The role of the amyloid precursor protein (APP) in protein homeostasis and neuroprotection

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Dekan: Prof. Dr. Michael Karas

Gutachter: Prof. Dr. Donat Kögel
Prof. Dr. Jochen Klein`

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To my parents, husband and daughter

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Abbreviations

% (v/v)	volume percent
% (w/v)	weight percent
× g	gravitation constant
°C	degree Celsius
aa	amino acids
Ab	antibody
AD	Alzheimer's disease
ADAM10	a disintegrin and metalloproteinase domain-containing protein 10
AICD	APP intracellular domain
APLP1	APP-like protein 1
APLP2	APP-like protein 2
APOE4	apolipoprotein allel E4
APP	amyloid precursor protein
APS	ammoniumpersulfate
ATP	adenosine triphosphate
Aβ	amyloid beta
BAG1	Bcl-2 associated athanogene 1
BAG3	Bcl-2 associated athanogene 3
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
Bim	Bcl-2-interacting mediator of cell death
BME	Basal Medium Eagle
BSA	bovine serum albumin
cDNA	complementary DNA
CNS	central nervous system
C-terminal	carboxyterminal
CTF	(APP) C-terminal fraction
DAPI	4'-6-diamino-2-phenylindol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
E1/E2	subdomains of sAPPα,the soluble ectodomain of APP
ECL	enhanced chemoluminescence
EOAD	early-onset AD
ex/em	extinction/emission
FACS	fluorescence-activated cell sorting
FAD	familial AD
FCS	fetal calf serum
FELASA	Federation of European Laboratory Animal Science Associations
G418	geneticine 418
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFLD	growth factor-like domain

GFP	green fluorescent protein
Gluc	glucose
GSK3β	glycogen synthase kinase 3 beta
HEK	human embryonic kidney (cells)
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HS	horse serum
IGF1	insulin-like growth factor 1
IGF1-R	insulin-like growth factor 1 receptor
IP	immunoprecipitation
I-R	insulin receptor
JNK	c-Jun N-terminal kinase
KD	knockdown
kD	kilo Dalton
KO	knockout
LB	Luria Bertani
LOAD	late-onset AD
Mcl-1	myeloid cell leukemia 1
MAP	microtubule-associated protein
MEFs	mouse embryonic fibroblasts fibroblasts
MEM	Minimum Essential Medium
MLK3	mixed lineage kinase 3
mRNA	messenger RNA
MW	molecular weight
NB	neurobasal
NFT(s)	neurofibrillary tangle(s)
NGF	nerve growth factor
NMDA	N-methyl-D-aspartic acid N-terminal aminoterminal
OTCs	organotypic (hippocampal) slice cultures
P2-4	postnatal day 2-4
P. pastoris	*Pichia pastoris*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC12	pheochromocytoma cells
PDGF	plateled-derived growth factor
pen/strep	penicillin/streptomycin
pGSK3α/β	phosporylated glycogen synthase kinase 3 alpha/beta
PI	propidium iodide
PI3K	phosphatidylinositide 3-kinase
PS	presenilin
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
RNA	ribonucleid acid
rpm	revolutions per minute
RT	room temperature
S, Ser	serine
sAPPα/β	soluble APP alpha/beta

SDS	sodium dodecyl sulfate
SEM	standard error of the mean
shRNA	small hairpin ribonucleic acid
T, Thr	threonine
TBS	Tris-buffer solution
TEMED	N,N,N‘,N‘-tetramethylendiamine
TM	transmembrane (region)
Tris	hydroxymethylaminoethane
Tween	polyoxyethylensorbitane monolaurate
UV	ultraviolet radiation
V	Volt
wt	wild type

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Overview and Summary

In “Section 1 – Introduction” the readers are introduced to the world of Alzheimer’s disease (AD) with a special focus on amyloid precursor protein (APP) involved in the disease. In this part, different kind of cell death mechanisms such as apoptosis, necrosis and autophagic cell death will also be discussed. The readers will also get a short overview of stress signaling pathways involved in neurodegenerative diseases and the putative role of APP in the survival signaling cascade.

AD is a chronic neurodegenerative disease that causes problems with memory, thinking and behavior. The pathophysiological hallmarks of AD are extracellular senile plaques and intracellular neurofibrillary tangles. Amyloid plaques mainly contain the amyloid-β (Aβ) peptide, which appears as a cleavage product of the APP. APP is a type I transmembrane protein with a large extracellular domain and a short cytoplasmic tail. It is expressed in variety of tissues e.g. in neuronal tissue (brain, spinal cord, retina), and non-neuronal tissues (kidney, lung, pancreas, prostate gland, and thyroid gland) (Dawkins and Small, 2014). APP has been studied because of its link to AD, however, its role in normal brain function is poorly understood. APP is processed by two different pathways, amyloidogenic pathway and non-amyloidogenic pathway. In physiological condition, the majority of APP is processed via the non-amyloidogenic, thus leading to the generation of the secreted N-terminal APP processing product sAPPα. sAPPα is formed due to the cleavage of APP by α-secretase. In previous studies, our group has shown that sAPPα produce potent neuroprotective effect by altering gene expression, as well as by antagonizing several different types of neurotoxic stress stimuli (Copanaki et al., 2010; Kögel et al., 2003, 2005; Milosch et al., 2014). Several studies have shown that protein degradation is reduced in AD (Hong et al., 2014; Lipinski et al., 2010) but the role of APP and its cleavage products in protein degradation is still unknown. This thesis discusses about the physiological functions of APP in neuroprotection and protein homeostasis.

“Section 2 – Materials” provides the list of chemicals, cell lines, medium, buffers etc. used during this study.

"Section 3 – Methods" explains in detail the different biochemical and cell biological techniques utilized in this work.

In the first part of "Section 4 – Result (4.1 - 4.4)", the neuroprotective properties of yeast derived sAPPα and E1 (N-terminal domain of sAPPα) were investigated under serum and glucose deprivation conditions. In previous work, it was shown that recombinant sAPPα evoked a significant decrease in serum deprivation triggered cell death in human SH-SY5Y neuroblastoma cells and mouse embryonic fibroblast MEF cells. It was also observed that sAPPα induces the phosphorylation of Akt which leads to neuroprotection (Milosch et al., 2014). This study investigated whether this neuroprotection is associated with altered expression of downstream intracellular Akt targets such as FoxO, Bim, Bcl-xL and Mcl-1 under stress conditions (Figure 1). Here it was shown that sAPPα prevents activation and nuclear translocation of FoxO. FoxO act as a transcription factor for different proapoptotic genes such as *Bim*. It was also observed that Bim protein and mRNA expression was significantly reduced with sAPPα and E1 treatment. The expression of antiapoptotic protiens such as Bcl-xL and Mcl-1 were also examined and it was observed that sAPPα and E1 increases expression of both these proteins. Furthermore, it was previously demonstrated that uncleaved holo-APP functionally cooperates with sAPPα to activate Akt and provide neuroprotection (Milosch et al., 2014). Therefore, to investigate the function of the APP in sAPPα regulated Akt downstream proteins expressions, MEF APP KO cells were used. E1 and sAPPα only showed neuroprotective modulatory effect on these Akt downstream targets in MEF wt cells, but not in APP KO cells. In addition, sAPPα also showed neuroprotection in primary wt hippocampal neurons under trophic factor deprivation. Cellular fractionation experiments were also done to determine the role of sAPPα in cytochrome c release from mitochondria. It was observed that sAPPα treatment can inhibit mitochondrial cytochrome c release in wt MEF cells.

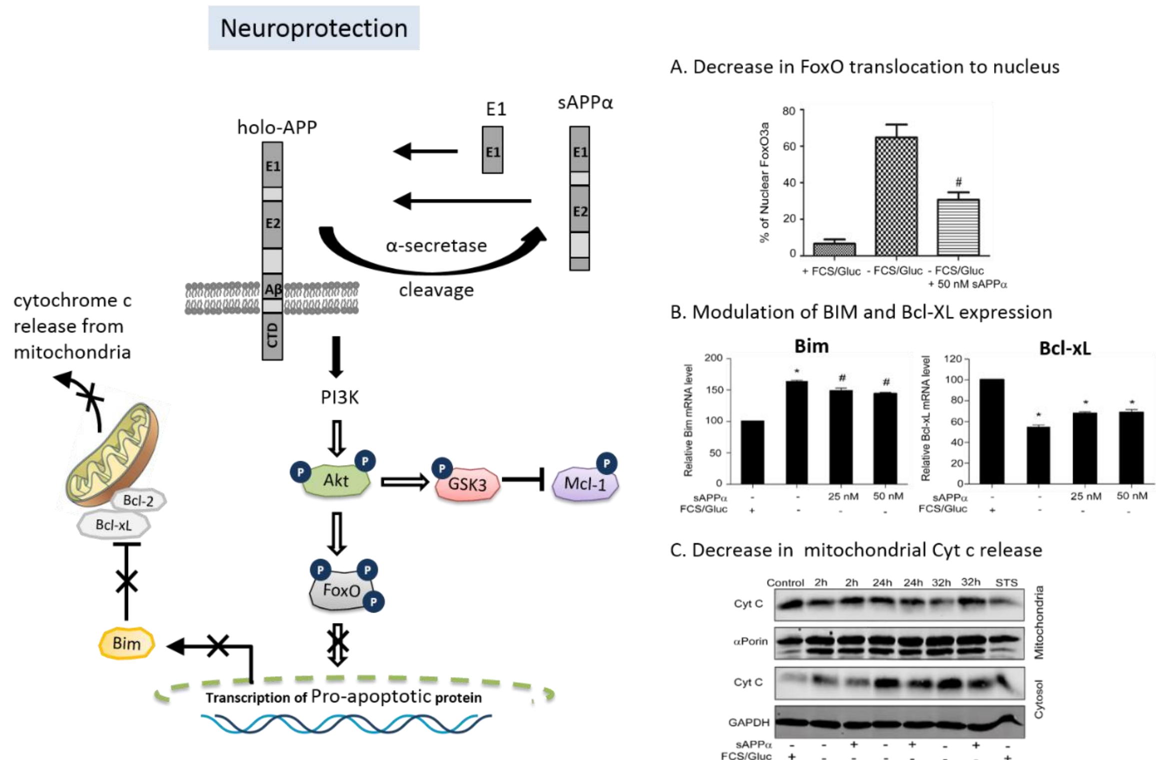


Figure 1. Neuroprotective function of sAPPα. (left) hypothetical model of APP/sAPPα mediated neuroprotection. (right) selective results showing the effect of sAPPα treatment on (A) nuclear translocation of FoxO, (B) pro-apoptotic Bim and anti-apoptotic Bcl-xL expression and (C) mitochondrial release of cytochrome c.

The second part of “Section 4 – Result (4.5 - 4.9)” discusses about the role of sAPPα in protein homeostasis (Figure 2). It was observed that sAPPα prevents proteotoxic stress induced BAG3 protein expression in SH-SY5Y and MEF cells. This was also observed in mRNA levels which indicate a transcriptional regulation. Furthermore, treatment with sAPPα was also shown to decrease aggresomes formation. Aggresomes are perinuclear aggregates which are formed due to accumulation of damaged and misfolded proteins and BAG3 plays important role in their formation and the transport of degradation prone proteins into these structures. The analysis of proteasomal activity showed a reduced accumulation of proteasomal substrate d2 by sAPPα under proteasomal stress. In proteasomal activity assay, sAPPα was shown to increase the degradation of proteasomal substrate SUC-LLVY-AMC and the fluorigenic signal was measured spectrophotometrically. The sAPPβ fragment which is generated via the amyloidogenic pathway was also examined for its role in BAG3 expression and proteasomal degradation. sAPPβ, which has almost similar structure as sAPPα,

only 17 amino acids at the C-terminus is missing, was failed to modulate BAG3 expression and proteostasis. This indicates that these biological effects are highly specific for sAPPα.

These sAPPα induced changes in protein homeostasis were unaltered in APP depleted SH-SY5Y cells and APP deficient MEF cells and primary hippocampal neurons. These observations indicate that holo-APP may not be required for this particular function of sAPPα. Other APP family members (APLP1 and APLP2) which were previously known to compensate different functions of APP (Anliker and Müller, 2006), may be involved in the modulation of protein homeostasis by sAPPα. sAPPα may also mediate this effect using other receptor proteins, like sorting protein related receptors SORLA (Hartl et al., 2013) or via alternative mechanisms such as APP internalization.

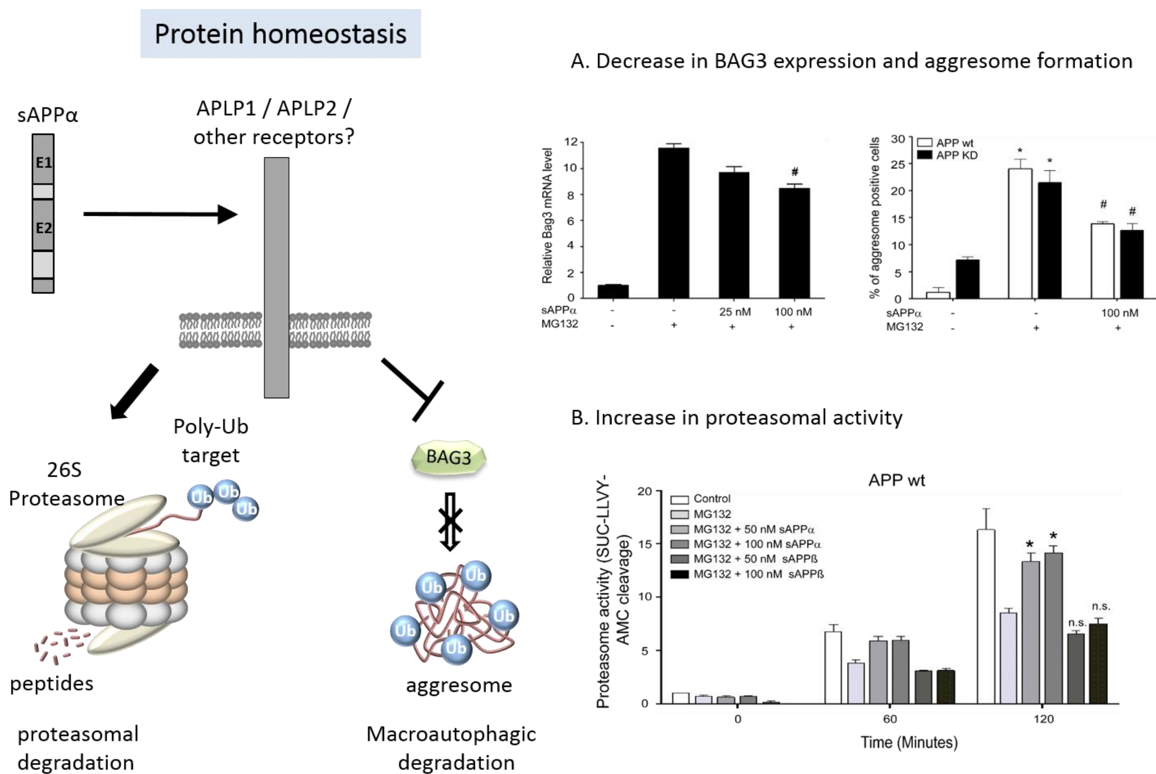


Figure 2. Role of sAPPα in protein homeostasis. (left) Hypothetical model of sAPPα mediated modulation of proteostasis. (right) selective results showing the effect of sAPPα treatment on (A) BAG3 expression, aggresome formation and (B) proteasomal activity.

In "Section 5 – Discussion" the role of sAPPα in neuroprotection and protein homestasis in the context of this study was discussed in details. Overall, an anti-apoptotic effect of sAPPα in presence of holo-APP on Akt downstream targets

such as FoxO, Bim, Bcl-xL, Mcl-1 was observed. The release of cytochrome c from mitochondria as observed during apoptosis was also shown to decrease with sAPPα treatment. In addition, this study demonstrates the effect of sAPPα on neuronal proteostasis under condition of proteasomal stress. The recombinant sAPPα significantly suppressed MG132 triggered expression of co-chaperone BAG3 and aggresome formation and also rescued proteasomal activity in dose dependent manner. These sAPPα induced changes were unaltered in APP deficient cells, suggesting that holo-APP may not be required for this particular function of sAPPα. Unlike sAPPα, sAPPβ failed to modulate BAG3 expression and proteasomal activity, which suggests that this function is specific for sAPPα.

Zusammenfassung

In “Abschnitt 1 – Introduction” wird der Leser in die Grundlagen von Morbus Alzheimer eingeführt, wobei ein spezieller Fokus auf dem Amyloid Precursor Protein (APP) liegt, welches bei dieser Krankheit involviert ist. In diesem Teil werden auch verschiedene Zelltodmechanismen wie Apoptose, Nekrose und der autophagische Zelltod diskutiert. Der Leser findet des weiteren eine kurze Übersicht verschiedener Stress-Signalwege, die bei neurodegenerativen Erkrankungen beteiligt sind, sowie Informationen über die Rolle von APP bei der Regulation der Zellüberlebens.

Morbus Alzheimer is eine chronische neurodegenerative Krankheit, die mit Gedächtnisproblemen, sowie verändertem Denken und Verhalten der Patienten einhergeht. Die pathophysiologischen Charakteristika von Morbus Alzheimer sind extrazelluläre senile Plaques und intrazelluläre neurofibrilläre Bündel. Amyloid Plaques bestehen hauptsächlich aus Amyloid-β (Aβ) Peptiden, welche ein Spaltprodukt von APP darstellen. APP ist ein Typ I-Transmembranprotein mit einer großen extrazellulären Domäne und einer kurzen zytoplasmatischen Domäne. APP wird in einer Reihe von Geweben exprimiert, z.B. im neuronalen Gewebe (Gehirn, Rückenmark, Retina) und nicht-neuronalen Gewebe (Leber, Lunge, Pankreas, Prostata und Schildrüse) (Dawkins and Small, 2014). APP wurde aufgrund seiner Verbindung zu AD untersucht. Seine Rolle bei normaler Gehirnfunktion ist jedoch noch nicht vollständig aufgeklärt. APP wird über zwei Hauptwege prozessiert, den sogenannten amyloidogenen Pathway und den nicht-amyloidogenen Pathway. Unter physiologischen Bedingungen wird der Großteil von APP über den nicht-amyloidogenen Weg prozessiert, wobei das sekretierte N-terminale APP Spaltprodukt sAPPα entsteht. sAPPα wird durch Spaltung von APP durch α-Sekretase gebildet. In vorangegangenen Studien konnte unsere Arbeitsgruppe zeigen, dass sAPPα durch Veränderungen der Genexpression und durch Antagonisierung verschiedener neurotoxischer Stress-Stimuli potente neuroprotektive Effekte entfaltet (Copanaki et al., 2010; Kögel et al., 2003, 2005; Milosch et al., 2014). Mehrere Studien konnten zeigen, dass die Proteindegradation bei Morbus Alzheimer gestört ist (Hong et al., 2014; Lipinski et al., 2010), doch die Rolle von APP und seiner Spaltprodukte bei der

Proteindegradation ist momentan unbekannt. Diese Dissertation widmet sich den physiologischen Funktionen von APP bei der Neuroprotektion und der Proteinhomöostase.

"Abschnitt 2 – Materialien" liefert Informationen zu den verwendeten Chemikalien, Zelllinien, Medien, Puffern etc., die in dieser Arbeit verwendet wurden.

In "Abschnitt 3 – Methoden" werden im Detail die verschiedenen biochemischen und zellbiologischen Methoden erläutert, die in dieser Arbeit verwendet wurden.

Im ersten Teil von "Abschnitt 4 – Results (4.1 - 4.4)", wurden die neuroprotektiven Eigenschaften von aufgereinigtem sAPPα und E1 (N-terminale Subdomäne von sAPPα, beide in *Pichia pastoris* exprimiert) unter Serum- und Glukoseentzug untersucht. Es konnte gezeigt werden, dass rekombinantes sAPPα einen signifikante Abnahme der durch Serumentzug ausgelösten Apoptose in humanen SH-SY5Y Neuroblastomzellen und murinen embryonalen Fibroblasten (MEF) bewirkte. Des weiteren wurde in Vorarbeiten beobachtet, dass durch sAPPα eine Phosphorylierung der Überlebenskinase Akt induziert wurde, deren Aktivierung zur Neuroprotektion führt (Milosch et al., 2014). In dieser Arbeit wurde untersucht, ob diese Neuroprotektion mit einer veränderten Expression von stromabwärts gelegenen intrazellulären Akt-Targets wie FoxO, Bim, Bcl-xL und Mcl-1 unter Stressbedingungen assoziiert ist (Abbildung 1). Es konnte gezeigt werden, dass sAPPα die Aktivierung und Kerntranslokation von FoxO verhindert. FoxO wirkt als Transkriptionsfaktor für verschiedene pro-apoptotische Gene wie *Bim* und es wurde beobachtet, dass die Protein- und mRNA Expression von Bim signifikant durch Behandlung mit sAPPα und E1 supprimiert wurde. Die Expression anti-apoptotischer Proteine wie Bcl-xL und Mcl-1 wurde ebenfalls untersucht und es konnte gezeigt werden, dass sAPPα und E1 die Expression beider Proteine aktiviert. Des weiteren wurde in Vorarbeiten demonstriert, dass ungespaltenes holo-APP funktionell mit sAPPα integagiert, um Akt und die Neuroprotektion zu aktivieren (Milosch et al., 2014). Um die mögliche Funktion von holo-APP bei der sAPPα-vermittelten Regulation von Akt Targets zu untersuchen, wurden APP-defiziente Zellen (MEF APP KO) verwendet. In der Tat bewirkten E1 und sAPPα ihre neuroprotektiven Effekte auf Akt Targets nur in MEF wt Zellen, nicht jedoch in APP KO Zellen. Außerdem konnten die durch sAPPα vermittelten

neuroprotektiven Effekte in primären hippokampalen Neuronen (APP Wildtyp) nach Entzug von trophen Faktoren bestätigt werden. Des weiteren wurden Fraktionierungsexperimente durchgeführt, um die Rolle von sAPPα bei der Regulation der Freisetzung von Cytochrom C aus den Mitochondrien zu analysieren. Es wurde beobachtet, dass die Behandlung mit sAPPα die Freisetzung von mitochondrialem Cytochrom C in MEF Wildtyp-Zellen verhindert.

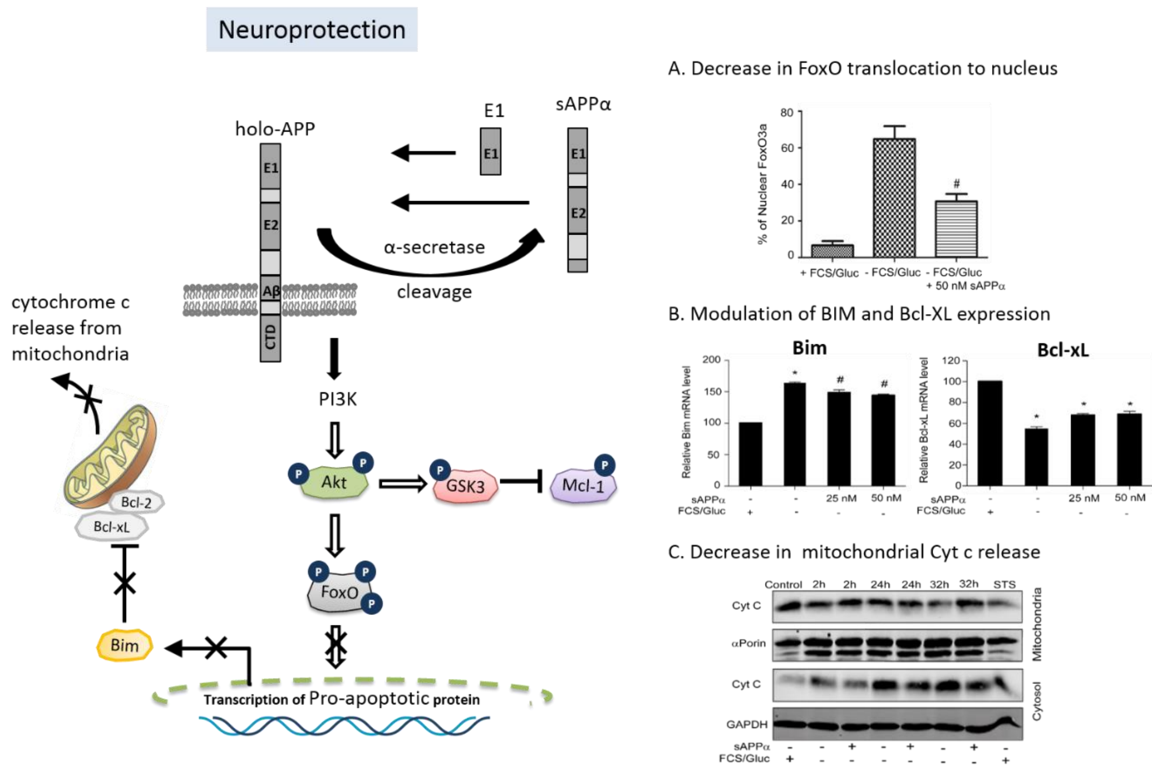


Abbildung 1. Neuroprotektive Funktion von sAPPα. (links) Hypothetisches Model von APP/sAPPα vermittelter Neuroprotektion. (rechts) Ausgewählte Ergebnisse zeigen den Effekt von sAPPα-Behandlung auf (A) Zellkern Translokation von FoxO, (B) pro-apoptotische Bim und anti-apoptotische Bcl-xL Expression und (C) mitochondriale Freisetzung von cytochrom c.

Im zweiten Teil von “Abschnitt 4 – Results (4.5 - 4.9)” wird die Rolle von sAPPα bei der Proteinhomöostase diskutiert (Abbildung 2). Es wurde beobachtet, dass sAPPα die durch proteasomalen Stress induzierte BAG3 Proteinexpression in SH-SY5Y und MEF Zellen inhibiert. Dieses Phänomen wurde auch auf mRNA-Ebene detektiert, womit eine transkriptionelle Regulation gezeigt wurde. Die Behandlung mit sAPPα zu einer Reduktion von Aggresomen. Aggresomen sind perinukleare Aggregate, die durch Anreicherung von geschädigten und nicht korrekt gefalteten Proteinen geformet warden. BAG3 spielt eine wichtige Rolle bei der Bildung von

Aggresomen und bei dem Transport von degradationsanfälligen Proteinen in diese Strukturen. Die Analyse der proteasomalen Aktivität zeigte eine sAPPα-vermittelte Reduktion der Akkumulation des Proteasomensubstrats d2 unter proteasomalem Stress. In proteasomalen Aktivitäts-Assays, bei denen das fluorigene Signal spektrophotometrisch und quantitativ ausgewertet wurde, war sAPPα in der Lage, die Degradation des Proteasomensubstrats SUC-LLVY-AMC signifikant zu induzieren. Das sAPPβ Fragment, das über den amyloidogenen Pathway gebildet wird, wurde ebenfalls im Hinblick auf BAG3 Expression und die proteasomale Degradation untersucht. sAPPβ, welches eine sehr ähnliche Struktur wie sAPPα aufweist (nur 17 Aminosäurereste am C-Terminus von sAPPα fehlen), war hingegen nicht in der Lage, die BAG3 Expression und die Proteostase zu modulieren. Diese Daten zeigen, dass die beobachteten Effekte spezifisch für sAPPα sind.

Die beschriebenen sAPPα-induzierten Veränderungen der Proteinhomöostase waren in APP-depletierten SH-SY5Y Zellen und APP-defizienten Fibroblasten und primären hippokampalen Neuronen unverändert. Diese Beobachtungen zeigen, dass holo-APP für diese spezielle Funktion von sAPPα nicht notwendig ist. Die beiden anderen APP-Familienmitglieder APLP1 und APLP2, die verschiedene Funktionen von APP kompensieren können (Anliker and Müller, 2006), könnten an der Modulation der Proteinhomöostase durch sAPPα beteiligt sein. sAPPα könnte diese Effekte außerdem über andere Rezeptoren vermitteln, wie z.B. den "sorting protein related receptor" SORLA (Hartl et al., 2013) oder mittels alternativer Mechanismen wie die sAPPα-Internalisierung.

In “Abschnitt 5 – Discussion” wird die Rolle von sAPPα bei der Neuroprotektion und Proteinhomöstase im Kontext mit dieser Studie im Detail erörtert. Zusammenfassend wurde ein anti-apoptotischer Effekt von sAPPα in Anwesenheit von holo-APP auf Akt Targets wie FoxO, Bim, Bcl-xL und Mcl-1 beobachtet.

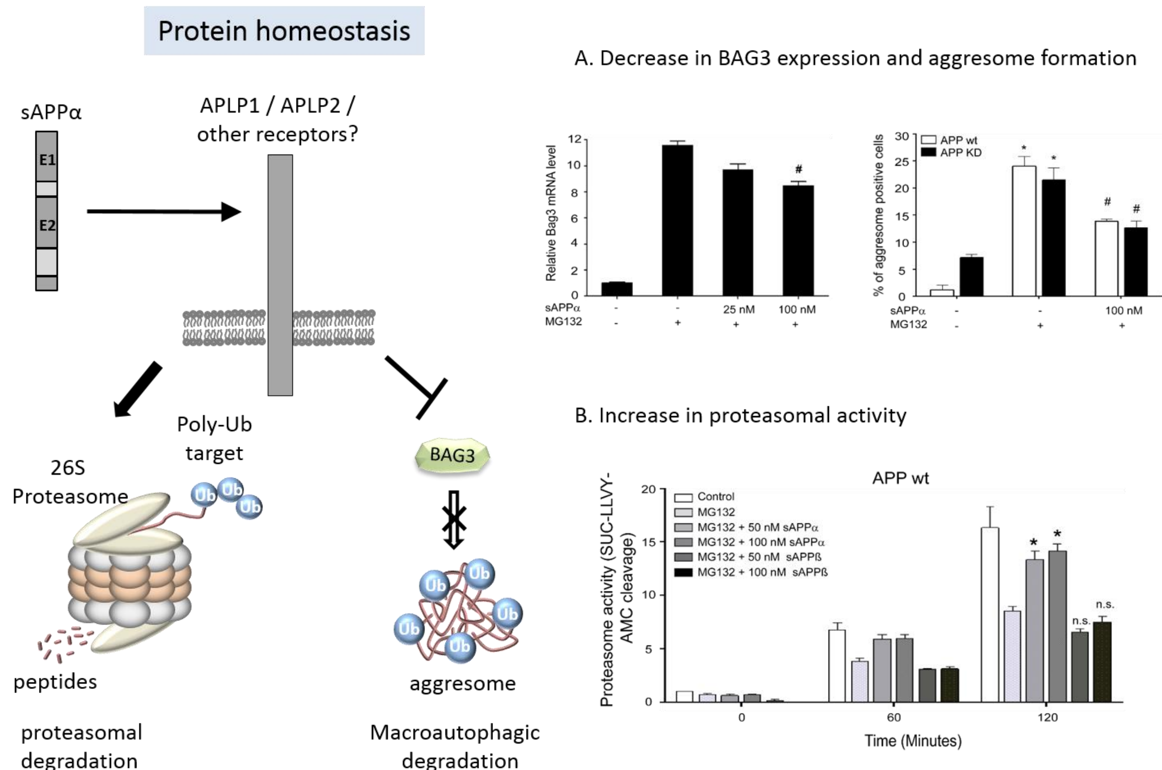


Abbildung 2. Bedeutung von sAPPα in der Protein Homöostase. (links) Hypothetisches Model von sAPPα vermittelter Proteostase. (rechts) Ausgewählte Er-gebnisse zeigen den Effekt von sAPPα-Behandlung auf (A) BAG3 Expression, Aggreosom-Bildung und (B) proteosomale Aktivität.

Die Freisetzung von Cytochrom C aus den Mitochondrien während der Apoptose wurde durch die Behandlung mit sAPPα vermindert. Zusätzlich zeigt diese Arbeit einen Effekt von sAPPα auf die neuronale Proteostase unter Bedingungen von subtoxischem proteasomalem Stress. Rekombinantes sAPPα konnte dosisabhängig die MG132-getriggerte Expression des Cochaperons BAG3 und die Aggresomenbildung signifikant supprimieren, die proteasomale Aktivität hingegen partiell wiedeherstellen. Diese sAPPα-induzierten Veränderungen waren in APP-defizienten Zellen unverändert, wodurch nahegelegt wird, dass holo-APP für diese spezielle Funktion von sAPPα nicht benötigt wird. Im Gegensatz zu sAPPα war sAPPβ nicht in der Lage, die BAG3 Expression und die proteasomale Aktivität zu modulieren, so dass diese spezifische Funktion spezifisch für sAPPα zu sein scheint.

