LRP1 modulates APP trafficking and APP metabolism within compartments of the secretory pathway

Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

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To my parents, Ralph and Christina, with love

Abstract

LRP1 modulates APP trafficking and metabolism within compartments of the secretory pathway

The amyloid precursor protein (APP) is the parent protein to the amyloid beta peptide (Aβ) and is a central player in Alzheimer's disease (AD) pathology. Aβ liberation depends on APP cleavage by β- and γ-secretases. To date, only a unilateral view of APP processing exists, excluding other proteins, which might be transported together and/or processed dependent on each other by the secretases described above. The low density lipoprotein receptor related protein 1 (LRP1) was shown to function as such a mediator of APP processing at multiple steps. Newly synthesized LRP1 can interact with APP, implying an interaction between these two proteins early in the secretory pathway. Therefore, we wanted to investigate whether LRP1 can mediate APP trafficking along the secretory pathway, and, if so, whether it affects APP processing. Indeed, we demonstrate that APP trafficking is strongly influenced by LRP1 transport through the endoplasmic reticulum (ER) and Golgi compartments. LRP1-constructs with ER- and Golgi-retention motifs (LRP-CT KKAA, LRP-CT KKFF) had the capacity to retard APP trafficking at the respective steps in the secretory pathway. Here, we provide evidence that APP metabolism occurs in close conjunction with LRP1 trafficking, highlighting a new role of lipoprotein receptors in neurodegenerative diseases.

Abstract

Increased AICD generation is ineffective in nuclear translocation and transcriptional activity

A sequence of amyloid precursor protein (APP) cleavages gives rise to the APP intracellular domain (AICD) together with amyloid β peptide (Aβ) and/or p3 fragment. One of the environmental factors identified favouring the accumulation of AICD appears to be a rise in intracellular pH. This accumulation is a result of an abrogated cleavage event and does not extend to other secretase substrates. AICD can activate the transcription of artificially expressed constructs and many downstream gene targets have been discussed. Here we further identified the metabolism and subcellular localization of the constructs used in this well documented gene reporter assay. We also co-examined the mechanistic lead up to the AICD accumulation and explored possible significances for its increased expression. We found that most of the AICD generated under pH neutralized conditions is likely that cleaved from C83. Furthermore, the AICD surplus is not transcriptionally active but rather remains membrane tethered and free in the cytosol where it interacts with Fe65. However, Fe65 is still essential in AICD mediated transcriptional transactivation although its exact role in this set of events is unclear.

Papers

This thesis is based on the following publications

Waldron E, Sebastian Jaeger, Pietrzik CU (2006): The functional role of the Low density lipoprotein receptor-related protein (LRP) in AD**.** *Neurodegenerative Diseases* 3(4-5):233-8

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Waldron E, Jaeger S, Martin AM, Weggen S, Pietrzik CU (2007): Increased AICD generation is ineffective in nuclear translocation and transcriptional activity. *Submitted*

Abbreviations

Aβ	Amyloid β-Protein
AD	Alzheimer's disease
ADAM	A Disintegrin and Metalloproteinase
AICD	APP Intracellular Domain
Amp	Ampicillin
Aph1	Anterior pharynx defective 1
APL-1	APP-like Protein 1
APLP1	Amyloid β Precursor-like Protein 1
APLP2	Amyloid β Precursor-like Protein 2
ApoER2	Apo-Lipoprotein-E-Receptor2
APPL	Amyloid Precursor Protein like
APP	Amyloid Precursor Protein
APPs	Amyloid Precursor Protein Ectodomain
APS	Ammonium persulfate
BACE	β-site APP cleaving enzyme
BCA	Bichinolin-4-carbonsäure
BSA	Bovine Serum Albumin
°C	Degrees Celsius
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovary
Cm	Centimeter
CO_2	Carbon dioxide
CTF	C-terminal fragment
Cu	Copper
$CuSO_4$	Copper sulfate
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylendiamintetraessigsäure
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FAD	Familial Alzheimer Disease
FKS/FBS	Fetal Bovine Serum
G	Gramm
GFP	Green Fluorescent Protein
H	hour
HEK	Human Embryonic Kidney
H_2O_{dd}	sterile distilled water
HRP	Horseradish Peroxidase
Hygro	Hygromycin
IgG	Immunglobulin G
IP	Immunoprecipitation

Abbreviations

KCl	Calcium chloride
kDa	kilo Dalton
KH_2PO_4	Calcium hydrogen phosphate
Ko	knock out
KPI	Kuniz-Type-Proteinase-Inhibitor
LB	Luria-bertani Broth
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LTP	Long terminal potentiation
M	Molar
mA	Milli ampere
MEF	Mouse Embryonic Fibroblast
MES	(2-(N-Morpholino)-ethansulfonsäure)
Mg	Milligramm
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
Min	Minute
Ml	Milliliter
Mm	Millimeter
mM	Millimolar
NaCl	Sodium chloride
NaH_2PO_4	Sodium hydrogen phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
Ng	Nano gramm
NICD	Notch Intracellular Domain
Nm	Nanometer
NMDAR	*N*-Methyl-D-aspartat-Receptor
NPxY	Asparagin-Prolin-x-Tyrosine-Motive
NSAID	Non-steroidal anti-inflammatory drug
NTF	N-terminal Fragment
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen-2	Presenilin enhancer 2
PHFs	Paired helical filaments
PID	Phosphotyrosine-Interactions domain
PM	Plasma membrane
PS	Presenilin
PSD-95	Postsynaptic Density Protein-95
RIP	Regulated intramembrane Proteolysis
Rpm	Rounds per minute

Abbreviations

RT	Room temperature
S	Second
SDS	Sodiumdodecylsulfate
SP	Signal Peptide
Sw	swedish mutation
TACE	TNF-alpha converting enzyme
TBS	Tris Bufferd Saline
TE	Tris-EDTA
TEMED	N,N,N'N'-Tetramethylethylendiamin
TM	Transmembrane domain
µg	Microgramm
µl	Microliter
µM	Micromolar
UV	ultra violet
V	Volt
VLDL	Very Low Density Lipoprotein
VLDL(R)	Very Low Density Lipoprotein Receptor
WB	Western Blot
Wt	Wild Typ

