 2004; Bonert, A., Marques, C. et al., 2004; Marques, C. A., Keil, U. et al., 2003) indicating that the the APPsw mutation, eventually via accumulation of insoluble intracellular $A\beta_{1-42}$, leads to disturbances in cellular energy metabolism resulting in cell death. This would be in accordance with previously described findings indicating that intracellular $A\beta_{1-42}$ might be the primary toxic species. Furthermore, one explanation for the low levels of soluble intracellular $A\beta_{1-40}$ and $A\beta_{1-42}$ might be that soluble forms do not accumulate intracellularly but are rapidly transported to the extracellular space, speaking for a relatively fast Aβ turnover in all HEK cells.

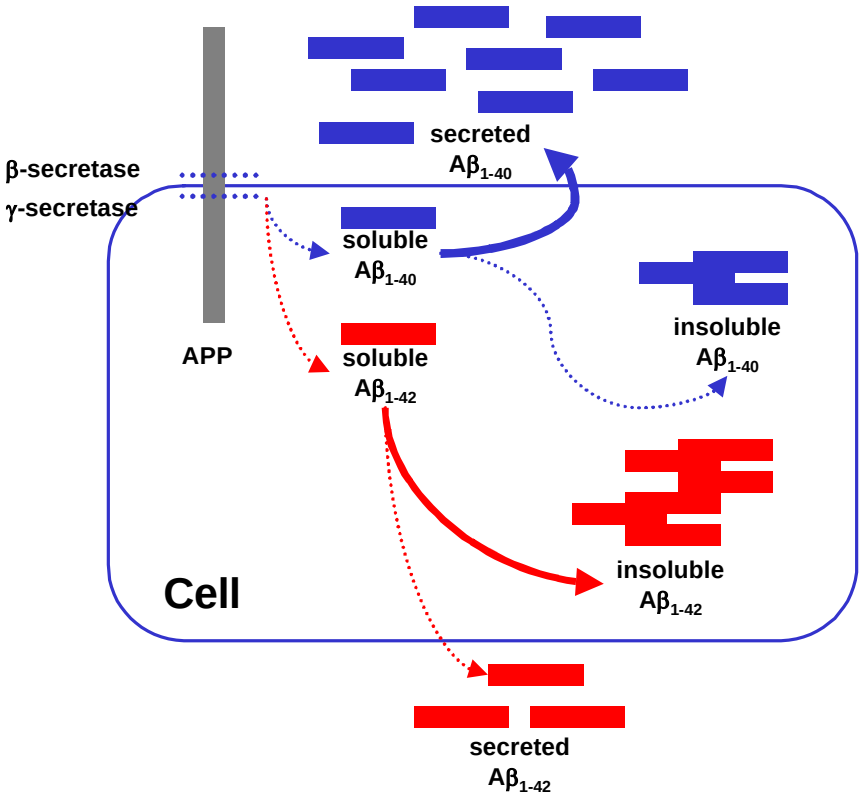

Figure 4.2: Model for the distribution of intracellular and secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPsw HEK cells. Generally, intracellular soluble $A\beta_{1-40}$ and $A\beta_{1-42}$ are in the same range. In contrast to this, the $A\beta_{1-40}$/ $A\beta_{1-42}$ ratio is shifted in favour of $A\beta_{1-42}$ in APPsw HEK cells, and $A\beta_{1-40}$ is secreted to a higher extent than $A\beta_{1-42}$.

Besides intraneuronal Aβ production, cellular uptake of Aβ from the environment is a second mechanism that contributes to intracellular Aβ accumulation. Selective intracellular accumulation of $A\beta_{1-42}$ was reported in cells treated with synthetic Aβ-peptides. This internalization could be prevented under conditions that do not allow endocytosis (Knauer, M. F., Soreghan, B. et al., 1992). At least some of the internalized Aβ accumulated in insoluble fractions and was resistant to degradation for several days in cell cultures (Ida, N., Masters, C. L. et al., 1996). This accumulation and degradation resistance seemed to be specific for $A\beta_{1-42}$, as internalized $A\beta_{1-40}$ and shorter peptides were eliminated with a half-life of about 1h (Burdick, D., Kosmoski, J. et al., 1997). The subcellular localization of internalized $A\beta_{1-42}$ seemed to be the endosomal/lysosomal system, where $A\beta_{1-42}$ induced a loss of lysosomal impermeability with concomitant membrane damage (Yang, A. J., Chandswangbhuvana, D. et al., 1998; Ditaranto, K., Tekirian, T. L. et al., 2001).

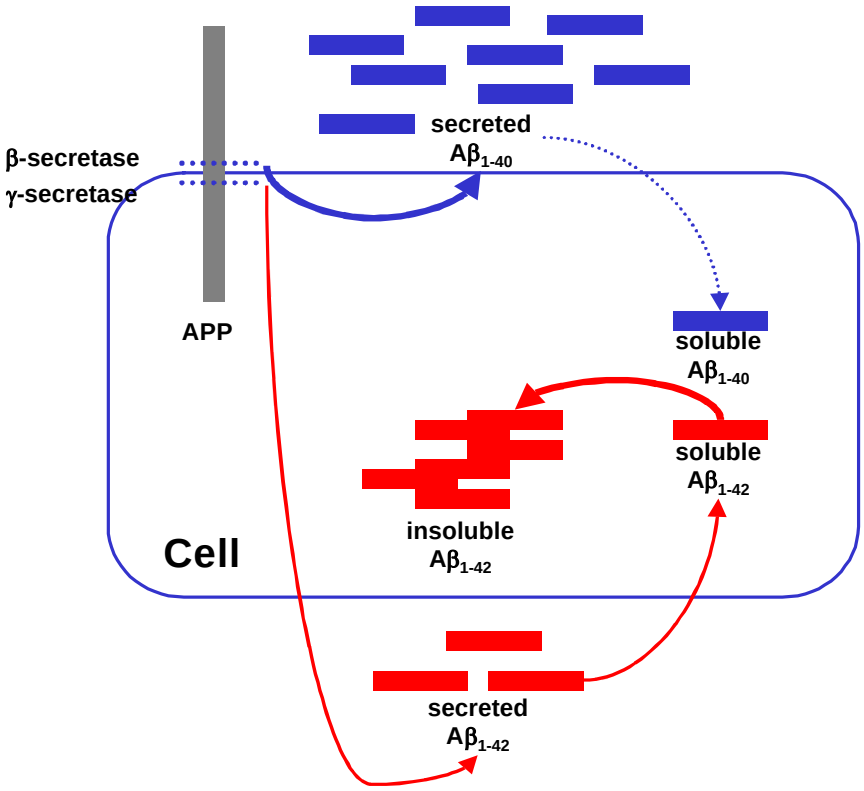

Figure 4.3: Alternative model for the distribution of intracellular and secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ in APPsw HEK cells.

4.1.3 Mitochondrial APP and Aβ

It has been reported recently that accumulation of full-length APP in the mitochondrial compartment in a transmembrane-arrested form caused mitochondrial dysfunction and impaired energy metabolism. APP is targeted to neuronal mitochondria under some physiological and pathological conditions, but its function in the mitochondrial compartment is still unknown (Anandatheerthavarada, H. K., Biswas, G. et al., 2003). Furthermore, as mitochondria may trigger the abnormal onset of neuronal cell death in Alzheimer's disease, it is noteworthy that presenilin 1 (PS-1), which is often mutated in familial forms of Alzheimer's disease, is located in mitochondria. It is hypothesized that presenilin mutations may sensitize cells to apoptotic stimuli mainly at the mitochondrial level. Also, dual targeting sequences (for endoplasmic reticulum and mitochondria) in NCT have been identified by immunoelectron microscopy. NCT, together with APH-1, PEN-2, and PS-1 form a high molecular weight complex located in mitochondria. γ-secretase activity in

isolated mitochondria was demonstrated generating an APP intracellular domain, and the activity was inhibited by γ-secretase inhibitors (Hansson, C. A., Frykman, S. et al., 2004). We could show in our own group by immunoelectron microscopy that APP is partly co-located with mitochondria (Keil, U., Bonert, A. et al., 2004). Here, we clearly demonstrated that indeed, full-length APP was present in the mitochondrial fractions of PC12 and HEK cells. Concluding, our data corroborate the finding of Anandatheerthavarada et al. that mitochondrial APP could potentially interact with transport proteins finally leading to mitochondrial dysfunction (Keil, U., Bonert, A. et al., 2004; Anandatheerthavarada, H. K., Biswas, G. et al., 2003). As mentioned above, intracellular Aβ appears to accumulate prior to the appearance of neurofibrillary tangles and senile plaques, and to be present in AD-affected areas of the brain. Furthermore, the toxic effects of intracellular Aβ on cells has been shown, in certain cases, to exceed those of extracellular Aβ (Kienlen-Campard, P., Miolet, S. et al., 2002). These data are consistent with the relevance of intracellular Aβ for disturbances of cellular function associated with AD. For this reason, we have sought possible intracellular targets of Aβ. It was demonstrated by others that Aβ is present in the mitochondria of AD brains and in brain mitochondria of transgenic mice (Lustbader, J. W., Cirilli, M. et al., 2004). The issue is extensively discussed in literature since it has been demonstrated that Aβ-binding alcohol dehydrogenase (ABAD) is a direct molecular link from Aβ to mitochondrial toxicity. These data were consistent with previous work (Yan, S. D., Roher, A. et al., 2000), demonstrating enhanced expression of ABAD in AD brain by immunoblotting with an anti-ABAD antibody. Aβ interacts with ABAD in the mitochondria of AD patients and transgenic mice. The crystal structure of Aβ-bound ABAD revealed a substantial deformation of the active site that prevents nicotinamide adenine dinucleotide (NAD) binding. Furthermore, transgenic mice overexpressing ABAD in an Aβ-rich environment manifest exaggerated neuronal oxidative stress and impaired memory.

The beneficial effects of ABAD on cellular functions under physiological conditions might be related to its sequestration of cyclophilin D in the mitochondrial matrix. By maintaining cyclophilin D in the matrix compartment, this chaperone molecule is unable to translocate to the inner mitochondrial membrane, where it could interact with other components of the MPT and, potentially, destabilize mitochondrial function. Taken together, these data are consistent with cytoprotective properties of

ABAD in health and disease. In an environment rich in Aβ, the protective properties of ABAD appear to be negated. Crystallographic studies demonstrated deformation of ABAD with loss of the NAD-binding site and a marked change in the enzyme's catalytic site. These structural changes were associated with inhibition of ABAD activity towards its diverse substrates. This leads to the hypothesis that decreased ABAD activity has negative effects on brain energetics and, possibly, other functions. For example, decreased ABAD activity might result in accumulation of toxic metabolites that could have deleterious effects. In addition, ABAD–Aβ interaction might displace cyclophilin D, resulting in its translocation to the inner mitochondrial membrane (Yan, S. D., Stern, D. M., 2005). Others have hypothesized that loss of ABAD's activity as a hydroxysteroid dehydrogenase might alter metabolism of estrogenic hormones involved in neuroprotection (He, X. Y., Merz, G. et al., 1999). These findings gave rise to the question whether full-length APP is further processed to Aβ in the mitochondrial compartment. The orientation of APP in the mitochondrial membrane leaves room to the question whether Aβ is released into the cytosolic compartment or accumulates in mitochondria. Our results obtained by ELISA provide evidence that $A\beta_{1-40}$ is present in the mitochondrial compartment of APPsw HEK cells (Fig. 3.8). Analysis of mitochondrial fractions of SY5Y and PC12 cells did not show similar results, which is not surprising since secreted $A\beta_{1-40}$ levels are significantly lower than that of HEK cells. Thus, we may deduct that for PC12 and SY5Y cells, mitochondrial Aβ is beyond the detection limit of the ELISA kit. The finding that $A\beta_{1-40}$ is found in the mitochondrial compartment of APPsw HEK cells is in accordance with the above-mentioned findings, indicating that in HEK cells, APP-processing secretases might be targeted to the mitochondria (Hansson, C. A., Frykman, S. et al., 2004). Furthermore, it has been shown that the N-terminus of APP is directed to the mitochondrial matrix, allowing secretases to correctly process APP. As a consequence, Aβ could be located in the mitochondrial matrix, potentially interacting with ABAD thus further promoting the generation of oxidative stress and failure of mitochondrial energy metabolism.