1 Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible, progressive, neurodegenerative brain disorder. This is the most common form of dementia, and currently millions of people worldwide are affected by this disease. Around 46.8 million people worldwide are affected by AD and due to higher life expectancy in civilized countries this number is dramatically increasing (Prince et al., 2015). The symptoms of this age related and currently incurable form of dementia includes a progressive decline of cognitive abilities, memory loss, mood swings, and communication problems. Early symptoms include short time memory loss which eventually worsens and finally death occurs due to impairment of different body functions and secondary diseases (Förstl and Kurz, 1999; Shankar and Walsh, 2009). In 1906, German psychiatrist Alois Alzheimer first identified and described the abnormality of the brain in a dementia patient. Neuropathological hallmarks of the disease that Alzheimer found in his patient's brain after her death included massive brain atrophy, neurodegeneration, senile plaques and neurofibrillary tangles (Figure 3; Alzheimer, 1907). The major component of these plaques is 40-42 amino acids long peptides known as β-amyloid (Aβ) (Glenner and Wong, 1984). Aβ peptides are derived by proteolytic cleavage of amyloid precursor protein (APP) (Kang et al., 1987).

There are two types of AD. The predominant form of AD is late onset AD (LOAD) or the sporadic form of AD occurs after the age of 65. The other one is early onset AD (EOAD) or familial AD (FAD) occurs between the ages of 40-65. Approximately 5-10% of AD are FAD which are caused by an autosomal dominant mutation in the genes encoding APP and two homologues molecules presenilin 1 (PS1) and presenilin 2 (PS2). 16 mutations in the *APP* gene on chromosome 21, 130 mutations of *PS1* gene on chromosome 14 and 10 mutations of *PS2* gene on chromosome 1 were identified (Rocchi et al., 2003; Shioi et al., 2007). If mutation occurs in the vicinity of β-secretase cleavage site such as Swedish mutation at amino acid 670/671, a higher production of Aβ occurs (Citron et al., 1992; Suzuki et al., 1994). In other cases, such as mutation in the γ-secretase cleavage site,

e.g. Florida-mutation at amino acid position 716, increases Aβ42 vs Aβ40 production (Rocchi et al., 2003). PS1 and PS2 are important component of a high molecular weight complex required for γ-secretase processing of APP. Mutation in these genes alter γ-secretase activity which in turn alters the proteolytic processing of APP (De Strooper and Annaert, 2000). In sporadic cases, the risk factors are primarily old age, plasma cholesterol level and diseases such as diabetes (Dosunmu et al., 2007; Jorm and Jolley, 1998; Toro et al., 2009). Genetic polymorphism in the gene for Apolipoprotein E4 (*ApoE4*) has also been identified as one of the risk for developing late onset AD (Turner et al., 2003).

The main pathophysiological hallmarks of AD are the formation of intracellular neurofibrillary tangles (NFTs) and the deposition of extracellular senile plaques. The main component of NFTs is paired helical filaments (PHFs), which consist of aggregated, abnormally phosphorylated microtubule associated protein (MAP) Tau (Delacourte and Defossez, 1986). Under physiological condition Tau is essential for axonal growth and development and play important role in axonal transport (Alonso et al., 2001). In normal cell, Tau stabilizes the cytoskeleton by interacting with tubulin which is controlled by its phosphorylation state (Mandelkow and Mandelkow, 2012; Weingarten et al., 1975). However, in AD, hyperphosphorylated Tau proteins forms tangles that are deposited within neurons located in the hippocampus and medial temporal lobe, the parietotemporal region, and the frontal association cortices. This deposition disrupts axonal transport which finally leads to neurodegeneration (Garcia and Cleveland, 2001). Besides NFTs, formation of extracellular senile plaques, which consist of Aβ peptide, is other pathophysiological characteristic of AD (Glenner and Wong, 1984). Overproduction of this peptide leads to self-aggregation in insoluble oligomers, which further accumulates and forms insoluble extracellular plaques and show synaptotoxicity, proteosomal inhibition and stimulation of inflammation in the brain (Hardy and Selkoe, 2002).

The first clinical manifestation of AD is memory loss. With the progression of the disease, patients experience greater memory loss and other cognitive difficulties. In the next stage, damage occurs in the brain areas that control language, reasoning, sensory processing, and conscious thought, therefore patients find

difficulty during conversation; they also experience difficulties to learn new things, carry out multistep tasks. Other signs and symptoms of the disease include psychiatric and behavioral disturbances (Alzheimer's Disease Fact Sheet, www.nia.nih.gov; McKhann et al., 1984). AD can be clinically determine by finding impaired activities of daily life, past medical problems, loss of specific cognitive functions such as language and motor skills (McKhann et al., 1984). Imaging techniques such as magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) are also used to diagnosis AD (Herholz, 1995; Scheltens, 1999). The current standard therapy against AD is the treatment with acetylcholinesterase inhibitors. In AD patient the level of acetylcholine is low due to diminished production of choline acetyl transferase. These inhibitors temporarily slowdown the loss of cognitive function by increasing the amount of free acetylcholine in the brain of AD patients. But these inhibitors could not cure or arrest the progression of dementia (Birks and Harvey, 2006; Pohanka, 2011). The noncompetitive NMDA receptor antagonist Memantine is also used for treatment of moderate to severe AD; it acts through inhibition of excitotoxic glutamate (Areosa Sastre et al., 2004). Furthermore, recent strategies to develop disease-modifying treatments against AD include the prevention of Aβ formation by vaccines, antibodies and inhibitors or modulators of γ- and β-secretase to modify APP cleavage. Developing agents directed against the tau protein as well as compounds such as serotoninergic 5-HT6 and histaminergic H3, acting as antagonists of neurotransmitter systems can also be used to modify AD (Godyń et al., 2016). The strategies are promising; but they need to be thoroughly investigated through several experiments and clinical trials before optimized for use as drugs in humans.

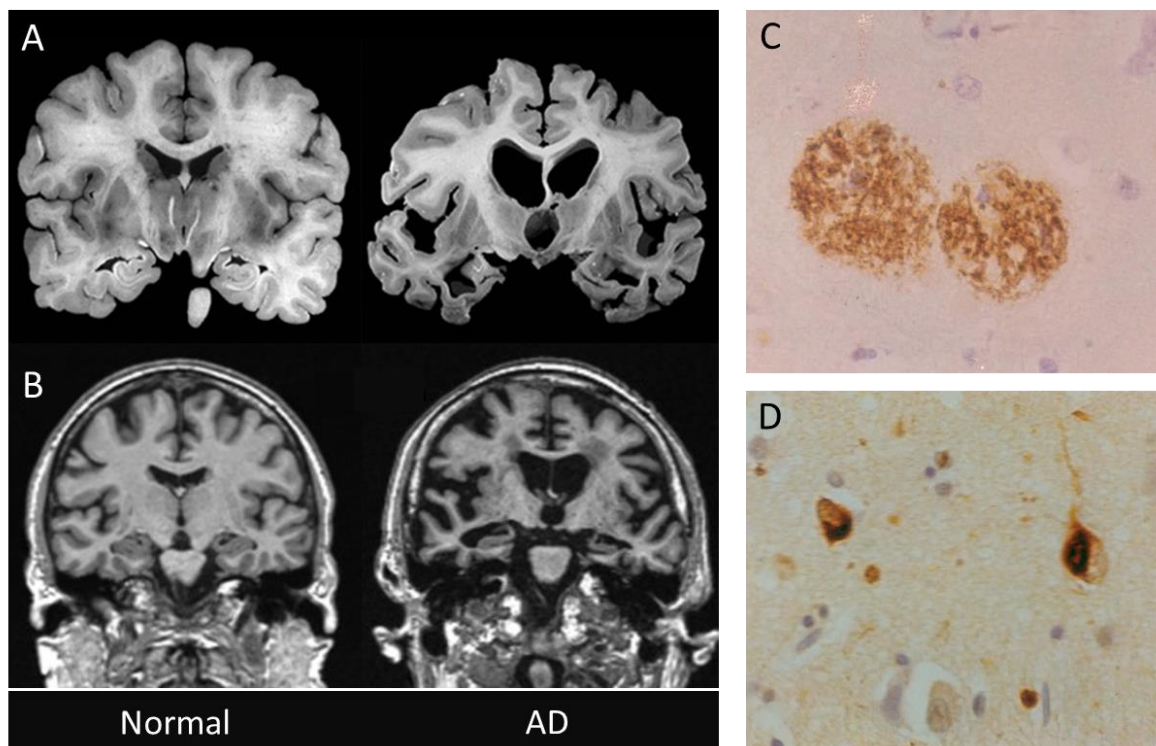


Figure 3. Alzheimer's disease brain. A. Healthy vs Alzheimer's disease (Wagoner, 2015); AD brain shows cortical atrophy and enlarged ventricle size, B. fMRI image of healthy vs Alzheimer's disease brain (Vemuri and Jack, 2010), C. Senile plaque formation which contains fibrilized Aβ (Armstrong, 2009), D. Intracellular neurofibrillary tangles (NTFs) consists of hyperphosphorylated Tau (Armstrong, 2009).

1.1.1 APP family members

The amyloid precursor protein (APP) and its family members are highly conserved type-I transmembrane protein family, which consists of APP, and the mammalian homologues APP-like Protein 1 and 2 (APLP1 and APLP2) as well as APPL in Drosophila and APL-1 in *C. elegans*. In humans, the *APP* gene is encoded on chromosome 21, APLP1 on 19, and APLP2 on 11(Zheng and Koo, 2006). APLP1 and APLP2 share a high sequence homology with APP, except the lack of an Amyloid β (Aβ) domain (Figure 4; Müller and Zheng, 2012; Walsh et al., 2007). Mammalian APP and APLP2 are expressed ubiquitously however APLP1 and Drosophila APPL expression are restricted to neurons (Müller and Zheng, 2012; Zheng and Koo, 2006). Single knock out of APP, APLP1, or APLP2 in mice caused only minor neurological abnormalities, whereas double knock out of APP/APLP2 or APLP1/APLP2 and triple knock out of APP/APLP1/APLP2 showed lethal phenotype (Heber et al., 2000; Herms et al., 2004; Zheng et al., 1995). In

contrust, APP/APLP1 knock out mice are viable (Heber et al., 2000). These studies indicate that different APP family members can compensate for each other.

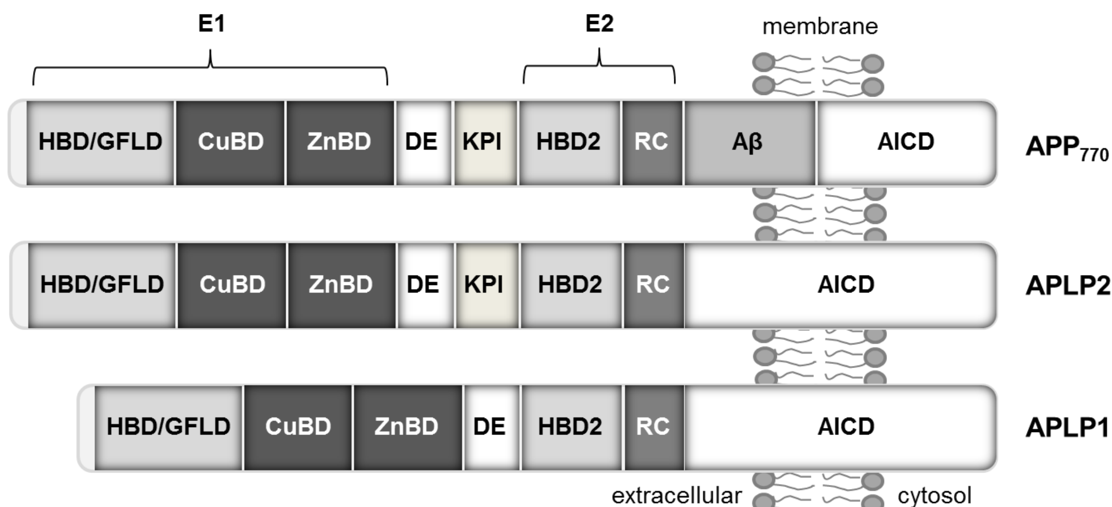


Figure 4. Schematic overview of APP family members. APP family members share a large sequence similarity: All APP family members contain a heparin binding domain/growth factor like domain (HBD / GFLD), copper binding domain (CuBD), zinc binding domain (ZnBD), acidic region (DE), random coil region (RC) and the APP intracellular domain (AICD). The E1 domain contains HBD / GFLD, CuBD and ZnBD. The E2 domain consists of another heparin binding domain (HBD2) and RC. The Kunitz-type protease inhibitor domain (KPI) is also shared by APP and APLP2, but not by APLP1. Aβ sequence is specific for APP. Modified from (Jacobsen and Iverfeldt, 2009).

1.1.2 APP structure

APP protein has a conserved structure with a large extracellular N-terminal domain and a short C-terminal cytoplasmic tail (Zheng and Koo, 2011). Uncleaved APP is located at the cell surface within cholesterol rich microdomains (lipid rafts) (Ehehalt et al., 2003); it is also present in the endoplasmic reticulum (ER), Trans-Golgi network (TGN) and in the membrane of mitochondria (Anandatheerthavarada et al., 2003; Hartmann et al., 1997; Selkoe, 1996). APP is encoded by a single gene of 18 exons; however, alternative splicing of APP mRNA generates several isoforms which range from 365 to 770 amino acids residues (Zheng and Koo, 2011). Three major isoforms of mammalian APP are APP695, APP751 and APP770. APP751 and APP770 contain a domain in the extracellular sequence which is homologues to the Kunitz-type serine protease inhibitors (KPI), and these are expressed in most tissues (Zheng and Koo, 2011).

APP695 lacks the KPI and OX-2 related domain and it is predominantly expressed in neurons (Sandbrink et al., 1994; Wertkin et al., 1993; Zheng and Koo, 2011). APP consists of a growth factor like domain (D1) with heparin and collagen binding properties, a zinc /copper binding domain (D2), and an acidic region (D3), a Kunitz protease inhibitor (KPI) domain (D4), an OX-2 related domain (D5), a carbohydrate domain (D6), and an intracellular domain (Figure 5; Corrigan et al., 2011). D1 and D2 domains together are referred as E1 domain (Soba et al., 2005).

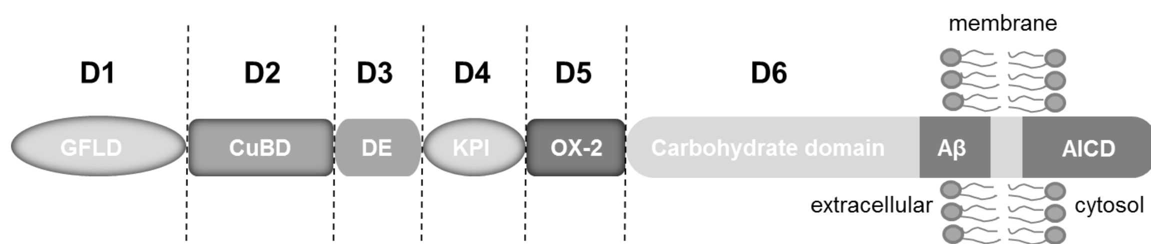


Figure 5. Different domains of APP. APP consists of a growth factor like domain (D1) with heparin and collagen binding properties, a zinc /copper binding domain (D2), and an acidic region (D3), a Kunitz protease inhibitor (KPI) domain (D4), an OX-2 related domain (D5), a carbohydrate domain (D6), and an intracellular domain. D1 and D2 domains together are referred as E1 domain.

1.1.3 Biochemical processing of APP

APP is processed by constitutive secretary pathway and post-translationally modified by N- and O-glycosylation (Haass et al., 2012; De Strooper and Annaert, 2000). Three proteinases termed α-, β-, and γ-secretases are involved in the sequential processing of full length APP (Zheng and Koo, 2011). There are two major pathways of APP processing, non-amyloidogenic pathway and amyloidogenic pathway (Figure 6; Jacobsen and Iverfeldt, 2009). Under physiological condition, the majority of APP is processed by non-amyloidogenic pathway. In this pathway, APP is initially cleaved by α-secretase within the Aβ sequence near the ectoplasmic side of the plasma membrane, which releases the neuroprotective N-terminal soluble ectodomain sAPPα and the membrane bound carboxyterminal fragment (CTF) C83. C83 is further processed by γ-secretase giving rise to a small 3 kD peptide p3 and the APP intracellular domain AICD (Jacobsen and Iverfeldt, 2009; Kögel et al., 2012a; Müller et al., 2008). In amyloidogenic pathway APP first cleaved by β-secretase, which produces

N-terminal ectodomain sAPPβ and a C-terminal fragment C99. C99 fragment is further processed by γ-secratase, generating Aβ40/42 or other Aβ species depending on the exact cleavage position and liberates the AICD (Wagner et al., 2014; Winkler et al., 2012; Zheng and Koo, 2006). Neuronal β-secretase enzyme BACE1 (β-site APP cleavage enzyme, also called Asp-2 and memapsin-2) is a transmembrane aspartyle protease (Vassar et al., 2014). BACE1 and γ-secretase cleavage produces different species of Aβ. The most toxic form is Aβ42, although it is produced at much lower level but it can aggregate faster by building fibrils due to its hydrophobic properties (Hardy and Selkoe, 2002; Shi et al., 2014). α-secretase is a zinc metalloproteinases that cleaves APP at Lys613-Lys614 bond within the Aβ domain and thereby inhibits the formation of Aβ and releases neuroprotective sAPPα (Kögel et al., 2012b; Roberts et al., 1994). This enzyme is a member of ADAM (a disintegrin and metalloproteinase) family. Two main α-secretases are ADAM10 and ADAM17, they are considered to have physiologically relevant α-secretase activity (Endres and Fahrenholz, 2012; Kuhn et al., 2010; Del Turco et al., 2014).

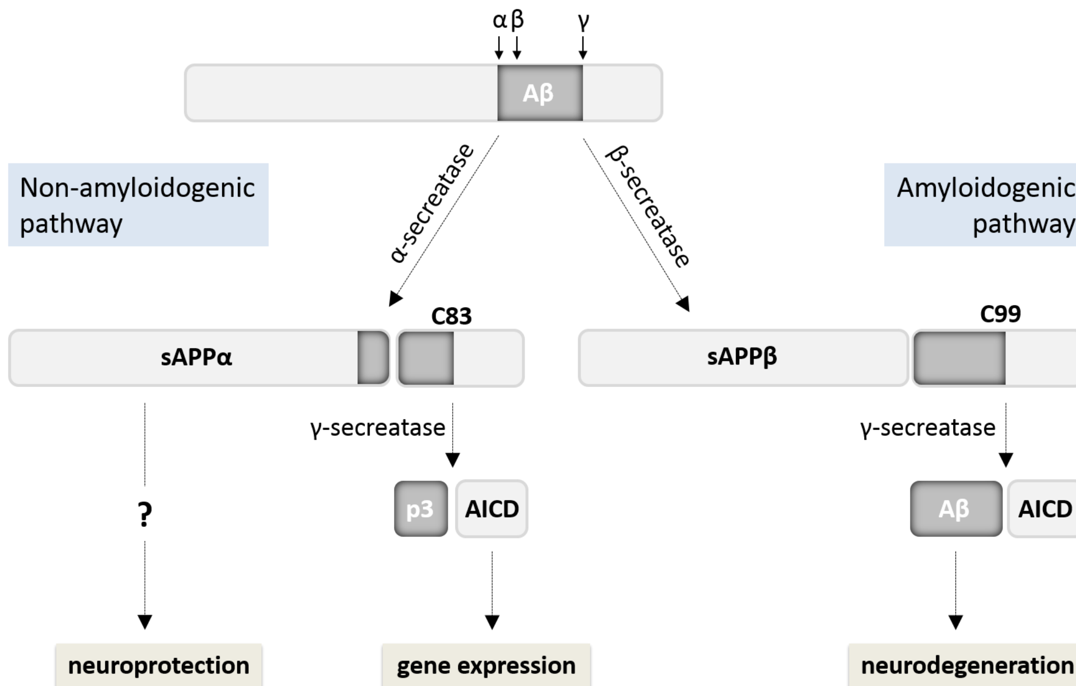


Figure 6. APP processing. The amyloid precursor protein is processed by three secretases (α, β, γ). Non-amyloidogenic pathway (left): α-secretase cleaves APP within Aβ sequence and generates soluble sAPPα and membrane-bound C83. γ-secretase cleaves C83 peptide into p3 and AICD. Amyloidogenic pathway (right): proteolysis by β-secretase releases sAPPβ and C99. Upon further cleavage of C99 by γ-secretase the APP intracellular domain (AICD) and the toxic Aβ fragment are liberated. Modified from (Zheng and Koo, 2006).

1.1.4 APP function

APP is the precursor protein for Aβ peptides, which forms the extracellular amyloid plaques in the brain of AD patients. However, several in vitro and in vivo studies have provided considerable amount of evidences for diverse physiological roles of APP both in the developing and adult nervous system. APP play important role in cell adhesion, neuronal survival, neuronal outgrowth, vesicular transport, neuronal migration, modulation of synaptic plasticity and synaptogenesis (Guo et al., 2012; Jacobsen and Iverfeldt, 2009).

One of the key physiological functions of APP is neuroprotection. In various experimental studies, APP and sAPPα were found to exert neuroprotective effects in vitro (Kögel et al., 2012a). APP expression was upregulated following traumatic brain injury (Corrigan et al., 2014; Van den Heuvel et al., 1999; Murakami et al.,

1998) and treatment with sAPPα was shown to decrease neuronal cell death and also had beneficial effects on cognitive and motor outcomes (Corrigan et al., 2012; Thornton et al., 2006). A number of studies have implicated the role of APP and sAPPα in proliferation of embryonic neural stem cells (NSCs). sAPPα bears a cysteine-rich domain that resembles domains in other growth factors, which suggests that sAPPα might function as a growth factor to stimulate intracellular signaling (Nicolas and Hassan, 2014).

According to Mattson et al. sAPPα mediated neuroprotection may be associated with rapid effects on ion channel function (Mattson et al., 1997). sAPPα was proposed to inhibit Ca^{2+} overloading by suppressing NMDA (*N*-Methyl-D-aspartic acid) currents and activating potassium channels, and thereby protecting neurons against excitotoxic stress (Camandola and Mattson, 2011; Furukawa and Mattson, 1998; LaFerla, 2002). However, it was observed that sAPPα mediated neuroprotection was only achieved by incubation with sAPPα over several h. These suggest that a prolonged activation of cell survival signaling pathways is necessary for sAPPα mediated neuroprotection (Kögel et al., 2012a; Milosch et al., 2014).

Several studies suggested that APP could act as a cell surface receptor similar to Notch (Selkoe and Kopan, 2003). Aβ peptide could bind to APP and serves as a candidate ligand for it (Lorenzo et al., 2000). Another studies provided evidences that the extracellular domain of APP could bind to F-spondin (Ho and Südhof, 2004) and Nogo-66 receptor which in turn affect the Aβ production and downstream signaling (Park, 2006). APP interacts with several adaptor proteins, including Fe65, JIP, Grb2, Dab1, and Numb through the YENPTY motif in the intracellular domain. This interaction depends on the phosphorylation of the intracellular domain of APP, either at Thr668 or Tyr682 (Jacobsen and Iverfeldt, 2009). Interaction with Fe65 affect APP trafficking and metabolism. This interaction also increases Aβ and sAPPα secretion. Gralle et al. suggested that enforced APP dimerization by APP N-terminal antibody 22C11 induces apoptosis. They also hypothesized that this homo-dimerization of APP could be disrupted by competitive binding of sAPPα to APP which is also necessary for sAPPα-mediated neuroprotection (Gralle et al., 2009). APP is able to form homo and

heterodimers with APLP1 and APLP2, which promotes cell-cell adhesion (Soba et al., 2005).

According to the findings mentioned above, loss of APP function may reduce neuronal survival, increased apoptosis, impaired neurite outgrowth, which provided evidences of prosurvival and neuroprotectiove functions of APP (Zheng et al., 1995). Despite these observations, the cellular receptor involved, the exact molecular mechanism and the intracellular downstream targets of sAPPα-dependent neuroprotective signaling is still unclear (Kögel et al., 2012a; Milosch et al., 2014).

1.2 Cell death

Cells are members of highly organized multi-cellular organism. The development and longevity of these multi-cellular organisms are tightly regulated by controlling the rate of cell division as well as by controlling the rate of cell death. Cell death is a biological event to remove no longer needed tissues or cells during development (e.g. sculpting of mouse paw, cell death in developing nervous system). It also removes damaged or mutated cells, and degenerated cells to prevent toxic effects on the organism (Alberts et al., 2002). Different type of cell death (apoptotic, necrotic, autophagic) is classified depending upon the biological mechanism and morphological appearance of the cells (Galluzzi et al., 2007; Kroemer et al., 2009).

1.2.1 Apoptosis

Apoptosis is a highly organized form of cell death that is important during development, tissue homeostasis, and elimination of damaged cells. Apoptosis eliminates unwanted cells from the body during development. In adult tissues, apoptosis helps to balance cell division. It is an active (requires energy/ATP) and highly regulated form of cell death, morphologically characterized by cell shrinkage, chromosome condensation, DNA fragmentation, membrane blebbing, and finally the formation of apoptotic bodies (Okouchi et al., 2007; Rami and Kögel, 2008; Skulachev, 2006). Apoptosis is induced by different stress stimuli,

such as DNA damage, reactive oxygen or nitrogen spices, nutrient or trophic factor deprivation (Okouchi et al., 2007).

One of the main biological features of apoptotic cell is the outward movement of the normal inward-facing phosphatidylserine of the lipid bilayer, on the outer layers of the plasma membrane (Galluzzi et al., 2012). This phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell which stimulates macrophages to engulf and digest the dying cells (Kroemer et al., 2005).

The intracellular machinery responsible for apoptosis depends on a family of proteases called caspases (cysteine aspartate-directed proteases) that have a cysteine residue at their active site and cleave their target proteins at aspartate residues. Ten major caspases have been identified and broadly categorized into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7) and inflammatory caspases (caspase 1, 4, 5) (Cohen, 1997; Rai et al., 2005). Caspases are synthesized in the cell as inactive precursors, or procaspases, which are activated by proteolytic cleavage (Würstle et al., 2012). Apoptotic stimuli first activates initiator procaspases (caspase 2, 8, 9, and 10) and active initiator caspases further cleave and activate downstream executioner procaspases (caspase 3, 6, and 7) (Fischer et al., 2003).

There are two main apoptotic signaling pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 7). In extrinsic pathway, procaspase activation is triggered from outside of the cell by activation of death receptors on the cell surface (D'Amelio et al., 2010). Upon activation by stress-inducing ligands such as Fas ligand (FasL), death receptors of the TNF/NGF super family, such as TRAIL, Fas, TNF-R1 are clustered to form death domains (de Almagro and Vucic, 2012; Gupta, 2001). These death domains are formed in the cytoplasomic tails of the receptors that interact with the adaptors protein such as FADD (Fas-Associated protein with Death Domain), forming a death receptor-induced signaling complex (DISC). DISC triggers the activation of downstream executioner procaspases. Furthermore, the extrinsic pathway can also initiates the cleavage of a pro-apoptotic BH3-only protein Bid that in turn

engages mitochondrial apoptotic signaling (D'Amelio et al., 2010; Okouchi et al., 2007).

When cells are stressed or damaged by different stress conditions, such as reactive oxygen species (ROS), radiation etc., they can also kill themselves by triggering intrinsic pathway of apoptosis. In this pathway, mitochondria are induced to release the electron carrier protein cytochrome c, which further binds and activates an adaptor protein called Apaf-1 (apoptosis protease activating factor-1). This activated Apaf-1 together with cytochrome c and other cofactors forms the apoptosome (Bao and Shi, 2007). Within this apoptosome activation of initiator procaspase 9 occurs, which further activates downstream executioner procaspases (Guégan et al., 2001; Pop et al., 2006).

The Bcl-2 (B-cell lymphoma 2) family of intracellular proteins helps to regulate the activation of procaspases. They have either pro- or anti-apoptotic functions. They control the release of cytochrome c from the mitochondria and bind to each other in various combinations to form homo- and heterodimers (Okouchi et al., 2007). Some members of the family like Bcl-2, Bcl-xL and Mcl-1 inhibit apoptosis, hence they are antiapoptotic proteins. They are located on the mitochondrial membrane and inhibit cytochrome c release and apoptosis. Other member of the family like Bax and Bak are proapoptotic and promote apoptosis. They trigger cytochrome c release from the mitochondria. BH3 only proteins (Bad, Bim, Bid, Puma, and Noxa) also have pro-apoptotic characteristics, inhibiting the activity of anti-apoptotic Bcl-2 proteins (Kurokawa and Kornbluth, 2009). Another important family of anti-apoptotic protein is the member of the IAPs (Inhibitor of apoptosis) family which are endogenous caspase inhibitors (Rami and Kögel, 2008). Caspase activation is antagonized by IAPs, which in turn can be inhibited by Smac/DIABLO proteins released from intermembrane space of damaged mitochondria (D'Amelio et al., 2010; Fuchs and Steller, 2011).

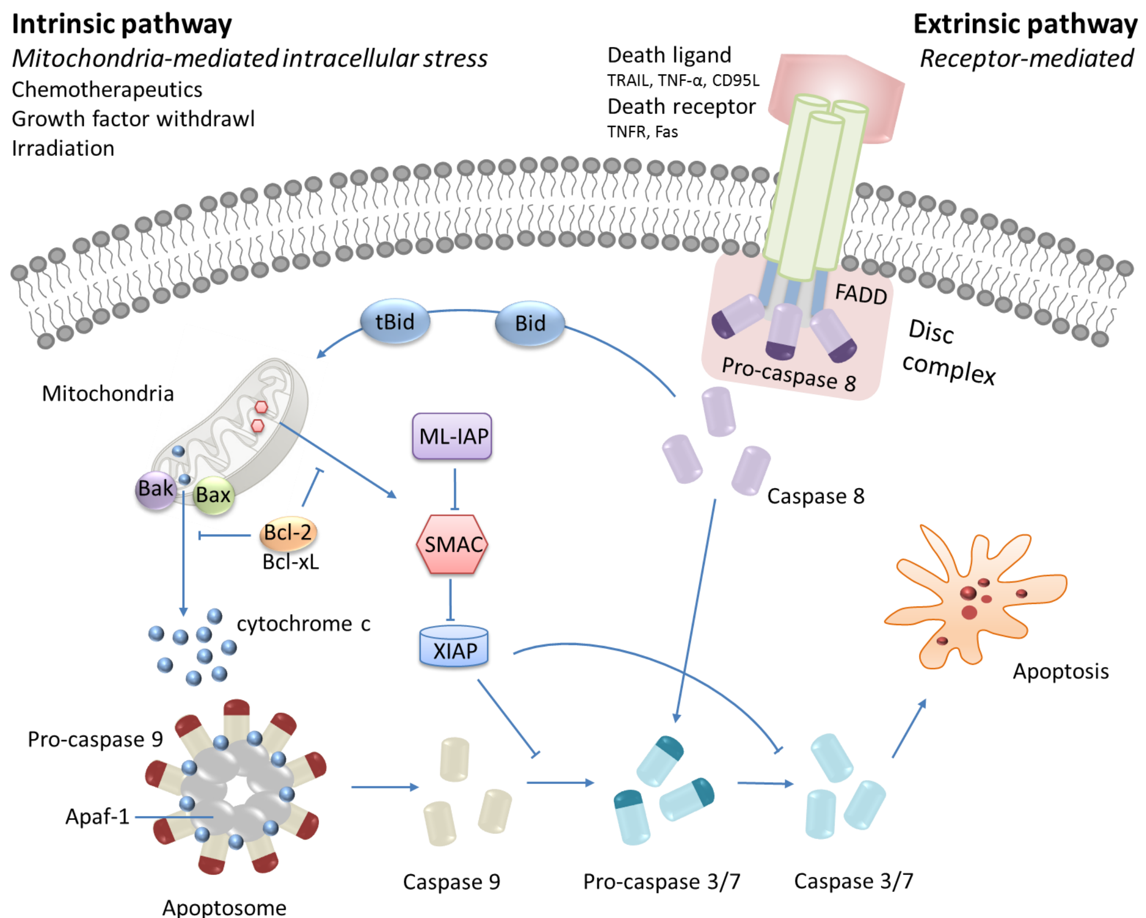


Figure 7. Extrinsic and intrinsic pathways of apoptosis. Apoptotic cell death can be induced through the extrinsic (receptor-mediated) or the intrinsic (mitochondria-mediated) signalling pathways. The extrinsic pathway involves death receptors activation by their ligands resulting in a sequential activation of caspase-8, and -3/7, which cleaves target proteins leading to apoptosis. This pathway is controlled by the anti-apoptotic protein XIAP that regulates activation of caspases. Intrinsic death stimuli, e.g. chemotherapeutics, growth factor withdrawl, or irradiation directly or indirectly activate the mitochondrial pathway by inducing release of cytochrome c and formation of the apoptosome, composed of Apaf-1 and caspase-9. Caspase-9 is activated at the apoptosome and, in turn, activates pro-caspase-3. This death pathway is largely controlled by the proapoptotic (e.g. Bax, Bak, Bid and Smac/DIABLO) and anti-apoptotic (e.g. Bcl-2, Bcl-xL and XIAP) proteins. Caspase-8 may also induce cleavage of Bid, which induces the translocation of Bax and/or Bak to the mitochondrial membrane and amplifies the mitochondrial apoptosis pathway. Modified from (de Almagro and Vucic, 2012).