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1 Introduction

1. Alzheimer's disease

Alzheimer's disease (AD) can be defined as a progressive neurodegenerative disorder with characteristic clinical and neuropathological features (Gauthier 2001). Clinically, it is characterized by gradual and progressive impairment in memory, language, calculation, visuospatial perceptions, and judgement; these impairments adversely affect the patient's functional abilities and are associated with changes in behaviour (Gauthier 2001). The disease was first described by the German psychiatrist, Alois Alzheimer just over 100 years ago. His reference patient was Auguste Deter, a 51 year old woman, who developed in her brain amyloid plaques, neurofibrillary tangles, and arteriosclerotic changes (Alzheimer 1907)(Alzheimer, Stelzmann et al. 1995). It is currently estimated that 24.3 million people have dementia today, with 4.6 million new cases every year (one new case every 7 seconds). Using these figures it can be predicted that the amounts will double every 20 years to 42 million by 2020 and 81 million by 2040(Ferri, Prince et al. 2005). Today, AD is the most common form of dementia, representing 40-70% of all cases of dementia and affecting 5-10% of the population over 65 years of age and more than 20% over the age of 80 (Fratiglioni, De Ronchi et al. 1999)(Fratiglioni, Launer et al. 2000)(Wimo, Winblad et al. 2003). Despite decades of research no prevention strategies or curative treatments exist and current medications provide only subtle symptomatic benefits for AD patients.

1.1 Risk Factors for AD

Several factors have been identified which contribute to the development of AD, the most important of which is, advancing age. In his original description, Dr. Alois Alzheimer described this disease as a type of accelerated aging and hence AD was originally referred to as presenile dementia, which is infact, early-onset AD observed in patients younger than 60-65 years (Alzheimer 1907; Alzheimer, Stelzmann et al. 1995). This disease was viewed as being different to senile dementia, which is late-onset AD typically observed in patients over the age of 80-85. However, since the 1970s, both forms have been redefined as being the same disease since both forms of dementia present themselves with similar symptoms and identical pathological hallmarks, despite age differences among patients (Chen and Fernandez 2001)(Blass 2002). While early-onset or familial AD (FAD) accounts for only

1-2% of all AD cases, late-onset or sporadic AD represents the most common disease form. However, whereas early-onset AD predominantly occurs from the inheritance of autosomal dominant mutations, demonstrating the role of genetics in AD etiology, the cause of late-onset AD remains largely unknown. Other risk factors which have gained attention include a person's educational level, gender, traumatic brain injury, vascular diseases, and genetic background (Zabar 2000)(Gauthier 2001)(Launer, Andersen et al. 1999)(Munoz and Feldman 2000)

1.2 Genetics of AD

There are several genetic loci associated with AD. Genes related to AD can be subdivided in to mutational genetics or susceptibility genetics based on how they contribute to the disease. Genes demonstrating mutations and follow a mendelian inheritance pattern include the amyloid precursor protein (APP), presenilin 1 and 2 (PS1 and PS2) i.e. mutational genetics. Genes which confer a higher degree of susceptibility to AD include apolipoprotein E (ApoE), alpha 2 macroglobulin (A2M), the low density lipoprotein receptor related protein 1 (LRP1) i.e. susceptibility genetics (Cacabelos 1999). While the latter genes are more associated with an increased predisposition to late-onset AD, the former genes are fundamentally linked to early-onset AD.

1.2.1 Amyloid Precursor Protein (APP)

The APP gene, located on chromosome 21 encodes a type-I integral membrane glycoprotein which is a member of an evolutionary conserved protein family. APP contains the amyloid beta peptide (Aβ) region (4kDa) extending 28 amino acids of the ectodomain and 11-14 amino acids of the adjacent transmembrane domain, which is released upon enzymatic processing of APP (Masters, Simms et al. 1985). Other family members include the amyloid precursor-like protein1 and 2 (APLP1 and 2)(Wasco, Bupp et al. 1992)(Wasco, Gurubhagavatula et al. 1993). Interestingly, the Aβ region is absent in the other family members (Walsh, Minogue et al. 2007). Alternative splicing of the APP gene gives rise to at least 10 isoforms with 3 predominant transcripts (APP_{695}, APP_{751}, APP_{770})(Yoshikai, Sasaki et al. 1990). APP_{695} is preferentially expressed in neurons while the others are expressed more predominately in the peripheral and CNS tissues (Yoshikai, Sasaki et al. 1990)(Kitaguchi, Takahashi et al. 1988)(Tanzi, McClatchey et al. 1988)(Kang

and Muller-Hill 1990). Several missense mutations residing within the vicinity of the Aβ region of APP give rise to early on-set AD and include the London APP_{717} mutation, the Swedish $APP_{670/671}$ double mutation, the Florida APP_{716} mutation, the Flemish APP_{692} mutation, the Dutch APP_{693} mutation, the Artic APP_{693} mutation, and the Italian APP_{693} mutation (Mullan, Crawford et al. 1992)(Hendriks, van Duijn et al. 1992)(Chartier-Harlin, Crawford et al. 1991)(Goate, Chartier-Harlin et al. 1991)(Nilsberth, Westlind-Danielsson et al. 2001)(Van Broeckhoven, Haan et al. 1990)(Levy, Carman et al. 1990)(Eckman, Mehta et al. 1997)(Tagliavini,F-italian, 1999). These mutations are characteristic features in AD pathology as they potentate the amount of Aβ released from APP. In addition, individuals with a duplication of the APP gene, such as patients with trisomy 21 (Down's syndrome) who have the potential to produce twice as much Aβ, also develop AD relatively early in life (Selkoe 1999)(Menendez 2005).

1.2.2 Presenilin 1 and 2

The genes encoding presenilin 1 and 2 (PS1 and PS 2) are located on chromosomes 14 and 1, respectively (Sherrington, Rogaev et al. 1995)(Levy-Lahad, Wasco et al. 1995; Levy-Lahad, Wijsman et al. 1995). Particularly, almost 50% of all FAD cases can be accounted for by more than 90 different mutations in the PS1 gene (Price, Tanzi et al. 1998)(Sherrington, Rogaev et al. 1995)(Bertram and Tanzi 2005)(Hyman 2006). In comparison, only a small number of PS2 mutations can account for autosomal dominant FAD (Price, Tanzi et al. 1998)(Rademakers, Cruts et al. 2003)(Bertram and Tanzi 2005). The majority of abnormalities in PS genes are missense mutations that enhance enzyme activity such that more of the toxic Aβ peptide ($A\beta_{42}$) is generated (Lemere, Lopera et al. 1996)(Scheuner, Eckman et al. 1996). The proteins encoded by these genes are multipass integral membrane proteins with at least eight transmembrane domains. PSs are rapidly processed to yield a 30kDa N-terminal and a 20kDa C-terminal fragments (Haass and De Strooper 1999)(Hyman 2006).