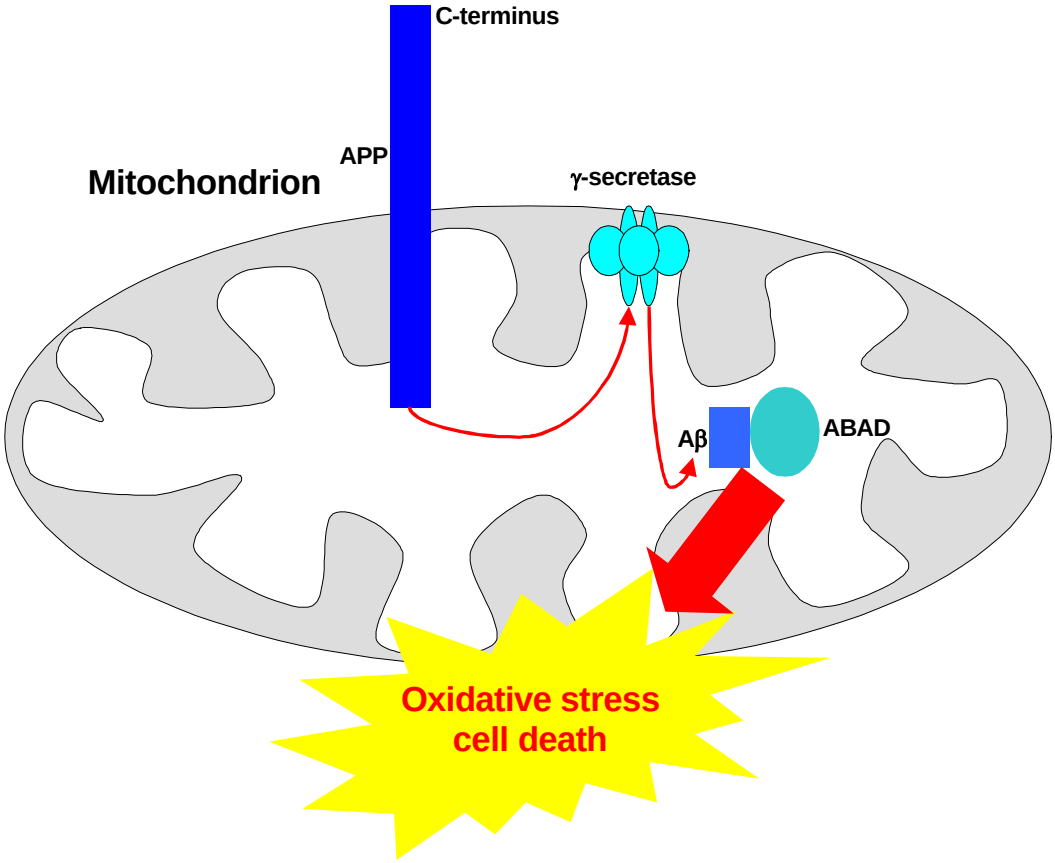

Figure 4.4: Hypothetical model for the impact of APP- and Aβ-mediated mitochondrial dysfunction.
Evidence is provided for the existence of γ-secretase in the mitochondrial fraction. Thus, mitochondrially processed Aβ might interact with ABAD contributing to enhanced levels of oxidative stress and cell death.

4.1.4 Aβ species and neurotoxicity

Genetic and pathological evidence strongly supports the amyloid cascade hypothesis of AD, which states that Aβ, a proteolytic derivative of the large transmembrane protein amyloid precursor protein (APP), has an early and vital role in all cases of AD. Aβ forms aggregates that are thought to initiate the pathogenic cascade, leading ultimately to neuronal loss and dementia (Hardy, J., Selkoe, D. J., 2002). Although Aβ is constitutively produced, for a long time it was assumed that only Aβ that is deposited in compact neuritic plaques is neurotoxic. Nevertheless, there is increasing evidence that Aβ oligomers contribute significantly to the pathological alterations underlying memory failure in AD. This oligomerization was observed to occur intracellularly (Walsh, D. M., Klyubin, I. et al., 2002; Walsh, D. M., Tseng, B. P. et al., 2000) and $A\beta_{1\text{-}42}$ oligomers turned out to be potent neurotoxins in neuronal organotypic cultures at nanomolar concentrations (Lambert, M. P., Barlow, A. K. et

al., 1998). These oligomers (also referred to as ADDLs, for Aβ-derived diffusible ligands) are further able to inhibit hippocampal long-term potentiation and disrupt synaptic plasticity (Lambert, M. P., Barlow, A. K. et al., 1998; Walsh, D. M., Klyubin, I. et al., 2002). Using antibodies against synthetic Aβ oligomers, these ADDLs have been found in brains of AD patients in concentrations up to 70fold higher than in control brains (Gong, Y., Chang, L. et al., 2003). These $A\beta_{1-42}$ oligomers were observed within abnormal processes and synaptic compartments in human AD brains (Takahashi, R. H., Almeida, C. G. et al., 2004). Dimeric Aβ peptides further rapidly accumulate in lipid rafts and were detected as sodium dodecyl sulfate soluble forms in brain tissue of APP transgenic mice. This begins as early as memory impairments become obvious, providing a further link to the assumption that Aβ oligomers are tightly linked to memory dysfunction in AD (Kawarabayashi, T., Shoji, M. et al., 2004). Investigating the formation of Aβ dimers and oligomers, an important observation was that APP-APP interactions can occur via the carbohydrate domain of the amyloid precursor protein. The physiological significance of APP-APP interactions remain to confirmed, since it is not understood whether and how APP dimerization occurs in vivo (Scheuermann, S., Hambsch, B. et al., 2001). It was additionally found that cellular APP exists as homodimers matching best with a two-site model. The generation of stabilized dimers by expressing mutant APP with a single cysteine in the ectodomain juxtamembrane region and the mutation of Lys624 to cysteine produced similar to 6-8fold more Aβ than cells expressing normal APP. These results suggest that Aβ production can in principle be positively regulated by dimerization in vivo and that dimerization could be a physiologically important mechanism for regulating the proposed signal activity of APP (Scheuermann, S., Hambsch, B. et al., 2001). Moreover, biochemical data implicate that Aβ dimers can easily aggregate into fibrils and may have originated from APP-homodimers by proteolytic processing (Schmechel, A., Zentgraf, H. et al., 2003). The presence of Aβ dimers in the cortex of patients with AD has been suggested to initiate the accumulation of Aβ in the human brain (Enya, M., Morishima-Kawashima, M. et al., 1999). In APP transgenic mouse brain two forms of sodium dodecyl sulfate-stable Aβ homodimers exist, species ending at $A\beta_{1-40}$ and $A\beta_{1-42}$. Spectroscopic analyses showed that engineered dimeric peptides ending at residue 42 displayed a much more pronounced β-structural transition than corresponding monomers (Schmechel,

A., Zentgraf, H. et al., 2003). Nonfibrillar sodium dodecyl sulfate-stable dimers have been characterized as neurotoxic species (Lambert, M. P., Barlow, A. K. et al., 1998) and selectively blocked hippocampal long-term potentiation in the absence of monomers, protofibrils or fibrils (Walsh, D. M., Klyubin, I. et al., 2002).

A very interesting model was proposed by Kayed et al., assuming that soluble oligomers are common to most amyloids and may represent the primary toxic species of amyloids. They could show that all of the soluble oligomers tested (insulin, α-synuclein, prion 106-126) display a common conformation-dependent structure that is unique to soluble oligomers regardless of sequence. The in vitro toxicity of soluble oligomers is inhibited by an oligomer-specific antibody. In human AD brain, soluble oligomers have a unique distribution that is distinct from fibrillar amyloid. Their results indicate that different types of soluble amyloid oligomers have a common structure and suggest they share a common mechanism of toxicity (Kayed, R., Head, E. et al., 2003).

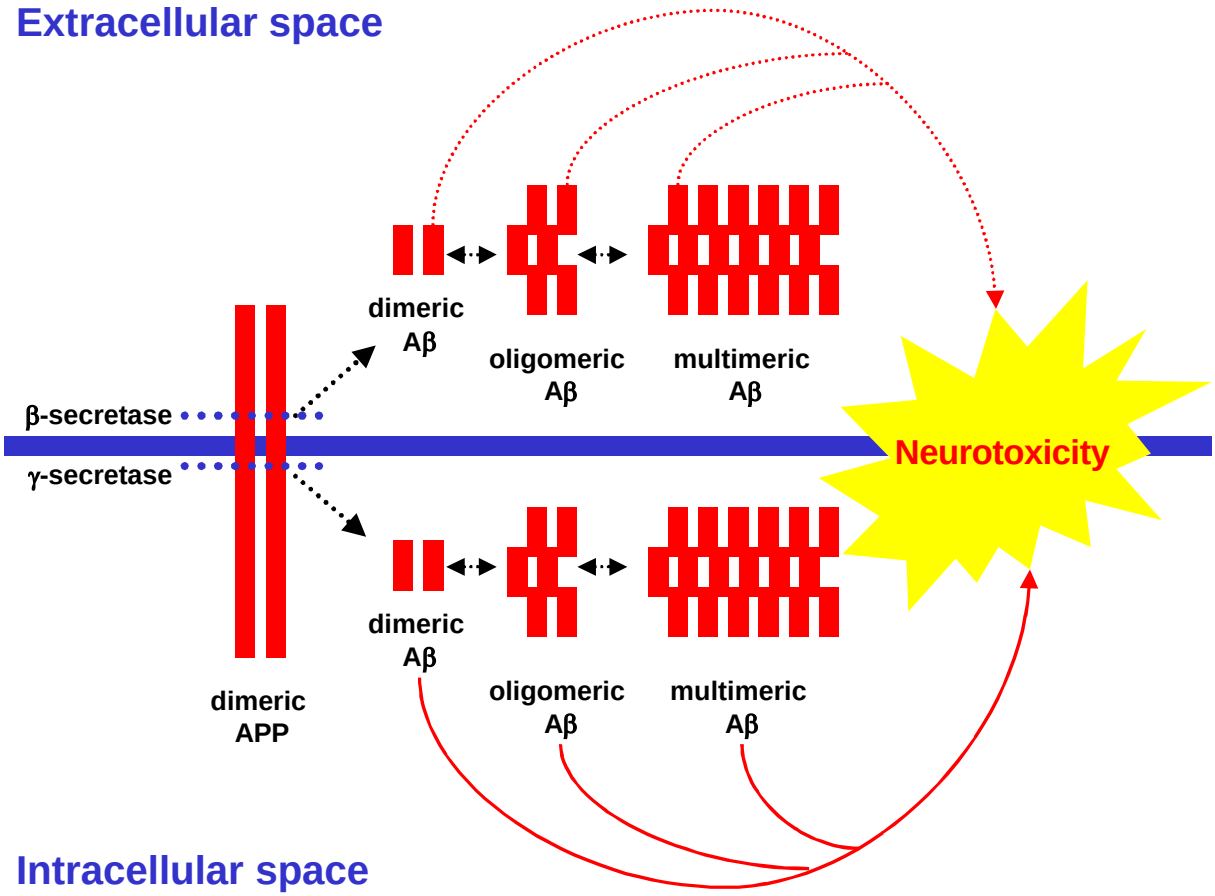

Figure 4.5: Model of Aβ-mediated neurotoxicity.

It has been found by several groups that dimeric as well as oligomeric Aβ isoforms exhibit highly neurotoxic effects. Furthermore, it has been proposed that dimers and oligomers exhibit stronger neurotoxic properties than multimeric/fibrillar Aβ. But still it is unknown, how dimeric, oligomeric or multimeric Aβ isoforms exert their neurotoxic effects.

Our data are in accordance with these previous findings since we could demonstrate that treatment with monomeric $A\beta_{1-42}$ did not lead to a decrease in MTT reduction potential, which implicates that the monomeric form does not exhibit neurotoxic effects (Fig. 3.13). In contrast to this, treatment with dimeric $A\beta_{1-42}$ resulted in a significant decrease in MTT reduction potential in both PC12 and SY5Y cells (Fig. 3.15). Furthermore, the neurotoxic effect of dimeric $A\beta_{1-42}$ was comparable to the neurotoxic effect achieved by the treatment with fibrillar $A\beta_{1-42}$ (Fig. 3.12). Most interestingly, treatment with dimeric $A\beta_{1-40}$ did only lead to a slight, but significant decrease in MTT reduction potential at 100 nM in SY5Y cells (Fig. 3.14). Again, this finding is in accordance with previous data since it has been found that not only monomeric, but also dimeric $A\beta_{1-42}$ exhibits a higher neurotoxicity than monomeric or dimeric $A\beta_{1-40}$ does. At least, in our model, dimeric $A\beta_{1-42}$ and multimeric/fibrillar $A\beta_{1-42}$ isoforms exhibit both the highest neurotoxic effects. Of note, we did not further characterize the critical components of multimeric/fibrillar $A\beta_{1-42}$, as this isoform was generated from commercially available $A\beta_{1-42}$. Thus, we can not definitely exclude that here, dimeric $A\beta_{1-42}$ is one of the major components. Moreover, we assessed the MTT reduction potential and ATP levels of APP and Aβ dimer producing SY5Y cells (SY5Y K623C) under baseline conditions and under H_2O_2-mediated oxidative stress (Fig. 3.16, 3.17). These cells represent a valuable model for the investigation of the neurotoxic effects mediated by dimeric APP and dimeric Aβ in comparison to control SY5Y cells. Under baseline conditions, we observed a decrease in MTT reduction potential as well a decreased ATP levels in SY5Y cells bearing the APP dimer mutation. This is in accordance with previous findings since it has been reported that APP dimer formation leads to a six- to eightfold increased Aβ dimer production (Scheuermann, S., Hambsch, B. et al., 2001). Increased Aβ dimer production thus decreases cellular viablility under baseline conditions as monitored by MTT reduction and a drop-down of ATP levels. Exposure to oxidative stress lead to a drastic decrease in MTT reduction potential and ATP levels in both APP dimer and control cells. Finally, we may conclude that not only extracellular, but also intracellular dimeric Aβ isoforms are neurotoxic. Our data further indicate that most probably both, intracellular and extracellular dimeric Aβ is involved in neurotoxicity: First, intracellular Aβ dimers lead to disturbances in mitochondrial function and cellular energy

metablolism, rendering the cell more vulnerable to oxidative stress. In the next step, secreted dimeric Aβ or extracellular Aβ in general might lead to the generation of ROS itself or enhance oxidative-stress-mediated neurotoxicity.