1.2.2 Necrosis

Necrosis is a passive, non-physiological, unregulated pathological form of cell death usually occurs after traumatic injury or infection. Apoptotic cell death is replaced by necrosis under severe ATP depletion. Necrosis is characterized by chromatin clumping and swelling of intracellular organelles followed by disintegration of cell organelles and membranes. During necrosis outer cellular

membrane is disrupted and the intracellular components are released into the intercellular space, resulting in an inflammation (Okouchi et al., 2007). Macrophages later eliminate these debris and exacerbate the inflammation by producing pro-inflammatory factors such as TNFα, which in turn trigger apoptosis (Khandelwal et al., 2011; Vakkila and Lotze, 2004).

Some studies indicate that certain cells can adopt a regulated form of necrosis called necroptosis (Okouchi et al., 2007). Under apoptotic deficient conditions, necroapoptosis is activated by ligands of death receptors and stimuli that induce the expression of death receptor ligands. This form of cell death is regulated by the kinase activity of RIP1, which further mediates the activation of two critical downstream mediators of necroptosis RIP3 and pseudo-kinase MLKL (Silke et al., 2015; Zhou and Yuan, 2014).

1.2.3 Autophagy

Autophagy is a self-digesting mechanism involved in the degradation of proteins, organelles and cellular survival during nutrient deficient conditions. As distinct from apoptosis and necrosis, it is a caspase-independent form of cell death where the cytoplasm and misfolded or damaged proteins and cell organelles are destroyed by lysosomal enzymes. Three types of autophagy have been identified: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. During macroautophagy an isolated membrane surrounds the cytoplasmic components, which eventually fuse to form a double membrane structure called autophagosome. Subsequently, the autophagosomes fuse with lysosomes and form autophagolysosomes, within this structure its contents are degraded by lysosomal hydrolases (Jaeger and Wyss-Coray, 2009; Rami, 2009). Autophagy provides a fundamental mechanism to regulate cellular homeostasis by recycling cellular components and balancing energy sources under normal and pathophysiological conditions (Ryter et al., 2013). However, accelerated and prolonged autophagy also contributes to several pathological events such as neuronal death in cerebral ischemia and aging, tumer development (as reduced autophagy is found in tumor cells) (Choi, 2012; Shi et al., 2012).

1.3 BAG1 and BAG3

In eukaryotic cells, two major pathways are involved in protein degradation; the ubiquitin- proteasome pathway and macroautophagy pathway (Figure 8). Impaired protein degradation pathways were observed in AD, this indicates disturbances in proteasomal activity and upregulation in macroautophagy (Hong et al., 2014; Lipinski et al., 2010). Bcl-2 associated athanogene 1 (*BAG1*) and Bcl-2 associated athanogene 3 (*BAG3*) are selective autophagy genes. These are co-chaperone of heat-shock protein 70 (Hsp70). BAG1 is associated with proteasomal degradation in young cells and BAG3 induces macroautophagy pathway during cellular aging and under stress condition (Gamerdinger et al., 2009). During cellular aging, a molecular switch from BAG1 to BAG3 expression and function was observed, which is accompanied by a reduced secretion of sAPPα (Gamerdinger et al., 2009; Kern et al., 2006). Autophagy is essential for neuronal survival, but excessive formation of autophagosomes and autolysosomes are observed in AD brains which is thought to hamper effective degradation of protein aggregates and increase γ-secretase activity (Lee et al., 2013; Ohta et al., 2010). The ubiquitin-proteasome-system (UPS) regulates protein homeostasis by conjugating misfolded or damaged proteins and substrates with ubiquitin, which are further degraded by 26S proteasomes (Lecker et al., 2006). In AD both degradation systems are involved in clearance of soluble tau and other Alzheimer-relevant proteins. Initially, the UPS act as a primary clearance system of pathological tau, but later under stress condition autophagy-mediated tau degradation play important role (Lee et al., 2013).

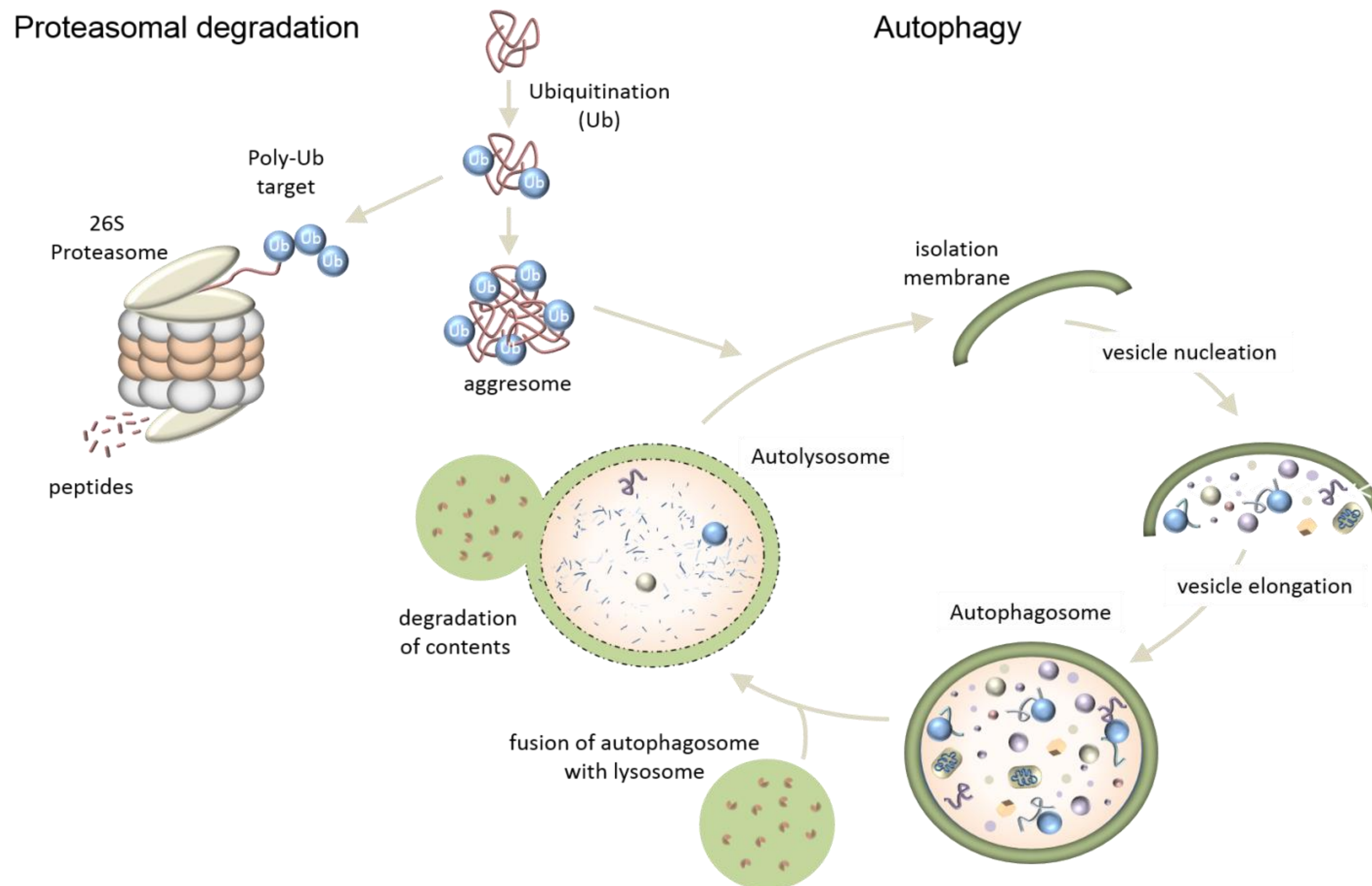


Figure 8. Protein degradation pathways. Proteins are degraded by two different pathways, the ubiquitin-proteasome pathway and macroautophagy pathway. In proteasomal pathway, misfolded or damaged proteins conjugated with ubiquitin and further degraded by 26S proteasomes. In macroautophagy pathway a small volume of cytoplasmic components along with aggregated damaged proteins are enclosed by the autophagic isolation membrane, which eventually results in the formation of an autophagosome. The outer membrane of the autophagosome then fuses with the lysosome to form autophagolysosome, where the cytoplasmic materials are degraded by lysosomal hydrolases. Modified from (Meléndez and Levine, 2009).

1.4 Role of stress signaling pathways in neurodegeneration and apoptosis

The c-Jun/N-terminal kinase (JNK) pathway is a central stress signaling pathway and play pivotal role in brain aging and neurodegeneration (Kögel et al., 2012a). Varity of stress stimuli, such as UV radiation, reactive oxygen species (ROS), inflammatory cytokines and aging were shown to activate JNKs (Moriguchi, 1997; Plotnikov et al., 2011; SONG and LEE, 2003). JNK is a subfamily of the mitogen activated protein kinase (MAPK) superfamily, which phosphorylates and activates JNK at Thr183 and Tyr185. After double phosphorylation activated JNKs relocate to the nucleus and phosphorylate transcription factors such as c-Jun (LIU and LIN, 2005; Plotnikov et al., 2011). JNK phosphorylates the c-Jun transcription factor in the N-terminal region at amino acid Ser63 and Ser73, which then forms homo or heterodimers (e.g. ATF2) and can activate the expression of target genes (Whitmarsh and Davis, 1996).

JNK/cJun signaling plays an important role in β-amyloid induced apoptosis in neuronal cells. There is evidence that β-amyloid induces ROS generation, which in turn oxidates thioredoxin-1 (TRX1) and glutaredoxin-1(GRX1) (Saitoh et al., 1998; SONG and LEE, 2003). After oxidation TRX1 and GRX1 dissociate from the apoptosis signal-regulating kinase 1 (ASK1) protein. This dissociation causes activation of the ASK1 protein, which in turn can trigger ASK1/JNK-dependent pro-apoptotic pathway (Figure 9; Liu et al., 2000; Tobiume et al., 2001). Other potential functions of JNK include activation of several pro-apoptotic proteins. The activation of JNK can lead to caspase 8-independent cleavage of Bid to jBid, which then translocate to the mitochondria and leads to the release of cytochrome c and Smac/DIABLO.

Activated JNK also phosphorylates Bim which in turn induce apoptosis, by inhibiting expression of different anti-apoptotic proteins, such as Bcl-xL and Bcl-w (Okouchi et al., 2007). Previous studies suggest that the activation of JNK patway is reduced by autocrine sAPPα signaling in APP overexpressing cells and also after addition of exogenous sAPPα (Kögel et al., 2012a). It was observed that APP play important role in transcriptional repression of c-Jun and JNK activity and

the application of sAPPα shows anti-apoptotic effect by inhibiting the stress-triggered JNK signaling pathway (Copanaki et al., 2010; Kögel et al., 2005).

An important molecule of stress signaling pathway is a serine/threonine kinase called glycogen synthase kinase 3 (GSK3). There are two isoforms of GSK3, GSK3α and GSK3β with a highly conserved N-terminal regulatory domain. GSK3α / β is phosphorylated by Akt at Ser21 / 9 residue which leads to inhibition of their kinase activity (Rossig et al., 2002). One of the functions of the GSK3 proteins is to inhibit the anti-apoptotic protein Mcl-1 by phosphorylation (Maurer et al., 2006). GSK3 is found to play critical role in AD pathogenesis by promoting Aβ production, neurofibrillary tangle (NFT) formation, tau hyperphosphorylation, and neuronal degeneration (Cai et al., 2012; Serenó et al., 2009). Inactivated GSK3 also plays important roles in Wnt and insulin signaling (McManus et al., 2005). It was demonstrated that the dose dependent sAPPα treatment inhibits the GSK3β stress signaling pathway supposedly through the IGF1 or insulin receptors. These data suggests that sAPPα mediated neuroprotection may require these receptors to stimulate the survival signal (Jimenez et al., 2011; Milosch et al., 2014).

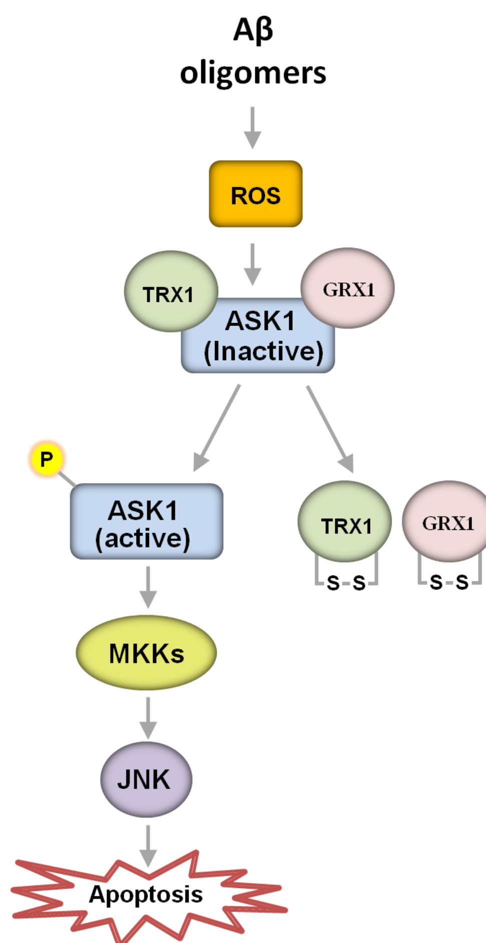


Figure 9. ASK1/JNK activation and apoptotic signalling induced by β-amyloid. β-amyloid generated ROS cause oxidation of TRX1 and GRX1, which dissociate from ASK1 and trigger ASK1/JNK pro-apoptotic pathway. Modified from (Okouchi et al., 2007).

1.5 Role of APP in survival cascade and stress signaling

As described before, several studies provide evidences that APP is a multifunctional protein involved in several biological processes like synaptic plasticity, neurite outgrowth, synaptogenesis and neuroprotection and implicated in various signaling pathways (Kögel et al., 2012a; Müller and Zheng, 2012; Nicolas and Hassan, 2014).

Activation of survival signaling cascade leads to the promotion of cell survival and the inhibition of apoptosis. The phosphatidylinositide 3-kinase (PI3K)/Akt pathway is a central cell survival pathway which can be activated by various growth factors and neurotrophins. Members of insulin-like growth factor (IGF) family proteins such as insulin-like growth factor 1 (IGF1) can trigger cell survival by binding to

their cognate cell membrane receptors. This binding activates phosphoinositide 3 kinase (PI3K) and downstream Akt (also called protein kinase B, or PKB) (Manning and Cantley, 2007). Akt is a serine/threonine kinase, which is activated by PI-3-kinase downstream proteins. Akt phosphorylates various target proteins and thereby activate or inhibit their functions, which leads to enhance cell survival and inhibit apoptosis (Mitsuuchi et al., 1998; Song et al., 2005).

After phosphorylation activated Akt can promote cell survival by inhibiting the pro-apoptotic proteins and activating anti-apoptotic proteins (Figure 10). Previous studies from Kögel and other labs suggested that sAPPα provide neuroprotection and holo-APP is required for this sAPPα dependent neuroprotection (Copanaki et al., 2010; Milosch et al., 2014). Based on experimental evidences it was observed that different synthetic inhibitors of PI3K can abrogate sAPPα mediated neuroprotection, which suggests sAPPα indeed can act as an activator of PI3K/Akt survival signaling pathway (Cheng et al., 2002; Copanaki et al., 2010; Eckert et al., 2011).

Although, the exact mechanisms of APP and sAPPα-mediated neuroprotection are not clearly established, especially the putative receptors and/or interactors involved in the neuroprotection is not fully understood. Therefore, further experimental proof is required to understand the complex mechanism of survival signaling pathways affected by APP and sAPPα.

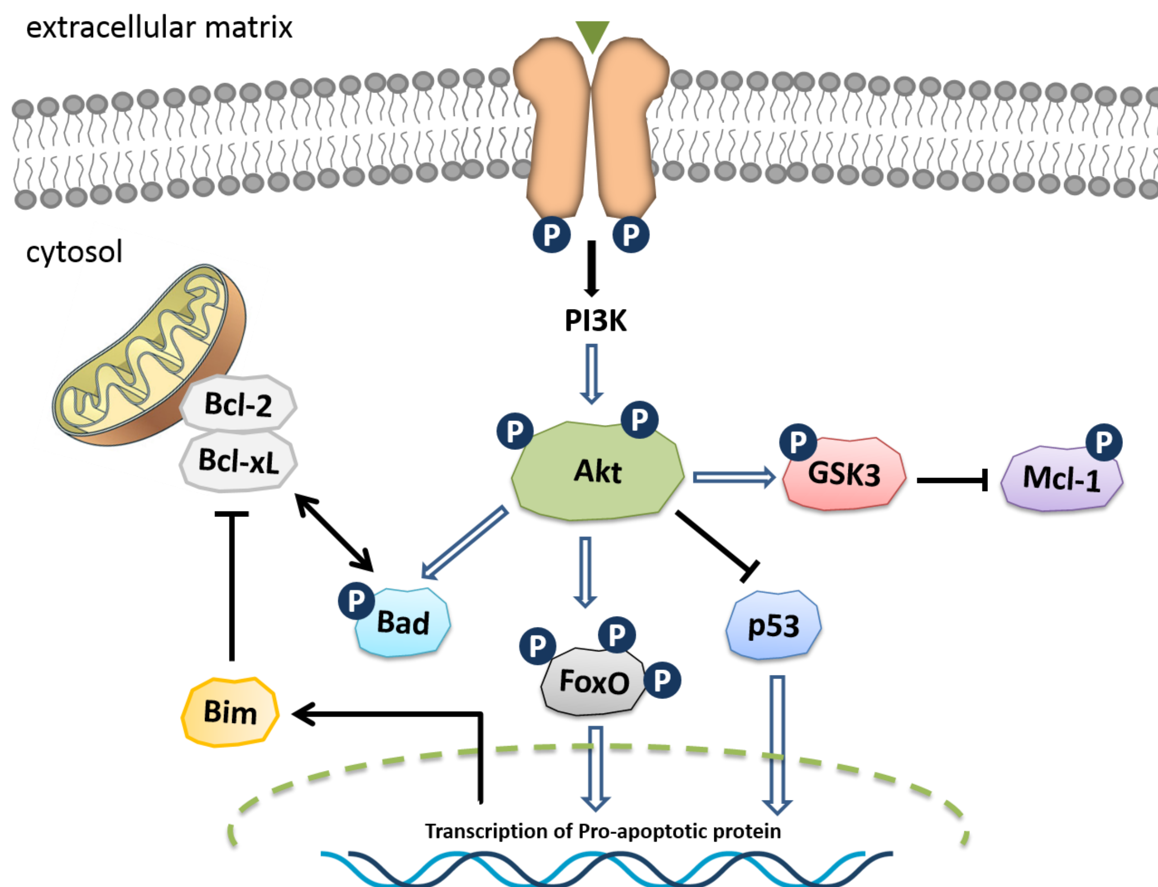


Figure 10. Schematic representation of the PI3K/Akt pathway. Survival signals trigger their cognate cell membrane receptors. It leads to activation of PI3K and downstream Akt. Akt is the key protein which switches off the activity of downstream proteins via phosphorylation. It inhibits Bad, FoxO, p53 and GSK3. These downstream proteins play roles in the apoptotic pathway. Therefore, Akt leads to survival by inhibiting these proteins.

1.6 Aim of the Thesis

Under physiological conditions, APP is cleaved by α-secretase via the non-amyloidogenic pathway. This cleavage generates a large soluble fragment of APP, sAPPα that plays crucial role in synaptogenesis, axonal growth, neuronal plasticity and neuroprotection (Zheng and Koo, 2011). The mechanisms behind sAPPα mediated neuroprotection are not clearly understood. It was proposed that sAPPα can disrupt membrane bound dimmers of holo-APP by competitive binding and thereby promoting cell survival (Gralle et al., 2009). Furthermore, in Drosophila, sAPPα orthologue sAPPL is neuroprotective only in the presence of membrane bound APPL (Wentzell et al., 2012). These findings suggest that APP

might act as a membrane receptor for sAPPα and their interaction initiates neuroprotective signaling.

Previous studies from our group showed an inhibitory effect of sAPPα on the c-Jun/JNK stress pathway which suggests influence of sAPPα on different signaling network involved in neuroprotection (Kögel et al., 2003, 2005). Several studies also postulate that the PI3K/Akt survival signaling pathway is involved in sAPPα mediated cytoprotection (Cheng et al., 2002; Copanaki et al., 2010; Eckert et al., 2011; Jimenez et al., 2011; Milosch et al., 2014). However, the relevant molecular mechanism of Akt survival signaling for sAPPα mediated neuroprotection are currently not well understood and needed to be further investigated. Therefore, one major goal of this thesis was to elucidate the effect of sAPPα on potential downstream intracellular signaling targets of Akt, such as FoxO, Bim, Bcl-xL and Mcl-1. The influence of sAPPα treatment on mitochondrial cytochrome c release was also investigated in the context of the stress conditions applied in this study.

Our recent study was able to demonstrate that maintenance of neuronal function by sAPPα is also exerted through modulation of protein degradation (Renziehausen et al., 2015). Disturbance of proteostasis is a key pathomechanism in aging and AD. During cellular aging, there is a molecular switch in the expression and function from the co-chaperone BAG1 to BAG3 occurs which is accompanied by a reduced secretion of sAPPα (Gamerdinger et al., 2009; Kern et al., 2006). This expressional shift in BAG1/3 expression evokes a change from proteasomal degradation to macroautophagy for the turnover of polyubiquitinated proteins. According to the above two observations, it has been postulated that in aged cells, a decrease in sAPPα secretion and an increase in the expression of BAG3 occurs. Therefore, an additional aim of this thesis was to investigate whether sAPPα inhibits BAG3 expression and BAG3 mediated aggresome formation under conditions of sublethal proteasomal stress. sAPPα dependent rescue of proteasomal activity was also studied. Finally, the effect of surface bound holo-APP on sAPPα mediated modulation of proteostasis was monitored.

During the work on this thesis, the principal molecular mechanisms of APP and sAPPα induced neuroprotection and proteostasis were analyzed. To this end, purification of recombinant APP fragments, sAPPα and its subdomain E1, is established in a yeast model (*P. pastoris*). Their neuroprotective abilities are examined in different cell models such as SH-SY5Y cells, MEFs and hippocampal primary neurons under various stress conditions by serum and glucose deprivation and sublethal proteasomal stress induced by MG132. Additionally, to further investigate the role of holo-APP, different knockdown (KD) and knockout (KO) models of APP were utilized. To address the principal aims of this thesis, a varied set of techniques is employed, which includes cell death and viability assays, protein and RNA expression analysis, immunostaining, fractionation and proteasomal activity assay.

2 Materials

2.1 Cell lines

Table 1. Cell lines

Cell line	Short description
SH-SY5Y	human Neuroblastoma
SH-SY5Y-6707	sh RNA TRC0000006707 mediated APP depletion in SH-SY5Y
MEF	mouse embryonic fibroblasts prepared from wt mice (from Prof. Ulrike Müller, University of Heidelberg, Germany)
MEF APP-KO	mouse embryonic fibroblasts prepared APP-KO mice (from Prof. Ulrike Müller, University of Heidelberg, Germany)

2.2 Animals

Table 2. Animals

Animal	Description
wild type mouse line	Mus musculus, C57Bl/6 (obtained from Prof. Ulrike Müller, University of Heidelberg, Germany)
transgenic mouse line	Mus musculus, C57Bl/6 APP-KO, lack membrane bound full length APP and all of its proteolytic fragments (obtained from Prof. Ulrike Müller, University of Heidelberg, Germany)

2.3 Cell culture medium

All media and media supplements were supplied in sterile and endotoxin-free condition (Invitrogen). After addition of supplements media were stored at 4^{0}C for 4 weeks, and pre-incubated at room temperature prior to use. Fetal calf serum (FCS) and horse serum (HS) were heat inactivated at 56^{0}C for 45 min.

Table 3. Media

Cell line	Media Composition
human SH-SY5Y neuroblastoma cells	RPMI-1640 Medium supplemented with: 10% FCS 1% L-glutamine (200 mM) 1% pen/strep
mouse embryonic fibroblast	Dulbecco's modified Eagle's medium (DMEM GlutaMAX) supplemented with: 10% FCS 1% L-glutamine (200 mM) 1% pen/strep
mouse embryonic fibroblast (for downstream signaling experiments)	DMEM GlutaMAX medium without glucose supplemented with: 1% L-glutamine (200 mM) 1% pen/strep
mouse hippocampal neurons	Neurobasal A Medium supplemented with: 2% B27 2% GlutaMAX 0.2% pen/strep 1% gentamycin
mouse hippocampal neurons (for downstream signaling experiments)	Neurobasal A Medium supplemented with: 2% GlutaMAX

	0.2% pen/strep 1% gentamycin
P. pastoris (BMGY culture medium)	10 g yeast 20 g peptone ddH_2O ad 700 ml autoclaved added freshly: 100 ml potassium phosphate buffer (see Buffers and Solutions) 100 ml 10x YNB (see Buffers and Solutions) 20 ml 50x biotin (see Buffers and Solutions) 100 ml 10x glycerol (see Buffers and Solutions)
P. pastoris (BMMY secretion medium)	10 g yeast 20 g peptone ddH_2O ad 700 ml autoclaved added freshly: 100 ml potassium phosphate buffer (see Buffers and Solutions) 100 ml 10x YNB (see Buffers and Solutions) 20 ml 50x biotin (see Buffers and Solutions) 100 ml 10x metha nol (see Buffers and Solutions)

2.4 Chemicals

Unless stated otherwise, all the chemicals were supplied in analytical grade purity from the following companies.

AppliChem GmbH, Darmstadt, Germany

Biozym Scientific GmbH, Hessisches Oldendorf, Germany

Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Fluka Chemie AG, Buchs, Switzerland

Invitrogen (Gibco), Karlsruhe, Germany

Merck KgaA, Darmstadt, Germany

Qiagen GmbH, Hilden, Germany

Serva Feinbiochemica, Heidelberg, Germany

Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany

2.5 Buffers and Solutions

Table 4. Buffers and Solutions

Buffer or Solution	Composition
SDS lysis buffer	2% SDS 68.5 mM Tris/HCl 10% glycerin 1 mM protease, phosphatase inhibitor cocktail
5% stacking gel (for SDS-PAGE)	5% bis acrylamide solution 120 mM Tris/HCl (1 M, pH 6.8) 0.1% SDS 0.1% APS 0.05% TEMED
10-15% running gel (for SDS-PAGE)	10-15% bis acrylamide solution 375 mM Tris/HCl (1 M, pH 8.8) 0.1% SDS 0.1% APS 0.05% TEMED

1x electrophoresis running buffer (for SDS-PAGE)	25 mM Tris 192 mM glycin 0.1% SDS
1x electrophoresis transfer buffer (for SDS-PAGE)	25 mM Tris 192 mM glycin 20% methanol
1x TBS	50 mM Tris 150 mM NaCl ddH$_2$0 ad 1000 ml pH 7.5
TBS-Tween	1x TBS 0.05% (v/v) polyethylenesorbitan monolaurate (Tween20) stored at RT, pH 7.4
5x SDS loading buffer	250 mM Tris/HCl, pH 6.8 10% SDS 30% glycerol 5% β-mercaptoethanol 0.02% bromophenol blue
protein marker (for SDS-PAGE)	Precision Plus Protein All Blue Standards (10-250 kD, Bio-Rad, Munich, Germany)
blocking solution (for immunoblotting)	5% skim milk or bovine serum albumin powder in 1x TBS-Tween
Coomassie stain	Imperial Protein Stain (Thermo Scientific, Schwerte, Germany)
10x YNB (for yeast culture)	134 g yeast nitrogen base (YNB, Invitrogen) with ammonium sulphate and without amino acids in 1000 ml water filter-sterilized, stored at 4 °C
10x biotin (for yeast culture)	20 mg biotin ddH$_2$0 ad 100 ml
10x methanol (for yeast culture)	20 ml methanol ddH$_2$0 ad 100 ml

	filter-sterilized, stored at 4 °C
10x glycerol (for yeast culture)	10 ml glycerol ddH_20 ad 100 ml filter-sterilized
1 M Potassium phosphate buffer (for yeast culture)	132 ml 1M K_2HPO_4 868 ml 1M KH_2PO_4 autoclaved, pH 6.0 (with KOH or phosphoric acid)
Buffered Glycerol/Methanol-complex Medium	1% yeast extract 2% peptone 100 mM potassium phosphate, pH 6.0 1.34% YNB 0.0004% biotin 1% glycerol or 2% methanol
10x LEW buffer (for protein isolation from yeast)	500 mM NaH_2PO_4 3 M NaCl ddH_20 ad 1 l filter-sterilized, pH 8.0 (with NaOH)
Imidazole (for protein isolation from yeast)	2 M imidazole in ddH_20 filter-sterilized, stored at 4 °C
Cytosol extraction buffer (CEB)	250 mM sucrose 70 mM KCl 137 mM NaCl 4.3 mM Na_2HPO_4 1.4 mM KH_2PO_4 100 µM PMSF 10 µg/ml leupeptin 2 µg/ml aprotinin 200 µg/ml digitonin ddH_20 ad 50 ml pH 7.2
Mitochondrial lysis buffer (MLB)	50 mM Tris/Cl pH 7.4

	150 mM NaCl 2 mM EDTA 2 mM EGTA 0.2% Triton X-100 0.3% NP-40 10 µg/ml leupeptin 2 µg/ml aprotinin ddH_20 ad 50 ml pH 7.4
Suc-Buffer 1	10 mM HEPES 10 mM Calciumacetate 1.45 mM Magnesiumacetate 1 mM Dithiothreitol (DTT) pH 7.6
Suc-Buffer 2	15 mM HEPES 130 mM Calciumacetate 1.5 mM Magnesiumacetate 1.5 mM $CaCl_2$ 2 mM DTT pH 7.6
5x loading buffer (for fractionation)	2M Tris/Cl pH 6.8 50% glycerin 10% SDS 1% DTT 0.04% bromophenol blue ddH_20 ad 25 ml
10x PBS	95.5 g PBS powder ddH_20 ad 1000 ml
HEPES buffer (for FACS)	10 mM HEPES 140 mM NaCl 5 mM $CaCl_2$ ddH_2O ad 500 ml
penicillin, streptomycin	antibiotics, 10000 µg/ml (Gibco)

(pen/strep)	
fixing solution	4% paraformaldehyde dissolved in PBS at 70°C
Fungizone solution	antimycotic (Gibco)
Trypan blue	1xPBS 0.4% (v/v) Trypan Blue (Sigma-Aldrich, Seelze)
Trypsin/EDTA solution	0.25% trypsin/EDTA (1x,Gibco)

2.6 Antibody

Table 5. Primary and Secondary antibodies

Primary antibody	**Host**	**Manufacturer**
Anti-APP (22C11)	Mouse monoclonal	Millipore
Anti-α-Porin	Rabbit polyclonal	Abcam
Anti-Bag3	Rabbit polyclonal	Abnova
Anti-Bak	Rabbit polyclonal	Proteintech
Anti-Bax	Rabbit monoclonal	Abcam
Anti-Bim	Rabbit monoclonal	Cell Signaling
Anti-Bcl-xL	Rabbit monoclonal	Cell Signaling
Anti-cytochrome c	Rabbit polyclonal	Abcam
Anti-GFP	Rabbit polyclonal	Abcam
Anti-Phospho-FoxO3a (S253)	Rabbit	Cell Signaling
Anti-Mcl-1	Rabbit monoclonal	Cell Signaling
Anti-FoxO3a	Rabbit monoclonal	Cell Signaling
Anti-Vimentin	Rabbit	Santa Cruz biotechnology
Anti-GAPDH	Mouse Monoclonal	Calbiochem

Secondary antibody	Type	Manufacturer
Anti mouse	IR-conjugated 680	LI-COR Bioscience
Anti mouse	IR-conjugated 800	LI-COR Bioscience
Anti rabbit	IR-conjugated 680	LI-COR Bioscience
Anti rabbit	IR-conjugated 800	LI-COR Bioscience