1.3 Neuropathology

The characteristic pathological features in an AD brain are the presence of senile or neuritic plaques and neurofibiillary tangles, in addition to amyloid angiopathy and

pronounced atrophy of the brain (Fig 1) (Braak and Braak 1997)(Gauthier 2001)(Dickson 1997). Neutitic plaques are predominantly made up of extracellular Aβ deposits as well as cellular components that include degenerating neuritic processes, astrocytes, and microglia (Dickson 1997). Dystophic neurites can exist within and surrounding the amyloid deposits. While microglia, are usually found within and adjacent to the amyloid core, astrocytes are typically found surrounding the outside of the plaque. The classical or typical type of neuritic plaque can be described as a central Aβ core surrounded by swollen neurites, amyloid bundles, and glial cells. In the late 1980s, a second type of plaque was identified and termed diffuse or preamyloid (Yamaguchi, Hirai et al. 1988)(Yamaguchi, Nakazato et al. 1989)(Selkoe 1999). These plaques are less fibrillar than neuritic plaques, and are mainly composed of $A\beta_{42}$ peptides, while neuritic plaques are composed of a mixed pool of $A\beta_{40}$ and $A\beta_{42}$. Furthermore, diffuse plaques lack prominent neuritic or glial changes. It has been proposed that the diffuse plaques are infact the precursor lesions to the neuritic plaques (Rozemuller, Eikelenboom et al. 1989)(Yamaguchi, Nakazato et al. 1989). An assumption made mainly on the basis that Down syndrome patients display diffuse plaques in their teenage years and then later in adulthood show neuritic plaques (Selkoe 1999)(Hyman 2006).

The second pathological hallmark in AD, are intracellular neurofibrillary lesions (Fig 1) (Braak and Braak 1997). These include neurofibrillary tangles (NFT) made up of paired helical filaments (PHF) in cell bodies and dendrites; neuropil threads, which are filamentous accumulations in dendrites; and dystrophic neuritis, which are axons with filamentous accumulations. These intracellular aggregates are relatively insoluble and can become extracellular tangles, what are otherwise known as ‘ghost tangles’ after neuronal death. The main component of the NFT is the highly phosphorylated aggregate of microtubule associated protein, tau, that self associates in to PHFs (Lee, Balin et al. 1991)(Lee, Goedert et al. 2001) (Goedert 2006)(Hyman 2006). The tau protein is responsible for the stabilization of microtubules. These microtubules form a skeletal network, which provides structure and organization with nerve cells. Hyperphosphorylated tau clogs up the microtubule so that motor proteins are incapable of binding, which ultimately leads to the starvation of the synapse and hence neuronal death. Independent studies have shown that the density of both plaques and tangles correlate with the degree of cognitive impairment shown by the patient at the time of death (Terry, Masliah et al. 1991)(Arriagada, Growdon et al. 1992; Arriagada, Marzloff et al. 1992)(Masliah, Mallory

et al. 1994)(Gomez-Isla, Hollister et al. 1997)(Ingelsson, Fukumoto et al. 2004)(Hyman 2006).

Finally, in addition to the aforementioned plaques and tangles, AD is characterized by neuronal loss, disruption of the neuritic cytoskeleton with altered corticocortical connectivity, and widespread synaptic loss (Zabar 2000).