4.2 APP processing, secretase inhibition and cellular viability

4.2.1 APP overexpression and secretase activity

APPsw cells (PC12, HEK) show a significant increase in β-secretase activity compared to control cells, which is in accordance with previous findings indicating that the APPsw mutation leads to an increase in β-secretase activity in cell culture (Haass, C., Lemere, C. A. et al., 1995b), a phenomenon which is probably also true for the situation in AD brain (Shen, Y., Yang, L. B. et al., 2002; Li, R., Lindholm, K. et al., 2004d; Holsinger, R. M. D., Mclean, C. A. et al., 2002). Of note, this finding underlines the important role of APPsw-transfected neuronal and non-neuronal cells as a valuable model for the investigation of β-secretase-mediated changes in AD pathology. In contrast to this, it was a very intriguing finding that γ-secretase activity was significantly elevated in APPsw-transfected PC12 cells compared to control cells, while in HEK cells, γ-secretase activity was at equal levels in APPsw and control cells (Fig. 3.10, 3.11). Thus, we conclude that a mutation-dependent increase in γ-secretase activity is an effect specifically observed in neuronal cell lines (PC12). In our experimental setting, we did not observe any significant differences in baseline values without the addition of β- and γ-secretase-specific substrates, leading to the conclusion that these baseline values without substrate do not have an impact on γ-secretase activity of PC12 and HEK cells, and that differences in secretase activity definitely result from the addition of secretase-specific substrates.

Regarding the Aβ secretion of APPsw PC12 and HEK cells, it seems that increased β-secretase, but not γ-secretase activity is responsible for the increased Aβ production and secretion. On the other hand, we may deduct that γ-secretase activity in control HEK cells under baseline conditions is at maximum level, due to the higher APP expression levels compared to PC12 cells. A further increase in the original substrate as monitored by APPsw-expression in HEK cells does not necessarily result in an increased γ-secretase activity.

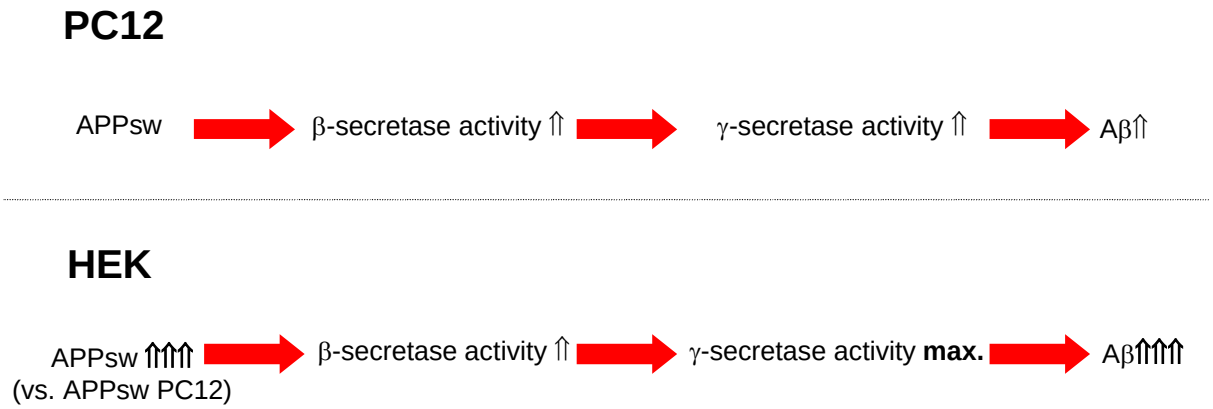

Figure 4.6: Proposed comparative model for β- and γ-secretase activity in APPsw PC12 and HEK cells.
In both cell models, the APPsw mutation leads to a significant increase in β-secretase activity. In APPsw PC12 cells, the APPsw mutation additionally leads to a significant increase in γ-secretase activity. This effect was not observed in APPsw HEK cells, since γ-secretase activity is maximum in control HEK cells.

4.3 AD-relevant mitochondrial alterations

There have been many links between mitochondrial function/dysfunction and Alzheimer's disease (AD) over the years (Arispe, N., Demazancourt, P. et al., 1994). Such a connection is rational, because meeting neuronal requirements for metabolic (especially energetic) homeostasis is a prerequisite for cognitive function. Defects in glucose utilization suggest possible abnormalities in mitochondrial function in AD. In support of this possibility, a range of reports have shown altered mitochondrial properties in AD. For example, decreased activity of complex IV (cytochrome c oxidase or COX) has been observed in the central nervous system, and even in peripheral tissues, such as platelets, of patients with AD (Kish, S. J., Bergeron, C. et al., 1992; Mutisya, E. M., Bowling, A. C. et al., 1994; Parker, W. D., Parks, J. et al., 1994; Maurer, I., Zierz, S. et al., 2000; Bosetti, F., Brizzi, F. et al., 2002). The latter is the most consistently noted mitochondrial abnormality in AD tissue. Indeed, studies with cybrids, in which mitochondria from patient tissue/cell samples (platelets) are transferred to a cell line depleted of mitochondrial DNA (thus, the only mitochondrial DNA is from the patient sample), have also demonstrated decreased COX activity (Khan, S. M., Cassarino, D. S. et al., 2000). These data are consistent with previous

findings of our own group indicating that PC12 cells bearing the Swedish double mutation suffer from reduced cytochrome c oxidase activity (Keil, U., Bonert, A. et al., 2004). In addition, others have shown a decrease in the area of intact mitochondria in AD brain, vs. control, and evidence of mitochondrial morphologic abnormalities associated with oxidative modification of guanosine (8-hydroxyguanosine) and increased levels of nitrotyrosine (Castellani, R., Hirai, K. et al., 2002). Still, studies in cell culture demonstrating a role for proapoptotic Bax in Aβ-induced apoptosis, mediated via Bax-induced increased permeability of the outer mitochondrial membrane, caspase activation and cell death (Giovanni, A., Keramaris, E. et al., 2000; Zhang, Y., McLaughlin, R. et al., 2002a), indicate that mitochondria remain important suspects as pathogenic contributors to Aβ-mediated cellular dysfunction. The counterpart of these studies on patient samples/intact cells are experiments using isolated mitochondria exposed to Aβ. For example, rat brain mitochondria incubated with Aβ showed decreased state 3 and 4 mitochondrial respiration (Casley, C. S., Canevari, L. et al., 2002a). COX-4, β-ketoglutatrate dehydrogenase and pyruvate dehydrogenase activities were also diminished in mitochondria incubated with Aβ (Casley, C. S., Canevari, L. et al., 2002a). In another study, liver mitochondria exposed to Aβ displayed a drop in membrane potential and increased susceptibility to matrix swelling, though brain mitochondria, by comparison, were less vulnerable to the effects of Aβ (Moreira, P. I., Santos, M. S. et al., 2002). As might be expected from studies using intact cells, in which Aβ can trigger apoptotic cascades (Loo, D. T., Copani, A. et al., 1993; LaFerla, F. M., Tinkle, B. T. et al., 1995; Mattson, M. P., Partin, J. et al., 1998), incubation of Aβ with brain mitochondria has been shown to cause cytochrome c release, as well as mitochondrial swelling (Kim, H. S., Lee, J. H. et al., 2002). The relevance of these results, from experiments in which isolated mitochondria were exposed to micromolar levels of synthetic Aβ peptides, to the in vivo situation is yet to be determined. Taken together, these studies indicate the likelihood that there is an association of mitochondrial dysfunction with AD.

4.3.1 Bcl-2 family proteins

Increasing evidence suggests that many neurons may undergo apoptosis in AD as high levels of activated pro-apoptotic proteins such as caspase-3 and Bax are present in particularly affected neurons (Mattson, M. P., Partin, J. et al., 1998). DNA damage and up-regulation of the proapoptotic proteins p53 and Bax occur in vulnerable neuronal populations at a relatively early stage in the disease process. Familial AD (FAD) mutations in presenilins render neurons vulnerable to apoptosis induced by Aβ, trophic factor deprivation and other stimuli (Mattson, M. P., Partin, J. et al., 1998), consistent with an apoptotic mode of neuronal death in patients with these presenilin mutations. APP mutations are also sufficient to trigger apoptosis in cultured cells (McPhie, D. L., Coopersmith, R. et al., 2003; Eckert, A., Keil, U. et al., 2003; Marques, C. A., Keil, U. et al., 2003). In our experiments, expression levels of mitochondrial pro-apoptotic factors differed markedly between PC12, HEK and SY5Y cells (Fig. 3.18, 3.19, 3.20). The most intriguing finding was that PC12 cells did not express the endogenous anti-apoptotic protein Bcl-2 (Fig. 3.18). This finding was corroborated by additional data of our group, indicating that Bcl-2 mRNA levels were not detectable in PC12 cells, but this phenomenon has also been described by others (Maroto, R., Perez-Polo, J. R., 1997). On the other hand, Bcl-xL is expressed in PC12 cells, and its intracellular distribution reveals that levels of cytosolic Bcl-xL are strongly reduced in APPwt and APPsw PC12 cells (Fig. 3.18, 3.28). This finding might be of in-vivo relevance since we observed a significant down-regulation of Bcl-xL levels without alterations of Bcl-2 in APPsw transgenic mice (Fig. 3.21, 3.24). We might resume that Bcl-xL, as another anti-apoptotic player in the cell death scenario might exert similar protective functions as Bcl-2 under certain circumstances. In contrast to the findings in PC12 cells, HEK and SY5Y cells, both of human origin, reveal detectable expression levels of Bcl-2 (Fig. 3.19, 3.20). Of note, Bcl-2 expression levels were reduced in HEK cells bearing the Swedish double mutation as well as in SY5Y cells stably transfected with APPwt, leading to reduced Bcl-2/Bax ratios (Fig. 3.23). It has been found that for many but not all apoptotic signals, the balance between these competing activities determines cell fate (Adams, J. M., Cory, S., 1998). Most interestingly, APPwt SY5Y cells exhibit significantly reduced Bcl-xL/Bax ratios (Fig. 3.23), indicating that reduced Bcl-xL levels are a characteristic