2.7 Oligoneucleotides for cDNA synthesis and qPCR

Table 6. Oligoneucleotides

Primers	Manufacturer
Random primer	Invitrogen
Oligo(dT)20-primer	Invitrogen
TaqMan^R Gen Expression Assay for BAG1	Applied Biosystem
TaqMan^R Gen Expression Assay for BAG3	Applied Biosystem
TaqMan^R Gen Expression Assay for Bcl2l11	Applied Biosystem
TaqMan^R Gen Expression Assay for Bcl2l1	Applied Biosystem
TaqMan^R Gen Expression Assay for Mcl1	Applied Biosystem
TaqMan^R Gen Expression Assay for Hsp 70	Applied Biosystem
TaqMan^R Gen Expression Assay for TATA box binding protein	Applied Biosystem

2.8 Enzymes and recombinant proteins

Table 7. Enzymes and recombinant proteins

Enzymes and recombinant proteins	**Manufacturer**
6His-sAPPα	Prepared from yeast *P. pastoris* (construct and protocol provide by Prof. Dr. Stefan Kins and Frederik Baumkötter, University of Kaiserslautern)
6His-sAPPβ	Prepared from yeast *P. pastoris* (construct and protocol provide by Prof. Dr. Stefan Kins and Frederik Baumkötter, University of Kaiserslautern)
6His-E1	Prepared from yeast *P. pastoris* (construct and protocol provide by Prof. Dr. Stefan Kins and Frederik Baumkötter, University of Kaiserslautern)
SuperScript^R II reverse transcriptase	Invitrogen

2.9 Inhibitors and drugs

Table 8. Inhibitors and drugs

Inhibitors and drugs	**Manufacturer**
aprotinin	Applichem
leupeptin	Applichem
MG132	Calbiochem
Phosphatase inhibitor cocktail	Roche
Pepstatin A	Applichem
Staurosporin (STS)	Alexis

2.10 Kits

Table 9. Kits

Use	Name (Origin, Cat. #)
PD-10 Desalting column	GE Healthcare
protein concentration	Pierce BCA Protein Assay (Thermo Scientific)
transfection	FuGENE Transfection Reagent (Roche Diagnostics, Mannheim, Germany)
RNeasy Mini Kit	Qiagen

2.11 Software

Table 10. Software

Use	Name (Origin)
immunoblot fluorescence scanning and blot quantification	Image Studio 3.1 (LI-COR Odyssey)
image editing and cropping	Photoshop 7.0 and Illustrator 7.0 (Adobe, San Jose, USA) ImageJ (NIH, Bethesda, MD, USA)
fluorescence microscopic imaging	NIS Elements AR 3.22 (Nikon, Düsseldorf, Germany)
protein sequences, alignments and BLAST	ApE – a plasmid editor (Wayne Davis, University of Utah, USA)
statistical analysis	Excel (Microsoft Corp., Redmond, USA) SPSS (IBM, Armonk, NY, USA)

2.12 Equipment and Other Instruments

Table 11. Equipment and Other Instruments

Item	Name (Origin)
autoclave	Varioklav (Biomedis, Gießen, Germany)
blotter	Trans-Blot SD Semi-Dry (Bio-Rad)
centrifuge tubes (for yeast supernatant)	Centrifugal Filter Units, Amicon Ultra-15 (10 K) (Millipore)
centrifuges	• table centrifuge Biofuge Fresco (Heraeus, Hanau, Germany) • Biofuge stratos (Heraeus) • Minispin (Eppendorf, Hamburg, Germany) • Sigma laboratory centrifuges 4-15C (Qiagen)
cold light emitter	Macrospot 1500 (Kaiser, Buchen, Germany)
FACS cytometer	BD FACS Canto II V96100345, cell wash and cleaning solutions (BD Biosciences, Heidelberg, Germany)
filter paper	Whatman 3 MM (Whatman, Kent, UK)
fluorescence plate reader	fluorescence reader GENios (Tecan, Mainz, Germany)
hemocytometer	Neubauer Improved (Optiklabor, Friedrichsdorf, Germany), volume: 0.0025 mm2, depth: 0.1 mm
horizontal rocker	3014 (GFL, Eppelheim, Germany)
horizontal shaker	KM-2 (Edmund Bühler AG, Hechingen, Germany)
ice machine	Scotsman AF80 (Enodis Deutschland GmbH, Herborn, Germany)
immunoblot fluorescence scanner/infrared imager	Odyssey LI-COR (LI-COR Odyssey)
incubators	• HeraCell (Kendro Laboratory Products GmbH, Langenselbold,

	Germany) • Binder (Tuttlingen, Deutschland)
liquid nitrogen tank	CBS Cryosystems 6000 series (Sanyo, Osaka, Japan)
magnetic stirrer and heat plate	Variomag Powertherm (IKA Werke GmbH & Co. KG, Staufen, Germany)
microscopes	• fluorescence microscope: Nikon Eclipse TE2000-S with Plan Fluor x4, x10 or x20 dry objectives, a 100 W mercury lamp and FITC (green, ex: 465-495 nm, dichroic mirror: 505 nm, em: 515-555 nm) or Texas Red (red, ex: 540-580 nm, dichroic mirror: 595 nm, em: 600-660 nm) excitation filters and a DS-5Mc cooled color digital camera (Nikon) • light microscope: Nikon Eclipse TS100 (Nikon) • stereomicroscope: Nikon SMZ645 (Nikon)
multistepper	HandyStep (Brand, Wertheim, Germany)
orbital incubator/shaker	Incubator Shaker Series 25 (New Brunswick Scientific, Edison, NJ, USA)
parafilm	(Pechiney Plastic Packaging, Menasha, WI, USA)
PD-10 columns (for protein purification from yeast)	PD-10 columns Sephadex G-25M (GE Healthcare)
pH meter	Lab850 (Schott AG)
photometer	Eppendorf Bio-Photometer (Eppendorf)
pipettes	• Eppendorf 2 µl, 10 µl, 100 µl, 1000 µl (Eppendorf) • Gilson 2 µl, 10 µl, 20 µl, 200 µl, 1000 µl (Gilson, Limburg an der Lahn, Germany)
pipettor	• Easypet (Eppendorf) • Pipetus (Hirschmann Laborgeräte, Eberstadt, Germany)

plasticizer-free reaction tubes (for virus stocks)	(Eppendorf)
power supply	Power Pac (Bio-Rad)
precision scales	TE3135-DS (Sartorius AG, Göttingen, Germany)
preparation instruments	scissors, spatulas, forceps and scalpels of different sizes (Fine Science Tools GmbH, Heidelberg, Germany)
protective gloves	Peha-soft nitrile (Hartmann, Heidenheim, Germany)
PVDF transfer membrane	Protran BA83, 0.2 µm pore size (GE Healthcare)
razor blades	single packed (Wilkinson, London, GB)
refrigerators and freezers	• 4 °C and -20 °C: Liebherr Comfort or Bosch economic/super (Liebherr, Biberach an der Riss, Germany; Bosch, Munich, Germany) • -80 °C: Thermo Electron HeraFreeze (Heraeus)
roller mixer	• SSRT9D (Stuart, Meckenheim, Germany) • RM5 (Hecht/Assistent, Sondheim, Germany)
scales	BL15000S (Sartorius)
scepter counter	Scepter Automated Cell Counter (Millipore)
SDS-PAGE running chamber and accessories	chamber, glass plates and spacers, 10-well-combs (Bio-Rad)
single-use pipettes , sterile	Costar Stripette 5 ml, 10 ml, 25 ml (Corning Life Sciences, Wiesbaden, Germany)
slice culture inserts	Millicell CM low height, pore size 0.4 µm (Millipore)
sonicator	UW/HD 2070 (Bandelin electronic GmbH & Co. KG,

	Berlin, Germany)
sterile benches	• HeraSafe laminar flow hood (Kendro Laboratories Products GmbH, Langenselbold, Germany) • 2F120-II GS (Integra Biosciences. Fernwald, Germany)
sterile filters	Steriflip (50 ml) and Steritop-GP (500 ml) 0.22 µm ExpressTM Membrane (Millipore)
sterilizor (for preparation tools)	Steri250 (Simon Keller AG, Burgdorf, Switzerland)
thermomixer	Eppendorf Thermomixer comfort (Eppendorf)
tissue chopper	McIlwain (Gabler, Bad Schwalbach, Germany)
UV crosslinker	CL-1000 UV crosslinker (UVP, Jena, Germany)
vacuum pump	Sonorex RK 100 H (Bandelin electronic GmbH & Co. KG, Berlin, Germany)
vortexer	Vortex-Genie 2 (Scientific Industries Inc., Bohemia, NY, USA)
water bath	• Köttermann Labortechnik (Uetze, Germany) • GFL
water destillator	Milli-Q Q-Gard 1 (Millipore)
X-ray film developer (ECL system)	Curix 60 with rapid fixer and developer solutions (Agfa, Mortsel, Belgium)

3 Methods

3.1 Cell biological techniques

3.1.1 Culture conditions

All cells used in the experiments were maintained in humidified incubators at 37 °C and 5% CO_2 in their respective media (see Materials). The cells were passaged every 2-3 days to prevent overgrowing. For this purpose, the culture medium was aspirated and the cell layer was washed with PBS (-Mg^{2+}/-Ca^{2+}). Then, trypsin-EDTA was added to detach the cells from the base of the culture flask. Afterwards, the detached cells were taken out into a 50 ml tube and centrifuged for 3 min at 1000 rpm. The supernatant was discarded and the pellet was resuspended in fresh medium and a definite amount of diluted medium was transferred back to the culture flask. Human SH-SY5Y APP-KD cells were treated with 1.5 µg/ml puromycin to select the cells carrying puromycin resistance. All cell lines were regularly checked for mycoplasma infection and discarded or treated with BM-Cyclin (Roche Diagnostics) following the manufacturer's protocol, if tested positive.

3.1.2 Determination of Cell Number and Seeding of Cells

To determine the number of cells in cell suspension, a Neubauer-type hemocytometer chamber was used. For this, 9 µl of homogeneous cell suspension were pipetted to the chamber assembled with a glass slide. Under the microscope, the cells within the four large squares were counted. The mean of counted cells were multiplied by 10^4 which correspond to the number of cells per ml cell suspension.

3.1.3 Cryopreservation and thawing of cells

For long time storage, the cells were first stored at -80 °C and then transferred to the liquid nitrogen tank where the cells were stored in vapor phase at about -120 °C. For this purpose, the cells were preserved in a medium mixture which contain 50% cell containing medium, 10% DMSO and 40% FCS. The cells were preserved in this mixture to avoid ice crystal formation.

For thawing the cells, the cryo tubes were quickly warmed to 37 °C in a water bath. The cell suspensions were resuspended in 9 ml pre-warmed fresh medium and pelleted at 1000 rpm for 3 min to remove all DMSO. Afterwards, the cell pellet were dissolved in fresh medium and transferred to the culture flasks containing fresh medium and cultured for at least 5 days before starting initial experiments.

3.2 Preparation of Hippocampal Neurons

Hippocampal neurons are very good neuronal cell model to analyze molecular interactions and signaling mechanisms. The isolation of hippocampus was done under a semi-sterile laminar flow hood using sterilized preparation instruments, surgical mask and cloths. The mice were maintained, bred and killed according to the FELASA (Federation of European Laboratory Animal Science Associations) and National Animal Experimental Ethics Committee Guidelines.

All preparations instruments (Figure 11A) were disinfected in a glass beads sterilizer and rinsed with sterile ddH_2O before use. The primary hippocampal neurons were prepared from hippocampus of P2-4 transgenic (APP-KO) and non-transgenic (wildtype) littermate mouse pups (see section 2.2, Figure 11B). At first, the mice were decapitated with scissors and the head was disinfected with ethanol. After that the skull was opened with pointy scissors and the brain was removed (Figure 11C) and transferred to a petri dish with ice-cold PBS (see section 2.5). Then the cerebellum was cut off and the brain hemispheres (Figure 11D) were separated with narrow spatulas. After that under a stereomicroscope, Thalamus (Figure 11D, Th) and basal ganglia were carefully removed. Then the “banana shaped” structure which makes up the hippocampus (Figure 11E, HC) was flipped out from each cortex cup. The hippocampi were transferred to an extra dish with ice-cold HBSS (-Mg^{2+}/-Ca^{2+}, Gibco) medium for approximately 30 min to slow down their metabolism. After that, the tissue was dissolved in 0.1% PBS/trypsin in a 15 ml tube on a horizontal rocker for 20-25 min. The trypsinization was inhibited by adding 6 ml HBSS medium supplemented with 10% FCS. The cells were completely dissociated by carefully triturating the solution with fire-polished Pasteur pipettes until no visible cell clumps remained. After centrifugation at 200 x g for 3 min, the cell pellet was resuspended in full

Neurobasal A medium (see section 2.3). The appropriate amount of cells were seeded into poly-D-lysine coated 6 well culture plates and kept at 37 °C in a 5% CO_2 incubator for at least 7 days prior to experiments. 50% of the medium was changed in every 2-3 days. To determine the ratio of glia to neuronal cells in the prepared culture, some cells were immunostained with antibodies against GFAP (glia marker), NeuN or MAP2 (neuronal markers). If many glia cells were present in a preparation, then cultures were treated with mitotic inhibitors (5 µM AraC, Calbiochem) to prevent further proliferation and overgrowing of neuronal cells.

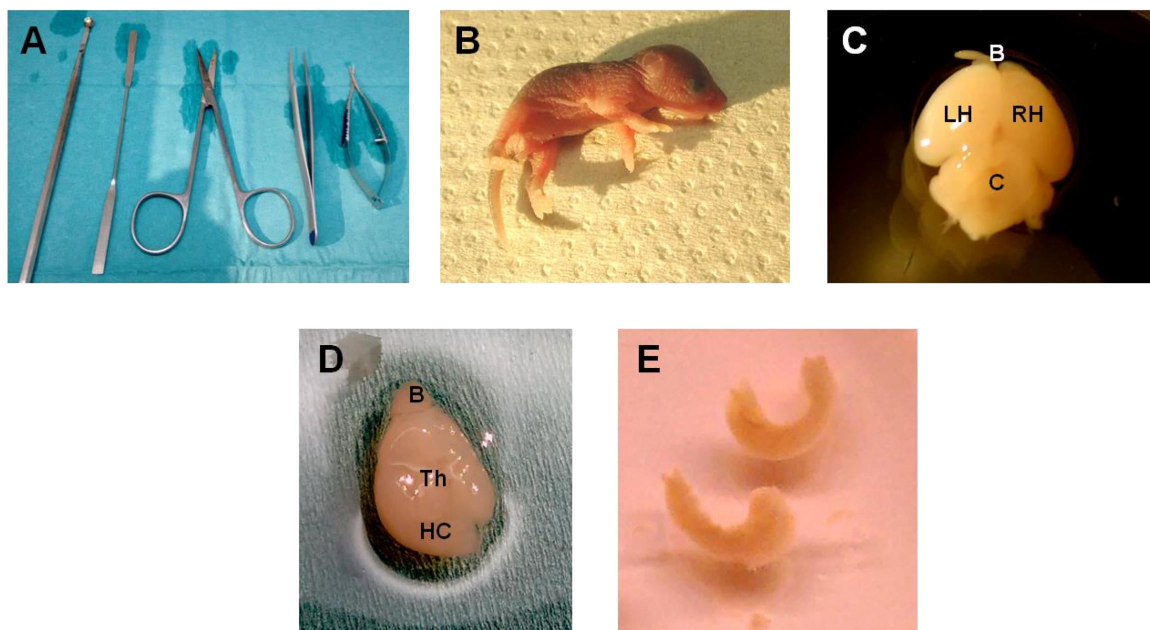


Figure 11. Preparation of hippocampal neurons. (A) Suitable instruments, from left to right: hollow spatula, round spatula, pointy scissors, pointy forceps, microsurgical scissors. (B) Mouse pup at age P3. (C) Freshly prepared mouse brain. B –bulbus olfactorius, RH – right hemisphere of the cortex, LH – left hemisphere of the cortex, C – cerebellum. (D) Right hemisphere with hippocampus (HC) and thalamus (Th). (E) Freshly prepared hippocampus.

3.3 Transfection of Plasmid

Transfection is a process of inserting a plasmid of choice into target cells. To obtain efficient transfection plasmid DNA can be complexed with cationinc lipid reagents, which leads to the formation of liposomes. Subsequently liposomes fuse with the cell membrane and transfer the plasmid DNA inside the cell (Behr et al., 1989). Here transfection of d2-GFP plasmid was performed in MEF wt and APP

KO cells using FuGENE Transfection Reagent (Roche Diagnostics) according to the manufacturer protocol.

Appropriate numbers of cells were seeded in 6 well plates to achieve 80% cell conflency on the following day. For each well to be transfected a mixture of 3 µl FuGENE and 97 µl serum free medium was prepared. After incubation for 5min at RT 1 µg plasmid DNA was added and the solution mixture was again incubated for 30 min at RT. Then serum fresh containing medium was added into the culture plate and 100 µl of FuGENE and DNA mixture was added into each well. Transfection rate were checked under microscope (plasmid containing fluorophore, EGFP) after 24-48 h and also confirmed in protein level using western blots.

3.4 Induction of stress

The cell death was induced by applying different stress stimuli. Trophic factor and nutrient such as serum and/or glucose deprivation from growth medium is known to activate stress responses and ultimately apoptosis in neuronal and non-neuronal cells (Mielke and Herdegen, 2000). The ubiquitin-proteasome system (UPS) mediates protein degradation and clearance of misfolded, accumulated and damaged proteins. For this reason, it is thought to play a major role during brain aging and neurodegenerative diseases such as AD (Copanaki et al., 2010; Glickman and Ciechanover, 2002). A potent reversible inhibitor of 26S complex, MG132 inhibits the proteasome and thereby mimics similar stress conditions as seen during brain aging and AD. This leads to an accumulation of damaged proteins, which causes activation of stress pathways and cell death (Meng et al., 1999). To induce proteasomal stress, the cells were seeded into appropriate culture dishes or plates and grown overnight. The hippocampal neurons were cultured for 5 to 7 days before performing the experiments. The cultures were then pretreated with 25 nM, 50 nM, or 100 nM recombinant yeast-derived sAPPα or sAPPβ for 24 h. Next day, the cells were washed with sterile PBS and fresh medium was added with 0.5 µM or 10 µM MG132 containing sAPPα/E1. DMSO served as negative control.

For experiments using trophic factor deprivation, the cells and the hippocampal neurons were seeded, grown and pretreated similarly as described above. After 24 h cultures were pretreated with either 25 nM or 50 nM recombinant yeast-derived sAPPα, E1 domain or 20 nM human IGF1 (Sigma Aldrich) for 24 h. IGF1 was used as a negative control as it is known to activate cell survival. On the following day, cells were washed with sterile PBS and changed into media lacking serum (FCS or HS) or trophic factors (B27) and glucose except for the control group that was changed into full medium. While growing in serum and glucose free medium, the same substances as administered during pretreatments were applied.

3.5 Analysis of proteins

3.5.1 Preparation of cell lysates

To prepare cell lysates cells were lysed with cell lysis buffer which containing 2X SDS, 1:100 PMSF, 1:1000 protease inhibitor and 1:10 phosphatase inhibitor. These lysed cells were scraped out from the culture dish using cell scraper and transferred to 1.5 ml eppendrof tube. Subsequently the cell lysate was homogenized with an ultrasonic homogenizer for 5 min and then incubate for 5 min at 95 °C. Lysates were either used freshly (especially for phospho-antibody probing) or stored at -20 °C.

3.5.2 Determination of protein concentration

The protein concentrations of different cell lysates were determined using Pierce BCA Protein Assay Kit (Thermo Fisher) according to the manual. For this purpose, 96 well plate was used. At first 150 µl of 0.9% NaCl was added per well, then 2 µl of samples, a BSA standard series and 2X SDS lysis buffer (used as blank) were pipetted per well. After that 150 µl of bicinchonine acid (BCA) solution was added per well. Then the plate was incubated for 30 min at 37 °C. Due to the reduction of Cu^{2+} by trypsine, tryptophan, cysteine and lysine residues of proteins and subsequent complex formation of Cu^{2+} with BCA, a color change occurred. This change was quantified by absorption measurement at 560 nM and the protein concentration of the lysates was determined using the BSA standard series.

3.5.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a standard method for protein analysis. In this method proteins are separated according to their molecular mass in an electrical field in a Tris buffer system (see section 2.5). The tertiary and secondary structure of proteins are broken due to the addition of sodium dodesyl sulphate (SDS), it also gives the protein an entire negative charge which draws them to the anode. Electrophoresis was performed in an electrophoresis chamber which was filled with western blot electrophoresis buffer (WEB, see section 2.5). Protein samples were loaded with 5X loading buffer (see section 2.5) containing 5% β-mercaptoethanol, which induces an additional break-up of disulfide bonds in proteins. Two gels were poured and polymerize to form a polyacrylamide separation matrix with varying pore size, which depends on the acrylamide concentration. At first protein samples were loaded in stacking gel which has a lower pH of 6.8 and a wide pore size due to its lower acrylamide concentration (5%), it accumulates stacks of proteins in the order of their mobility. Then proteins were separated in lower gel (separation gel) with a higher pH of 8.8 and smaller pore size (10-15% acrylamide). Proteins were run in the stacking gel at a constant voltage of 90 V and then separated in the separation gel at a constant voltage of 120 V. For determination of protein size, a pre-stained protein ladder was loaded as reference.

3.5.4 Western Blot

In Western blot proteins that were previously separated by gel electrophoresis, were transferred to a nitrocellulose membrane. A semi-dry method was used, in which the nitrocellulose membrane and SDS gel were equilibrated for 5 min in transfer buffer (see section 2.5) with 20% methanol. Whatman papers were also soaked in transfer buffer with 20% methanol. In the blot apparatus the nitrocellulose membrane and the SDS-separation gel, were flanked by Whatman paper, the nitrocellulose membrane was placed towards the anode and the SDS-separation gel were positioned towards the cathode. The proteins were blotted from the gel to the membrane at a constant current of 3 mA/cm^2 gel and at a constant voltage of 15 V for 40 min.

3.5.5 Coomassie staining of polyacrylamide gels

To check equal protein loading and transfer quality, SDS-separation gel was incubated overnight in Coomassie-based Imperial Protein Stain (Thermo Scientific) and destained afterwards with ddH_2O until the bands became visible.

3.5.6 Immunodetection

To decrease unspecific binding and to increase specific binding of primary antibody to each antigen, free binding sites were blocked on the membrane. This was achieved by incubating the membrane in blocking solution (5% milk powder or 5% BSA diluted in 0.05% TBS/Tween-20) for 1 h at RT on a roller. After that the membrane was incubated overnight at 4 °C in optimally diluted primary antibody solution (antibody + 5% BSA in TBS/Tween-20). Antibodies and dilutions are listed in section 2.6. Then, membrane was washed with TBS buffer (see section 2.5) with 0.05% Tween-20 and secondary antibody (diluted in 5% BSA in 0.05% TBS/Tween-20) was added to the membrane and incubated for 1 h on a roller. Secondary antibodies were coupled to fluorescent dyes in infrared range; therefore the specifically bound antibodies can be detected in LI-COR Odyssey Infrared Imager system (LI-COR Biosciences). Western blot quantifications were performed with LI-COR Odyssey Image Studio 3.1 software.

3.6 Immunostaining

For immunostaining cells were grown on glass cover slips in 24 wells plate. After treatment cells were washed with PBS and then 4% paraformaldehyde solution (see section 2.5) was added to fix the cells and shaken in a shaker for 20 min at RT. Then cells were permeabilized with 0.1% Triton X-100 in PBS for 2 min at RT. After washing with PBS, cells were treated with blocking solution (5% HS in 0.3% Triton X-100 in PBS) for 1 h at RT on a shaker. After that the membrane was incubated overnight on a shaker at 4 °C in optimally diluted primary antibody solution (antibody + 3% HS in 0.3% Triton X-100 in PBS). Antibodies and dilutions are listed in section 2.6. Then cells were washed twice with PBS and secondary antibody solution (diluted in 3% HS in 0.3% Triton X-100 in PBS) was added. After incubation at RT for 1 h on a shaker, cells were washed again for 2 times and

mounted on a glass slide with mounting solution. Afterwards wells were observed under fluorescent microscope.

3.7 Nucleic acid techniques

3.7.1 Isolation of RNA

Before the RNA was isolated, all work surfaces and equipments were cleaned with RNase decontamination solution. For the isolation of total RNA, the RNasy Mini Kit from Qiagen was used and the RNA was isolated by following the manufacturer's protocol. Possibly DNA was also isolated together with RNA which was hydrolyzed by treating the solution for 15 minutes with Dnase I.

3.7.2 Determination of RNA concentration

The concentration of RNA solution was measured spectrophotometrically at a wavelength of 260 nM.

3.7.3 cDNA synthesis

In qPCR DNA is used as a template, therefore cDNA was synthesized at first using reverse transcriptase enzyme. The isolated RNA was used as template for synthesis of complementary DNA (cDNA) by reverse transcriptase. For the preparation of cDNA 1 µg RNA, 1 µl Oligo(dT)20- primer, 1 µl Random primer, and 1 µl 10 mM dNTPs were used. Random primers can bind to the RNA complementary region and serve as a starting point for the reverse transcriptase and dNTPs are the building blocks for the cDNA synthesis. At first RNA was denatured at 65 °C for 5 min. After that the PCR tubes were placed in ice and First-strand buffer and 0.1 M DTT were added and incubated for 2 min at 40 °C. Subsequently, 1µl SuperScript III reverse transcriptase was added and cDNA program was started in PCR machine. The PCR program consists of slow heating (5 mins at 25 °C, 5 mins at 30 °C, 5 mins at 35 °C) this is required for the binding of primers and the reverse transcriptase to the RNA, after that elongation phase starts (50 mins at 40 °C) in which the cDNA is synthesized. Ultimately, the synthesis was terminated by inactivation of reverse transcriptase (15 mins at 70 °C).

3.7.4 Quantitative real-time polymerase chain reaction (qPCR)

The real-time quantitative polymerase chain reaction (qPCR) is based on the conventional amplification of DNA by PCR, which is used to amplify and simultaneously quantify the target mRNA or its cDNA molecule. For qPCR 25 ng cDNA, 1µl 1xTaqMan Gene Expression Assay primer (for amplification of respective target genes) and 10µl 1x FastStart Universal Probe Master-mix were used. The PCR program began with ten minutes of heating to 95 °C, in this temperature the FastStart Taq DNA polymerase was activated, then the amplification was started (15 sec at 95 °C) and at last quantification (60 sec at 60 °C) was done. This cycle was repeated for forty times. In TaqMan qPCR experiment, a fluorogenic probe was used which is complementary to the target sequence. This probe is an oligonucleotide with a reporter dye (6-FAM-phosphoramidite) attached to the 5' end and a quencher dye attached to the 3' end. During PCR, the Taq DNA polymerase carries out the extension of the primer and replicates the template. During extension the 5' exonuclease activity of the polymerase cleaves the probe, releasing the reporter molecule away from the close vicinity of the quencher and thereby increases the fluorescence intensity of the reporter dye. The fluorescence intensity of the samples was normalized to the amplification value of the reference gene TATA-box binding protein (*TBP*).

3.8 SUC-LLVY-AMC Assay

SUC assay was performed to measure proteasomal activity. For this assay, the cells were grown in a 6 well plate and treated accordingly. After that, the cells were detached from the plate using trypsin-EDTA and collected in a tube and centrifuged at 1200 x g for 4 min at RT. The cell supernatant was discarded and the cell pellet was dissolved in PBS and again centrifuged at 600 x g for 4 min at 4 °C. After that, the cell pellet was resuspended in 100-200 µl SUC-Buffer-1(containing 1 mM DTT, see 2.5) and incubated for 30 min at 4 °C. After incubation, the cell suspension was passed ten times through a 25 gauge needle and centrifuged at 640 x g for 5 min at 4 °C and supernatant was collected in a new eppendrof tube. After that, Calcium acetate was added to the supernatant at a final concentration of 90 mM and centrifuged again for 20 min at 10000 x g at 4

°C. The supernatant was collected in a new eppendrof tube and used for BCA Protein Assay. After protein concentration measurement, 10 µg protein, SUC-Buffer-2 (containing 2 mM DTT, see 2.5), 8 mM ATP and 4 µl of 10 mM SUC-LLVY-AMC proteasomal substrate were added in an eppendrof tube. Then, 100 µl of above mentioned solution was added in each well of a black 96 well plate. The samples were assayed in triplicate. The plates were then measured in a fluorescence plate reader at 37 °C, an extinction wavelength of 360 nm and emission at 460 nm for 2 h recording data every 10 min. The data analysis was performed plotting the absorbance as a function of substrate cleavage against time.

3.9 FACS Analysis

Flow cytometry or fluorescence activated cell sorting (FACS) analysis is a technique for sorting of cells in a heterogeneous mixture of biological cells. It is a highly accurate and high throughput cell analysis. The cytometer measures single cells in suspension depending on their fluorescence and characteristic light scattering. The laser passing through a single cell is scattered depending on the size (forward scatter) and structure (side scatter) of the cell. If the cells are stained with specific fluorescent dye (e.g. MTDR, PI), and they are sorted and quantified according to the wavelength of the dye. To determine the mitochondrial mass, FACS based approach is used by staining the cells with widely used mitochondria selective probe MitoTracker® Deep Red (MTDR).

The cells were seeded into 24-well-plates and treated, collected by trypsinization and transferred to single use FACS tubes. After centrifugation at 2000 x g for 4 min, cell pellets were resuspended in 50 µl HEPES buffer and stained with 10 nM MTDR (Life Technologies) or 0.8 µg/ml PI (Sigma-Aldrich). The cells were then analyzed with a FACS cytometer (BD FACSCanto). Prior to use, the cytometer was calibrated according to the manufacturer's instructions. For every treatment, four samples were measured. The results were analyzed with the corresponding software BD FACSCanto. The mitochondrial mass and PI positive cells were calculated by measuring the percentage of MTDR or PI positive cells.

3.10 Subcellular fractionation and immunodetection of cytochrome c

The subcellular fractionation was done to separate cytosolic and mitochondrial fraction. For this, appropriate amount of each cell line were seeded in175 cm^2 cell culture flask and pretreated with 50 nM sAPPα for 24 h. After that the cells were transferred into serum (FCS) and glucose free medium for 2 h, 8 h, 24 h and 32 h. In control culture, the cells were grown in serum (FCS) and glucose containing medium. After incubation, the floating cells were collected and spinned down at 1000 rpm for 3 min at room temperature (RT). The adherent cells were trypsinized and transferred into a 15 ml falcon tube. The cell pellets of the floating cells were resuspended in ice-cold PBS and added to the scraped cell suspensions. The combined cell suspensions were centrifuged for 3 min at 1000 rpm at RT and the pellets were washed with 1 ml ice-cold PBS. The subsequent lysis and fractionation was carried out with cytosol extract buffer (CEB; see 2.5) and mitochondrial lysis buffer (MLB; see 2.5). The cells were lysed with 100-300 µl ice-cold CEB lysis buffer by pipetting up and down using a 1 ml pipette tip and incubation for 15 min at RT on an end-over-end shaker. Afterwards the cell lysates were centrifuged at 1000 x g for 5 min at 4 °C and the resulting supernatants which contained the cytosolic fractions were carefully transferred into 1.5 ml eppendrof tubes. The remaining cell pellets were disrupted with 50-150 µl MLB lysis buffer by pipetting up and down using a 1 ml pipette tip. The resulting lysates were incubated at RT for 10 min and vortexed sometimes in between to break the clumps completely. After that lysates were centrifuged for 10 min at 4 °C and 10000 × g and supernatants were transferred into fresh 1.5 µl eppendrof tubes. For western blot analysis of cytochrome c, equal protein amount (30 µg) for each treatment, cell line and fraction were loaded on 15% SDS gels. The cytochrome c was detected with a rabbit polyclonal antibody 1:1000 (ab90529, Abcam). Equal loading was controlled by a rabbit polyclonal α-Porin antibody 1:800 (ab15895 Abcam) as a mitochondrial marker protein and a mouse monoclonal GAPDH antibody 1:10000 (CB 1001, Calbiochem) as a cytosolic marker.

3.11 Purification of sAPPα, sAPPβ and E1 from yeast *P. pastoris*

The recombinant his-tagged sAPPα, sAPPβ and APP-E1 domain were purified from the yeast *P. pastoris* (Baumkötter et al., 2014; Milosch et al., 2014). The yeast stocks and isolation protocols were developed by Frederik Baumkötter, (Division of Human Biology and Human Genetics, University of Kaiserslautern, Germany, Prof. Stefan Kins lab). The coding sequences of human sAPPα (aa 18-611 of APP695), sAPPβ (aa 18-596 of APP695) and E1 (aa 18-189 of APP695) were cloned as 6xHis-tagged fusions in a pBLHIS-SX derived expression vector and expressed in *P. pastoris* strain GS115. The yeast derived APP ectodamins undergoes eukaryotic modifications. The asparagine residue at position 467 of APP695 (glycosylation site) was replaced with serine by site directed mutagenesis. Therefore, the secreted ectodomains were non-glycosylated. Since these secreted APP ectodomains carry polyhistidin tag, they could be isolated by affinity resins (sepharose beads) containing bound bivalent nickel ions that bind histidine with micromolar affinity. This purification process yields pure proteins, as confirmed by crystallography experiments in the Kaiserslautern lab.