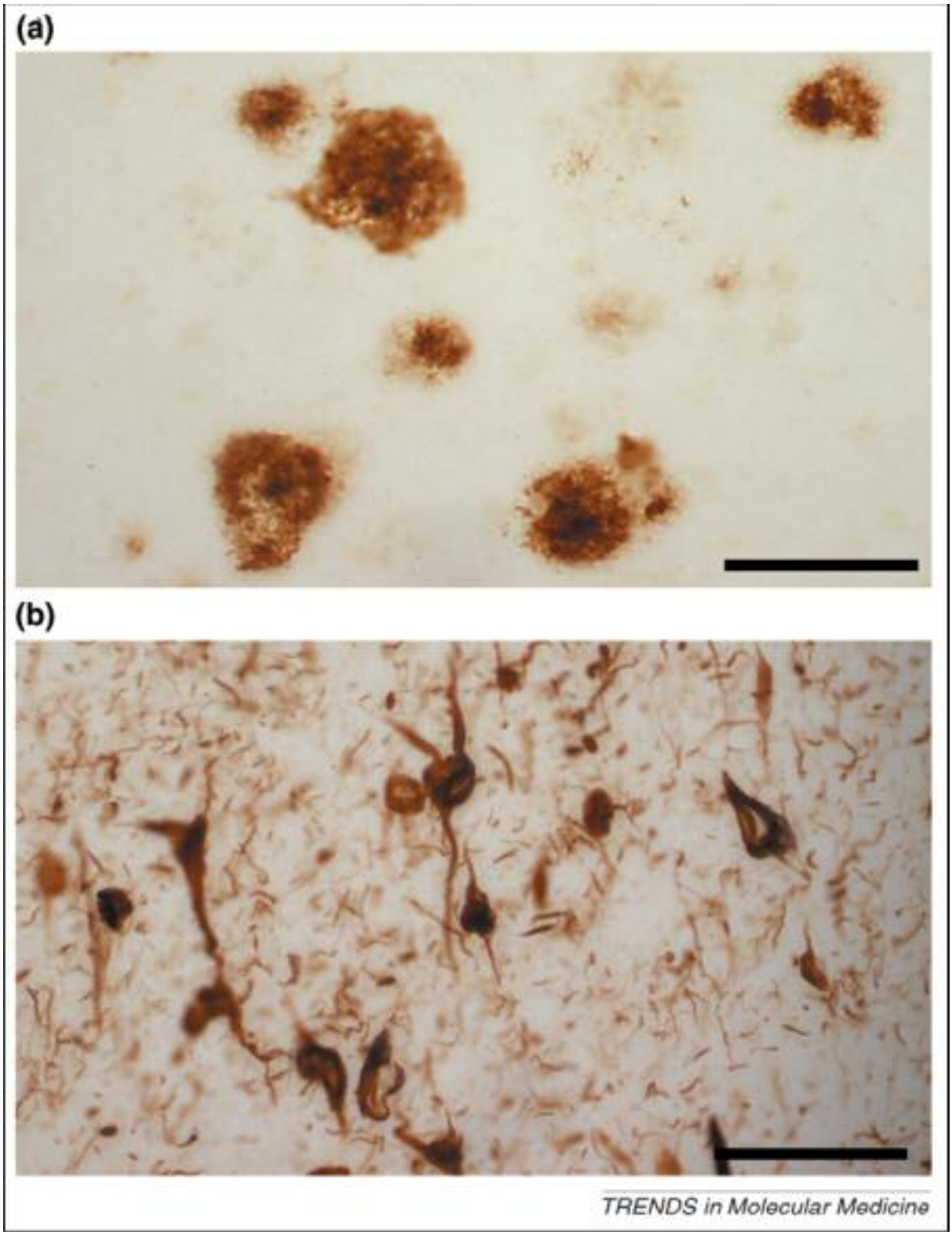

Fig. 1 Plaques and tangles in the AD brain. (A) A microphotograph showing amyloid plaques in the AD brain. Scale bar: 125µM (B) A microphotograph showing neurofibrillary tangles in the AD brain. Scale bar: 125µM. (LaFerla and Oddo 2005)

1.4 Pathobiology

1.4.1 Amyloid Hypothesis

The extraction and sequencing of the highly insoluble amyloid fibrils of the neuritic plaque proteins paved the foundation for the amyloid hypothesis. According to this hypothesis, it is the accumulation and extracellular deposition of insoluble fibrillar Aβ in the form of neuritic plaques which causes a cascade of neurotoxic effects that eventually culminates in neurodegeneration and development of AD (Fig 2) (Selkoe 1991)(Hardy, Chartier-Harlin et al. 1992)(Hardy 2002). In strong favour of this hypothesis, patients with trisomy 21 (Down syndrome) possess an extra copy of the APP gene and show the same pathology as that of AD. The Aβ deposits from AD patients are pathologically identical to the deposits from Down syndrome patients (Olson and Shaw 1969)(Glenner and Wong 1984)(Hyman 2006). The amyloid hypothesis gained further support following the identification of mutations in APP and PS1 and 2 and since their discovery studies with transgenic mouse models have shown that animals with these mutations produce excessive Aβ (Citron, Oltersdorf et al. 1992)(Cai, Golde et al. 1993)(Duff, Eckman et al. 1996)(Citron, Westaway et al. 1997)(Tomita, Maruyama et al. 1997). Furthermore, the degree of Aβ deposition correlates well with the severity of the disease in both AD patients and animal models (Hsiao, Chapman et al. 1996)(Naslund, Haroutunian et al. 2000)(Gordon, King et al. 2001). Fibrillar Aβ has been shown to be neurotoxic in vitro and capable of mediating neurotoxic effects, inflammatory responses and abnormal tau phosphorylation (Yankner, Duffy et al. 1990)(Akiyama, Barger et al. 2000)(Lewis, Dickson et al. 2001)(Hyman 2006). Although this hypothesis has been accredited with a profound amount of evidence, definitive proof that Aβ initiates the degenerative cascade is lacking. This uncertainty has spawned argumentative debate between the degrees to which, Aβ contributes to AD pathology over NFTs (Hyman 2006).

A number of studies have questioned the relationship between plaque load and the severity of AD (Arriagada, Growdon et al. 1992)(Roses 1994)(Neve and Robakis 1998). However, recent data revealing a twist on the Aβ hypothesis of AD might partially put to rest the imperfect relationship between Aβ levels and cognitive impairment. Researchers have found a positive relationship with soluble Aβ as opposed to insoluble Aβ and mature Aβ deposits with AD severity (Lue, Kuo et al. 1999)(Klein, Krafft et al. 2001)(Klein

2002)(Tucker and Estus 2002)(Hyman 2006). For example, one study found that soluble Aβ levels correlate better with disease severity than do plaque levels, and a second study showed that high pathology control subjects (i.e. patients who displayed high levels of insoluble Aβ and mature Aβ deposits) who did not exhibit measurable cognitive deficits also did not show significant increases in soluble Aβ levels (McLean, Cherny et al. 1999)(Lue, Kuo et al. 1999). The twist in the Aβ hypothesis rather suggests that the sequence of pathophysiological events may have more to do with the Aβ form i.e. soluble Aβ oligomers as opposed to monomers and insoluble amyloid fibrils (Klein, Krafft et al. 2001)(Tucker and Estus 2002)(Hyman 2006).