feature of neuronal cells of over-expressing APP, as similar results were found in Thy-1 APP transgenic mice (Fig. 3.24). Concluding, we can say that a shift of Bcl-2/Bax or Bcl-xL/Bax ratios in favour of Bax represents a characteristic hallmark of cells suffering from the over-expression of APP. Concluding, we may state that our data corroborate the involvement of Bcl-2 family proteins in Aβ-mediated apoptosis. From a more general point of view, the role of Bcl-2 family proteins in AD-associated neurotoxicity has been confirmed by former findings showing that Bcl-2 directly interacts with presenilin 1 (Alberici, A., Moratto, D. et al., 1999) and that presenilin 2 and its mutations regulate the expression of Bcl-2 family proteins (Alves, d. C., Paitel, E. et al., 2002). Moreover, several groups report that transfection with the anti-apoptotic Bcl-2 gene rescued PC12 cells from apoptotic death induced by Aβ, and they suggest that NF-κB plays a role in Bcl-2-mediated protection against Aβ-induced apoptosis through augmentation of cellular antioxidant capacity (Jang, J. H., Surh, Y. J., 2004). Tan et al. showed that Bcl-xL significantly inhibits both early-stage apoptosis and late-stage apoptosis/necrosis caused by Aβ treatment, suggesting that Bcl-xL exhibits both anti-necrotic as well as anti-apoptotic roles in Aβ-mediated cell death (Tan, J., Town, T. et al., 1999). Other groups could clearly demonstrate that intracellular $A\beta_{1-42}$ is selectively cytotoxic to human neurons through the p53-Bax cell death pathway (Zhang, Y., McLaughlin, R. et al., 2002b). Analysis of the anti-apoptotic factor Bcl-xL and the pro-apoptotic factor Bax in cytosolic and mitochondrial fractions of PC12 cells under baseline conditions and after oxidative injury induced by H_2O_2 lead to the following conclusions: Reduced cytosolic Bcl-xL levels in APPwt and APPsw PC12 cells compared to control cells as well as a shift in the Bcl-xL/Bax ratio towards Bax under baseline conditions might be involved in the higher sensitivity of APPwt and APPsw cells to mitochondrial failure. During oxidative injury, cytosolic Bcl-xL levels remained unaltered indicating that dyshomeostasis in Bcl-xL/Bax ratio was not compensated by an increase cytosolic Bcl-xL levels (Fig. 3.28). Of note, there was no alteration of baseline mitochondrial levels of monomeric, dimeric and heteromeric Bcl-xL in PC12 clones which remained unchanged during oxidative stress leading to the conclusion that mitochondrial Bcl-xL did not counteract apoptosis induction (Fig. 3.29). Furthermore, we did not observe a baseline up-regulation of Bax nor did we observe alterations in cytosolic and mitochondrial Bax levels of APPwt and APPsw compared to control cells (Fig. 3.30). The temporary general increase in cytosolic Bax levels in all clones, namely dimeric Bax (Fig. 3.31),

might pave the way for the translocation of Bax into the outer mitochondrial membrane leading to the release of pro-apoptotic mitochondrial factors into the cytosol by PTP opening or by Bax pore formation. We finally conclude that moderate, chronic APP over-expression and exposure to Aβ results in a shift of Bcl-xL/Bax ratio in favour of Bax thus increasing cellular vulnerability, but there was no evidence for a protective action of Bcl-xL during oxidative injury. It was rather difficult to compare our findings with that of other groups as most workers in the field investigate the effects of extracellular Aβ on apoptosis in cells over-expressing Bcl-2, Bcl-xL or Bax. Naturally, it was found that Bcl-2 over-expression protects against secondary injury and that Bax-over-expression results in increased apoptosis rate, but the in-vivo relevance has to be questioned as these models are not close to physiological conditions. In contrast, PC12 cells as a model of moderate APP over-expression and Aβ secretion levels in the physiological range could mimic the physiological situation in an early stage of the disease, as reduced cytosolic Bcl-xL levels precede a reduction in Bcl-xL expression levels observed in cells massively over-expressing APP.

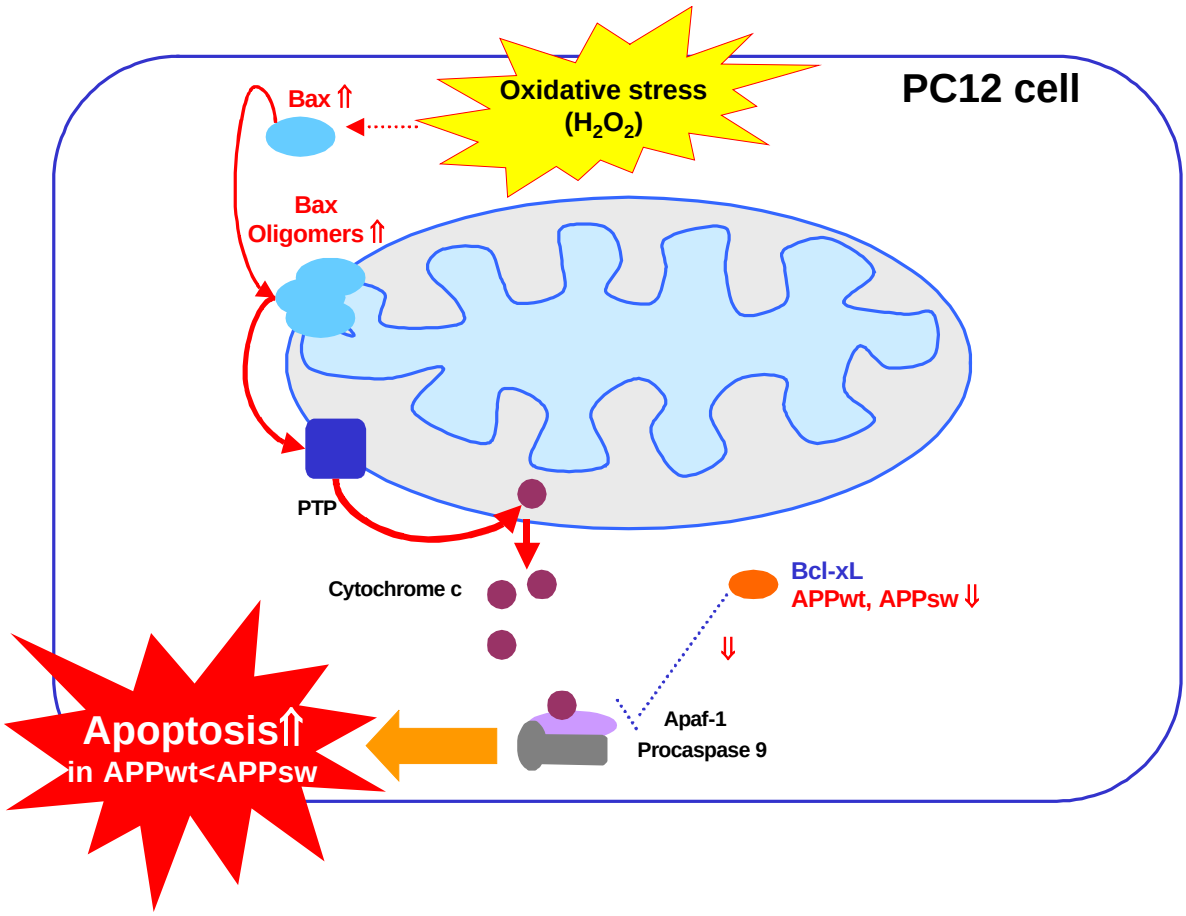

Figure 4.4: A hypothetical role for Bcl-2 protein family members in oxidative stress-induced apoptosis in PC12 cells.

Increased cytososlic Bax oligomerization is implicated in apoptosis-induction in PC12 cells. Since Bcl-2 is not expressed in PC12 cells, Bcl-xL is the only pro-survival protein to counteract cytochrome c release as a trigger of the apoptotic cascade. Reduced cytosolic levels of Bcl-xL in APPwt and APPsw PC12 cells could be implicated in their increased vulnerablilty against oxidative stress.

Furthermore, previous findings of our group describe the consequences of Aβ-induced mitochondrial dysfunction as follows: Under baseline conditions, the chronic low levels of intracellular Aβ (picomolar range) in APPsw PC12 cells lead to reduced ATP levels and reduced metabolic activity (Keil, U., Bonert, A. et al., 2004). Intriguingly, PC12 cells seem to counteract energy deficiency, since no enhanced baseline apoptosis has been observed. If this adaptation occurs through a upregulation of glycolysis needs to be determined e.g. by measuring the activation of the energy charge-sensitive AMP-activated protein kinase or the 6-phosphofructo-1-kinase, the master regulator of glycolysis. This shift in the Bcl-xL/Bax ratio towards the pro-apoptotic Bax, and the energy deficiency conduct APPsw cells to be more

vulnerable against mitochondrial membrane potential changes and ATP reduction after oxidative stress. The consequence is an activation of the intrinsic apoptotic pathway, releasing cytochrome c into the cytosol, resulting in an activation of the apoptotic protease-activating factor-1/caspase 9 apoptosome complex.

4.3.2 Pro-apoptotic mitochondrial factors

The release of pro-apoptotic factors from mitochondria into the cytosol is considered as a crucial step in oxidative stress-induced apoptosis. Indeed, cytochrome c, Smac/DIABLO and AIF are time-dependently released upon secondary injury induced by H_2O_2 treatment. Our data are consistent with previous findings reporting that cytochrome c/ Smac/DIABLO co-release precedes the translocation of AIF from mitochondria into the nucleus (Fig. 3.32, 3.34, 3.36) (Arnoult, D., Parone, P. et al., 2002). Furthermore, it is a generally accepted that Bax oligomerization and translocation into the outer mitochondrial membrane induces the release of pro-apoptotic factors (Arnoult, D., Gaume, B. et al., 2003). This finding is corroborated by our data showing an increase in cytosolic Bax monomers and dimers when the release of cytochrome c and Smac/DIABLO into the cytosol is initiated during oxidative injury (Fig. 3.30, 3.32, 3.34). Cytochrome c is released in a time-dependent pattern with a maximum release in APPwt and APPsw PC12 cells (Fig. 3.32). We speculate that APP over-expression, results in an increased cytochrome c liberation from the mitochondrion. Feedback amplification loops of pro-apoptotic factors and caspases have been described before: Caspase-3 potentiates activation of upstream caspases (-2, -6, -8) in this pathway and also participates in a feedback amplification loop involving caspase-9 (Creagh, E. M., Martin, S. J., 2001; Marzo, I., Susin, S. A. et al., 1998; Slee, E. A., Harte, M. T. et al., 1999). Here, maximum cytochrome c release appeared shortly after maximum Smac/DIABLO release potentially leading to a mutual potentiation of their pro-apoptotic effect resulting in increased or prolonged caspase-3 activation, an effect potentially being enhanced by APP over-expression (Marques, C. A., Keil, U. et al., 2003). Our experiments additionally confirmed former findings as treatment with caspase-2 and -8 inhibitors and a JNK inhibitor diminished cytochrome c release from mitochondria into the cytosol, eventually by interrupting the feedback amplification loop (Fig. 3.33). This indicates that caspase-2 and –8 as

well as JNK act upstream mitochondria, and by their inhibition, cytochrome c release as a further crucial step in the apoptotic cascade, could be prevented.

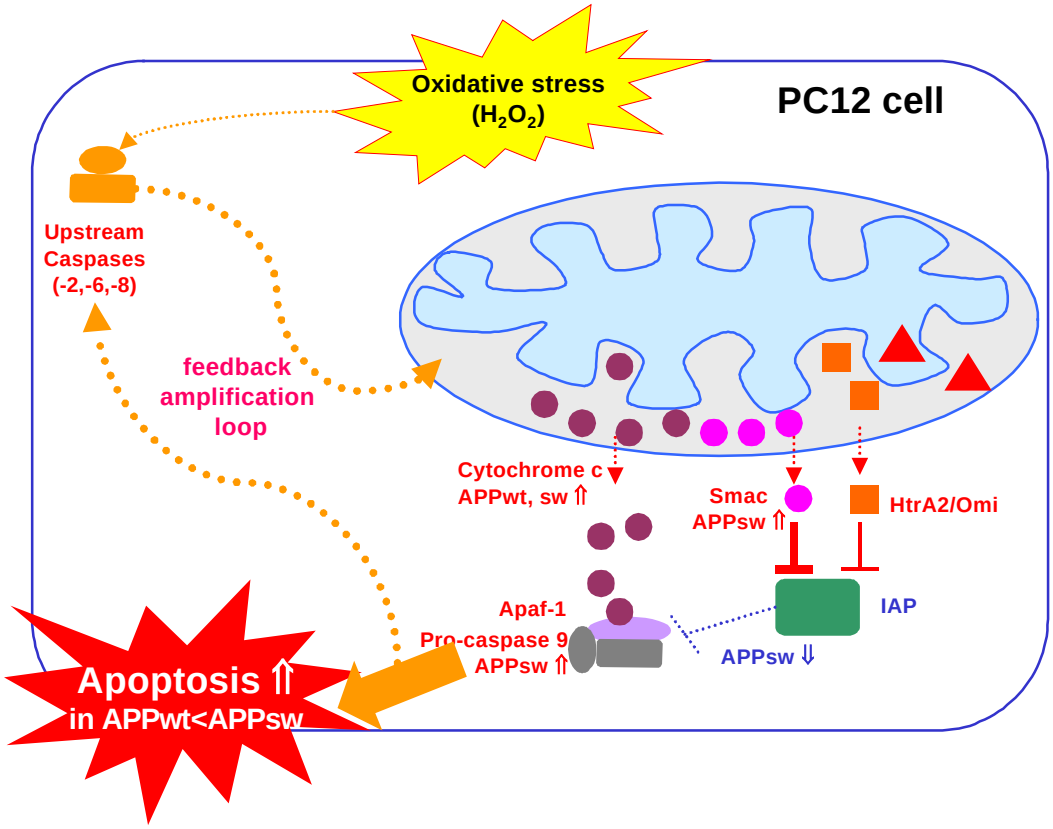

Figure 4.7: A hypothetical response to oxidative stress of Smac/DIABLO and cytochrome c and the implication of upstream caspases in the feedback amplification loop.