The yeast cells were cultured in buffered glycerol complex (BMGY) medium. Approximately 80-100 µl of yeast culture was added into 1000 ml BMGY medium and incubated at 30 °C for 48 h at 250-300 rpm in sterile baffled flasks in a horizontal shaker. The porous filter membranes were used to seal the flasks which produce ideal oxygen ventilation and sterile atmosphere. After 48 h, the turbid yeast culture solution was harvested by centrifugation at 1500 x g for 5 min and resuspended with 250 ml buffered methanol complex medium (BMMY). The addition of methanol induces the protein secretion. The cultures were then incubated for overnight at 30 °C and 250-300 rpm.

After incubation the supernatant of cultured medium was collected by centrifugation at 1500 x g for 10 min. sAPPα, sAPPβ and APP-E1 domain were purified from the supernatant using Ni sepharose beads or Ni affinity chromatography (FPLC). For the purification using beads, ~10 ml Ni-sepharose beads were equilibrated in 1x LEW buffer by washing and centrifuging at 1500 rpm three times for 5 min. The yeast culture supernatant was sterile filtered and

distributed to 50 ml falcon tubes. 2-2.5 ml equilibrated Ni-beads were added to each of these 50 ml falcon and incubated overnight in an over-head roller at 4 °C. Next day, the solutions were pelleted at 1500 rpm for 5 min at 4 °C and pellets were washed with ice-cold 1x LEW buffer three times to remove unspecifically bound proteins. The elution of the bound proteins was performed with 1x LEW buffer containing 500 nM imidazole for 30 min in an overhead roller at 4 °C. Fresh elution buffer was added to the beads two more times until all three fractions were combined and concentrated by ultracentrifugation. For ultracentrifugation, 50 ml centricon tubes (Centrifugal Filter Units Amicon Ultra-15, Millipore) were equilibrated with 1x LEW buffer by centrifugation at 3000 rpm. Afterwards, the eluted protein solution was centrifuged at 3000 rpm and 4 °C for ~30 min or until the end volume in each tube was less than 2.5 ml.

The PD10 desalting columns (GE Healthcare) were used to remove imidazole from the protein solution. The columns were first equilibrated wit PBS. The desalting was performed according to the product's manual. The final volume of the protein solution was approximately 3.5 ml. 10 % sterile glycerol were added to the purified protein samples and shock frozen in liquid nitrogen and stored at -80 °C. The protein concentration was determined with a photometer (Nanodrop). The protein amount and purity was also assessed in Coomassie gels in comparison to BSA standards.

4 Results

4.1 Purification of sAPPα, sAPPβ and E1 from *P. pastoris*

Previous data from our group provided evidences that sAPPα derived from different sources shows neuroprotective effects (Copanaki et al., 2010; Eckert et al., 2011). Both secreted sAPPα from the conditional supernatant medium of wt APP overexpressing HEK cells as well as purified sAPPα derived from COS7 cell were shown to exhibit neuroprotection under stress conditions. However, these sources of sAPPα, and especially commercially available *Escherichia coli* derived sAPPα had shown some unfavorable limitations such as inefficient production yield, inadequate purity and inconsistent reproducibility. Therefore, a new technique was followed to produce recombinant yeast-derived sAPPα, APP-E1 and sAPPβ with reliable performance. The His-tagged sAPPα, APP-E1 and sAPPβ was expressed and purified from *P. pastoris* yeast strains (provided by Prof. Dr. Stefan Kins and Frederik Baumkötter, University of Kaiserslautern) for further use in neuroprotection assays. *P. pastoris* expression system produces high yield and purified APP cleavage products with eukaryotic modifications (Cereghino and Cregg, 2000). The yeast derived sAPPα showed similar biological and neuroprotective fuctions as sAPPα purified from mammalian COS7 cells (Copanaki et al., 2010; Milosch et al., 2014). It was observed that APP-E1 domain produced in similar manner was also able to induce neuroprotection (Milosch et al., 2014). Additionally, the effect of these recombinantly expressed APP cleavage products (sAPPα and sAPPβ) on protein homeostasis was also investigated. The maintenance of protein homeostasis is necessary for neuronal health and survival. In this work, it was observed that sAPPα plays important role in modulation of proteostasis. sAPPβ has been previously shown to increase neurite outgrowth, induces neural differentiation, and decreases cell adhesion (Chasseigneaux and Allinquant, 2012; Freude et al., 2011). But our group could show that it does not play any role in neuroprotection (Copanaki et al., 2010) or protein homeostasis (Kundu et al., 2016).

The purified sAPPα, APP-E1 and sAPPβ were analyzed by using Coomassie staining (Figure 12) and Western blot. The molecular weight of sAPPα is 76 kD

(Figure 12A, upper left panel) and the molecular weight of sAPPβ is 67 kD (Figure 12B, upper right panel). The additional bands on the gel are most likely not from yeast but might correspond to higher order sAPP products, may be formed due to covalent modifications. APP-E1 domain was detected as a single band (~20 kD) on Coomassie gel (Figure 12C, lower panel). The purity and concentration of each protein batch were always confirmed spectrophotometrically and on Coomassie gels before experiments.

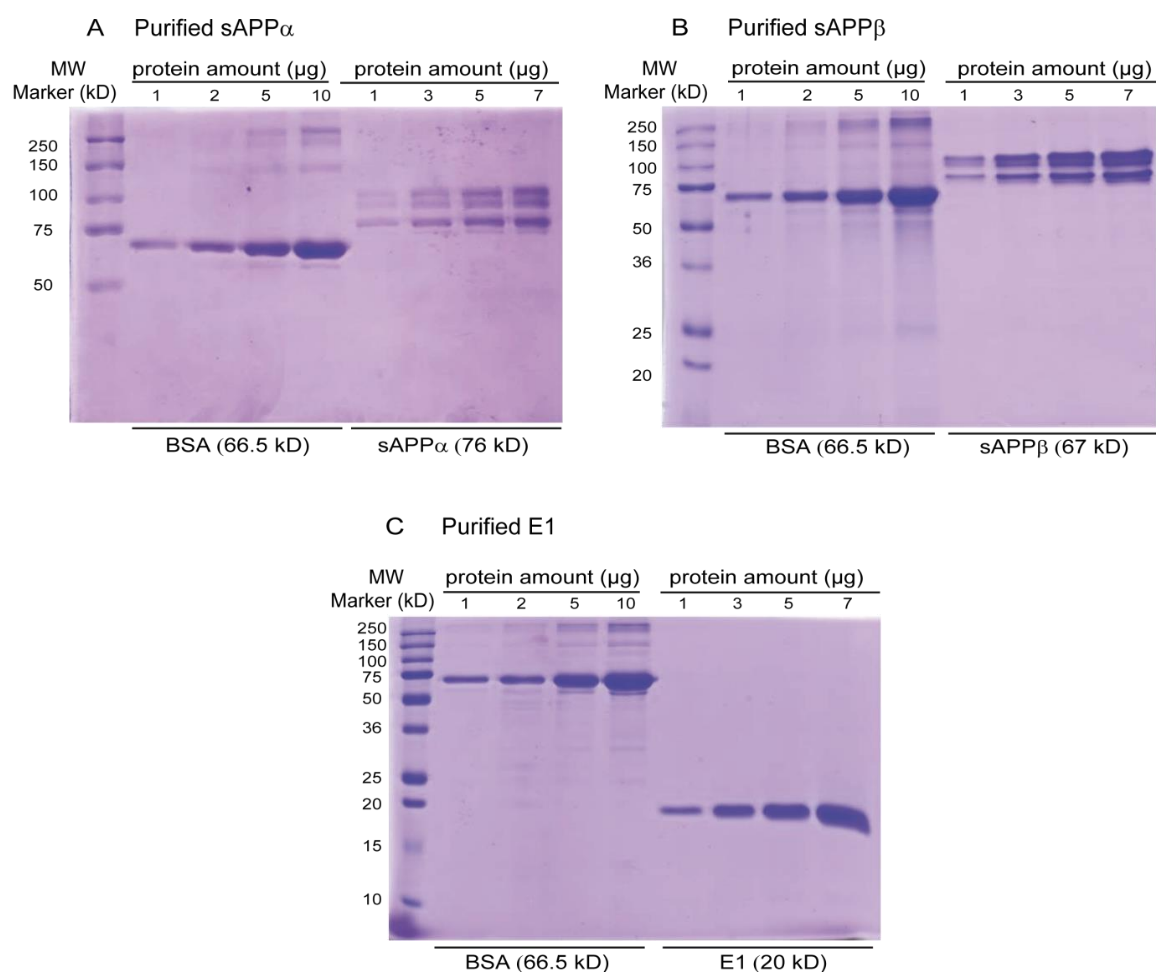


Figure 12. Purified APP cleavage products from *P. pastoris*. Recombinant sAPPα, sAPPβ or APP-E1 domains were purified from yeast supernatants and tested on Coomassie gels at different dilutions to evaluate purity and concentration. Bovine serum albumin (BSA, 66.5 kD) was loaded at concentrations ranging from 1-10 µg to compare band intensities to the loaded APP domains. Recombinant sAPPα (A) and sAPPβ (B) usually appeared at 76 kD and 67 kD respectively. Purified E1 (C) appeared highly pure and ran as a single band at ~20 kD. kD = kilo daltons.

4.2 Neuroprotective properties of sAPPα

In several studies it was established that sAPPα has neroprotective properties (Corrigan et al., 2012; Mattson et al., 1997; Thornton et al., 2006). As previously mentioned, our group could show that sAPPα present in the conditioned supernatant from APP overexpressing HEK cells provide protection to the PC12 cells under proteasomal stress condition (Copanaki et al., 2010; Eckert et al., 2011). To confirm that yeast derived sAPPα also provide protection under stress conditions, human SH-SY5Y cells were subjected to proteasomal stress (10 µM MG132, 24 h) and simultaneous treated with sAPPα. In accordance with previous results, sAPPα was able to exert anti-apoptotic effects under proteasomal stress conditions (Figure 13A; performed by diploma student Andreas Zymny). It was also observed that sAPPα exerted protective effects under trophic factor and glucose deprivation. It is now widely acknowledged that loss of trophic factors and a diminished glucose metabolism may contribute to brain aging and the pathogenesis of AD (Furst et al., 2012; Hyman and Yuan, 2012; Pluta et al., 2013). Therefore, trophic factor withdrawal as induced by serum and glucose deprivation (-FCS/-Gluc) was used to induce cell death in the utilized cell cultures. The non-neuronal cell model, mouse embryonic fibroblasts (MEFs) derived from wt mice were subjected to trophic factor and glucose deprivation and simultaneous treatment with sAPPα. The cell death was analyzed by FACS analysis of PI uptake (Figure 13B). Human IGF1 was used as a positive control for activation of cell survival. It was observed that recombinant yeast derived sAPPα exerted protective effect in wt MEF cells subjected to trophic factor and glucose withdrawal.

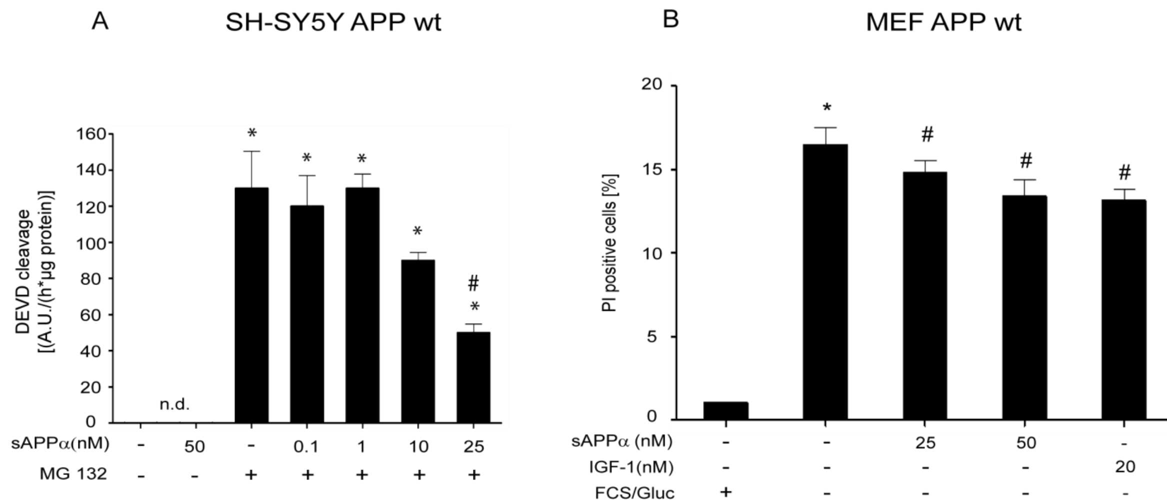


Figure 13. Recombinant sAPPα antagonizes cell death and caspase induction under different stress conditions. (A) Human wt SH-SY5Y cells were pretreated with increasing doses of yeast-derived sAPPα for 24 h followed by treatment with10 µM MG132 or equal volume of DMSO as a negative control for 24 h to induce proteasomal stress. Then cells were lysed and subjected to a caspase-3-like activity assay. (A) was performed by diploma student Andreas Zymny. (B) MEF wt cells were grown in full medium (+FCS/+Gluc) or in medium lacking trophic factors and glucose (-FCS/-Gluc) for 24 h to induce cell death. In parallel, cells were also treated with increasing doses of recombinant 6-His-sAPPα purified from yeast or IGF1 as positive control activating cell survival. PI cells were measured in a FACS cytometer and normalized to DMSO-treated controls Data from (A) and (B) are means from four independent cultures ±SEM. Statistical significance: *$p<0.05$ compared to controls (DMSO); #$p<0.05$ compared to MG132-treated cultures in the absence of sAPPα.

APP is a transmembrane protein that can form homo and heterodimers and may function as membrane bound signaling receptor (Baumkötter et al., 2012; Soba et al., 2005). It was suggested that sAPPα act as a competitive inhibitor disrupting membrane-bound APP homodimers, thereby exerting its neuroprotective function (Gralle et al., 2009). The cellular receptor necessary for sAPPα mediated downstream survival signaling and intracellular protein homeostasis is still unknown.

To find out the possible involvement of holo- APP in sAPPα induced neuroprotection and modulation of proteostasis, a stable lentiviral knockdown of APP was performed in human SH-SY5Y cells by diploma student Andreas Zymny (Zymny, 2010). After the selection of clonal knockdown lines, the expression levels of the APP protein were analyzed by western blot (Figure 14).

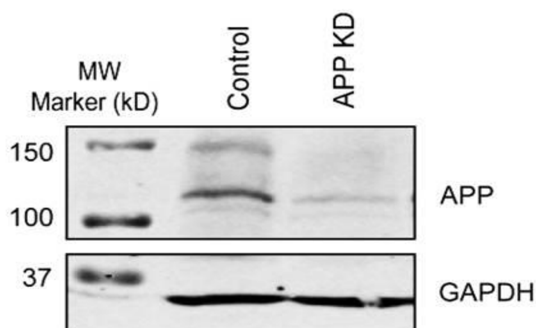


Figure 14. APP knockdown in SH-SY5Y cells. Stable knockdown of APP in wt human neuroblastoma SH-SY5Y cells was obtained by infecting lentiviral particles to express shRNAs directed against human APP. As a control empty vector SH-SY5Y cells were used. The blot shows a representative single cell clone with efficient suppression of APP expression was utilized in this work.

To investigate the effect of endogenous APP on sAPPα induced neuroprotection cell viability or cell death was analyzed in various assays, e.g. by quantification of ATP levels (Figure 15A and B) and microscopical evaluation of propidium iodide (PI) uptake (Figure 15C and D). Human IGF1 was used as a positive control for activation of cell survival. Both sAPPα and E1 failed to exert protective effect in cells lacking endogenous APP under stress condition. This result suggests that the expression of holo-APP is necessary for the sAPPα mediated neuroprotection (Figure 15B and D; was performed by Dr. Nelli Milosch and published in Milosch et al., 2014). Moreover, the recombinant sAPPα and its subdomain APP-E1 exerted protective effects in wt human SH-SY5Y subjected to trophic factor withdrawal (Figure 15A and C; A was performed by master student Gaye Tanriöver; C was performed by Dr. Nelli Milosch and published in Milosch et al., 2014).

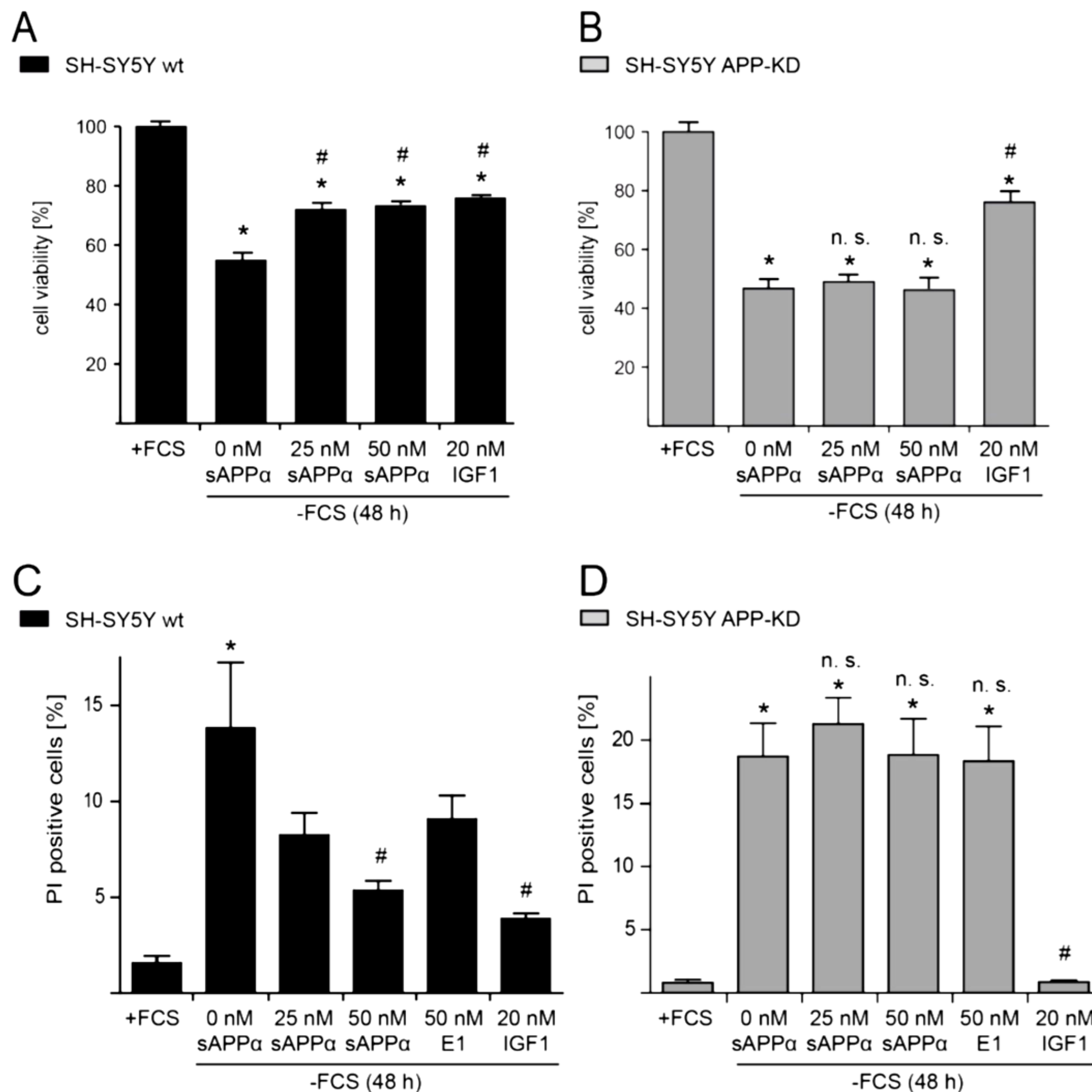


Figure 15. Cell survival promoted by sAPPα and E1 only in the presence of endogenous holo-APP. Human SH-SY5Y wt and KD neuroblastoma cells were cultured in full medium (+FCS) or in medium lacking trophic factors (-FCS) for 48 h to induce cell death (A and B). In parallel, cells were also treated with increasing doses of recombinant 6-His-sAPPα purified from yeast or 20 nM IGF1 as positive control activating cell survival. Cell viability was measured photometrically in a bioluminescence assay by quantifying ATP levels. Serum deprived SH-SY5Y wt (C) or APP-KD (D) cells were treated with increasing doses of 6-His-sAPPα or 50 nM recombinant E1 or 20 nm IGF1. PI-stained dead cells was counted microscopically in three random visual fields (> 150 cells) and calculated as a percentage of the total number of visualized cells (Hoechst staining) to assess cell death. Data are means from four to ten cultures ±SEM. Statistical significance: *P<0.05 compared with controls (+FCS); #P<0.05 compared with serum withdrawal in the absence of sAPPα/E1/IGF1; NS = not significant. (A) was performed by Gaye Tanriöver and (B), (C) and (D) were performed by Dr. Nelli Milosch (Milosch et al., 2014).

4.3 Recombinant sAPPα and APP-E1 domain activate the PI3K/Akt survival pathway

As outlined in the introduction, earlier studies (Copanaki et al., 2010; Eckert et al., 2011) suggested that the PI3K/Akt survival signaling pathway is involved in mediating the protective function of sAPPα. Previous studies provided evidences that synthetic inhibitors of PI3K can abolish sAPPα mediated neuroprotection (Cheng et al., 2002; Copanaki et al., 2010; Eckert et al., 2011). However, the putatively involved cellular receptors and the exact molecular mechanisms underlying sAPPα dependent neuroprotective signaling are still unknown.

After phosphorylation activated Akt can promote cell survival by inhibiting the pro-apoptotic protein Bad, which triggers cell death in its nonphosphorylated state. Activated-Bad is thought to induce cell death possibly via the formation of heterodimers with Bcl-xL and the generation of BAX homodimers. When Akt is activated, it blocks Bad mediated cell death by phosphorylating Bad at S136. The inactive Bad releases the anti-apoptotic proteins, which can further block apoptosis and promote cell survival (Datta et al., 1997; del Peso et al., 1997).

Akt also inhibits other pro-apoptotic BH3-only proteins expression by inhibiting transcription factors, such as FoxO (Manning and Cantley, 2007). When FoxO is phosphorylated by Akt, it stays in the cytosol as an inactive complex and cannot go into the nucleus; therefore, it cannot initiate downstream gene expression. By this way Akt inhibits transcription of FoxO downstream target genes that promote apoptosis, cell-cycle arrest, and metabolic processes (Manning and Cantley, 2007; Sanphui and Biswas, 2013). FoxO regulates the expression of the pro-apoptotic BH3-only protein Bim. It was observed that in response to nerve growth factor (NGF) deprivation; Akt-mediated phosphorylation of FoxO is inhibited, which results in its nuclear translocation and induction of Bim (Sanphui and Biswas, 2013).

Recent studies from our group demonstrated that in the absence of endogenous holo-APP, Akt activation by sAPPα was completely abolished. The E1 domain of APP alone can also activate Akt under conditions of serum deprivation, but this effect could not be observed in APP depleted cells. APP also contain a G-protein

interaction domain and sAPPα mediated neuroprotection was lost upon depletion of this motif. This suggests that APP serves as a receptor to trigger G-protein dependent activation of PI3K (Milosch et al., 2014). This APP/sAPPα mediated activation of Akt might leads to further activation or inhibition of Akt downstream anti-apoptotic or pro-apoptotic proteins. In this thesis, the effects of sAPPα or E1 treatment on Akt downstream proteins expression and cytochrome c release from mitochondria were investigated. The role of APP in sAPPα or E1 mediated modulation in Akt downstream proteins expression and mitochondrial function were also focused during this study.

4.4 sAPPα alters the expression levels of Akt downstream proteins only in the presence of holo-APP

Since sAPPα activated the PI3K/Akt pathway, it might also affect the apoptotic pathway and alter the phosphorylation and/or expression levels of proteins involved in the regulation of apoptosis which are closely related to the PI3K/Akt pathway. Therefore, Bcl-xL (anti-apoptotic), Bim (pro-apoptotic), Mcl-1 and FoxO were further investigated under serum and glucose deprivation.

4.4.1 sAPPα induces the phosphorylation of FoxO3a and inhibits its nuclear translocation

FoxO3a, a transcription factor belonging to Forkhead box, class ‘O’ (FoxO) subfamily is an important mediator of cell death, including neuronal apoptosis. In response to growth factor or insulin stimulation FoxO3a is phosphorylated by Akt and localized in the cytosol as an inactive complex bound with 14-3-3 protein. Several stress kinases can activate and thereby translocate FoxO3a into the nucleus (Sanphui and Biswas, 2013). To investigate whether sAPPα can induces FoxO3a phosphorylation and inhibits its nuclear translocation we preincubated MEF wt and APP KO cells with 25 nM and 50 nM sAPPα for 24 h and then cultured the cells for another 24 h in serum and glucose free medium. Then cells were lysed for western blot analysis or fixed for immunostaining. MEF wt cultures subjected to serum and glucose withdrawal showed decrease in FoxO3a phosphorylation which was induced in the presence of recombinant sAPPα. This effect was not observed in APP KO cells (Figure 16A and B).

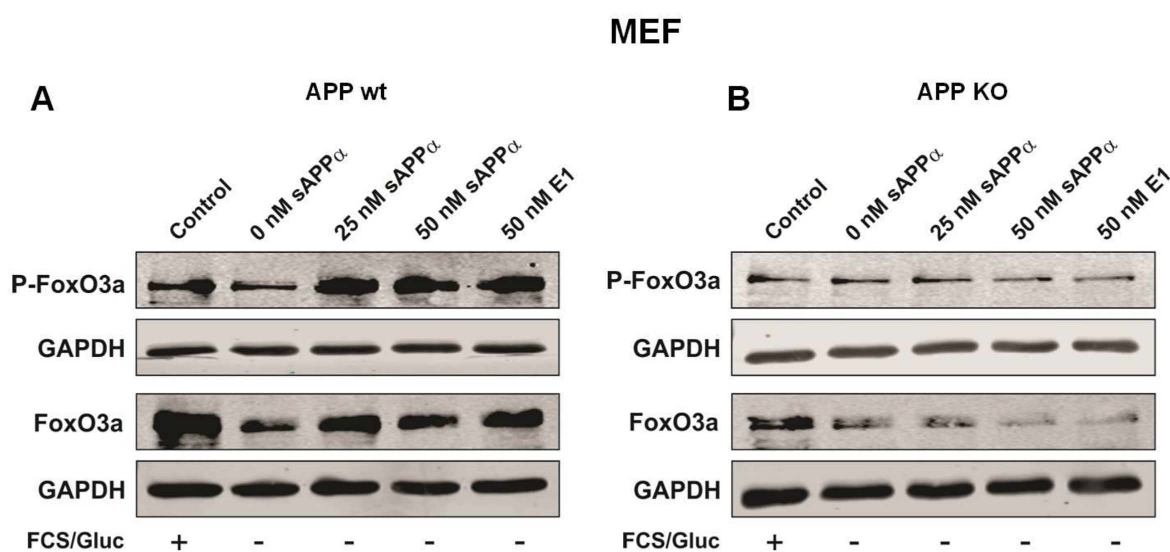


Figure 16. Serum and glucose deprivation triggered decrease of FoxO3a phosphorylation was inhibited by sAPPα and E1 only in the presence of holo-APP. MEF wt (A) and APP KO (B) cells were pretreated with 25 nM and 50 nM sAPPα and 50 nM E1 prior to the FCS and glucose deprivation for 24 h. Afterwards, the cells were harvested and total cell lysates were analyzed for P-FoxO3a and FoxO3a protein levels by western blot analysis. GAPDH was used as a control for equal protein loading. Phosphorylation of FoxO3a was decreased with -FCS/-Gluc removal and treatment of cells with sAPPα and E1 increased FoxO3a phosphorylation in MEF wt cells. In APP KO cells this effect was not observed.

In immunostaining, a pronounced increase of FoxO3a positive nucleus in serum and glucose deprived cultures was observed. This was significantly reduced in the presence of recombinant sAPPα. Importantly, this effect was also only visible in wt cultures and not in APP KO cultures (Figure 17).

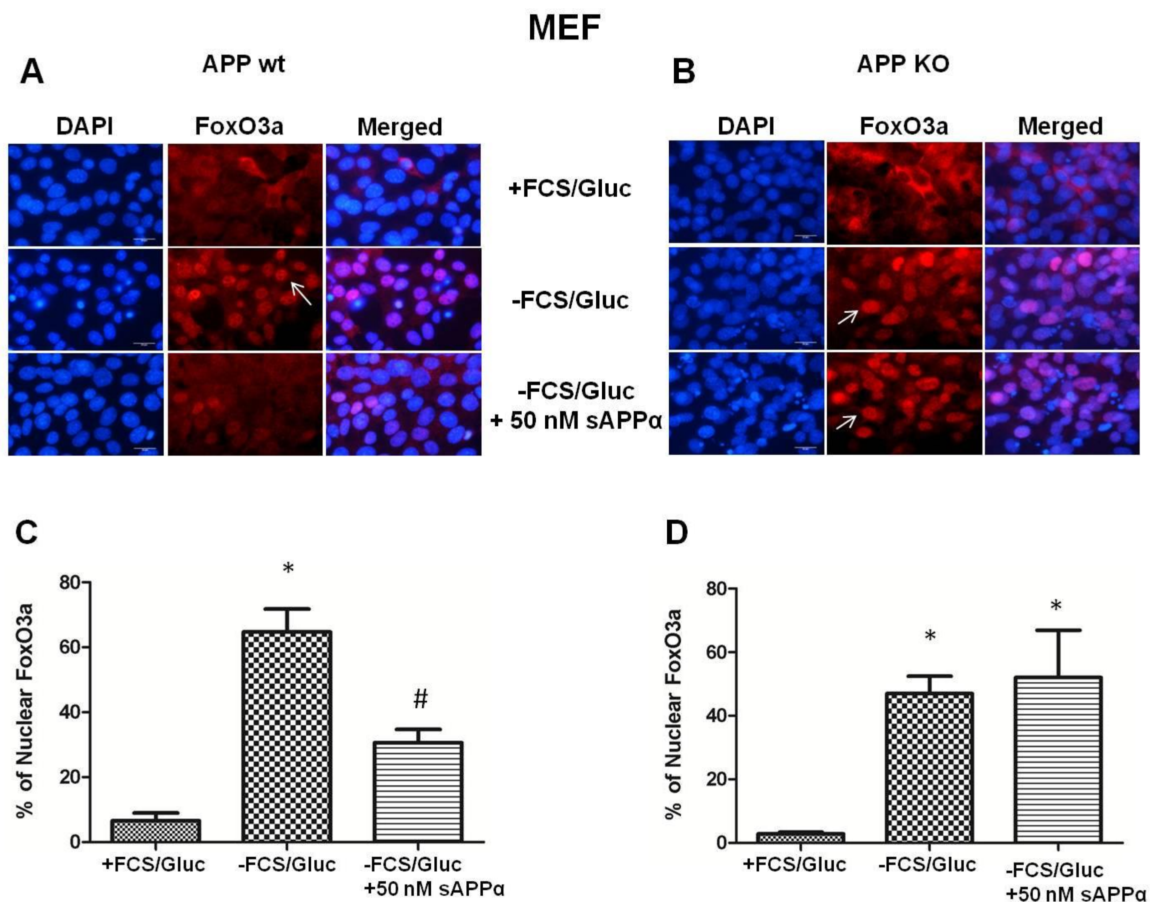


Figure 17. sAPPα prevents FoxO3a nuclear translocation in APP wt cells. MEF wt (A) and APP KO (B) cells were princubated with 50 nM sAPPα and stressed by serum and glucose deprivation for 24 h.The cells were fixed, immunostained with an anti-FoxO3a antibody, and analyzed microscopically. Examples of FoxO3a-positive nucleus are marked with arrows. (C and D) FoxO3a nuclear translocation was assessed microscopically by counting FoxO3a-stained nucleus in three random visual fields (300 cells) and calculated as a percentage of the total number of visualized cells (DAPI staining). Data represent mean ±SEM from n=300 cells. Statistical significance: *$p<0.05$ compared with controls (+FCS/+Gluc); # $p<0.05$ compared with –FCS/-Gluc withdrawal in the absence of sAPPα **.**

4.4.2 sAPPα and E1 decreases Bim expression under serum and glucose deprivation

The active PI3K/Akt pathway decreases the expression level of Bim. We examined whether sAPPα or E1 can decreases the Bim expression under serum and glucose deprivation (-FCS/-Gluc). For this experiment, MEF wt and APP KO cells were treated with 25 nM and 50 nM sAPPα or 50 nM E1 for 24 h and then serum and glucose were removed from the medium for another 24 h. After that cells were lysed for western blot analysis. Both sAPPα and E1 decreased the –FCS and -Gluc induced Bim expression level in wt cells but not in APP KO cells (Figure 18).