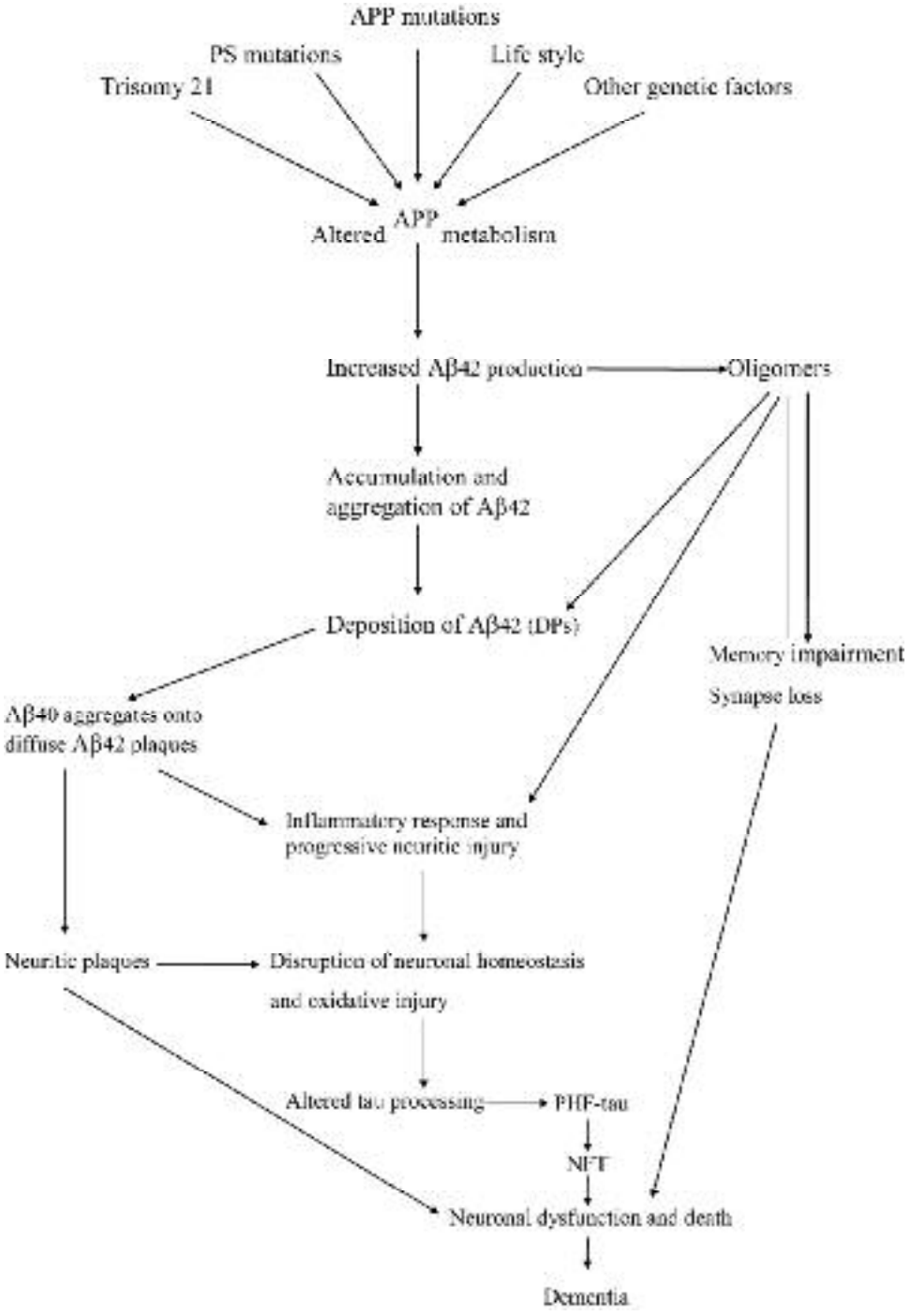

Fig. 2 Proposed sequence of pathogenic events denoting Aβ as the primary casual factor leading to dementia. The right hand side of the diagram shows various factors which promote Aβ formation, which results in the formation of toxic oligomers. These events can conduce to synaptic loss, memory impairment, direct neuronal dysfunction and death, and also activation of the inflammatory response in the brain. On the left, accumulation of $A\beta_{42}$ subsequently leads to diffuse plaque formation, eventual neuritic plaque formation, and ultimately neuronal death. PS=presenilin; APP=amyloid precursor protein; PHF-tau=paired helical filament tau protein; NFT=neurofibrillary tangle. Adapted from (Rojas-Fernandez, Chen et al. 2002)

1.4.2 Amyloid Precursor Protein (APP) Metabolism

APP is an integral membrane glycoprotein with a large extracellular component, a single transmembrane domain, and a short cytoplasmic tail. No definitive role(s) for APP have been elucidated, but several putative functions have been proposed. APP isoforms with the kunitz protease inhibitor domain can inhibit serine proteases and factor XIa of the clotting cascade, whereas others can function in cell-to-cell interactions (Dodart, Mathis et al. 2000). They may act as autocrine factors and exhibit neuroprotective effects, in addition to possessing possible neurotrophic properties. Recently through the use of reverse genetics, whereby APP fragments were expressed in an APP KO background, the authors could show that knocked in APP fragments were sufficient to rescue the phenotype of APP KO mice i.e. learning deficits and impaired long term potentiation (LTP). The APP fragments expressed in the APP KO mice were either APPsα or APP lacking the last fifteen residues of the C-terminal domain. Since APPsα lacks the TMD and C-terminus, APPsα alone may be the sole metabolite of APP contributing a physiological role. Despite the argument against AICD not having any significant physiological role, this data does not support the latter, as the intracellular domain (ICD) may still be generated from either APLP1 or APLP2 and thus assume the function of AICD (Ring, Weyer et al. 2007). Additionally proposed functions include maintenance of neuronal viability, growth, and functional plasticity; a likely role in learning and memory; and participation in normal brain development (Dodart, Mathis et al. 2000).

APP is the parent protein of Aβ, which is released via a proteolytic mechanism that involves a complex interplay of three proteases, known as α-, β-, and γ-secretases (Fig 3) (Seubert, Oltersdorf et al. 1993). γ-secretase is made up of a complex of proteins as opposed to being a single protein (Selkoe and Kopan 2003)(Li, Ma et al. 2003)(Kimberly, LaVoie et al. 2003)(Iwatsubo 2004)(Ma, Li et al. 2005). It consists at least of PS, nicastrin, Aph-1, and Pen-2, and all four proteins are necessary for functional enzyme activity (De Strooper 2003). APP can be cleaved by one of two pathways. The first pathway known as the non-amyloidogenic pathway involves α- and γ-secretase. Transmembrane APP is cleaved within its Aβ domain at the lysine-16 site by α-secretase, leading to the production of a secreted 100-120kDa form of APP (APPsα) and precluding Aβ formation (Fig 4) (Esch, Keim et al. 1990)(Sisodia 1992)(Roberts, Ripellino et al. 1994)(Postina, Schroeder et al. 2004)(Fahrenholz and Postina 2006). α-secretase cleavage also leaves behind a