Increased release of Smac/DIABLO by APPsw and cytochrome c by APPwt and APPsw PC12 cells lead to an increased reponse to oxidative stress and the initiation of the apoptosis cascade. The feedback amplification loop, mediated by caspases upstream mitochondria, leads to an enhanced and prolonged apoptotic signaling.

The release of AIF independently from caspase-activation is still controversely discussed (Penninger, J. M., Kroemer, G., 2003; Cande, C., Cohen, I. et al., 2002; Cande, C., Vahsen, N. et al., 2004). Nevertheless, AIF is involved in oxidative stress-induced cell death in a two-step, time-dependent manner. Increased accumulation of AIF in the mitochondrial compartment has been observed to be part of the early events in apoptosis, similar to the relase of cytochrome c and Smac/DIABLO into the cytosol (Fig. 3.35). Of note, overexpression of the APPsw mutations results in an increased accumulation of AIF compared to APPwt and control cells. Thus, the APPsw mutation has an impact on caspase-dependent and independent apoptosis, but it is open to discussion whether these effects might be finally interrelated. Eventually, increased accumulation of AIF in the mitochondrial compartment is

dependent on other pro-apoptotic factors or on the opening of PTP, whereas its release finally is caspase-independent. The release of AIF, accompanied by the cleavage of the protein, is a late event in apoptosis (Fig. 3.36). The occurence of a cleaved fragment in AIF release has been described before (Nicholson, D. W., 2001) and this finding gave rise to the question whether AIF might be an unidentified caspase after all, since inactive pro-caspase-2 and -9 are located in the mitochondrion before induction of apoptosis and cleaved to their active form in the cytosol (Susin, S. A., Lorenzo, H. K. et al., 1999b; Susin, S. A., Daugas, E. et al., 2000; Katoh, I., Tomimori, Y. et al., 2004). Moreover, as AIF is characterized as a functional oxidoreductase its janus-faced function in the modulation of apoptosis is focus of intensive investigation and its implication in Alzheimer´s disease is yet not fully elucidated.

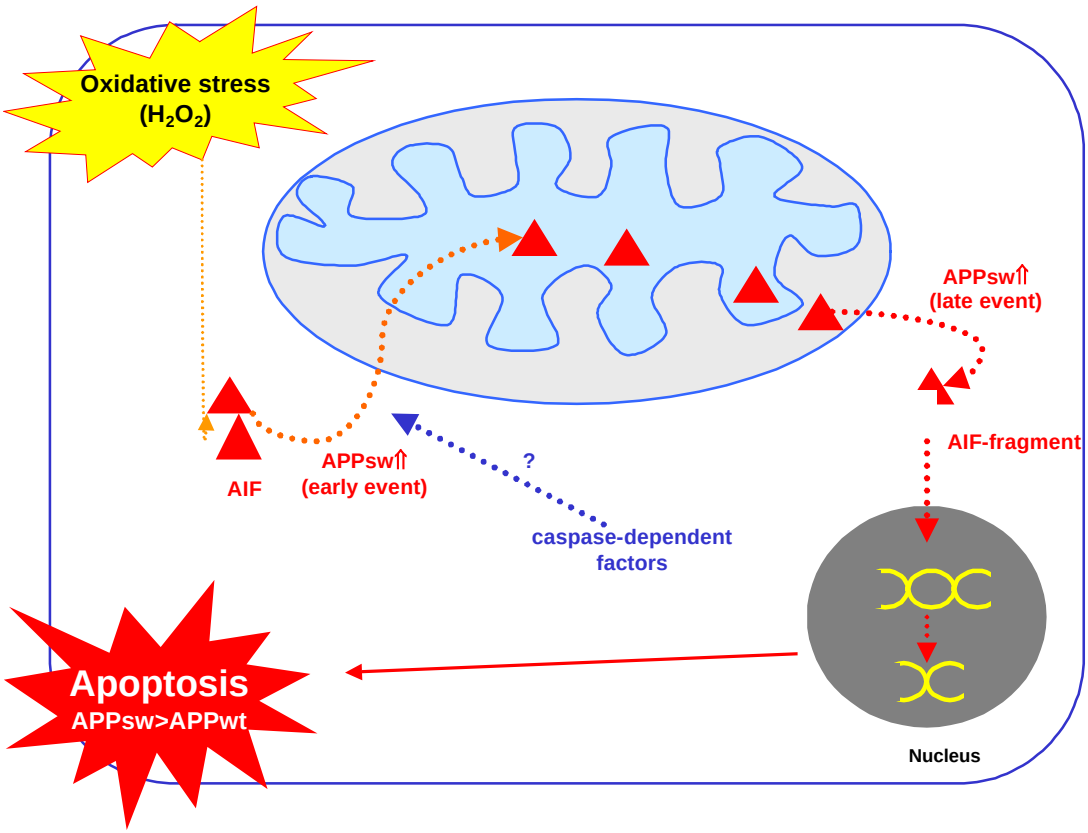

Figure 4.8: A hypothetical response to oxidative stress of the pro-apoptotic mitochondrial factor AIF. Translocation of AIF into the mitochondrial compartment is generally considered as an early event of apoptosis. Accumulation of AIF is considered to be a caspase-independent process, but further investigation is required. Upon its release, cleaved AIF translocates to the nucleus where it initiates nuclear fragmentation and chromatin condensation.

4.4 Disease-modifying strategies

4.4.1 Secretase inhibition

Most of the worldwide resources for anti-amyloid therapy have focused on the inhibition of Aβ production through secretase inhibitors. Since 1992, it has been known that cells that endogenously express β- and γ-secretase enzymes secrete significant quantities of Aβ into the culture medium when they are transfected to over-express APP (Haass, C., Schlossmacher, M. G. et al., 1992; Shoji, M., Hosokawa, M. et al., 1992).

APP over-expressing HEK cells represent a valuable tool to study both the effect of modulation of APP processing and secretase inhibition on cell survival (Vandermeeren, M., Geraerts, M. et al., 2001; Edbauer, D., Kaether, C. et al., 2004; Su, Y., Ryder, J. et al., 2004). Furthermore, it has been established recently that expression of APPsw in non-neuronal cells more closely mimics processing of APPwt in neurons (Forman, M. S., Cook, D. G. et al., 1997). Large evidence is provided in literature for the γ-secretase inhibiting effects of DAPT and L-685458 in cell culture and transgenic mouse models (Lanz, T. A., Himes, C. S. et al., 2003a; Dovey, H. F., John, V. et al., 2001). This is in accordance with our findings, in which we observed a drastic reduction of both $A\beta_{1-40}$ and $A\beta_{1-42}$ during DAPT treatment (Fig. 3.40-3.42) and a modest reduction of both Aβ species for L-685458 (Fig. 3.43). Interestingly, at low concentrations (0.2 µM), treatment with the γ-secretase inhibitor L-685458 resulted in a slight increase in secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in APPwt HEK cells. This effect has formely been described for other γ-secretase inhibitors, but the reason for that phenomenon requires further explanation (Lanz, T. A., Hosley, J. D. et al., 2004). For DAPT, we did not observe a similar effect. Testing the β-secretase inhibitor II for its Aβ-reducing activity resulted only in a modest, but significant decrease in $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion at the highest concentration (10 µM) in APPsw HEK cells (Fig. 3.47). Treatment with the β-secretase inhibitor II did not decrease secreted $A\beta_{1-40}$ levels in APPwt HEK cells, and secreted $A\beta_{1-42}$ levels rose slightly during exposure to the inhibitor. Inhibition of β-secretase by commercially available, peptidic inhibitors did not result in an increase in MTT reduction potential.

Of note, the β-secretase inhibitor II exhibited highly toxic side effects as monitored by MTT assay after 48h treatment with 5 and 10 µM (Fig. 3.46), which we assume to reflect side effects due to the lack of enzyme specificy. Our findings further emphasize the need for more potent and less toxic β-secretase inhibitors, as most of the commercially available β-secretase inhibitors are of peptidic nature and do not meet the requirements for an effective drug-like enzyme inhibition. Thus, promising research is currently undertaken to inhibit β-secretase cleavage by alternative non-peptidic structures such as ribozymes (Nawrot, B., Antoszczyk, S. et al., 2003; Nawrot, B., 2004) or small inhibitory RNA interference molecules (Kao, S. C., Krichevsky, A. M. et al., 2004; Lavery, K. S., King, T. H., 2003) specifically targeting β-secretase. L-685458 and DAPT differ in their nature of γ-secretase inhibition: L-685458 is described as a highly specific transition-state analogue of γ-secretase, directly binding to the active site of the enzyme (Li, Y. M., 2001). Originally, L-685458 has been synthesized to mimick the amino acid sequence of βAPP in proximity to the γ-secretase cleavage site. Although it has been hypothesized that the substance would exhibit a higher inhibitory potency against γ-secretase, it showed a loss of activity in enzyme inhibition (Nadin, A., Owens, A. P. et al., 2003). Testing the L-685458 inhibitor in HEK cells stably expressing βAPP and delta-Notch revealed a reduced effect on cleavage inhibition of both substrates. Furthermore, a non-competitive mechanism of enzyme inhibition has been proposed for L-685458 (Tian, G. C., Ghanekar, S. V. et al., 2003). In contrast, DAPT is described as a highly potent functional non-transition state analogue, and its site of action is yet not fully characterized (Dovey, H. F., John, V. et al., 2001; Lanz, T. A., Himes, C. S. et al., 2003b). Our data corroborate the previous findings since we observed in our model that DAPT dose- and time-dependently reduced secreted Aβ levels (Fig. 3.41, 3.42) earlier and to a higher extent than L-685458 (Fig. 3.43). Although we can not finally exclude other mechanisms, the increased potency and rapid action of Aβ secretion inhibition by DAPT (1 µM) might result in the significant reconstitution of MTT reduction potential in APPsw HEK cells after 48h treatment (Fig. 3.38). This finding is in coherence with the formerly observed significant increase in ATP levels and reconstitution of membrane potential during 48h DAPT treatment in APPsw PC12 cells (Keil, U., Bonert, A. et al., 2004). Furthermore, it has been reported that the inhibition of intracellular $A\beta_{1-42}$ formation by DAPT increases survival of rat primary

cortical neurons, an effect which has not been achieved by the treatment with other secretase inhibitors (Kienlen-Campard, P., Miolet, S. et al., 2002). Consequently, we might deduct that DAPT could inhibit intracellular Aβ formation to a higher extent than that of secreted Aβ, since it has been reported that their formation is spatially distinct (Hartmann, T., Bieger, S. C. et al., 1997; Xu, H. X., Sweeney, D. et al., 1997; Greenfield, J. P., Tsai, J. et al., 1999). Alternatively, it has been described by others in photoaffinity labeling assays that DAPT succesfully competed with a photoactivatable transition state analogue at high concentrations but could not displace the photoprobe at concentrations with which a transition state analogue can compete. This suggests that DAPT binds to the active site of γ-secretase, which is distinct but overlaps with the catalytic sites bound by the transition state analogue (Kornilova, A. Y., Wolfe, M. S., 2003). Others report differential effects of DAPT and L-685458 on Aβ production from CTFs 1-48 and 1-49 transiently expressed in HEK cells, leading to the conclusion that the γ-secretase inhibitors have distinct binding sites on presenilins (Funamoto, S., Morshima-Kawashima, M. et al., 2004). We may assume that L-685458, by specifically inhibiting presenilins in other vital functions may cause a loss in cellular viability. We monitored a significant decrease in MTT reduction potential (Fig. 3.39) after 48h treatment with concentrations significantly reducing $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion levels (Fig. 3.43). This finding might indicate that L-685458 affects other presenilin-mediated mechanisms at concentrations succesfully inhibiting γ-secretase, and that reduction of Aβ levels per se does not necessarily result in a reconstitution of cellular viability. For example, it has been reported that presenilins are implicated in the PI3K pathway thus inhibiting GSK-3 activation and tau over-phosphorylation (Baki, L., Shioi, J. et al., 2004). Others suggest that presenilins affect processing and nuclear functions of γ-protocadherins (Haas, I. G., Frank, M. et al., 2005) and contribute to calsenelin-mediated apoptosis (Jo, D. G., Chang, J. W. et al., 2003). It has been reported by some groups that APP is cleaved not only in the middle of the membrane (γ-cleavage) but also at alternative cleavage sites close to the membrane/cytoplasmic boundary (ε-cleavage), releasing APP intracellular domains (AICDs) (Gu, Y. J., Misonou, H. et al., 2001; Sastre, M., Steiner, H. et al., 2001; Weidemann, A., Eggert, S. et al., 2002). In a general assumption, ε-cleavage precedes Aβ production and ε-cleavage determines the preference for the final Aβ species. Funamoto et al. deduced from that observation

the presence of a novel enzyme being additionally involved in ε-cleavage, sensitive to L-685458, but insensitive to DAPT (Funamoto, S., Morshima-Kawashima, M. et al., 2004). Concluding, we may assume that in the last years, considerable investigative efforts have been accomplished in search of γ-secretase inhibiting substances. Recently, it has become clear that not all γ-secretase inhibitors block the active site of the enzyme in the same way. This is not surprising since γ-secretase is a multimeric enzyme complex with potentially more than one active site, but distinct modes of inhibition might become crucial for the further drug design of γ-secretase inhibitors. γ-secretase inhibitors structurally similar to DAPT might be favourable since a reconstitution of cellular viability has been observed by our group and by others (Keil, U., Bonert, A. et al., 2004; Bonert, A., Marques, C. et al., 2004; Kienlen-Campard, P., Miolet, S. et al., 2002).