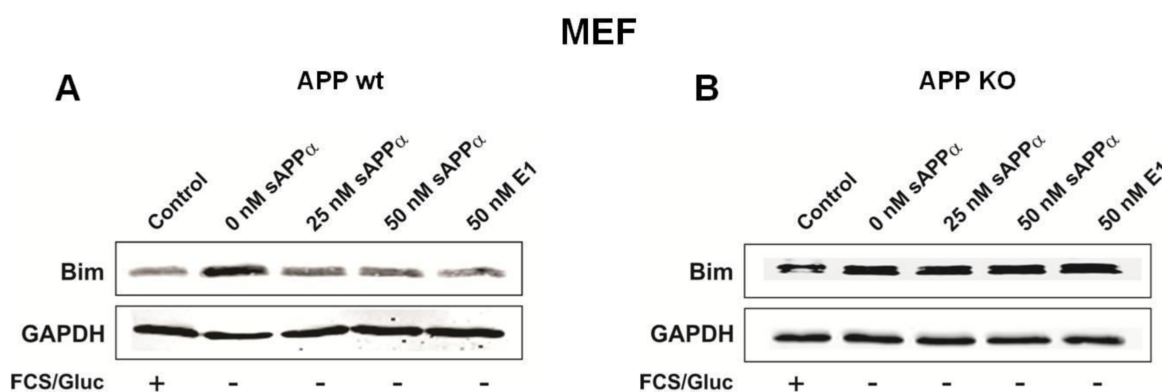


Figure 18. sAPPα and E1 mediated suppression of Bim induction depends on the presence of holo-APP. MEF from (A) wild-type (wt) and (B) APP knockout (KO) cells were cultured in full medium (+FCS/+Gluc) or in medium lacking trophic factor (-FCS/-Gluc) for 24 h to induce stress. In parallel cells were treated with 25 nM and 50 nM sAPPα or 50 nM E1 to activate cell survival. Afterwards, the cells were harvested and total cell lysates were analyzed for Bim protein levels by western blot. GAPDH was used as a control for equal protein loading.

To confirm these results we isolated primary hippocampal neurons from wt and APP KO mice and treated them with sAPPα or E1 for 24 h and then culture them in serum and glucose free (-HS/-Gluc) medium for 24 h. The cell lysates were then analyzed by Western blot. As in MEF cells, this result also confirmed the similar effect of sAPPα and E1 on Bim protein levels (Figure 19).

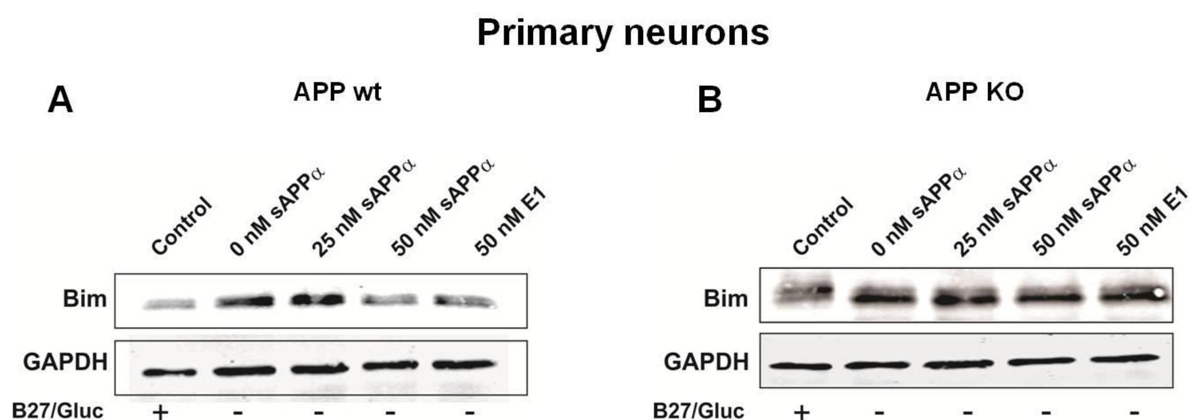


Figure 19. sAPPα reduces Bim induction in primary hippocampal neurons from wt mice but not from APP deficient mice. Primary hippocampal neurons of wt (A) and (B) APP knockout (KO) neurons were cultured into serum and glucose free neurobasal A medium with 25 nM, 50 nM sAPPα or 50 nM E1 for 24 h. Serum and glucose deprivation triggered induction of Bim expression was reduced in the presence of sAPPα.

We also investigated the effect of sAPPα on Bim expression on the transcriptional level. The cells were treated similarly as above and then lysed for RNA isolation and qPCR. qPCR analysis showed a significant reduction of Bim messenger RNA (mRNA) level upon sAPPα treatment in wt cells under trophic factor deprived stress condition, indicating transcriptional regulation of Bim expression by sAPPα (Figure 20).

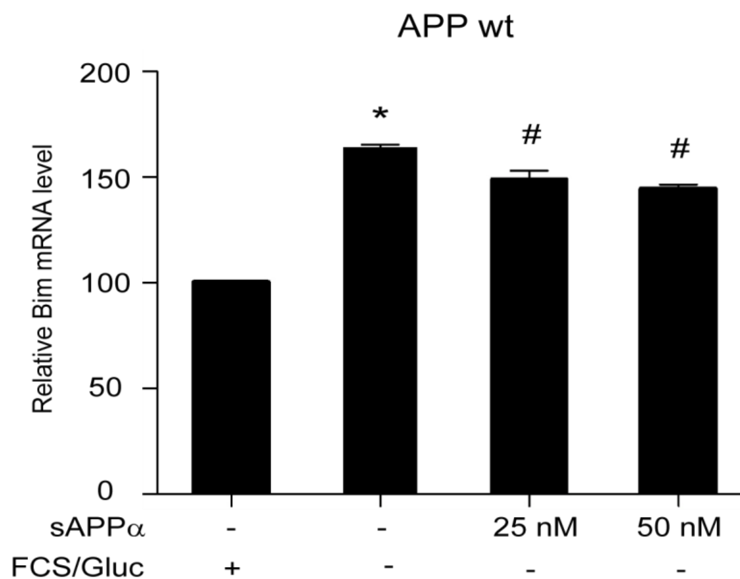


Figure 20. Effect of sAPPα pretreatment on Bim expression on the transcriptional level. MEF wt cells were treated with 25 nM and 50 nM sAPPα and subsequently serum and glucose were withdrawn from the medium for another 24 h. After that cells were harvested for mRNA isolation. The relative mRNA levels were determined by quantitative PCR analysis. Data represent mean ±SEM from quadruplicate determinations. Significant Bim induction was observed in APP wt cells compared to +FCS/+Gluc control are marked by asterisks:* $p<0.05$. Significant differences between sAPPα treated and –FCS/-Gluc cultures are indicated by hashtags: # $p<0.05$.

4.4.3 sAPPα and E1 increases Bcl-xL and Mcl-1 expression under serum and glucose deprivation

Bcl-xL is an anti-apoptotic protein which resides in the outer mitochondrial membrane and inhibits cytochrome c release. Mcl-1 is also a member of an anti-apoptotic Bcl-2 protein family that is essential for cell survival. Different Mcl-1 isoforms reside in distinct mitochondrial locations and exhibit different functions. A Mcl-1 isoform reside on the outer mitochondrial membrane and acts as an anti-apoptotic molecule (Perciavalle et al., 2012). The expression of the anti-apoptotic proteins Bcl-xL and Mcl-1 are correlated with Akt phosphorylation. sAPPα and E1 triggered the increase in Bcl-xL and Mcl-1 expression level compared to the serum and glucose deprived control (Figure 21). These data suggest that sAPPα and E1 might inhibit the mitochondrial pathway of apoptosis through the PI3K/Akt signaling cascade.

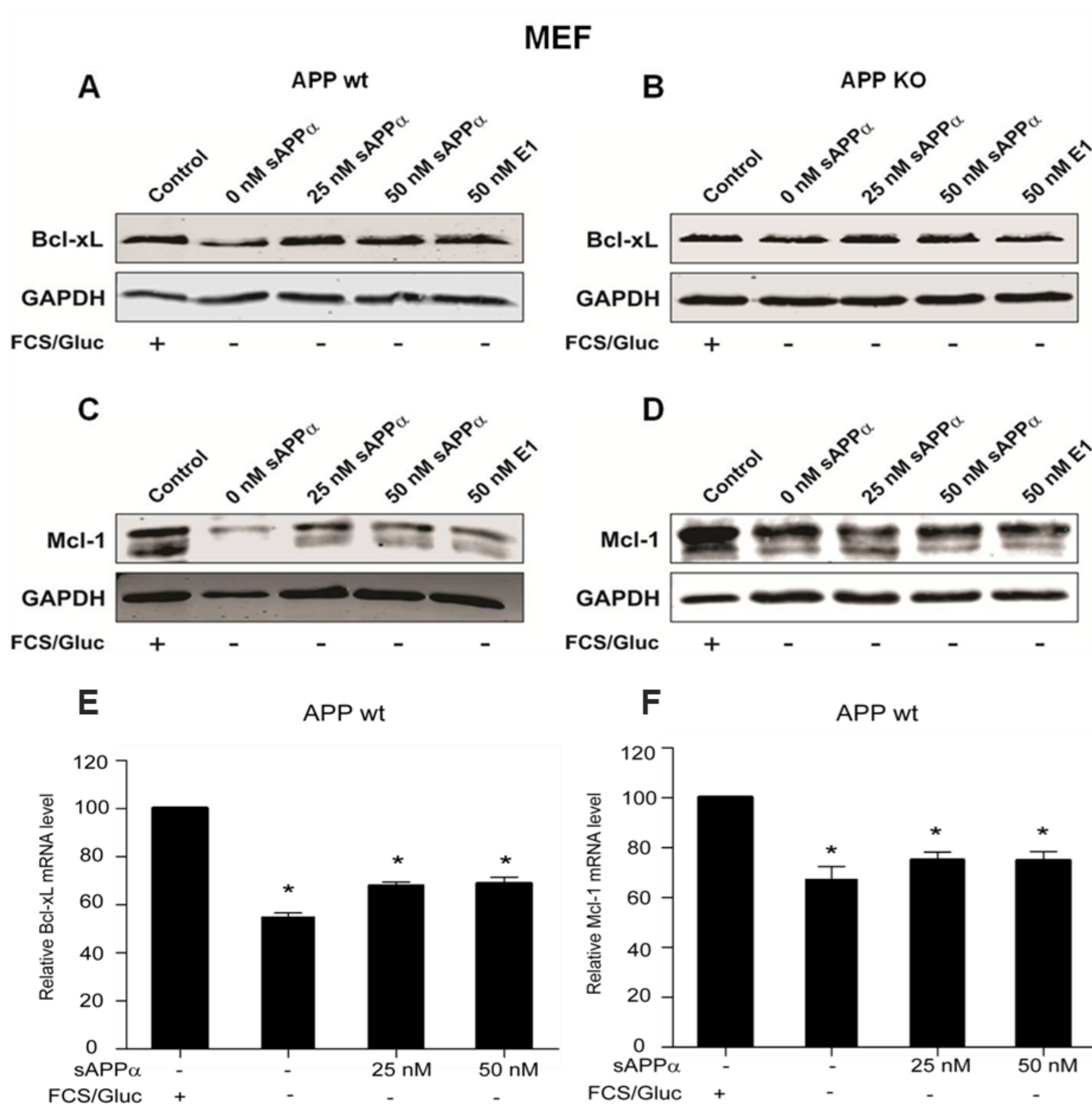
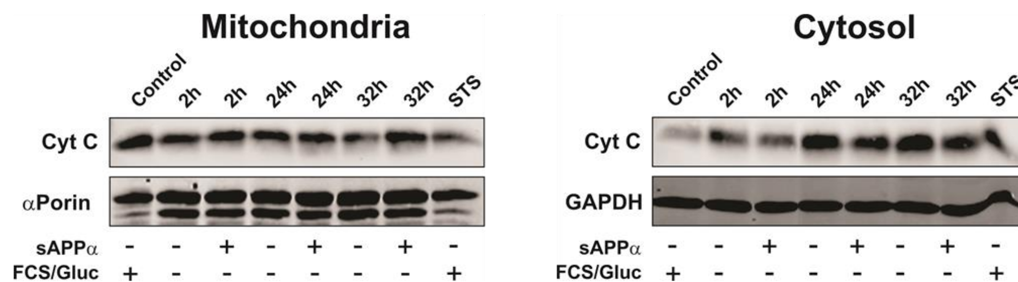


Figure 21. sAPPα and E1 induces anti-apoptotic Bcl-xL and Mcl-1 expression in APP wt cells. MEF from APP wt and APP KO cells were cultured in full medium (+FCS/+Gluc) or in medium lacking trophic factor (-FCS/-Gluc) for 24 h to induce stress. In parallel cells were treated with 25 nM and 50 nM sAPPα or 50 nM E1 to activate cell survival. Afterwards, the cells were harvested and total cell lysates were analyzed for Bcl-xL and Mcl-1 protein levels by western blot. GAPDH was used as a control for equal protein loading. Under –FCS/-Gluc deprived condition both Bcl-xL and Mcl-1 expression was decreased which was further increased by sAPPα or E1 treatment in APP wt cells (A, C) but not in APP KO cells (B, D). Under trophic factor deprived condition, Bcl-xL and Mcl-1 expression on transcriptional level was significantly suppressed in MEF wt cells treatment with sAPPα also evoke a minor but not significant increase of Bcl-xL and Mcl-1 mRNA expression (E, F).

4.4.4 Effect of sAPPα treatment on cytochrome c release from mitochondria

The pro and anti-apoptotic Bcl-2 family members tightly regulate the mitochondrial pathway of apoptosis. Since we observed that sAPPα shows effect on some Bcl-2 family members (e.g. Bim, Bcl-xL, Mcl-1), therefore we try to investigate whether it can also affects the cytochrome c release from mitochondria. To investigate the cytoprotective physiological function of sAPPα on cytochrome c release we used MEF wt cells and APP KO cells. The cells were pretreated the cultures for 24 h with 50 nM sAPPα. After that, similar stress condition (-FCS/-Glu) was used and cells were lysed at different time points (2 h, 24 h and 32 h). STS which is a known apoptotic inducer was used as a positive control. In MEF wt cells mitochondrial release of cytochrome c was decreased with sAPPα treatment at 24 h and 32 h. Slight decrease was also observed at 2 h. But no such decrease in cytochrome c relase was observed in APP KO cells with sAPPα treatment. (Figure 22).

A MEF APP wt



B MEF APP KO

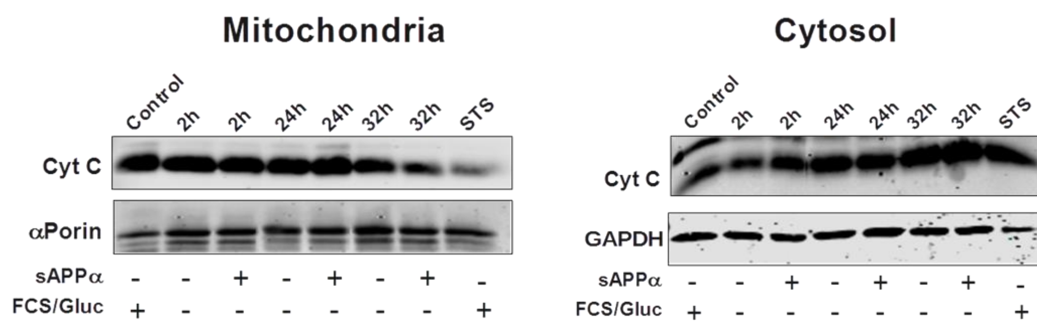


Figure 22. Effect of sAPPα treatment on mitochondrial cytochrome c release in APP wt and APP KO cells. MEF (A) wild-type (wt) and (B) APP knockout (KO) cells were cultured in full medium (+FCS/+Gluc) with or without 50 nM sAPPα for 24 h. Then, cells were cultured in full medium (+FCS/+Gluc) or in medium lacking trophic factor (-FCS/-Gluc) for 2 h, 24 h and 32 h. Afterwards, the cells were harvested at respective time points and mitochondrial and cytosolic cell lysates were analyzed for cytochrome c levels by western blot. GAPDH and αPorin was used as a control for equal protein loading.

4.4.5 Effect of sAPPα and E1 on Bax-Bak activation

The Bax-like proteins (Bax and Bak) trigger mitochondrial permeabilization, which is required for the cytochrome c release from mitochondria and subsequent activation of initiator caspase 9 and downstream effector caspases (Kroemer et al., 2007). Under different stress conditions, activated cytoplasmic Bax and Bak convert into pore-forming proteins by changing their conformation and assemble into oligomeric complexes in the mitochondrial outer membrane (Westphal et al., 2011). This Bax and Bak oligomers form pores in the mitochondrial outer membrane, which is a crucial event in the mitochondrial apoptosis pathway (Klinkenberg et al., 2010). Since we observed an effect of sAPPα treatment on

cytochrome c release from mitochondria in APP wt cells therefore we tested the effect of sAPPα treatment on Bax and Bak expression level. Our results show that the treatment with sAPPα or E1 did not alter Bax and Bak expression in serum and glucose deprived MEF APP wt or KO cells (Figure 23). Further research has to be performed to confirm these findings.

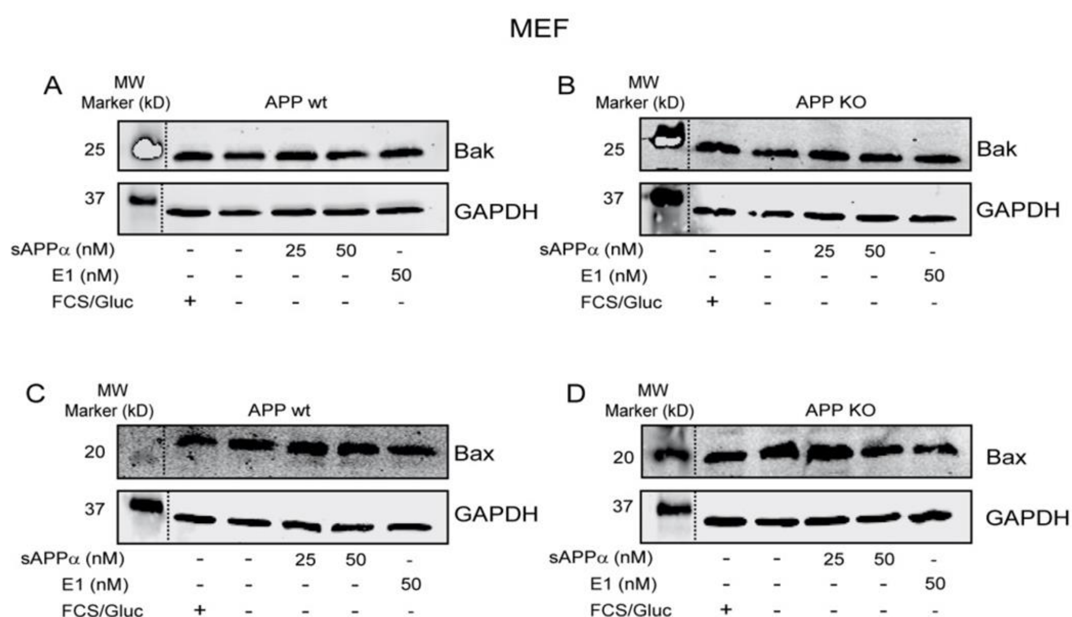


Figure 23. sAPPα and E1 treatment has no effect on Bax and Bak expression. MEF cells from APP wt and APP KO were cultured and treated similarly as mentioned in Figure 18. Afterwards, the harvested cells were lysed to analyze Bax and Bak protein levels by western blot. GAPDH was used as a control for equal protein loading. Under –FCS/-Gluc deprived condition no change was observed in Bak or Bax expression compared to the +FCS/+Gluc treated culture. There was also no further changes occur after sAPPα or E1 treatment in both wt (A and C) and KO (B and D) cell line.

All these results suggest that sAPPα and E1 show the neuroprotective effects via the PI3K/Akt survival pathway only in the presence of endogenous holo-APP and via the activation or inhibition of several downstream anti-apoptotic (Bcl-xL, Mcl-1) or proapoptotic (Bim, FoxO) target molecules,depending on the APP expression. sAPPα also modulate the release of cytochrome c from mitochondria in cooperation with holo-APP.

4.5 Modulation of protein homeostasis by sAPPα

As discussed above sAPPα dependent neuroprotection involves activation of PI3K/Akt survival signaling pathway and inhibition of stress triggered JNK/cJun

pathway (Copanaki et al., 2010; Eckert et al., 2011; Milosch et al., 2014; Sarker et al., 2009). Interestingly, the inhibition of JNK signaling leads to the decrease of expression of the co-chaperone protein BAG3. BAG3 is a member of Bcl-2 associated athanogene family that is stimulated under stress conditions, such as oxidative stress or proteasomal inhibition. In previous study it was observed that the expression of BAG3 increases during aging, and a shift from BAG1 to BAG3 expression was observed (Gamerdinger et al., 2009). BAG1 is involved in proteasomal degradation in young cells and BAG3 induces selective macroautophagy pathway of protein degradation during aging and under stress conditions (Gamerdinger et al., 2009). The maintenance of protein homeostasis is essential for neuronal health. One of the key pathomechanism of aging and Alzheimer's disease is disturbances in protein homeostasis. In a recent study it was observed that sAPPα exerts important role in modulation of protein degradation under stress conditions by suppressing stress triggered BAG3 expression and aggresome formation and partially rescuing the proteasomal activity (Renziehausen et al., 2015). In this study I have investigated the correlation between the two beneficial aspects of sAPPα, its role in activation of neuroprotective survival signaling and its modulatory effect on proteostasis.

4.5.1 sAPPα prevents upregulation of BAG3 expression after proteasomal stress

In recent study, it was demonstrated that recombinant yeast derived sAPPα decreases proteasomal stress induced BAG3 expression in young IMR90 fibroblast cells (Renziehausen et al., 2015). Therefore, in this thesis the effect of recombinant sAPPα on BAG3 expression in APP wt human neuroblastoma SH-SY5Y cell and MEF cell under proteasomal stress was studied. A well-known proteasomal inhibitor MG132 was used to trigger BAG3 expression. The cells treated with sublethal doses of proteasomal inhibitor MG132 was found to induce BAG3 expression and treatment with sAPPα suppresses this stress triggered BAG3 expression (Figure 24A and B). In transcriptional level significant induction of BAG3 expression with MG132 treatment and consequently sAPPα mediated reduction of BAG3 expression was also observed (Figure 24C).

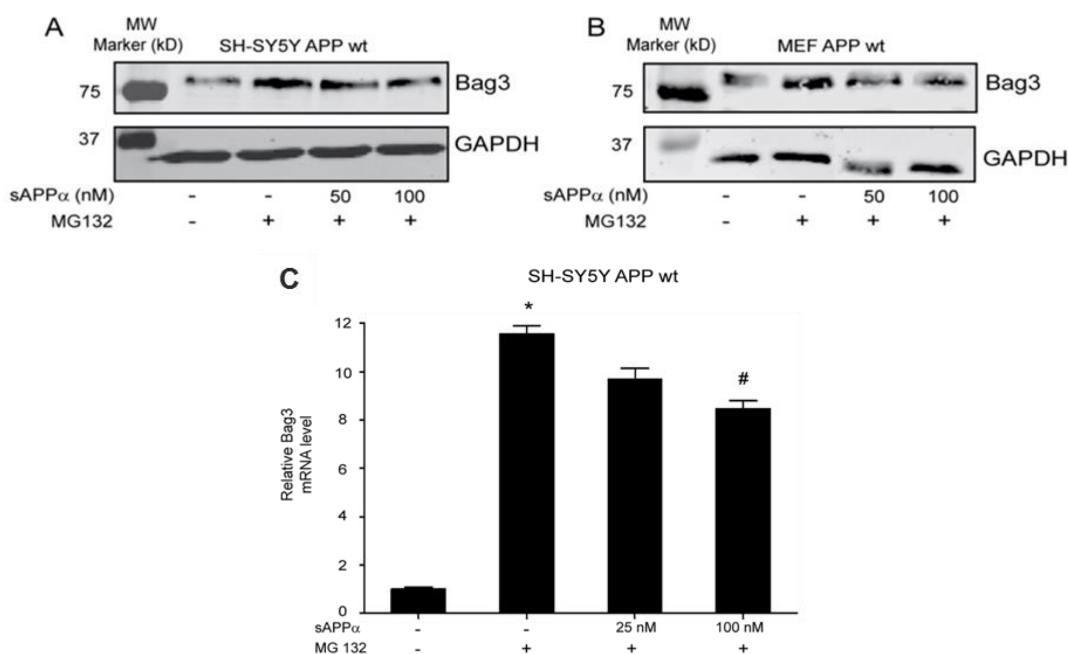


Figure 24. Effect of sAPPα treatment on BAG3 expression under proteasomal stress. SH-SY5Y wt (A) and MEF wt (B) cells were pretreated with 50 nM and 100 nM sAPPα for 24 h and subsequently treated with 0.5 µM MG132 for another 24 h. After that the cells were harvested and total cell lysates were analyzed for BAG3 protein levels by western blot analysis. GAPDH was used as a control for equal protein loading. (C) cells were similarly treated as (A) and (B) with 25 nM and 100 nM sAPPα and treated with 0.5 µM MG132. Afterwards, cells were harvested for mRNA isolation. The relative mRNA levels were determined by quantitative PCR analysis. Data represent mean ±SEM from quadruplicate determinations. Significant BAG3 induction compared to DMSO controls are marked by asterisks:* $p<0.05$. Significant differences between sAPPα treated and MG132 only treated cultures are indicated by hashtags: # $p<0.05$.

4.5.2 Role of sAPPα and APP in the regulation of BAG3 expression under proteotoxic stress

Previous experiments from our group demonstrated that holo-APP is essential to mediate the sAPPα dependent activation of PI3K/Akt survival pathway (Milosch et al., 2014). A novel function of sAPPα in modulation of protein homeostasis was also identified (Renziehausen et al., 2015). Therefore, to address the question whether common molecular mechanism may involve in these two beneficial roles of APP and to study the potential function of holo-APP in mediating the effects of sAPPα on proteostasis, the possible involvement of holo-APP in the modulation of BAG3 expression by sAPPα was investigated in APP KD and APP KO cells. SH-SY5Y wt and APP KD cells were pretreated with different concentrations (25 nM, 50 nM and 100 nM) of His-tagged sAPPα for 24 h and subsequently treated with 0.5 µM MG132 for another 24 h. In control condition cultures were treated with DMSO. After treatment cells were lysed for western blot. Analysis of the blots indicates that the stress induced increase in BAG3 expression was significantly decreased with sAPPα treatment in dose dependent manner in both wt and APP KD cells (Figure 25). These results demonstrate that holo-APP is not required for sAPPα mediated BAG3 expression under proteasomal stress condition.

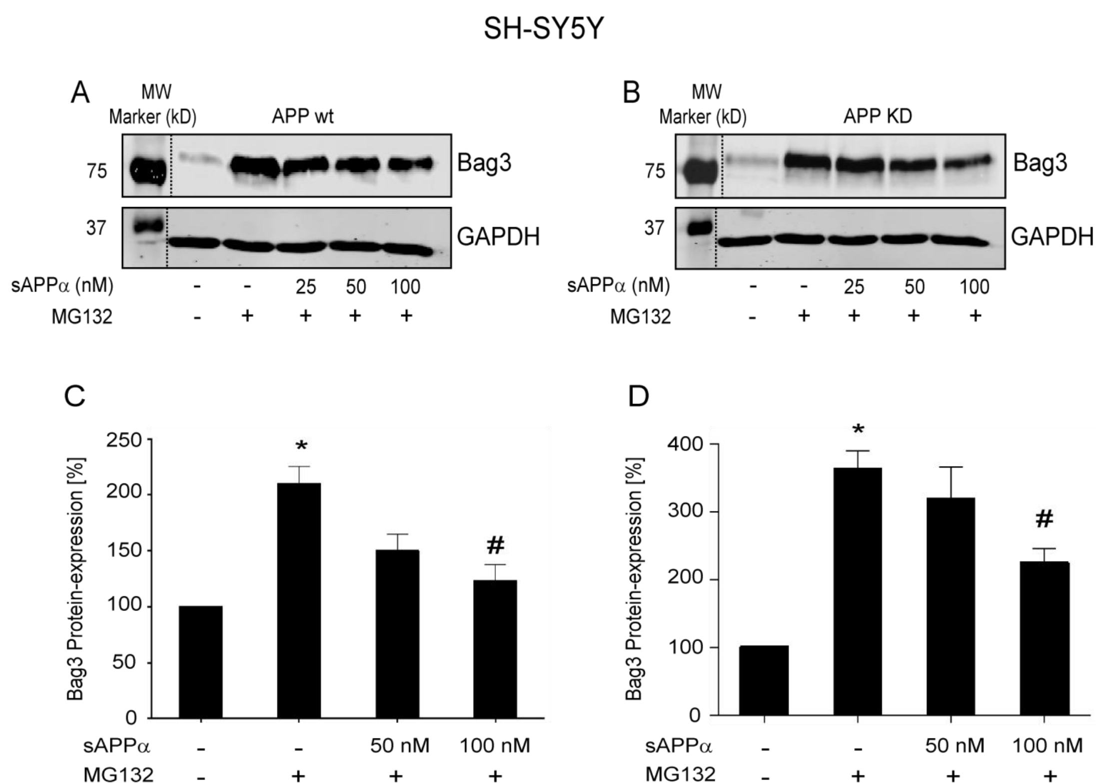


Figure 25. sAPPα prevents BAG3 induction under conditions of proteotoxic stress. Stable transfected SH-SY5Y cells expressing (A) APP or (B) shRNA directed against APP (KD) were pretreated with 25 nM, 50 nM, and 100 nM sAPPα prior to the administration of 0.5 µM proteasome inhibitor MG132 for 24 h. Afterwards, the cells were harvested and total cell lysates were analyzed for BAG3 protein levels by western blot analysis. GAPDH was used as a control for equal protein loading. (C, D) Quantitative fluorescence signal analysis of immunoblots in which BAG3 immunoreactive signals were first normalized to GAPDH and the resulting values were normalized to DMSO controls, which were set as 100%. Significant BAG3 induction compare to DMSO controls are marked by asterisks: *p<0.05. Significant differences versus MG132 only treated cultures: #p<0.05. Data represent mean ±SEM from triplicate determinations.

4.5.3 sAPPα prevents BAG3 upregulation in primary hippocampal neurons in wt and APP KO cells

In order to investigate the influence of sAPPα on differentiated neuronal cells, we have employed primary neurons isolated from the hippocampus of wt and APP KO animals. The hippocampal cultures were pretreated with 25 nM, 50 nM and 100 nM sAPPα. After 24 h the cells were exposed to 0.5 µM proteotoxic stress MG132 for another 24 h. After treatment cells were lysed for western blot. Our results demonstrated a significant induction of MG132 induced BAG3 expression in primary hippocampal neurons from both wt and KO animals, which were significantly prevented by sAPPα (Figure 26A and B). As in SH-SY5Y cells, this

result also confirmed the similar effect of sAPPα on BAG3 protein levels irrespective of the presence of endogenous APP.

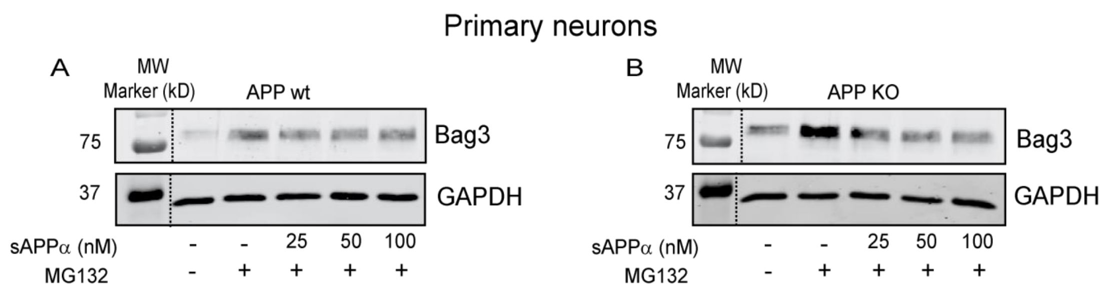


Figure 26. sAPPα prevents MG132 induced BAG3 expression in primary hippocampal neurons. Primary hippocampal neurons of wt (A) and (B) APP knockout (KO) neurons were pretreated with 25 nM, 50 nM, and 100 nM sAPPα prior to the administration of 0.5 µM proteasome inhibitor MG132 for 24 h. Afterwards, the cells were harvested and total cell lysates were analyzed for BAG3 protein levels by western blot analysis. GAPDH was used as a control for equal protein loading.