membrane-anchored stub or C-terminal fragment (CTF), in this case referred to as C83 because of the number of amino acids which comprise it. C83 then becomes the substrate for γ-secretase which executes two cleavages, one which releases the non-toxic peptide, p3, extracellularly and the second cleavage releases the cytoplasmic tail or intracellular domain (ICD/AICD) of the CTF, intracellularly (Fig 4) (Cao and Sudhof 2001). The latter cleavage was coined ε-cleavage ($A\beta_{49}$) (Sastre, Steiner et al. 2001)(Weidemann, Eggert et al. 2002). Recent data provide support for a sequential cleavage model in which ε-cleavage serves as the initial cleavage site followed by δ- and γ-processing within the membrane (Zhao, Cui et al. 2005)(Qi-Takahara, Morishima-Kawashima et al. 2005)(Zheng and Koo 2006). Thus the ε-cleavage of APP may represent the primary processing event (Zheng and Koo 2006). The second route is referred to as the amyloidogenic pathway and involves β- and γ-secretase. In this pathway, β-secretase cleaves just outside the Aβ domain, so that, the extracellular domain is released, here referred to as APPsβ, and in doing so leaves behind a membrane anchored stub, C99 (Hussain, Powell et al. 1999)(Vassar, Bennett et al. 1999). This stub like C83 then becomes the substrate for γ-secretase. The latter enzyme then cleaves C99 at the C-terminus of the Aβ domain leads and depending on the exact site Aβ peptides of varying lengths are released (Cai, Wang et al. 2001). Aβ1-40 is the most abundant form while Aβ1-42 is produced to a lesser extent (Vigo-Pelfrey, Lee et al. 1993)(Shinkai, Yoshimura et al. 1995). However, Aβ1-42 is more closely associated with disease pathogenesis and the senile plaques found in diseased brains are comprised mainly of Aβ1-42(Iwatsubo, Odaka et al. 1994)(Lemere, Blusztajn et al. 1996). Furthermore, in vitro Aβ1-42 has been shown to form fibrils more readily than Aβ1-40 (Masters, Multhaup et al. 1985)(Roher, Lowenson et al. 1993)(Iwatsubo, Odaka et al. 1994). In addition, C99 can also be cleaved by γ-secretase such that its ICD is released (Cao and Sudhof 2001). Under normal conditions, the cleavage of APP by α-secretase predominates over that achieved by β- and γ-secretase (Cordell 1994)(Mattson 1997). Pathogenic mutations in APP can affect either the β-secretase cleavage site such that more of the Aβ containing CTF, C99 is produced and hence more substrate for Aβ production becomes available for γ-secretase leading to elevated levels of Aβ1-40 and Aβ1-42(Mullan, Crawford et al. 1992)(Cai, Golde et al. 1993). Other pathogenic APP mutations rather affect the γ-secretase cleavage site leading to elevated Aβ1-42 levels, specifically (Lemere, Lopera et al. 1996)(Scheuner, Eckman et al. 1996)(Hyman 2006).

Since the discovery of APP as a secretase substrate, many other integral membrane proteins have been identified as substrates for the secretases (Lee, Jung et al. 2002)(May, Reddy et al. 2002)(von Arnim, Kinoshita et al. 2005)(Bohm, Seibel et al. 2006)(Nyborg, Ladd et al. 2006). One of the most important ones identified to date is Notch (De Strooper, Annaert et al. 1999). Notch controls cell fate decisions during embryogenesis through the release of its ICD by γ-secretase (NICD). The NICD translocates to the nucleus and activates the transcription of genes involved in early development (De Strooper, Annaert et al. 1999)(Selkoe and Kopan 2003)(Iwatsubo 2004)(Barrick and Kopan 2006). Therefore, γ-secretase inhibition would not only prevent Aβ formation but also NICD release and thus lead to unwanted side effects.

Fig. 3 Cleavage sites of the APP processing secretases, α, β, and γ. The Aβ domain is shown in red, the transmembrane domain (TMD) is underlined and the intracellular domain (ICD) is shown in blue. (Haass 2004)