4.4.2 Pro-apoptotic mitochondrial factors: potential therapeutic targets in AD therapy?

Inhibition of caspases as a key and central component of the biochemical pathway that mediates apoptotic cell death represents an attractive therapeutic strategy. There are efforts to induce caspase activation for the treatment of disorders where insufficient apoptosis occurs (e.g. cancer). In HIV infection efforts to activate caspases in infected cells exist e.g. gene therapy with a TAT-caspase-3 construct containing a HIV-protease recognition motif selectively induces apoptosis in HIV infected cells only (Vocero-Akbani, A. M., Heyden, N. V. et al., 1999). Preliminary experiments in animal models using non-selective caspase inhibitors such as Z-VAD(OMe)-CH2F, have shown in-vivo efficacy in ischemic and hypoxic brain injury (Cheng, Y., Deshmukh, M. et al., 1998; Daemen, M. A., van, '., V et al., 1999). Nevertheless, the development of selective caspase-inhibitors remains a big challenge in AD therapy. Recently, efforts have been undertaken inhibit pro-apoptotic factors up-stream caspase-3 for the development of anti-apoptotic drugs. Several groups have focused on IAPs (inhibitor of apoptosis proteins) as the most promising target: XIAP's capacity to maintain inhibition of caspase-9 within the Apaf-1 apoptosome is influenced by its ability to simultaneously inhibit active caspase-3, depending on the apoptotic stimulus, inhibition of caspase-9 or 3 is essential for

XIAP's anti-apoptotic activity (Bratton, S. B., Lewis, J. et al., 2002). Interestingly, only the IAPs have been demonstrated to be endogenous repressors of the terminal caspase cascade. In turn, the caspase inhibiting activity of XIAP is negatively regulated by at least two XIAP-interacting proteins, XAF1 and Smac/DIABLO. In addition to the inhibition of caspases, recent discoveries from several laboratories suggest that XIAP is also involved in a number of other biologically significant cellular activities including modulation of receptor-mediated signal transduction and protein ubiquitination. The multiple biological activities of XIAP, its unique translational and post-translational control and the centrality of the caspase cascade make the control of XIAP expression an exceptionally promising molecular target for modulating apoptosis. Therapeutic benefits can be derived from both the suppression of inappropriate cell death such as in neurodegenerative disorders and ischemic injury (Holcik, M., Gibson, H. et al., 2001). Thus, it seems at least possible that Smac/DIABLO might be a potential target for apoptosis-inhibition, since it selectively blocks the binding of IAP to the apoptosome. Recently, an essential serine protease with pro-apoptotic activity, named HtrA2/Omi, was identified to interact with Aβ in yeast two-hybrid assays. Co-immunoprecipitation assays further confirmed the interaction of Aβ and the protease in cell culture experiments (Park, H. J., Seong, Y. M. et al., 2004). Considerung this fact, HtrA2/Omi-inhibiting substances could be of great interest for AD drug development

Concerning AIF, a recent report provides new insights into the mechanisms of neurodegeneration. In a mouse mutant with progressive cerebellar and retinal degeneration, the expression of the apoptotic protein AIF was found to be downregulated leading paradoxically to apoptosis of neurons that is associated with an imbalance in free radical metabolism and cell cycle re-entry (Bonni, A., 2003). This controversial finding indicates that intensive investigative efforts have to be undertaken to elucidate the complex role of AIF in cell death decision before AIF inhibitors might be considered as potential therapeutic target in AD therapy.

4.4.3 Growth factors in AD

The ability of neurotrophic factors to regulate developmental neuronal survival and adult nervous system plasticity suggests the use of these molecules to treat neurodegeneration associated with human diseases. Solid rationales exist for the use of NGF and neurotrophin-3 in the treatment of neuropathies of the peripheral sensory system, insulin-like growth factor and ciliary neurotrophic factor in motor neuron atrophy, and NGF in Alzheimer's disease. Growth factors have been identified for neurons affected in Parkinson's disease, Huntington's disease, and acute brain and spinal cord injury. Various strategies are actively pursued to deliver neurotrophic factors to the brain, and develop therapeutically useful molecules that mimic neurotrophic factor actions or stimulate their production or receptor mechanisms (Hefti, F., 1994). In a clinical trial with three patients, intrathecal NGF infusion resulted in a slight cognitive benefit, but also led to significant back pain and weight loss (Jonhagen, M. E., 2000). Working on the assumption that these side effects could be avoided by delivering the factor to precise cellular targets, a small clinical Phase I trial of NGF gene therapy in AD was completed. The results were interpreted as being encouraging enough for larger, controlled trials of NGF gene delivery to commence (Tuszynski, M. H., Smith, D. E. et al., 1998; Tuszynski, M. H., Thal, L. et al., 2005). Nevertheless, NGF therapy for AD seems unlikely to produce clinical benefits as striking as described for glial cell line-derived neurotrophic factor infusion in Parkinson’s disease (Gill, S. S., Patel, N. K. et al., 2003), where only a localized set of neurons needs protection/rescue. Therefore, efforts are currently undertaken to develop drug with NGF-like activity or with NGF-enhancing properties.

4.4.4 NGF therapy

Aβ is reported to cause a loss of cholinergic neurons in the basal forebrain, particularly in the nucleus basalis Meynert of AD patients. An up-regulation of $p75^{NTR}$, the neurotrophin affinity receptor, has been reported to correlate with β- amyloid sensitivity in vitro and in vivo, suggesting an implication in Aβ-mediated cell death. Blocking the interaction of Aβ with $p75^{NTR}$ by NGF abolished Aβ-induced apoptotic cell death (Kuner, P., Schubenel, R. et al., 1998). As $p75^{NTR}$ and TrkA receptors are expressed in PC12 cells, it is noteworthy that APP expression and secretion is regulated by the binding of NGF to these receptors. NGF stimulates APP processing in favour of the sAPPα secretory pathway in a dose-dependent pattern (Rossner, S., Ueberham, U. et al., 1998b). Consequently, it is generally supposed that treatment with NGF could ameliorate cortical cholinergic function in AD (Rossner, S., Ueberham, U. et al., 1998a). One AD treatment strategy is to protect or repair the neurons affected by the disease process, and neurotrophic factor treatments provide such an approach, but only little research has focused on the application of neurotrophin-like substances or growth factor enhancers in AD therapy.

4.4.5 Substituted pyrimidines as potential AD-relevant drugs ?

Substituted pyrimidines similar to KP544 have modest neurotrophin-like activities (Awaya, A., Kobayashi, H. et al., 1993; Ikeuchi, T., Nakatani, A. et al., 1998) and their neuroprotective effect has been studied in models of cerebral ischemia, Parkinson and Huntington´s disease (Matsumoto, K., Yamamoto, K. et al., 2003; Fyfe, J. A., Beauchamp, L. M. et al., 2004). It has been demonstrated before that KP544 promotes neurite outgrowth, but little investigative work has been done on the effect of KP544 on cell viability and mitochondrial activity. Evidence is provided that oxidative stress is an AD relevant secondary insult representing an early pathogenic key event of the disease. APPsw PC12 cells, besides their reduced mitochondrial activity under baseline conditions, showed a concentration-dependent decrease in the cellular ability to reduce MTT when exposed to H_2O_2 for 6h (Fig. 3.48). The significant protective effect of KP544 on APPsw PC12 cells under baseline conditions implicates that KP544 might rescue mitochondrial activity of PC12 cells suffering from

chronic APPsw exposure in a NGF-independent pattern. After massive exposure to oxidative stress (H_2O_2, 500 µM), KP544 exhibited significant protective effects on APPsw and control cells while elevating MTT reduction of APPsw cells on the level of H_2O_2 -treated control cells (Fig. 3.48).

In terminally differentiated PC12 cells, KP544 significantly promoted mitochondrial activity (Fig. 3.49). In differentiated PC12 cells suffering from trophic factor withdrawal, the protective effect was most pronounced in the APPsw clone (Fig. 3.49). As KP544 showed protective effects in undifferentiated and terminally differentiated APPsw cells, we supposed that that besides the promoting effect of KP544 on $p75^{NTR}$- and TrkA-mediated signaling, which is mainly tied to the presence of NGF, other enzymes involved in APP processing might be the original target of KP544. A recent study corroborated our theory revealing that KP544 functions within the cell at a site downstream or independent of MAPK and ERK1/2 pathway (Fyfe, J. A., Beauchamp, L. M. et al., 2004). As PI3K activation stimulates Akt which in turn phosphorylates GSK-3, a multifunctional serine/threonine kinase playing a pivotal role not only in the regulation of cell proliferation and neurite outgrowth, but also in APP processing, post-translational modification and transport (Doble, B. W., Woodgett, J. R., 2003; Aplin, A. E., Jacobsen, J. S. et al., 1997). GSK-3β is considered to be the connecting link between the two main hallmarks of AD, as abnormal tau phosphorylation as well as altered APP processing is GSK-3β-mediated (Kaytor, M. D., Orr, H. T., 2002). In siRNA experiments, reduced GSK-3β protein expression levels promote $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion, which is consistent with our data since we observed reduced GSK-3β expression in APPsw compared to control cells, while $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion is significantly increased in APPsw cells (Keil, U., Bonert, A. et al., 2004). Of note, GSK-3β expression levels were not affected by the treatment with NGF or NGF/KP544 for 1h or 48h (Fig. 3.50). Moreover, after 1h treatment with NGF/KP544, the phosphorylation status of GSK-3β at Ser9 is significantly increased in APPsw cells compared to control cells (Fig. 3.50). In contrast to this, NGF treatment alone resulted in a slightly increased level of phospho-GSK-3β, while the combined treatment with KP544 resulted in a significant increase in phospho-GSK-3β compared to untreaed APPsw PC12 control. Lithium, a non-selective GSK-3β inhibitor, reduces the secretion of $A\beta_{1-40}$ and $A\beta_{1-42}$ while promoting the α-secretory pathway (Sun, X., Sato, S. et al., 2002). In an other

experimental setting, GSK-3β linked neuroprotection by 17β-estradiol to key AD processes (Goodenough, S., Schafer, M. et al., 2003). Briefly, in a hippocampal slice culture model of kainic acid-induced neurotoxic cell death as well as in in vivo models, estrogen caused phosphorylation and hence inactivation of GSK-3β having implications for estrogen receptor modulation as a target for neurodegenerative disorders. In our setting, APPsw PC12 cells showed a significant reduction in $A\beta_{1-40}$ levels when exposed to NGF, KP544 or NGF/KP544 (Fig. 3.51). Of note, the early (1h) and significant increase in phospho-GSK-3β of APPsw cells could be one reason for the strong effects of KP544 on $A\beta_{1-40}$ reduction, which is probably due to the influence of KP544 on GSK-3-mediated APP processing.

Refering to the results of Fyfe et al. (Fyfe, J. A., Beauchamp, L. M. et al., 2004), we resume that in our setting, KP544 acts additively but not synergistically on the APP processing pathway. However, treatment with a combination of KP544 and NGF resulted in reduced $A\beta_{1-40}$ levels comparable to those achieved by the commercial γ-secretase inhibitor DAPT. As presenilins directly interact with GSK-3β leading to a modulated APP processing (Van Gassen, G., De Jonghe, C. et al., 2000), we suppose that an altered interaction of GSK-3β and the presenilins due to increased phosphorylation of GSK-3β might reduce secreted $A\beta_{1-40}$ levels.