4.5.4 sAPPα suppress MG132 induced BAG3 expression on the transcriptional level

The influence of sAPPα on BAG3 expression might be exerted on the transcriptional level. To confirm this quantitative real-time PCR (qPCR) were performed in SH-SY5Y wt and APP KD cells. The cells were preincubated with 25 nM and 100 nM sAPPα for 24 h prior to the treatment with 0.5 µM MG132 for another 24 h. The cells were then lysed for RNA isolation and qPCR. qPCR analysis showed a significant reduction of BAG3 messenger RNA (mRNA) level by sAPPα treatment in both wt and APP KD cells under proteasomal stress, indicating transcriptional regulation of BAG3 expression by sAPPα (Figure 27A and B). BAG1 expression was also investigated but no induction of BAG1 mRNA level was observed after proteotoxic stress treatment (Figure 27A and B).

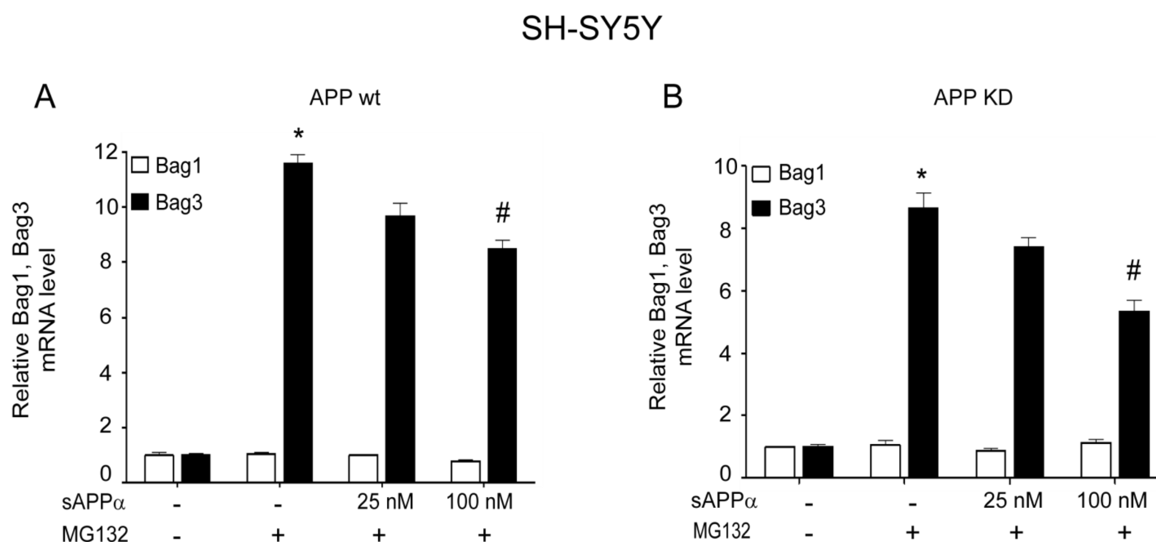


Figure 27. Effect of sAPPα pretreatment on BAG3 expression on the transcriptional level. SH-SY5Y wt (A) and APP KD (B) cells were treated with 25 nM and 100 nM sAPPα and subsequently treated with 0.5 µM MG132 for another 24 h. After that cells were harvested for mRNA isolation. The relative mRNA levels were determined by quantitative PCR analysis. Data represent mean ±SEM from quadruplicate determinations. Significant BAG3 induction compared to DMSO controls are marked by asterisks:* $p<0.05$. Significant differences between sAPPα treated and MG132 only treated cultures are indicated by hashtags: # $p<0.05$.

4.5.5 Effect of sAPPα treatment on BAG3 binding partner Hsp70

BAG3 is a co-chaperone and binding partner of heat shock protein 70 (Hsp70). It has been suggested that proteasomal impairment induces the upregulation of the stress-induced chaperone Hsp-70 expression (Gamerdinger et al., 2009; Goldbaum and Richter-Landsberg, 2004). In this study, the effect of sAPPα on Hsp70 mRNA expression was also examined. For this, cells were preincubated with 25 nM, 50 nM and 100 nM sAPPα for 24 h and subsequently treated with 0.5 µM MG132 for 24 h. The cells were then lysed for RNA isolation and qPCR. After treatment with MG132, Hsp70 expression was increased ~50 fold in both wt and APP KD cells but no additional effect of sAPPα treatment was observed (Figure 28).

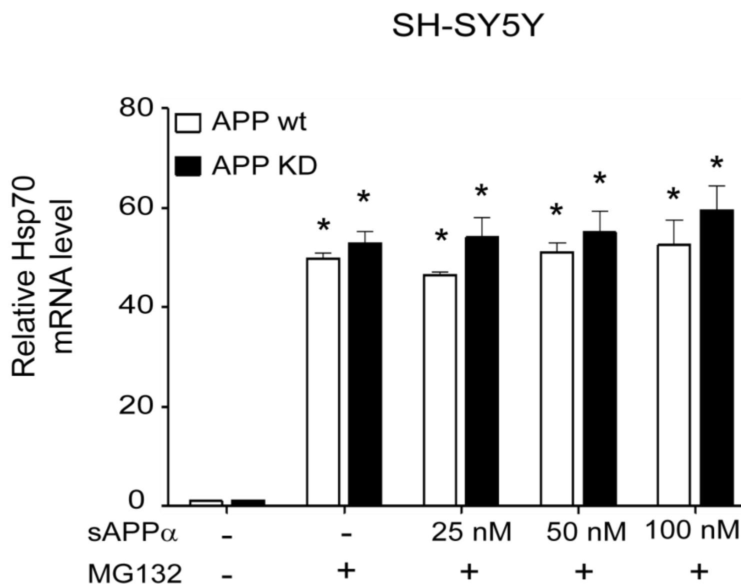


Figure 28. sAPPα has no influence on Hsp70 expression. SH-SY5Y wt (A) and APP KD (B) cells were treated with 25 nM, 50 nM and 100 nM sAPPα and subsequently treated with 0.5 μM MG132 for another 24 h. Afterwards cells were also harvested for mRNA isolation. Induction in Hsp70 mRNA expressions were observed in both wt and APP KD cells but no additional effect of sAPPα treatment was observed. The relative mRNA levels were determined by quantitative PCR analysis. Data represent mean ±SEM from quadruplicate determinations. Significant Hsp70 induction compared to DMSO controls are marked by asterisks:* $p<0.05$.

4.6 Investigating the possible role of sAPPα on mitophagy

Mitophagy is a process of selective degradation of damaged mitochondria by autophagy. It is crucial for maintaining cellular functions. Since a role of sAPPα on macroautophagy under proteasomal stress condition was observed, therefore the influence of sAPPα on mitophagy was also investigated under MG132 treated condition. At first FACS of MTDR (Mito Tracker Deep Red) was performed to quantify the michondrial mass. Previous result had suggested that MG132 inhibits the removal of mitochondria via mitophagy (Chan et al., 2011). In this experiment, a minor increase in mitochondrial mass (~0.3%) was observed upon MG132 treatment. This indicates that basal mitophagy was blocked by MG132. Treatment with sAPPα did not cause any changes in mitochondrial mass (Figure 29A). As a positive control for mitophagy, some cultures were treated with 10 μM FCCP (Carbonyl cyanide 4(trifluoromethoxy)phenylhydrazone)), which is a well known inducer of mitophagy and observed a significant reduction of mitochondrial mass (Figure 29B).

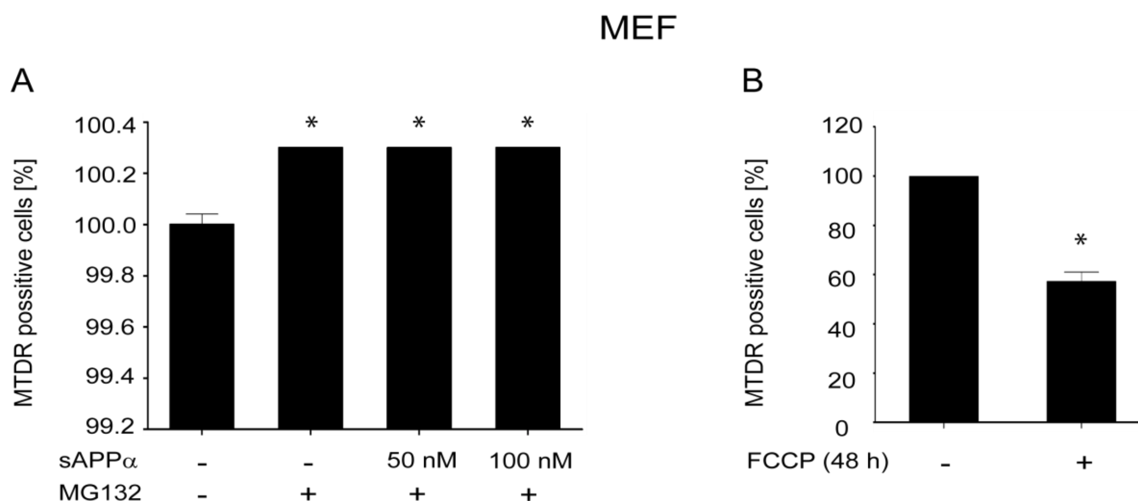


Figure 29. MG132 treatment inhibits mitophagy. (A) MEF wt cells were preincubated with 50 nM, 100 nM sAPPα for 24 h followed by treatment with 10 µM MG132 or equal volume of DMSO as negative control for 24 h. MTDR positive cells were measured by a FACS cytometer.(A) significant increase in MTDR positive cells were observed in MG132 treated cultures compared to DMSO control. As a positive control for mitophagy, cells were treated with 10 µM FCCP for 48 h (B). Data are means from four independent cultures ±SEM. Statistical significance:* p<0.05 compared to control (DMSO).

4.7 sAPPα prevents MG132 induced perinuclear aggresome formation

Aggresome are large perinuclear structures which are formed by accumulation of damaged or misfolded proteins. Upon inhibition of the proteasomal degradation pathway, aggresomes and aggresome targated proteins are degraded by autophagy. BAG3 and vimentin, are well known markers of aggresome due to their direct localization into these structures. BAG3 plays important role in the formation of aggresome and the transport of degradation prone proteins into these structures (Behl, 2011; Gamerdinger et al., 2011). In this study, the effect sAPPα on aggresome formation in SH-SY5Y wt and APP KD cells was examined after induction of proteasomal stress with MG132. The immunostaining of aggresomal marker vimentin was used to detect aggresome formation. Under proteasomal stress, a strong aggregation and redistribution of vimentin from cytosol to discrete enrichment areas in the perinuclear space was observed for both wt and APP KD cells, indicating the formation of perinuclear aggresome. The cells were first preincubated with 100 nM sAPPα for 24 h before administration of 10 µM MG132. After 8 h cells were fixed, immunostained with anti-vimentin antibody, and

analyzed microscopically. The cells with clustered, vimentin-positive perinuclear aggregates are marked with arrow (Figure 30A). In MG132 treated cultures only the cells with perinuclear inclusions were counted. In DMSO treated cultures the vimentin stain were much more dispersed which represents the intermediary filaments of the cells. The higher magnification of a DMSO treated cell and a MG132 treated cell were shown in the Figure 30B. The number of cells with perinuclear aggresome was significantly increased after MG132 treatment; whereas co-treatment with sAPPα significantly decreased the MG132 induced accumulation of vimentin positive aggregates in both cell lines (Figure 30C). These results demonstrate that holo-APP is not required for mediating this sAPPα induced effect on aggresomes.

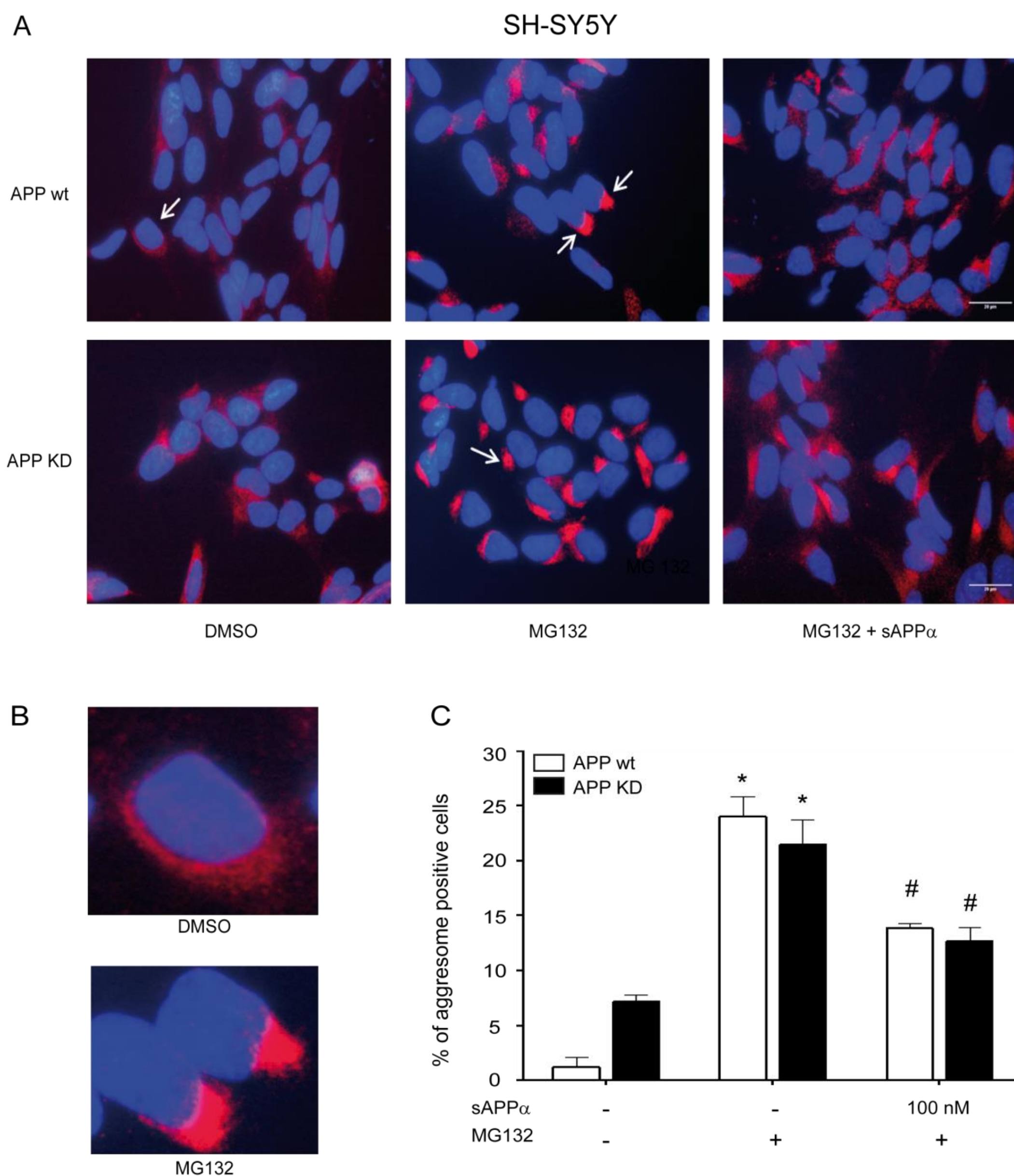


Figure 30. sAPPα prevents MG132 induced perinuclear aggresome formation. SH-SY5Y cells stably transfected with (A) empty vector or shRNA directed against APP were pretreated with 100 nM sAPPα for 24 h before administration of 10 µM MG132. After 8 h, the cells were fixed, immunostained with an anti-vimentin antibody, and analyzed microscopically. Examples of aggresome-positive cells are marked with arrows. (B) Higher magnification of the two cells marked with arrows shown in the upper panel of Figure (A). (C) Quantification of cells with vimentin positive aggresomes was done by counting in four random microscopic fields. Data represent mean ±SEM from n=400 cells. Statistically significant induction of aggresome formation is indicated by asterisks: *$p<0.05$. Significant differences versus MG132 only treated cultures are indicated by hashtags: # $p<0.05$.

4.8 sAPPβ unable to suppresses BAG3 induction under proteasomal stress

sAPPβ is an ectodomain of APP, which is generated after β- secretases cleavage in the amyloidogenic pathway of APP processing. sAPPα and sAPPβ have almost similar structure, except the absence of 17 amino acids motif from C-terminal in sAPPβ. Previous studies have shown that unlike sAPPα, sAPPβ lacks neuroprotective properties (Copanaki et al., 2010). In this study, the effect of sAPPβ treatment on proteostasis was investigated. To analyze the function of sAPPβ on BAG3 expression, purified recombinant sAPPβ was applied on MEF wt and APP KO cells for 24 h and then the cells were treated with 0.5 μM MG132 for another 24 h. sAPPβ treatment did not suppresses MG132 induced BAG3 induction in MEF wt and APP KO cells (Figure 31A and B). These results indicate that the modulation of proteaostasis under proteasomal stress condition is specific for sAPPα. Further the effect of sAPPα and sAPPβ on LC3-I/II, a prominent autophagosomal marker triggering the fusion of autophagosomes with lysosomes, was also investigated. The western blot analysis of LC3-I/II indicated a slight increase LC3-I/II expression after MG132 treatment but no additional effect of sAPPα or sAPPβ treatment was observed (Figure 31A and B).

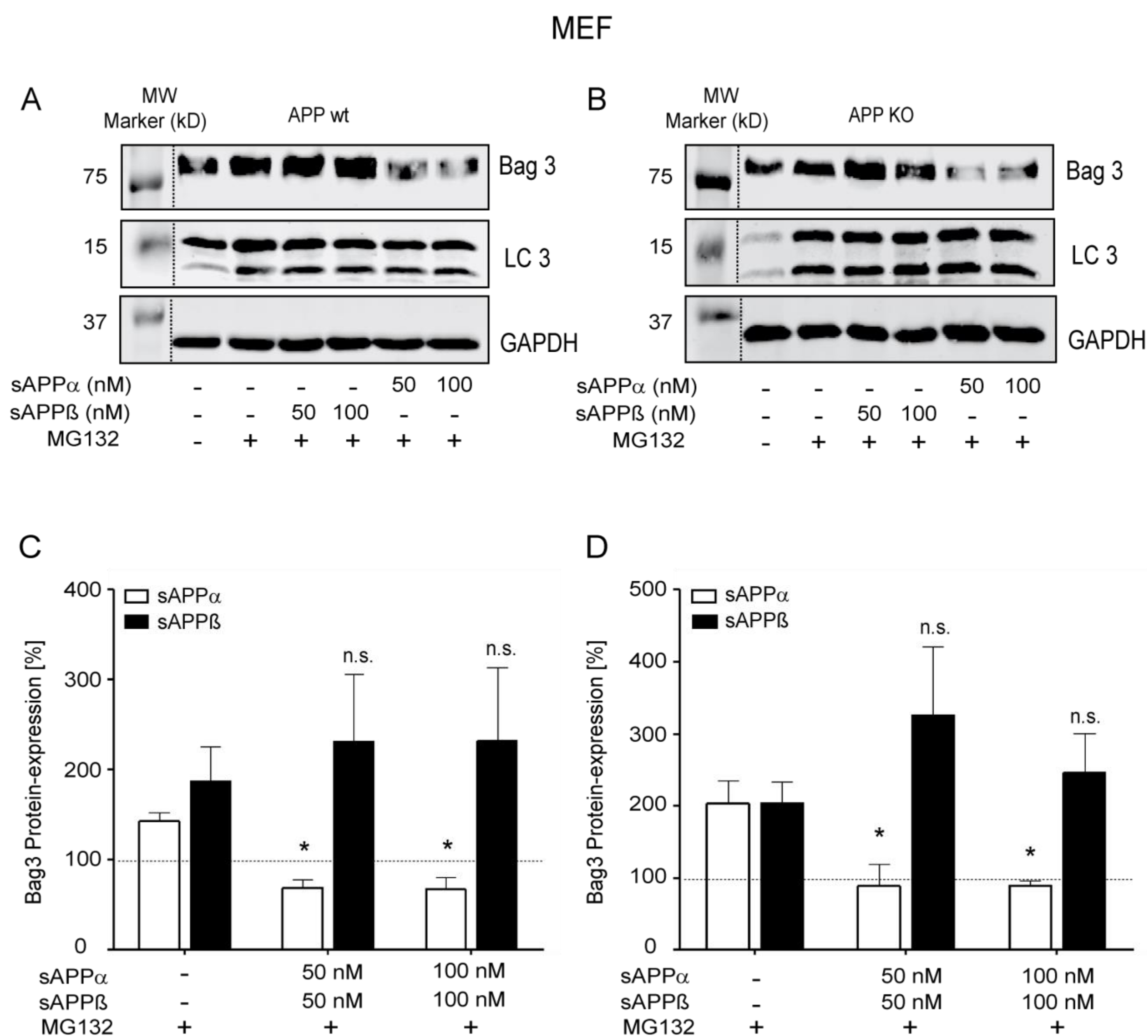


Figure 31. sAPPα but not sAPPβ, suppresses BAG3 induction under proteasomal stress. MEF from (A) wild-type (wt) and (B) APP knockout (KO) cells were pretreated with 50 nM and 100 nM sAPPα or sAPPβ prior to the administration of 0.5 μM MG132 for 24 h. Afterwards, the cells were harvested and total cell lysates were analyzed for BAG3 and LC3-I/II protein levels by western blot. GAPDH was used as a control for equal protein loading. (C, D) Quantitative fluorescence signal analysis of immunoblots in which BAG3 immunoreactive signals were first normalized to GAPDH and the resulting values were normalized to DMSO controls, which were set as 100%. Statistically significant differences versus cells treated with MG132 alone: #p<0.05. n.s. not significant. Data represent mean ±SEM from triplicate determinations.

4.9 sAPPα, but not sAPPβ increases the activity of the proteasome underproteasomal stress

An increase in macroautophagy and enhanced turnover of polyubiquitinated proteins occurs under proteasomal stress condition. Here the role of sAPPα in ubiquitin proteasome system (UPS) activity was examined. For this, MEF wt and APP KO cells were transfected with GFP-based UPS reporter (d2-GFP) plasmid. The degradation of d2-GFP was blocked by MG132 and pretreatment with sAPPα induced an increase in degradation of d2-GFP (Figure 32A and B). To further analyze the role of sAPPα in activating proteasomal activity, which may serve as a compensatory mechanism under conditions of sublethal proteasomal stress, a well-established substrate for 20S proteasome, SUC-LLVY-AMC was used. The activity of 20S proteasome was measured by monitoring the consumption rate of this substrate. The cells were pretreated with sAPPα or sAPPβ for 24 h and subsequently treated with 0.5 µM MG132 for 24 h. After treatment, the cells were lysed, mixed with SUC-LLVY-AMC substrate and proteasomal activity was measured. Similar to the modulation of BAG3 expression, the MG132 induced decrease of proteasomal activity was rescued by sAPPα, but sAPPβ treatment was failed to induce any increase of SUC-LLVY-AMC cleavage (Figure 32C).

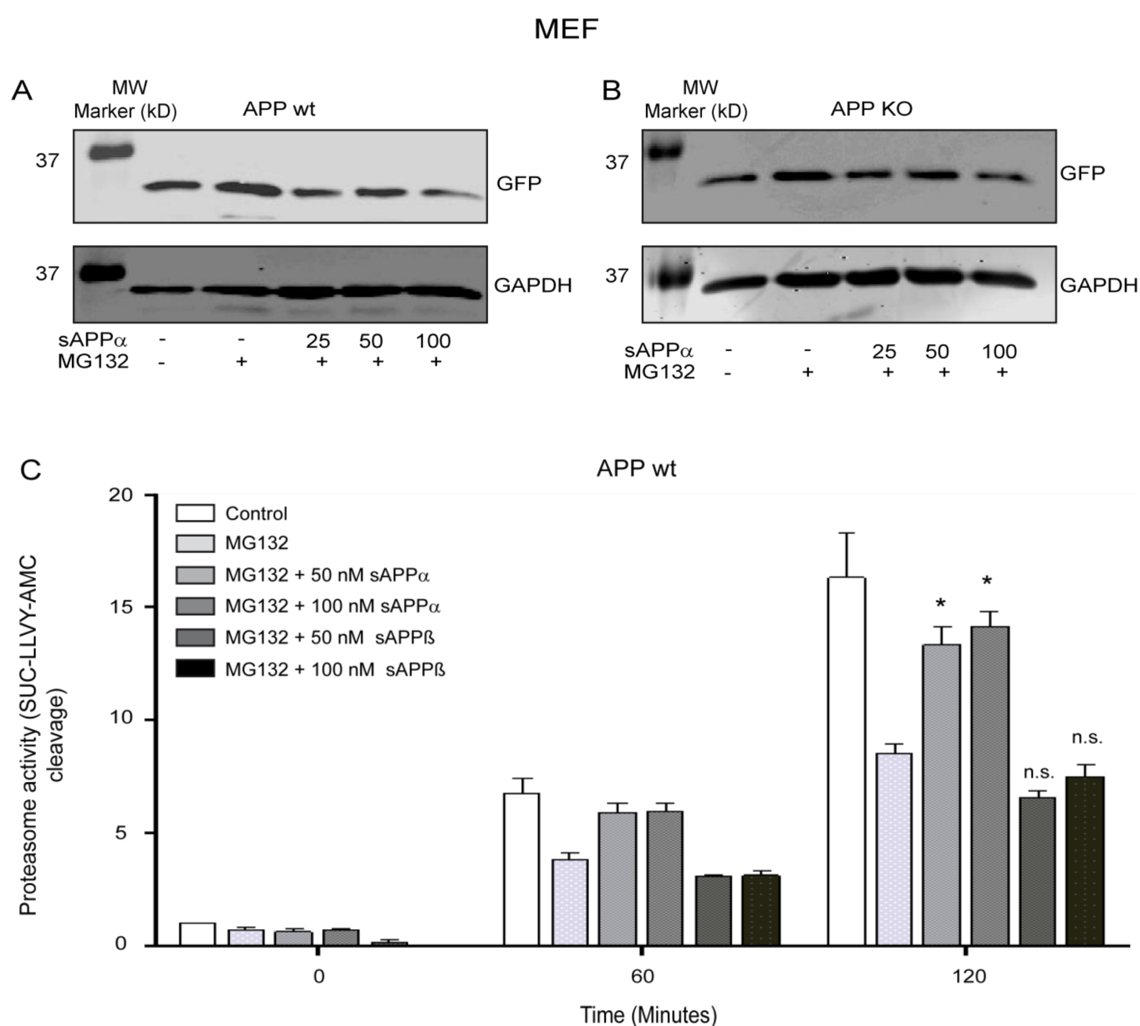


Figure 32. sAPPα, but not sAPPβ increases the activity of the proteasome under MG132 treatment. (A) MEF wt and (B) APP KO cells were transfected with d2-GFP reporter plasmid and pretreated with 50 nM, 100 nM sAPPα prior to addition of 0.5 μM MG132. Cells were harvested and total cell lysates were analyzed by Western blotting using antibody against GFP. (C) Measurement of proteasomal activity by SUC assays, representing the rate of SUC-LLVY-AMC cleavage as a proteosomal substrate in MEF wt cells. Data are means ± SEM from three independent cultures. Statistically significant differences versus cells treated with MG132 alone: #p<0.05. n.s. not significant.

5 Discussion

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases and so far no cure has been found for it. The main risk factor of the AD is the age and as a consequence of the increasing life expectancy in civilized countries, more and more people are suffering from this disease (Ferri et al., 2005; Kern and Behl, 2009).

In the histopathology of AD, deposits in the form of NFTs from hyperphosphorylated tau and amyloid plaques can be found (Alzheimer, 1907; Maurer et al., 1997). A major component of these plaques is Aβ, a cleavage product of APP. Although, the physiological role of APP in normal brain function is not clearly understood, it is known that APP can be proteolytically cleaved via two different pathways. Under physiological conditions the majority of APP is processed by α-secretase, ADAM10, via the nonamyloidogenic pathway, thus leading to the formation of the secreted N-terminal APP fragment sAPPα. APP processing via the amyloidogenic pathway leads to the production of Aβ by β- and γ-secretase cleavage (Chow et al., 2010; Kögel et al., 2012a; Nunan et al., 2001; Vassar et al., 1999).

APP and its metabolism play fundamental roles in the pathophysiology of Alzheimer’s disease (AD). The accumulation of Aβ results in the formation of Aβ plaques in the brain of AD patients. At least two downstream amyloidogenic processing fragments, Aβ and AICD, have largely been linked to neurodegeneration and apoptosis (Kögel et al., 2012b; Nhan et al., 2015; Wang et al., 2014). The loss of physiological functions of APP, in particular reduction of the secreted N-terminal ectodomain sAPPα, may also contribute to neurodegeneration and to cognitive deficits seen in AD (Reinhard et al., 2005; Ring et al., 2007; Turner et al., 2003; Zheng and Koo, 2006). The processing of APP via the amyloidogenic pathway is increased during brain aging and generates sAPPβ and the membrane bound C-terminal fragment CTFβ that can be further processed by γ-secretase to yield Aβ (Zhang et al., 2011). The formation of the toxic Aβ peptide is prevented by α-secretase cleavage within the Aβ region, which liberates the neuroprotective soluble ectodomain sAPPα (Endres

and Fahrenholz, 2012). Previous studies suggested that the nonamyloidogenic APP cleavage product sAPPα exerts potent neuroprotective effects (Furukawa et al., 1996; Kögel et al., 2005, 2012a; Mattson et al., 1993). APP and sAPPα also play important role in different physiological processes, such as synaptogenesis, neuronal plasticity and axonal growth (Zheng and Koo, 2011). Furthermore, it was shown in a mouse model that the expression of sAPPα can reverse the deficits of an APP -/- mouse (Ring et al., 2007) and during aging, the secretion of sAPPα decreases (Kern et al., 2006). This led to the hypothesis that not only the accumulation of Aβ but also the absence of sAPPα can lead to AD. However, the molecular mechanism underlying the beneficial effects of sAPPα remain elusive and may involve a complex interplay between survival and stress pathways, and interaction of sAPPα with surface receptors and other targets.

Two N-terminal regions, E1 and E2 domains of sAPPα are proposed to be responsible for the neuroprotective and the neurotrophic effects of sAPPα. Both of these domains contain heparin sulfate proteoglycan (HSPG) motifs which are thought to be responsible for this influence of sAPPα (Corrigan et al., 2011, 2014; Furukawa et al., 1996). Corrigan et al. have shown that the HSPG of the E1 domain is protective in traumatic brain injury and Furukawa et al. showed that the HSPG of the E2 domain increases cell survival in glutamate and Aβ induced neurotoxicity (Corrigan et al., 2011, 2014; Furukawa et al., 1996; Rossjohn et al., 1999). In contrast to sAPPα and E1, sAPPβ which is formed during the amyloidogenic processing of APP and is only 5 to 17 amino acids shorter than sAPPα, was observed to mediate either no or only minor biological effects in regard to neuroprotection (Brinkmalm et al., 2013; Chasseigneaux and Allinquant, 2012; Copanaki et al., 2010; Furukawa et al., 1996). Furukawa et al., 1996 proposed that sAPPα has an additional C-terminus heparin binding motif which is not present in sAPPβ, this motif may be crucial for neuroprotective properties of sAPPα (Furukawa et al., 1996).

Previous studies have shown that exogenous sAPPα treatment mediates neuroprotection against oxidative, proteasomal and excitotoxic stress (Clement et al., 2008; Kögel et al., 2003, 2005; Milosch et al., 2014; Schubert and Behl, 1993). This suggests that sAPPα plays a role in the activation of survival signaling such

as PI3K/Akt, ERK and NF-κB pathways under different stress conditions (Cheng et al., 2002; Eckert et al., 2011; Greenberg et al., 1995; Jimenez et al., 2011; Nizzari et al., 2007; Venezia et al., 2006). Interestingly, studies from our group reported that the stress induced activation of stress signaling JNK/c-Jun pathway and subsequent apoptosis were reduced in cells overexpressing wt APP and by exogenous sAPPα treatment (Kögel et al., 2005). Studies have also shown that sAPPα-mediated neuroprotection involves the activation of PI3K/Akt survival signaling pathway (Copanaki et al., 2010; Eckert et al., 2011; Jimenez et al., 2011). The PI3K/Akt signaling is a central cell survival pathway that can be activated by various growth factors and neurotrophins, such as insulin-like growth factor (IGF) family proteins. These proteins bind to their cognate cell membrane receptors and trigger the Akt cell survival pathway by activating PI3 kinase and downstream Akt (Manning and Cantley, 2007). The phosphorylation of Akt promotes cell survival by inhibiting pro-apoptotic proteins such as FoxO, Bim and by activating anti-apoptotic proteins such as Bcl-xL, Mcl-1 (Manning and Cantley, 2007; Yoshimoto et al., 2001). Additionally, activated Akt can inhibit JNK upstream kinases MLK3 and ASK1, which consequently inhibit JNK stress signaling pathway. This suggests a certain crosstalk between stress and survival pathways (Barthwal et al., 2003; Kögel et al., 2012a).