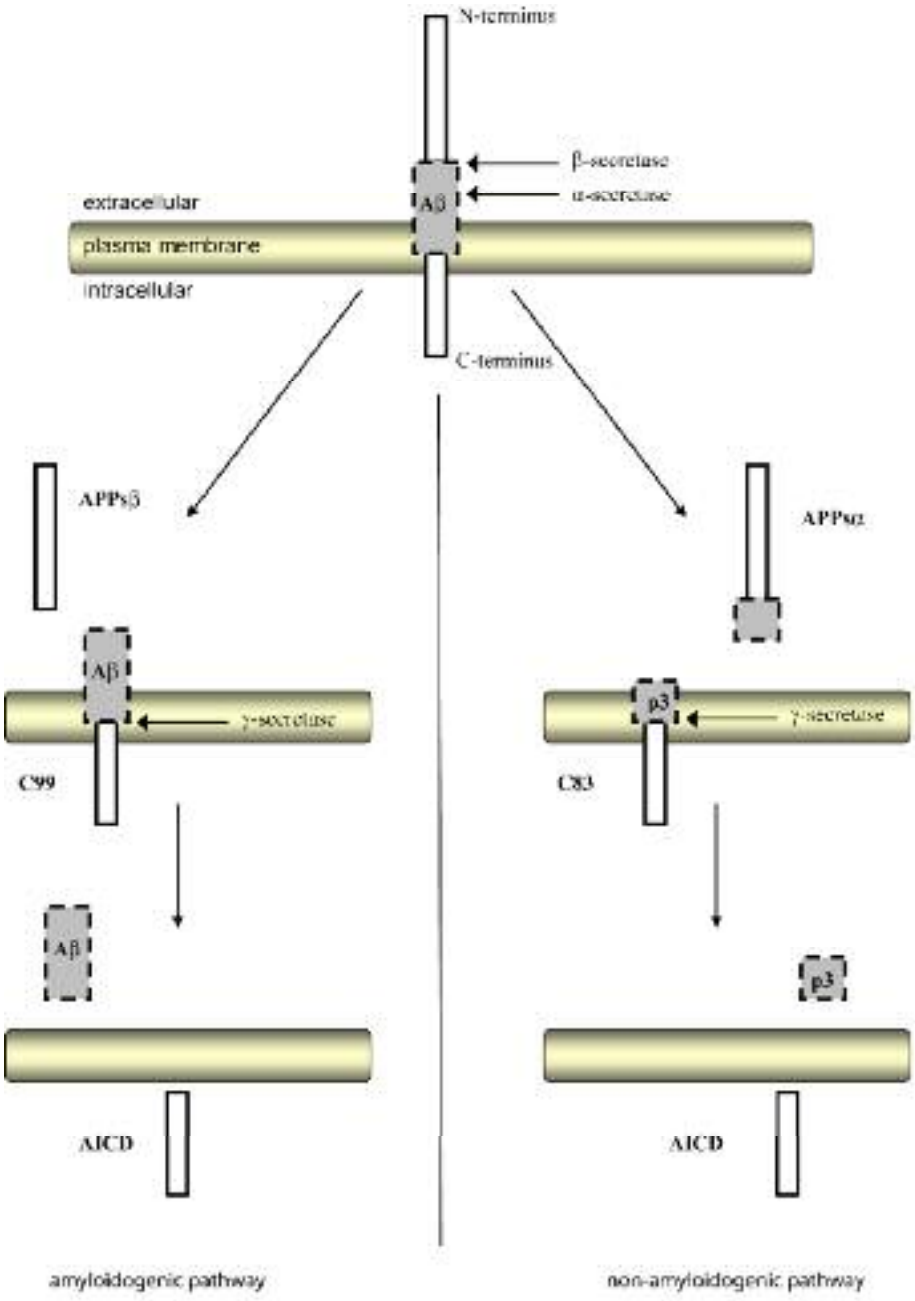

Fig. 4 The amyloidogenic and non-amyloidogenic processing routes. On the left hand side, APP is first cleaved by β-secretase at the N-terminus of the Aβ region. This cleavage event results in the release of the APP soluble ectodomain, APPsβ with the concomitant generation of the membrane anchored stub, C99. γ-secretase executes two sequential cleavages on C99, which result in the release of Aβ and AICD. The latter sequence of cleavages is collectively referred to as the amyloidogenic pathway. The right hand side depicts the non-amyloidogenic pathway and as the name implies this set of cleavages are non-Aβ generating. α-secretase cleaves within the Aβ region, which thus prevents Aβ production. This cleavage results in the release of the APP soluble ectodomain, APPsα and the concomitant generation of the membrane bound stub, C83. Like C99, C83 then becomes the substrate for γ-secretase, which cleaves twice to release the non-toxic peptide, p3 and the AICD.

1.4.2.1Amyloid Precursor Intracellular Domain (AICD)

The importance of Notch and particularly the release of its ICD (NICD) and its vital signalling role in early embryonic development suggested that the ICD released from APP may also play an important functional role. In addition, the high degree of sequence conservation between the ICDs of APP, APLP1, and APLP2 predicts that it is a critical domain regulating APP function. Since the identification of this domain as being a cleavage product analogous to the NICD many important functions have been assigned to it (Zheng and Koo 2006).

1.4.2.2 Phosphorylation

APP is subject to phosphorylation at multiple sites in both its extracellular and intracellular domains (Hung and Selkoe 1994). However, one of the most noteworthy sites is at threonine residue (Thr^{668}) in intracellular domain, of which several kinases have been claimed to be instrumental in i.e. cyclin-dependent kinase 5 (CDK5), glycogen synthase kinase 3β (GSK3β), c-jun N-terminal kinase 3 (JNK3) (Iijima, Ando et al. 2000)(Kimberly, Zheng et al. 2005)(Muresan and Muresan 2005). While this phosphorylation event may regulate APP localization in neurons, the Thr^{668} phosphorylation event has been shown to result in the preferential localization of APP to the nerve terminals (Muresan and Muresan 2005). Furthermore, Thr^{668} phosphorylated APP fragments are increased in AD, but not in control subjects (Lee, Kao et al. 2003). The prolyl isomerise Pin1 can also bind to phosphorylated Thr^{668} an event which induces its isomerisation and results in an AICD conformational change and abrogated APP processing (Pastorino, Sun et al. 2006). The AICD has been shown to interact with many adaptor proteins including Fe65 (as well as Fe65 like proteins Fe65L1 and Fe65L2) and Mint-1/X11a. Interestingly phosphorylation at Thr^{668} has been reported to result in a conformational change, which negatively regulates APP binding to Fe65 and reduces the stability of AICD (Ando, Iijima et al. 2001)(Ramelot and Nicholson 2001). This phosphorylation has also been reported to modulate APP interaction with Mint-1/X11a (Taru and Suzuki 2004)(Zheng and Koo 2006).

1.4.2.3 Cell signaling

Similarities between the processing of Notch and APP prompted the notion that APP may play an analogous signalling role. It has been shown that AICD complexed with Fe65, a nuclear adaptor protein and Tip60, a histone acetytransferase, form a nuclear complex and can regulate the expression of artificial expression constructs in transfected cells (Cao and Sudhof 2001)(Zheng and Koo 2006). The exact path taken by AICD and the part played by the other proteins involved have not been clearly demonstrated. It was later shown that translocation of AICD to the nucleus was in fact not necessary for transcriptional activation. The authors rather showed that membrane tethered AICD as opposed to AICD free in the cytosol, recruits and activates Fe65 and upon cleavage by γ-secretase, both AICD and active Fe65 translocate to the nucleus (Cao and Sudhof 2004). In addition, Fe65 appears to stabilize the highly labile AICD fragment (Kimberly, Zheng et al. 2001). Although many putative target genes for AICD have been documented, much controversy surrounds the part played by AICD in the transcription of these genes (Baek, Ohgi et al. 2002)(von Rotz, Kohli et al. 2004)(Pardossi-Piquard, Petit et al. 2005)(Hebert, Serneels et al. 2006). Overall, a potential nuclear signaling activity remains to be established. Other important roles assigned to this domain include cell migration and synapse remodelling, apoptosis, and axonal transport (Zheng and Koo 2006).