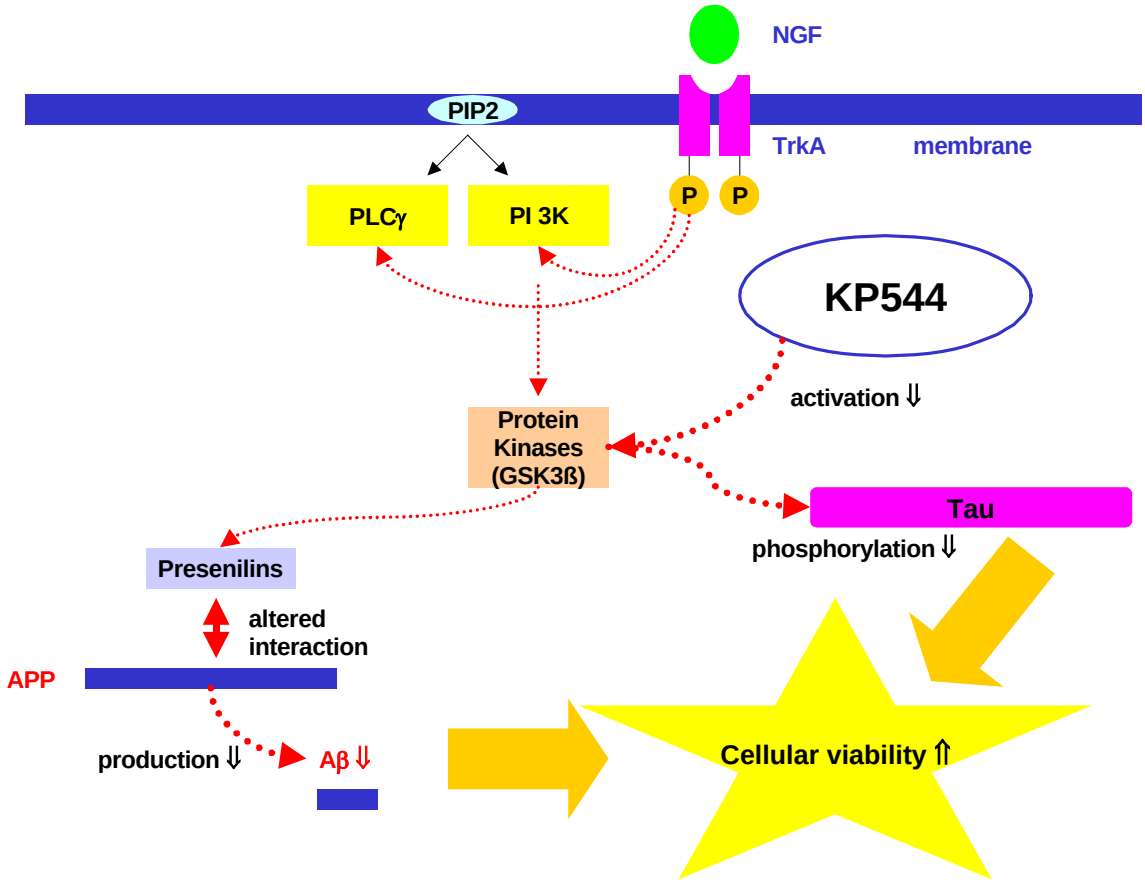

Figure 4.9: Proposed model for the impact of KP544 on APP processing and cellular viability.

In our model, treatment with KP544 results in a decreased activation of GSK-3β. A decrease in GSK-3β results in decreased tau phosphorylation and an altered interaction between presenilins and APP, finally leading to reduced Aβ levels.

5 SUMMARY

5 Summary

Alzheimer's Disease (AD) is the most common neurodegenerative disorder marked by progressive loss of memory and cognitive ability. The pathology of AD is characterised by the presence of amyloid plaques, intracellular neurofibrillary tangles and pronounced cell death. The amyloid plaque is composed of amyloid beta (Aβ) peptide. Aβ is derived from the amyloid precursor protein (APP) through an initial β-secretase cleavage followed by an intramembraneous cut of γ-secretase. Autosomal dominant forms are caused by mutations in APP, presenilin 1 and presenilin 2, mainly associated with the early onset of AD. These mutations alter APP processing with respect to an enhanced Aβ production and are related to an increased vulnerability to cell death. However, the underlying mechanisms responsible for the massive neurodegeneration in the brain from AD patients are still not completely understood.

The aim of this thesis was to investigate pathways involved in the Aβ cascade of neurodegeneration. Since novel findings indicate that already this Aβ species exerts neurotoxic effects long before hyperphosphorylated tau, neurofibrillary tangles and extracellular Aβ plaques appear, the investigations were accomplished with specific regard to the effects of intracellular Aβ.

In the first part of this thesis, APP processing was studied in different in-vitro models of AD in order to characterize the cells with regard to APP expression, Aβ secretion, intracellular Aβ contents and distribution. For this purpose, PC12, HEK and SY5Y cells stably transfected with APP wild-type (APPwt) or APP bearing the Swedish mutation (APPsw) were investigated. The Swedish double mutation in the APP gene (K670M/N671L) results in six- to eightfold increased Aβ production of both $A\beta_{1-40}$ and $A\beta_{1-42}$ compared to human wildtype APP cells (APPwt). Data obtaine from PC12 cells indicate that it is possible to specifically increase the Aβ load without enhancing APP expression levels. The amount of secreted $A\beta_{1-40}$ followed the subsequent order: HEK APPsw >> HEK APPwt = SY5Y APPwt > PC12 APPsw > PC12 APPwt. On the basis of these findings, it seemed possible to investigate dose-dependent effects of Aβ in multiple experimental designs. These assay designs were created in order to mimick different in-vivo situations that are discussed to occur in AD patients: APPsw PC12 cells exhibit low physiological concentrations of Aβ within picomolar range in

contrast to APPsw HEK cells, expressing Aβ levels within the nanomolar range. Of note, the APPsw HEK cells showed a specific and highly significant increase in the intracellular accumulation of insoluble $A\beta_{1-42}$. Moreover, an intracellular accumulation of Aβ and APP was found in the mitochondria of the HEK APPsw cells suggesting a direct impact on mitochondrial function on these cells. This effect might finally lead to disturbances in the energy metabolism of the cell or to increased cell death. Furthermore, baseline γ- and β-secretase activity was assessed since these enzymes represent promising therapeutic targets to slow or halt the disease process. As expected, β-secretase activity was significantly elevated in all APPsw cell lines. This might be due to the proximity of the Swedish double mutation next to the N-terminus of the Aβ sequence. Interestingly, γ-secretase activity was similarly increased in PC12 APPsw cells. In addition, the toxicity of different Aβ species was investigated in SY5Y and PC12 cells with regard to their effect on cellular viability mirrored by mitochondrial activity using MTT assay. Here, it turned out that not monomers, but already dimers are neurotoxic correlates. Fibrillar Aβ species showed the highest toxicity. In the next step, SY5Y cells forming endogenous, dimeric APP and Aβ (SY5Y APPK623C) were investigated. In accordance with previous findings, these cells showed a decreased MTT reduction potential in comparison to APPwt and control SY5Y cells reflecting a decrease of cellular viability. The impaired energy metabolism of the cells was even more drastically mirrored by reduced baseline ATP levels.

In the second part of this thesis, the expression and intracellular distribution of Bcl-2 family proteins and pro-apoptotic mitochondrial factors under baseline conditions and during oxidative stress were analyzed in the APPwt and APPsw bearing cells, since recent findings of our group indicate the pivotal role of mitochondria in Aβ-induced cell death (Keil, U. et al., 2004; Marques, C. A. et al., 2003), Of note, these factors might potentially represent valuable therapeutic targets for the reduction of increased cell death associated with the AD progress. The most prominent finding was the reduction of expression levels of the anti-apoptotic factor Bcl-xL in the cytosolic fractions of APPwt and APPsw PC12 cells. This might indicate that a lack of anti-apoptotic factors or their altered intracellular distribution, rather than an increase in caspase-dependent pro-apoptotic factors, could be responsible for the increased vulnerability of APPwt- and APPsw-transfected PC12 cells against oxidative stress. Since total Bcl-xL expression was unaffected in PC12 cells, in contrast to APPwt and

APPsw-expressing SY5Y and HEK cells revealing significantly decreased Bcl-xL expression levels. Thus, alterations in Bcl-xL distribution seem to be an early event in the disease process. Increasing Bcl-xL expression might potentially be one promising strategy for AD modification.

In the last part of the thesis, disease-modifying strategies were assessed. PC12 and HEK cells bearing APPsw or APPwt were treated with the potent γ-secretase inhibitor DAPT. Of note, DAPT did not only efficiently block Aβ production, but additionally led to an elevation of the MTT reduction potential, reflecting an increase in cellular viability. In contrast to DAPT, a structurally distinct γ-secretase inhibitor, L-685458, did not increase MTT reduction potential, but rather decreased MTT reduction potential at high concentrations indicating that under these conditions, the γ-secretase inhibitor exhibits toxic side effects surmounting the potential beneficial effect of a decrease in Aβ production. The development of γ-secretase inhibitors similar to DAPT seems thus to be a promising strategy for AD therapy. Furthermore, cells were treated with the β-secretase inhibitor II. Unfortunately, the β-secretase inhibitor II did block Aβ production only at extremely high concentrations while exhibiting toxic side effects reflected by a decrease in MTT reduction potential. This indicates that the commercially available inhibitor is not suitable for therapeutic use.

As another disease-modifying strategy, several efforts are undertaken to ameliorate AD-relevant symptoms by the treatment with nerve groth factor (NGF). Since NGF is rapidly degraded and is not able to cross the blood brain barrier, it seems a reasonable strategy to apply substances increasing the NGF action in the brain. Generally, it is known that substituted pyrimidines have modest growth-promoting effects. Here, KP544, a novel substituted pyrimidine, was characterised. The exact mode of action of KP544 is not known. This drug increased MTT reduction potential in terminally differentiated and undifferentiated PC12 cells. Furthermore, treatment with KP544 led to a reduction in Aβ40 secretion. In addition, glycogen synthase kinase-3β (GSK-3β) was inactivated in the presence of KP544. This is very interesting since GSK-3β does not only modulate APP processing, but is also involved in the hyperphosphorylation of tau, another pathological hallmark of AD. Thus, one may conclude that the target of KP544, GSK-3β, represents a connecting link between the two main pathological hallmarks of AD and might thus be a very promising therapeutic target for AD.

6 ZUSAMMENFASSUNG

6 Zusammenfassung

Alzheimer Demenz (AD) ist die häufigste neurodegenerative Erkrankung, welche durch einen kontinuierlichen Verlust der Gedächtnisleistung und der kognitiven Fähigkeiten charaktersiert ist.

Typische pathologische Merkmale der Erkrankung sind Amyloid-Plaques, neurofibrilläre Bündel und gesteigerter Zelltod. Die Amyloid-Plaques bestehen aus Amyloid beta-Peptid (Aβ), welches aus dem Amyloid-Vorläufer-Protein (APP) durch eine initiale β-Sekretase-Spaltung, gefolgt von einem intermembranären Schnitt der γ-Sekretase, gebildet wird. Autosomal-dominante Formen werden durch Mutationen im APP-, Präsenilin-1- und Präsenilin-2-Gen verursacht. Diese Mutationen beeinflussen die APP-Prozessierung, so dass vermehrt Aβ gebildet wird. Auβerdem werden sie mit einer gesteigerten Empfindlichkeit von Neuronen gegenüber Zelltod in Zusammenhang gebracht. Bis heute jedoch sind die molekularen Mechanismen, die für die massive Neurodegeneration in Gehirnen von AD-Patienten verantwortlich sind, nicht vollständig aufgeklärt.

Das Ziel dieser Arbeit war die Untersuchung zellulärer Signaltransduktionswege, die an der Amyloidkaskade beteiligt sind. Hier wurde ein besonderes Augenmerk auf intrazelluläres Aβ gerichtet, da neuere Ergebnisse belegen, dass diese Aβ-Spezies neurotoxische Effekte auslöst, und zwar lange bevor hyperphosphoryliertes Tau und extrazelluläre Aβ-Ablagerungen auftreten.

Im ersten Teil der Arbeit wurde die APP-Prozessierung und Aβ-Produktion in verschiedenen in-vitro-Modellen untersucht. PC12-, HEK- und SY5Y-Zellen, die stabil mit der Schwedischen APP-Doppelmutation (APPsw) und mit wildtyp APP (APPwt) transfiziert wurden, dienten als Zellkulturmodelle. Die Schwedische Doppelmutation auf dem APP-Gen (K670M/N671L) führt zu einer sechs- bis achtfach erhöhten Produktion von $A\beta_{1-40}$ und $A\beta_{1-42}$ im Vergleich zu APPwt-Zellen.

Anhand der Daten aus PC12-Zellen konnte abgeleitet werden, dass durch diese Mutation die sekretierten Aβ-Spiegel ohne Beeinflussung der APP-Expression gesteigert werden. Die sekretierten Aβ-Spiegel nehmen in der folgenden Reihenfolge ab: HEK APPsw >> HEK APPwt = SY5Y APPwt > PC12 APPsw > PC12 APPwt.