Age plays a very important role in AD (Lobo et al., 2000). The protein quality control (PQC) and the associated degradation of misfolded and aggregated proteins is a very important process in the organism, which changes in the course of aging. Due to the decreasing capacity of PQC in old age, rather defective proteins such as Aβ and NFTs can accumulate and influence cell function (Cuervo and Dice, 2000). Studies have shown that increased protein degradation has beneficial effects on age-related diseases such as AD (Komatsu et al., 2006).

In the present work, the influence of sAPPα on Akt downstream targets such as FoxO, Bim, Bcl-xL, Mcl-1 as well as on PQC was investigated.

5.1 Neuroprotective properties of recombinantly expressed sAPPα and E1

As discussed above sAPPα which is generated via cleavage of APP by α-secretase enzyme along the nonamyloidogenic pathway shows neuroprotective properties. The subdomain of sAPPα, E1 also evokes neuroprotection under stress conditions (Mattson et al., 1993; Milosch et al., 2014; Müller et al., 2012). After brain injury, the injection of sAPPα or sAPPα domain had beneficial effects on motor and cognitive outcome (Corrigan et al., 2011; Thornton et al., 2006). It also exerts protective effects during ischemic brain injury (Smith-Swintosky et al., 1994). The role of sAPPα in rescuing neuronal death induced by hypoglycemia (Mattson et al., 1993) and trophic factor deprivation (Cheng et al., 2002) has also been studied. The neuroprotective mechanism of sAPPα may be associated with modulation of ion homeostasis through rapid effect on ion channel function and delayed activation of transcription dependent survival signaling processes (Furukawa et al., 1996; Kögel et al., 2012a; LaFerla, 2002; Mattson et al., 1997).

Recombinant sAPPα and subdomain APP-E1 purified from yeast *P. pastoris* were reported to be neuroprotective in various cell models under proteasomal stress condition or trophic factor deprivation (Milosch et al., 2014). It was already reported from our group that recombinant sAPPα is able to protect PC12 cells and neurons from apoptosis induced by epoxomicin, an irreversible inhibitor of the 20S proteasome (Copanaki et al., 2010). sAPPα and even its subdomain APP-E1 alone were shown to inhibit cell death triggered by proteasome inhibition with MG132 (Milosch et al., 2014). The inhibition of the proteasome by epoxomicin or MG132 leads to an accumulation of damaged proteins, which activate different stress pathways and trigger cell death and mimics similar stress conditions as seen during brain aging and AD. The deprivation of trophic factors such as serum and / or glucose represents another simple method to induce stress in cells. The neuronal and non-neuronal cells require a steady support by trophic factors to promote cell survival and cell growth. Glucose is the brain's main energy source and its deprivation cause metabolic stress condition which arrest cell growth and survival (Kögel et al., 2006; Russo et al., 2004; Wang et al., 2002). It was observed that loss of trophic factors and a diminished glucose metabolism may

contribute to brain aging and the pathogenesis of AD (Furst et al., 2012; Hyman and Yuan, 2012; Pluta et al., 2013). It was established from our group that treatment with yeast derived recombinant sAPPα and APP-E1 potently antagonized serum and / or glucose deprived cell death in a dose and time dependent manner in different cell and tissue cultures such as in mouse fibroblasts (MEFs), human neuroblastoma cells (SH-SY5Y) as well as in primary (hippocampal) neurons and organotypic slices (Milosch et al., 2014).

Our group also demonstrated that sAPPα and E1 activate neuroprotective survival signaling cascade through activation of Akt survival signaling pathway and subsequent inhibition of GSK3β stress pathway in the presence of holo-APP (Milosch et al., 2014). Moreover, we provided evidences that holo-APP is required for sAPPα mediated neuroprotection and in the absence of holo-APP, sAPPα mediated activation of PI3K/Akt pathway is completely abolished (Milosch et al., 2014). E1 domain alone can also rescue Akt activity under stress conditions in wt cells. In APP deficient cells both sAPPα and E1 failed to rescue Akt activity under stress conditions. This study suggests that the E1 domain, which contains the growth factor like domain is the active neuroprotective part of sAPPα (Milosch et al., 2014).

The role of APP C-terminal domain in sAPPα mediated neuroprotection was also investigated. It was observed that the last 15 amino acids of APP C-terminal domain are essential for neuroprotective effect of sAPPα (Milosch et al., 2014). It was also shown that the YENPTY motif of APP is not required for mediating this neuroprotective effect, which suggested that YENPTY independent interactors are involved in sAPPα mediated neuropeotection. It was also determined that APP KO cells with G-protein interaction motif (PEER) deletion mutant were unable to rescue sAPPα induced Akt activation after serum starvation (Milosch et al., 2014). Collectively all these data indicate that sAPPα mediated neuroprotection occurs through membrane bound APP, where APP serves as a receptor and triggers G-protein dependent activation of PI3K which leads to further activation of downstream prosurvival kinase Akt and subsequent inhibition of proapoptotic kinase GSK3β (Milosch et al., 2014).

5.2 Downstream intracellular signaling targets of Akt involved in APP-sAPPα mediated neuroprotection

As previously discussed, Akt pathway is a well known cell survival pathway, which promotes neuroprotective pathways under different stress conditions involved in neurodegenerative disorders (Manning and Cantley, 2007; Rickle et al., 2004). Akt is a serine/threonine kinase, which is activated by PI-3-kinase. Upon activation, Akt phosphorylates various target proteins to activate or inhibit their functions and enhance cell survival and growth (Mitsuuchi et al., 1998). Our group could previously show that sAPPα treatment increases Akt phosphorylation at Ser 473, which is the key phosphorylation site for fully active Akt (Sarbassov et al., 2005). The phosphorylated, active form of Akt promotes cell survival by inhibiting pro-apoptotic proteins, such as Bad, FoxO, Bim and activating anti-apoptotic proteins, such as Bcl-xL, Mcl-1 (Manning and Cantley, 2007; Yoshimoto et al., 2001). In this thesis, the effect of sAPPα on these Akt downstream targets was further investigated under serum and glucose deprived condition. The aim of this study was to elucidate the role of Akt downstream targets in sAPPα activated Akt survival signaling pathway and neuroprotection. Therefore, different Akt downstream intracellular signaling targets such as FoxO, Bcl-xL, Bim, Mcl-1 were investigated under stress condition. These molecules were studied as putative signaling components involved in sAPPα or E1 mediated neuroprotection.

As previously mentioned, that GSK3β which is a major target of Akt, were observed to be inactivated following sAPPα treatment in serum deprived SH-SY5Y cells (Milosch et al., 2014). The inhibition of GSK3β occurs due to its phosphorylation by Akt kinase induced by sAPPα (Milosch et al., 2014). Another established pro-survival function of Akt is to inhibit the expression of proapoptotic BH3 only proteins, by deactivating transcription factors such as, FoxO. FoxO controls the expression of BH3 only protein Bim, which stimulate cell death (Dijkers et al., 2002). After phosphorylation by activated Akt, FoxO excluded out from the nucleus and is degraded through ubiquitination. Thus it cannot initiate gene expression of downstream molecules such as, Bim (Sanphui and Biswas, 2013). In this way, Akt blocks FoxO mediated transcription of target genes that promote apoptosis, cell cycle arrest and cell death (Manning and Cantley, 2007).

Akt also antagonizes the mitochondrial pathway of apoptosis by inhibiting proapoptotic protein Bad, which triggers cell death by holding antiapoptotic proteins Bcl-2 or Bcl-xL in an inactive state (Datta et al., 1997; del Peso et al., 1997). Since it was previously shown that sAPPα and E1 mediated neuroprotection occurs through the activation of PI3K/Akt pathway (Milosch et al., 2014). Therefore one of the main focuses of this study was to investigate the effect of sAPPα and E1 treatment on apoptotic pathway proteins closely related to the PI3K/Akt pathway. For this the expressions of transcription factor FoxO, pro-apoptotic protein Bim and anti-apoptotic proteins Bcl-xL and Mcl-1 after sAPPα and E1 treatment under serum and glucose deprivation were monitored. It was observed that in wt cultures phosphorylation of FoxO3a (which is an inactive state) increased after sAPPα and E1 treatment. Indeed, the expression of the proapoptotic protein Bim is also correlated to Akt phosphorylation with sAPPα and E1 treatment under stress conditions. Both Western blot and qPCR results confirmed that Bim expression decreased in presence of sAPPα and E1. Moreover, sAPPα and E1 treatment increased the expression of antiapoptoic proteins Bcl-xL and Mcl-1. In contrast, these effects of sAPPα or E1 on Akt downstream signaling molecule were not observed in APP KO cultures. This suggests that the neuroprotective effect of sAPPα and E1 regulates both proapoptotic BH3 only proteins and the mitochondrial pathway of apoptosis through PI3K/Akt pathway in presence of APP.

All these data presented here provides an understanding of key Akt downstream signaling components involved in sAPPα mediated neuroprotection. They suggest that sAPPα and E1 are capable to activate Akt and downstream intracellular signaling cascade through membrane bound APP.

5.3 sAPPα prevents release of cytochrome c from mitochondria in MEF wt cells

As discussed above, in this work we observed that sAPPα treatment can modulate expression of different pro and anti-apoptotic Bcl-2 family proteins (e.g. Bim, Bcl-xL, Mcl-1). It is also well known that these Bcl-2 family proteins tightly regulate the mitochondrial pathway of apoptosis (Kroemer et al., 2007; Kuwana and Newmeyer, 2003). Therefore, in this study we also investigate the role of sAPPα on cytochrome c release from mitochondria. The release of cytochrome c was monitored at different time point and it was observed that sAPPα treatment can reduce mitochondrial cytochorome c release after 24 and 32 h of serum and glucose deprivation. This effect was only observed in wt cells but not in APP KO condition. Since Bax and Bak triggers mitochondrial permeabilization which is required for the release of cytochrome c from mitochondria (Kroemer et al., 2007), we also studied the effect of sAPPα treatment on Bax and Bak expression. But we did not observe any changes in Bax and Bak expression level after sAPPα treatment. These results indicate that sAPPα may modulate Bax/Bak independent mechanism of cytochrome c release such as serine proteases dependent mechanism (Mizuta et al., 2007) of cytochrome c release.

5.4 The potential role of sAPPα in the maintenance of the intracellular protein homeostasis

In addition to its role in promoting cell survival, sAPPα also has effects on neuronal proteostasis under conditions of proteasomal stress (Renziehausen et al., 2015). The degradation of damaged and unfolded proteins is essential for maintenance of cellular viability and function. The ubiquitin proteasome system (UPS) and the macroautophagy pathway are the two major pathways of cellular protein degradation. These pathways are essential for stress induced and constitutive protein turnover (Morawe et al., 2012). Both systems provide a fine tuned regulation of the cellular protein quality control by removal of damaged and aggregated proteins. These two clearance systems have partial overlapping functions and their tightly regulated crosstalk is crucial for cellular viability and proteostasis. The impairment of protein degradation and proteostasis might lead

to an accumulation of damaged and unfolded proteins in the cell. This plays a pivotal role in brain aging and neurodegenerative diseases such as AD (Morawe et al., 2012; Nixon and Yang, 2012; Tanaka and Matsuda, 2014). The important members of UPS and macroautophagy pathway are co-chaperone proteins BAG1 and BAG3 (Gamerdinger et al., 2011). A prevalence of macroautophagy pathway over UPS is observed in naturally aged cells and brain tissue (Gamerdinger et al., 2009). A molecular switch in the expression of co-chaperone BAG1 to BAG3 is also observed during cell and organismal aging (Gamerdinger et al., 2009). Kern and colleagues (Kern et al., 2006) demonstrated that biochemical processing of APP is downregulated during aging, which is accompanied by a reduced secretion of sAPPα. Therefore, it has been suggested that the aging associated increase of BAG3 expression may have some correlation with reduction of sAPPα levels in aging brain (Endres and Fahrenholz, 2012). The present study established a sublethal proteasomal stress induced by low doses (0.5 μM) of proteasome inhibitor MG132, a condition that leads to a robust induction of BAG3 at the mRNA and protein levels. Higher doses of MG132 also affect the γ-secretase processing of APP and generation of APP intracellular domain fragment AICD, with subsequent AICD associated gene expression (Gersbacher et al., 2013). However, in this study no differences in the induction of BAG3 expression were observed in APP KD and KO cells, suggesting there is no major impact of endogenous APP and γ-secretase derived cleavage products on BAG3 activation. Furthermore, this study revealed that sAPPα can suppress MG132 induced BAG3 expression. It also demonstrates that this suppression of BAG3 induction by sAPPα do not require the presence of endogenous holo-APP. In contrast to the role of sAPPα and holo-APP in modulation of survival signaling pathway, the functional interaction between sAPPα and holo-APP is dispensable for the newly indentified role of sAPPα in proteostasis. Findings in the thesis support the notion that this biological activity represents a completely novel, independent function of sAPPα that is mediated via alternative signaling pathway, possibly involving distinct surface receptors and membrane domains, or alternative mechanisms such as internalization of APP.

5.5 sAPPα reduces accumulation of aggresomes

During macroautophagy, damaged, misfolded proteins are concentrated and isolated from other cytoplasmic components and sequestered in a perinuclear compartiment called aggresome (Gamerdinger et al., 2011). In the case of intensive protein accumulation, e.g. under proteasomal stress, perinuclear aggresomes form in the cells, which contain aggregated proteins that cannot be degraded immediately. Through the formation of aggresomes, cells isolate damaged proteins until their degradation. Previous investigations indicated that BAG3 is actively transported with misfolded proteins to the aggresome (Gamerdinger et al., 2011). BAG3 localisation was observed in perinuclear aggresomes induced by the proteasomal inhibitor MG132 (Gamerdinger et al., 2011; Renziehausen et al., 2015). Gamerdinger et al. have shown that in one hand vimentin, which is a well-known aggresomal marker, accumulates in aggresomes and on the other hand BAG3 also increases in the area of the aggresomes (Gamerdinger et al., 2011). Immunofluorescence staining with antibodies against vimentin could provide information about the number and localization of the aggresomes. Vimentin tends to locate aggresomally near the cell nucleus after proteasomal stress with MG132. In this work, immunocytochemistry analysis of the number and distribution of vimentin in the cells revealed a strong accumulation of perinuclear vimentin positive aggresomes in wt and APP KO MEF cells after MG132 treatment. It was observed that proteasomal inhibition increased the number of vimentin-positive nuclei was increased significantly compared to the untreated control cells (Figure 30A and C) Subsequent treatment with sAPPα significantly reduced the number of vimentin positive nucleus and it was mostly distributed over the cytoplasm (Figure 30A, B and C). In APP KD cells accumulation of vimentin positive aggresome were also observed after proteasomal inhibition and there was further reduction of aggresome with sAPPα treatment. This finding also confirmed that the role of sAPPα on BAG3 expression and aggresome formation occurs through a distinct mechanism which is independent of endogenous holo-APP.

Overall, these results suggest that sAPPα reduces selective macroautophagy and the number of perinuclear aggresomes under proteotoxic stress. It was expected that proteasomal inhibition and reduced autophagy leads to an increased of aggresome formation. On the other hand decrease in the number of aggresome may lead to accumulation of damaged proteins in the cells which in turn reduce cell viability. However, our studies indicate that survival of the cells is increased with sAPPα (Milosch et al., 2014; Renziehausen et al., 2015). Therefore, it is more likely that sAPPα affects protein homeostasis via a different pathway and thus facilitating degradation of the damaged proteins.

5.6 sAPPα-induced increase in proteasomal activity may prevent the induction of BAG3 protein

The increased cell viability and the reduced formation of aggresomes in the presence of sAPPα suggest that no further accumulation of proteins occur in the cells, despite the inhibition of proteasome and reduced autophagy. For this reason, the activity of the proteasome was examined. The protreasomal degradation of misfolded or damaged proteins may serve as a compensatory mechanism under conditions of sublethal proteotoxic stress. In this study, the role of sAPPα on proteasomal activity was investigated in wt and APP KO cells. First, the degradation of the proteasome substrate d2 in transiently transfected MEF wt and APP KO cells was investigated. It was previously shown by Gamerdinger et al. that after the inhibition of proteasome, with high concentration of proteasomal inhibitor (25 µM MG132) more d2-GFP accumulated in the cells, which could not be degraded due to the inhibition of proteasome (Gamerdinger et al., 2009). Similar results was observed in our study, as accumulation of d2-GFP was observed in both wt and APP KO cells which was reduced with sAPPα treatment (Figure 32A and B). This suggests that sAPPα can increase proteasomal activity even in the presence of low doses of the proteasomal inhibitor MG132. To verify this result, the activity of the proteasome was then measured by the Suc-LLVY-AMC assay. sAPPα can significantly increases the proteasomal activity, which was reduced by MG132 in both wt and APP KO cells (Figure 32C).

As discussed before in our study we observed that sAPPα treatment can reduce BAG3 expression under proteotoxic stress which suggest an alternative interpretation that sAPPα induced increase in proteasomal activity may be associated with or even prevent the induction of BAG3 expression. The autophagic pathway assists in protein degradation when cellular levels of damaged and misfolded proteins overwhelm the disposal capacity of proteasome. Under conditions of increased proteasomal activity cell may not need to induce BAG3-mediated autophagy. In conclusion, our results suggest that in addition to sAPPα-dependent suppression of BAG3 induction, sAPPα treatment has also an effect in modulation of proteasomal activity. Our results confirm that sAPPα increases proteasomal activity under stress conditions and thus reduces the number of poly-ubiquitinated proteins and increases cell viability.

5.7 Regulatory effects of sAPPα in primary neuronal cells

All of our studies have been performed in human neuroblastoma (SHSY5Y) and mouse embryonic fibroblast (MEF) cells. Since AD is a neurodegenerative disease, the protein analysis was additionally performed in primary neuronal cells. It was previously shown in our experiments that BAG3 expression was reduced by sAPPα after proteasomal stress in SH-SY5Y and MEF cells. Similar effect of sAPPα was also demonstrated in wt as well as APP KO hippocampal neurons which are physiologically more relevant models (Figure 26).

5.8 Recombinant sAPPβ fails to modulate BAG3 expression and proteostasis in APP-proficient wt cells

sAPPβ which is generated via β-secretase cleavage of APP lacks only 17 amino acids at the C-terminus in comparison to sAPPα. Earlier data from our and other groups suggest that sAPPβ lacks the neuroprotective properties of sAPPα (Copanaki et al., 2010; Furukawa et al., 1996). sAPPβ can actively regulate gene expression but it failed to rescue perinatal lethality and synapse defects of APP and APLP2 KO animals (Li et al., 2010). Similar to sAPPα, sAPPβ also carries the E1 domain, which was shown to induce neuroprotection (Milosch et al., 2014). Therefore, this lack of neuroprotective properties of sAPPβ in comparison to

sAPPα is difficult to explain. This may involve conformational differences between these two molecules. The last 17 amino acids of the second HSPG domain and a C-terminus heparin binding domain are absent in sAPPβ which may play crucial biological function in neuroprotection or the interaction of both HSPGs of E1 and E2 domains is necessary for neuroprotective property (Chasseigneaux et al., 2011; Freude et al., 2011; Furukawa et al., 1996). It was already shown by Renziehausen et al., that the E1 domain alone has no effect on protein homeostasis and therefore it was important to check whether sAPPβ can influence protein homeostasis (Renziehausen et al., 2015).

For observing the effect of sAPPβ on BAG3 expression and proteostasis, MEF cells were utilized. Data obtained in this study demonstrate that in contrast to sAPPα, sAPPβ did not exert any detectable effects on BAG3 expression and proteasomal activity (Figure 31 and Figure 32C). The exact molecular mechanisms behind this interesting observation and these differences between sAPPα and sAPPβ will have to be addressed in further studies.

5.9 The role of APP protein family on protein homeostasis

The signaling cascade through which sAPPα modulates protein homeostasis has not been elaborately studied yet. It is also largely unexplained how the extracellular sAPPα can exert intracellular effects. In addition to APP, the IGF1 and the insulin receptor have so far been identified as potential receptors of sAPPα (Jimenez et al., 2011). The two APLPs would also have to be considered as potential receptors since studies have shown that these can form homo and heterodimers with APP via their extracellular domains (Soba et al., 2005), and subsequently soluble processing products can also bind to these (Müller and Zheng, 2012). In addition, the AICD can bind to transcription factors such as Fe65, Tip60 and act as a transcription regulator (Cao and Südhof, 2001; Müller et al., 2008). Gralle et al., reported that sAPPα might modulate membrane-tethered APP dimerization, which might subsequently trigger the intracellular signaling (Gralle et al., 2009). In this thesis, the role of APP on the modulation of protein homoeostasis by sAPPα was studied. For this, the experiments were performed in both APP wt and stable APP KD SH-SY5Y and APP KO MEF cells, which

endogenously express the other APP family members APLP1 and APLP2. It was observed that in both APP wt and APP KD or KO cells, sAPPα can significantly reduced BAG3 protein expression (Figure 25 and 31). Since APP is not involved in the role of sAPPα-mediated modulation of protein homoeostasis, it can be argued that other APP family members (APLP1 and APLP2) may influence this effect of sAPPα. A compensatory role of other APP family members in mediating APP functions has already been described in previous studies (Anliker and Müller, 2006). Overall, it can be inferred that in APP KD or KO conditions, sAPPα may influence the protein homeostasis in the presence of other APP family members and thus APLP1 or APLP2 in this case could be the receptor of sAPPα. However, further studies are required to decipher the role of other APP family members (APLP1 and APLP2) in the underlying intracellular molecular mechanisms of sAPPα mediated protein homeostasis.

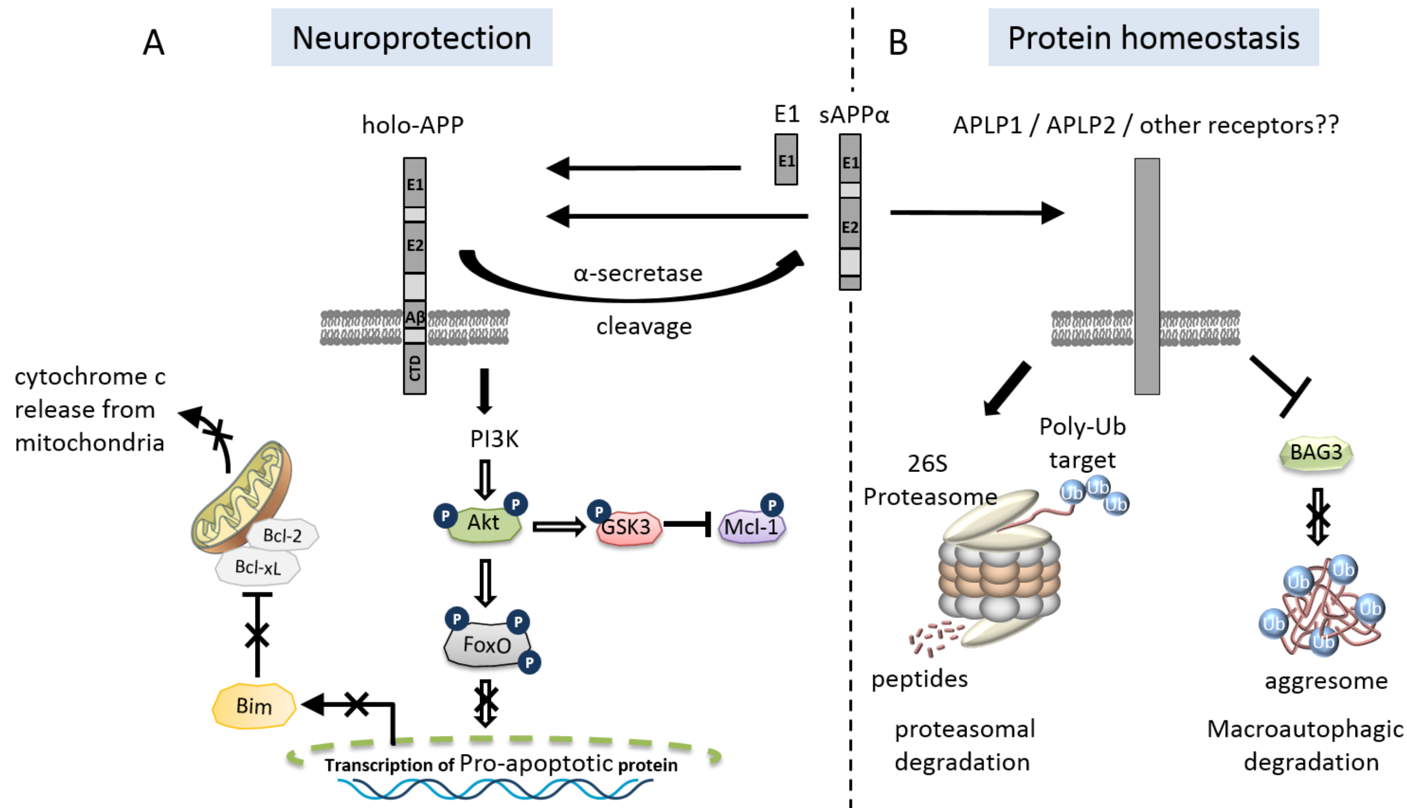


Figure 33. Hypothetical model of sAPPα function in neuroprotection and protein homeostasis. (A) In the nonamyloidogenic pathway, APP is cleaved by α-secretase generating sAPPα that can activate the PI3K/Akt survival pathway. Its subdomain E1 was also shown to mediate the same effects. This activation depends on the presence of holo-APP. PI3K/Akt phosphorylates and inactivates downstream FoxO, which in turn inhibits its translocation to the nucleus and inhibits transcription of proapoptotic gene *Bim*. This lead to the increase in expression of anti-apoptotic protein Bcl-2 and Bcl-xL and decrease of cytochrome c release from mitochondria. Activated Akt phosphorylates and inhibits GSK3β expression, which subsequently inhibits Mcl-1 phosphorylation and thereby prevents neurodegeneration and cell death. (B) sAPPα modulates protein homeostasis using an alternative signaling pathway possibly involving distinct surface receptors or other APP family members (APLP1/APLP2). sAPPα suppresses BAG3 expression and BAG3 mediated aggresome formation. sAPPα increase proteasomal activity and thereby facilitates proteasomal protein degradation to maintain protein homeostasis within the cells, under sublethal proteasomal stress.

5.10 Conclusion and Outlook

In conclusion, this thesis aimed at determining the key signaling components and mechanisms involved in sAPPα mediated neuroprotection and a completely novel function of sAPPα in modulation of the co-chaperone BAG3 and proteostasis. A neuroprotective role of sAPPα is well established and has been constantly observed in several different studies (Corrigan et al., 2011; Kögel et al., 2012a). Recently, it has been demonstrated that sAPPα and holo-APP functionally cooperate to induce the PI3K/Akt survival pathway and thereby inhibit stress triggered cell death (Milosch et al., 2014).

This study demonstrated that sAPPα protects neuronal cells death induced by serum and glucose deprivation via the modulation of the key downstream signaling targets of the PI3K/Akt survival pathway. These include central pro- and anti-apoptotic signaling components such as FoxO, Bim, Bcl-xL, Mcl-1 etc. sAPPα reduces expression of pro-apoptotic proteins FoxO and Bim and it induces anti-apoptotic Bcl-xL and Mcl-1 expression. The investigations made in this thesis also provide evidence that sAPPα reduces cytochrome c release from mitochondria, and thereby inhibits apoptotic cell death. While no changes were observed in the expression levels of Bax and Bak, which are responsible for triggering mitochondrial permeabilization required for the release of cytochrome c from mitochondria (Kroemer et al., 2007). Bax and Bak are mainly activated on the posttranslational level and under stress condition they convert into a pore forming proteins and assemble into oligomeric complexes in the mitochondrial outer membrane (Westphal et al., 2011). Therefore, future studies should further elucidate the exact mechanism, how sAPPα modulate cytochrome c release from mitochondria. In addition, the potential effect of sAPPα on other downstream targets such as Bad, PUMA, Bcl-2, Bcl-w, TRIB3, mTor etc. should also be investigated.

Additional experiments could be conducted to analyze the protective properties of other APP fragments, such as E2 and sAPPβ. As discussed earlier, studies with the APP ectodomain sAPPβ will be of particular interest, as sAPPβ also carries the E1 domain like sAPPα.

GSK3β in active form was found to induce tau hyperphosphorylation, which is one major pathological condition in the development of neurodegeneration and AD (Bradley et al., 2012; Hernandez et al., 2013). Therefore, another interesting topic could be to investigate the effect of sAPPα on GSK3β mediated inhibition of tau phosphorylation in both wt and APP-KO models.

Furthermore, the future studies should take into account the in vivo relevance of APP/sAPPα mediated neuroprotection. Several previous studies have demonstrated that cerebral ischemia may contribute to the pathogenesis of AD (Pluta et al., 2013) and sAPPα was also shown to have protective effects after cerebral ischemia due to traumatic brain injury (Corrigan et al., 2011, 2012, 2014; Smith-Swintosky et al., 1994; Thornton et al., 2006). Therefore, it will be important to further analyze APP/sAPPα mediated neuroprotection and changes in stress signaling pathways after acute ischemia/trauma. Similarly, the effect of sAPPα treatment after ischemic events in AD mouse model and control animals could also be an interesting study.

Additionally, this thesis also focused on sAPPα dependent suppression of BAG3 induction. It could be observed that the rescuing effect of sAPPα on proteasomal activity under proteotoxic stress conditions do not require the presence of endogenous holo-APP. In contrast to modulation of survival signaling, a functional interaction of sAPPα and APP is therefore dispensable for the newly identified role of sAPPα in proteostasis. Data from this study suggest that this biological activity of sAPPα represents a completely novel function of sAPPα that is mediated via alternative signaling events possibly involving other APP family members (APLP1 / APLP2) or distinct surface receptors and membrane domains such as sorting protein related receptors containing LDLR class A repeats (SORLA or LR11) (Hartl et al., 2013) or via alternative mechanisms such as internalization of sAPPα. However, further experiments are required to characterize the intracellular molecular mechanisms behind the role of sAPPα in protein homeostasis. In contrast to sAPPα, sAPPβ did not exert any detectable effect on BAG3 expression and proteasomal activity in our experiments. As discussed before, this difference may be related to a crucial biological function of the 17 aa motif in sAPPα (which is not present in sAPPβ) or major conformational differences between sAPPα and

sAPPβ. The exact molecular mechanism behind this difference will have to be addressed in future studies.

Collectively, the data from this thesis provide novel mechanistic insights into the detail understanding of APP's physiological function. This work also shed light on the effect of sAPPα on protein homeostasis under proteotoxic stress in a physiological context. Finally, this study indicated that the treatment of AD could be achieved by induction of neuroprotective pathways or by inhibition of stress signals. This in a wider frame could contribute for the development of therapeutic approaches against AD (Crews and Masliah, 2010).

6 References

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8 Curriculum Vitae

Personal Data

Name	Arpita Kundu
Nationality	Indian
Date of birth	05 February 1984
Place of birth	Kolkata (India)
Marital status	Married

School

03/1991-05/2001	Primary education (Jodhpur Park Girls High School and Jadavpur Sammilani Girls High School)
07/2001-07/2003	High school (Jadavpur Sammilani Girls High School)

University Education

08/2003-08/2006	Bachelor Degree in Microbiology University of Calcutta (India).
08/2006-09/2008	Master Degree in Microbiology University of Calcutta (India).
10/2009-01/2012	Master Degree in Interdisciplinary Neuroscience Frankfurt Goethe University (Germany).
06/2012-today	PhD student in Prof. Donat Kögel group, Experimental Neurosurgery, Neuroscience Center, Frankfurt University Clinics, Frankfurt Goethe University (Germany).

Academic Teachers

Mrs. Rupa Roy, Mrs. Sumana Das, Dr. Dipak K. Kar, Dr. Soma Halder, Mr. Nirmalya Chakroborty, Dr. Rukhsana Chowdhury, Prof. Dr. Abdelhaq Rami, Prof. Dr. Donat Kögel.

Complete list of publications

1. Modulation of BAG3 Expression and Proteasomal Activity by sAPPα Does Not Require Membrane-Tethered Holo-APP.
   Kundu A, Milosch N, Antonietti P, Baumkötter F, Zymny A, Müller UC, Kins S, Hajieva P, Behl C, Kögel D.
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2. The cleavage product of amyloid-β protein precursor sAβPPα modulates BAG3-dependent aggresome formation and enhances cellular proteasomal activity.
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