Basierend auf diesem Ergebnis erscheint es zumindest möglich, dosisabhängige Aβ-Effekte in verschiedenen experimentellen Ansätzen zu untersuchen. Diese Versuchsdesigns sollten möglichst authentisch verschiedene Stadien in Alzheimer-Gehirnen abbilden: Beispielsweise dient das APPsw PC12-Zellmodell als Modell für niedrige physiologische Aβ-Konzentrationen im picomolaren Bereich, im Gegensatz zu APPsw HEK-Zellen, die Aβ-Spiegel im nanomolaren Bereich zeigen. APPsw HEK-Zellen zeigten eine spezifische und hochsignifikante Anreicherung von unlöslichem $A\beta_{1-42}$ im intrazellulären Kompartiment.

Auβerdem wurde eine Akkumulation von Aβ und APP in Mitochondrien von APPsw HEK-Zellen beobachtet. Dies läβt eine direkte Beeinflussung mitochondrialer Funktionen vermuten, welche letztendlich zu Störungen im Engergiehaushalt der Zelle und zu gesteigertem Zelltod führen könnte. Desweiteren wurden die basalen Aktivitäten der γ- und β-Sekretase untersucht, da diese Enzyme vielversprechende therapeutische Angriffspunkte darstellen, um den Verlauf der Erkrankung zu verlangsamen bzw. aufzuhalten. Erwartungsgemäβ war die β-Sekretase-Aktivität in allen APPsw-Klonen erhöht. Dies läβt sich durch die Nähe der Schwedischen Doppelmutation zum N-Terminus der Aβ-Sequenz erklären. Interessanterweise war die γ-Sekretase-Aktivität in PC12-Zellen ebenfalls signifikant erhöht.

Die Toxizität verschiedener Aβ-Spezies wurde in SY5Y- und PC12-Zellen im Hinblick auf die zelluläre Viabilität, dargestellt durch die Messung der mitochondrialen Aktivität im MTT-Assay, untersucht. Hier zeigte sich, dass nicht Monomere, sondern Dimere erste toxische Korrelate darstellen, und dass fibrilläres Aβ die höchste Toxizität aufweist.

Im nächsten Schritt wurden SY5Y-Zellen untersucht, die dimeres APP und Aβ (SY5Y APPK623C) bilden. In Übereinstimmung mit anderen Daten besaβen diese Zellen im Vergleich zu APPwt und Kontroll-Zellen ein erniedrigtes MTT-Reduktionspotential und somit eine reduzierte zelluläre Viabilität. Der gestörte Energiehaushalt der Zellen konnte durch erniedrigte basale ATP-Spiegel noch drastischer dargestellt werden.

Im zweiten Teil der Arbeit wurde die Expression und die intrazelluläre Verteilung von Proteinen der Bcl-2-Familie und pro-apoptotischer mitochondrialer Faktoren untersucht. Die Proteine wurden unter basalen Bedingungen und unter oxidativem Stress in APPwt und APPsw Zellen untersucht, da neuere Ergebnisse unserer Arbeitsgruppe eine zentrale Rolle der Mitochondrien bei Aβ-vermitteltem Zelltod

zeigten (Keil, U. et al., 2004; Marques, C. A. et al., 2003). Diese Faktoren könnten potentiell wichtige therapeutische Angriffpunkte zur Reduktion des gesteigerten, Alzheimer-assoziierten Zelltods darstellen.

Interessanterweise sind die Expressionsspiegel des anti-apoptotischen Faktors Bcl-xL in zytosolischen Fraktionen von APPwt und APPsw Zellen erniedrigt. Dies könnte darauf hinweisen, dass ein Mangel an anti-apoptotischen Faktoren oder ihre veränderte intrazelluläre Verteilung, eher noch als eine Erhöhung caspase-abhängiger, pro-apoptotischer Faktoren, für die gesteigerte Empfindlichkeit der APPwt- und APPsw-transfizierten Zellen gegenüber oxidativem Stress verantwortlich ist. Da die Bcl-xL-Expression in APPsw PC12-Zellen, im Gegensatz zu APPsw HEK und APPwt SY5Y unverändert war, könnten Veränderungen in der intrazellulären Bcl-xL-Verteilung ein frühes Ereignis im Krankheitsverlauf darstellen. Somit würde eine Erhöhung der Bcl-xL-Expression eine potentielle krankheitsmodulierende Strategie darstellen.

Im letzten Teil der Arbeit wurden krankheitsmodulierende Strategien untersucht. PC12- und HEK-Zellen wurden mit dem potenten γ-Sekretase-Inhibitor DAPT behandelt. Die Behandlung mit DAPT führte nicht nur zu einer effizienten Hemmung der Aβ-Produktion, sondern auch zu einer Steigerung des MTT-Reduktionspotentials, welche eine gesteigerte zelluläre Viabilität wiederspiegelt. Im Gegensatz zur DAPT-Exposition erhöhte die Behandlung mit einem strukturell verschiedenen γ-Sekretase-Inhibitor, L-685458, das MTT-Reduktionspotential nicht. Im Gegenteil, L-685458 führte zu einer Reduktion der zellulären Viabilität, bedingt durch toxische Nebeneffekte, welche die protektiven Eigenschaften der γ-Sekretase-Inhibition überlagerten. Die Entwicklung von DAPT-ähnlichen γ-Sekretase-Inhibitoren könnte somit eine vielversprechende Strategie in der Behandlung der Alzheimer Demenz darstellen. Auβerdem wurden Zellen mit dem β-Sekretase-Inhibitor II behandelt. Unglücklicherweise hemmte der β-Sekretase-Inhibitor II die Aβ-Produktion nur in sehr hohen Konzentrationen, bei denen massive toxische Nebeneffekte im MTT-Assay beobachtet wurden. Somit scheint dieser kommerziell erhältliche Inhibitor nicht zur therapeutischen Anwendung geeignet zu sein.

Es wurden schon einige Versuche unternommen, AD-relevante Symptome durch die Behandlung mit Nervenwachstumsfaktor (NGF) zu behandeln. Da NGF sehr rasch abgebaut wird und die Blut-Hirn-Schranke nicht passieren kann, erschien es sinnvoll, Substanzen zu entwickeln, welche die NGF-Wirkung im Gehirn fördern.

Es ist allgemein bekannt, dass substituierte Pyrimidine wachstumsfördernde Eigenschaften besitzen. In dieser Arbeit wurde KP544, ein neuartiges substituiertes Pyrimidin, näher charakterisiert. Der genaue Wirkmechanismus von KP544 ist unbekannt. Die Behandlung mit KP544 führt zu einer Steigerung des MTT-Reduktionspotentials in undifferenzierten und differenzierten PC12-Zellen sowie zu einer verminderten $A\beta_{1-40}$-Sekretion. Zusätzlich wurde eine Inaktivierung der Glykogen-Synthase-Kinase-3β (GSK-3β) in Gegenwart von KP544 beobachtet. Dieser Befund ist sehr interessant, da GSK-3β nicht nur die APP-Prozessierung, sondern auch die Hyperphosphorylierung von Tau, einem weiteren Merkmal der Alzheimer Demenz, moduliert. Daher könnte man schlussfolgern, dass das Target von KP544, GSK-3β, ein Bindeglied zwischen den zwei Hauptmerkmalen der Alzheimer Demenz darstellt und somit ebenfalls ein sehr interessanter therapeutischer Angriffspunkt darstellt.

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7 References

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8 ABBREVIATIONS

8 Abbreviations

AD	Alzheimer´s disease
ABAD	Aβ-binding alcohol dehydrogenase
ADAM	A disintegrin and metalloproteinase
ADAPT	Alzheimer´s disease prevention trial
ADDL	Aβ-derived diffusible ligands
ADP	Adenosine diphosphate
AGE	Advanced glycation end products
AICD	APP intracellular domain
AIF	Apoptosis inhibiting factor
Apaf-1	Mammalian homologue of ced-4
APH	Anterior pharynx
APLP	Amyloid precursor-like protein
Apo E	Apolipoprotein E
APP	Amyloid precursor protein
APPK623C	dimeric APP
APPsw	APP bearing the Swedish mutation
APPwt	wild-type APP
Aβ	Amyloid beta
ATP	Adenosine triphosphate
BACE	β-secretase, β-site amyloid precursor cleaving enzyme
BSA	Bovine serum albumne
°C	degree Celsius
cdk	Cyclin-dependent kinase
Ced-4	Caenorhabditis elegans protein-4, pro-apoptotic
COX-1/-2	Cyclooxygenase 1/2
COX-4	Cytochrome c oxidase
CSF	Cerebrospinal fluid
CT	Computer toograhy
CTF	C-termina fragment

dB-cyclic AMP	N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate
Delta-1	delta-like protein-1
DMEM	Dulbecco´s modified eagle medium
DMSO	Dinethylsulfoxide
DNA	Desoxyribonucleic acid
DSM-IV	Diagnostic and Statistic manual of mental disorders
DTT	Dithiothreitol
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
EOAD	Early-onset AD
ER	Endoplasmatic reticulum
ERAB	Endoplasmatic reticulum-associated amyloid beta binding protein
ErbB4	v-erb-a erythroblastic leukaemia viral oncogene homologue-4
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FA	Formic acid
FAD	Familial Alzheimer´s disease
$FADH_2$	Flavine adenine dinucleotide
FCS	Fetal calf serum
FDA	Food and drug administration
g	Gram
g	gravitation
G418	Geneticine sulfate
GSK-3	Glycogen synthase kinase-3
GTP	Guanyl triphosphate
h	Hours
H_2O_2	Hydrogen peroxide
HEK	Human embryonic kidney

HIV	Human immunodeficiency virus
IAP	Inhibitor of apoptosis proteins
IDE	Insulin-degrading enzyme
Jag-2	Jagged-2
JNK	c-Jun N-terminal kinase
KP544	[2-amino-5-(4-chlorophenylethynyl)-4-(4-trans-hydroxycyclohexylamino)pyrimidine]
l	Litre
LDH	Lactate dehydrogenase
LOAD	Late-onset Alzheimer´s disease
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
min	Minutes
ml	Millilitres
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NFkB	Nuclear factor kB
NGF	Nerve growth factor
NIA	National institute of aging
NIH	National institute of health
NINCDS-ADRDA	National Institute of neurologic and and communicative disorders and stroke-alzheimer´s disease and related disorders association
NMDA	N-methyl-D-aspartate
NSAIDs	Non-steroidal anti-inflammatory drugs
$p75^{NTR}$	Low affinity neurotrophin receptor
PBS	Phosphate buffered saline
PC12	Rat pheochromocytoma
PCR	Polymerase chain reaction

PEN-2	Presenilin enhancer-2
PET	Positron-emission tomography
PHF	Paired helical filaments
PI3K	Phosphoinositide-3 kinase
PMSF	Phenoylmethysulfonylfluoride
PPAR-γ	Peroxisome proliferator activated receptor-γ
PS	Presenilin
PTP	Permeability transition pore
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sAPP	soluble APP
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Seconds
Smac	Second mitochondrial activator of caspases
SY5Y	Human neuroblastoma
t	Time
TACE	Enzyme cleaving members of the TNF receptor family
Trk	Tyrosine kinase
vs	Versus

9 INDEX OF FIGURES

9 Index of Figures

10 BIBLIOGRAPHY

10 Bibliography

10.1 Orignal publications

Bonert A, Müller WE, Eckert A
A novel 5-phenylpyrimidine derivate promotes survival and reduces secretion of amyloid beta in PC12 cells bearing Alzheimer's APP mutation
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10.2 Reviews

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10.3 Oral presentations

Astrid Bonert
The impact of mutant APP on members of the Bcl-2 family and on the release of mitochondrial factors in oxidative stress-induced cell death 5th Int Conference on Cell and Molecular Biology of Alzheimer's disease, Hamburg, Germany , Nov 2003

Astrid Bonert
Bcl-2 family proteins and pro-apoptotic mitochondrial factors in Alzheimer´s disease
Abschlusssymposium des Graduiertenkollegs für Arzneimittelentwicklung und Analytik , Oestrich am Winkel, Januar 2004

10.4 Posters

Astrid Bonert, Uta Keil, Franz Müller-Spahn, Walter E. Müller, Anne Eckert
Effects of γ- and β-secretase inhibition on mitochondrial viability in APPsw PC12 and HEK cells
35th Annual Meeting Soc. Neuroscience, Washington D.C., USA, 2005. Soc. Neurosci., Abstracts 2005

A. Bonert, C. Marques, U. Keil, W.E. Müller and A. Eckert
The impact of mutant APP on members of the Bcl-2 family and on the release of mitochondrial factors in oxidative stress-induced cell death
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Impact of ß-amyloid on mitochondrial failure in Alzheimer's disease

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11 Danksagung

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12 CURRICULUM VITAE

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