1.5 The Low Density Lipoprotein Receptor (LDLR) Gene Family

In mammals the LDLR gene family is a family of endocytic receptors and consists of seven core members (Fig 5). These include the LDL receptor, the LDL receptor related protein 1 (LRP1), a closely related receptor termed LRP1b, megalin, the VLDL receptor (VLDLR), Apolipoprotein E receptor-2 (ApoER2), and a multiple epidermal growth factor (EGF) repeat containing protein, MEGF7 (Fig 5). Core members can be identified by the presence of all of the following five distinct domains or sequence motifs: (A) ligand binding type cysteine-rich repeats followed by, (B) epidermal growth factor type cysteine-rich repeats, (C) YWTD domains that form a fold of β-pleated sheets resembling a propeller structure, (D) a single membrane spanning segment, and (E) a cytoplasmic tail containing one or more NPxY (Asp-Pro-any amino acid-Tyr) amino acid motifs (Herz and Bock 2002)(Springer 1998)(Jeon, Meng et al. 2001). Distant relatives of the LDLR family

do not possess all five domains which characterize a core family member but rather harbour one or more of the repeats and domains. Distant family members include LRP5, LRP6, and LR11/SORLA (Fig 5) (Herz and Bock 2002)(Brown, Twells et al. 1998)(Hey, Twells et al. 1998)(Dong, Lathrop et al. 1998)(Jacobsen, Madsen et al. 1996)(Yamazaki, Bujo et al. 1996)(Morwald, Yamazaki et al. 1997).

Aside from their structural resemblance, members of the LDLR family also share a common property they are all capable of binding the receptor associated protein (RAP) (Bu, Geuze et al. 1995)(Bu 1998). RAP behaves as an endoplasmic reticulum (ER) chaperone, in that, it binds to LDLR members such that no other ligands may bind to the receptors (Willnow, Armstrong et al. 1995)(Willnow, Rohlmann et al. 1996). It may also assist in their folding (Bu, Geuze et al. 1995)(Obermoeller-McCormick, Li et al. 2001).

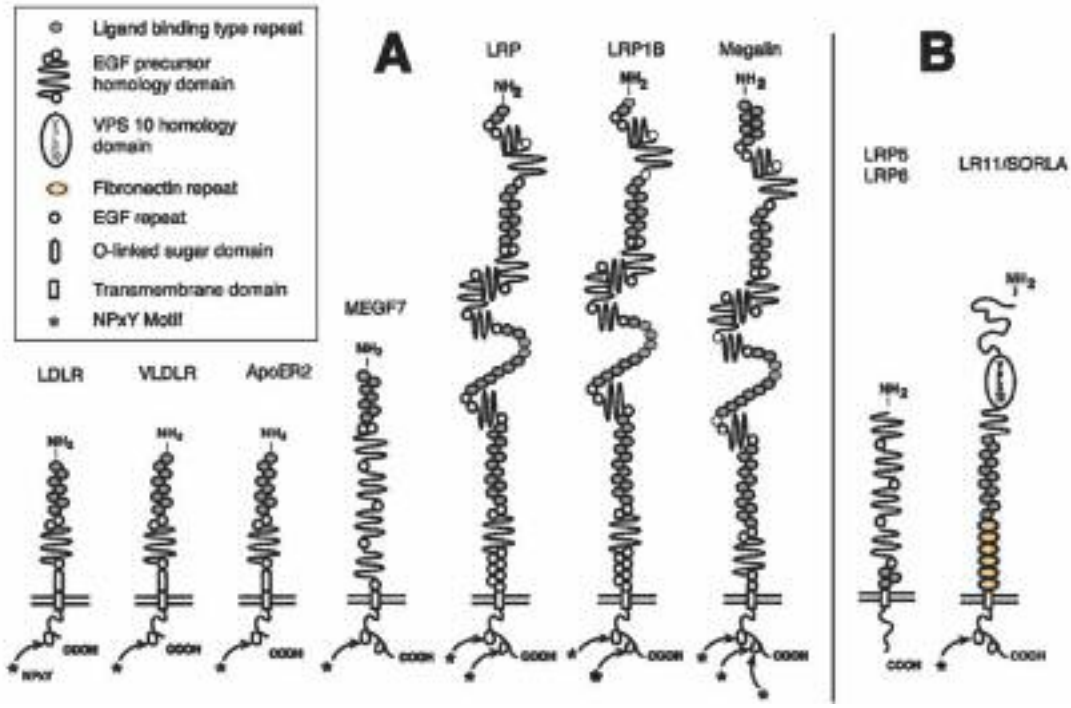

Fig. 5 The mammalian LDL receptor gene family. The core of the gene family is shown in A, while the far-out members, LRP5, LRP6, and LR11/SORLA are shown in panel B. (Herz and Bock 2002)

1.5.1 The Low Density Lipoprotein Receptor Related Protein 1 (LRP1)

LRP1 is one of the largest cell surface receptors identified to date. It is synthesized as a 600kDa protein but is later cleaved in the trans-Golgi network by furin to form a heterodimer (Herz, Hamann et al. 1988)(Herz, Kowal et al. 1990)(Herz and Strickland 2001). Among the LDLR family members, LRP1 has the fastest rate of endocytosis (Zerbinatti and Bu 2005)(Waldron, Jaeger et al. 2006). The extracellular domain, also referred to as the α-chain (~515kDa), contains multiple EGF and GF repeats and four distinct ligand binding sites which remains associated to the c-terminal domain or β-chain (~85kDa)(Herz, Hamann et al. 1988)(Cam and Bu 2006). The β-chain contains two NPxY sites, the second of which overlaps with a YxxL domain, that direct endocytosis of the receptor (Chen, Goldstein et al. 1990)(Li, Marzolo et al. 2000)(Zerbinatti and Bu 2005)(Waldron, Jaeger et al. 2006). It is most abundantly expressed in hepatocytes and neurons but can be found in almost all cell types to some degree. The first known function for LRP1 was that of a chylomicron remnants receptor i.e. large triglyceride-rich lipoproteins that carry lipids from the gut to the liver. Since the latter discovery, it appears that LRP1 possesses quite a diverse array of physiological functions outside lipoprotein metabolism as can be seen from the wide spectrum of its functionally distinct ligands (Herz and Bock 2002). LRP1 ligands identified to date include apoE, activated alpha-2-macroglobin (α2M*), tissue-type plasminogen activator (tPA) and complexes of tPA and plasminogen activator inhibitor type I (PAI-1), isoforms of APP containing the Kunitz protease inhibitor domain (KPI)(Willnow 1999)(Herz and Strickland 2001)(Li, Cam et al. 2001). Its sheer functional necessity can be appreciated especially in embryogenesis since conventional LRP1 knockout mice die during various stages in early and mid-gestation (Herz, Clouthier et al. 1992)(Herz, Couthier et al. 1993)(Waldron, Jaeger et al. 2006)

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In addition to its role in endocytosis of a variety of ligands, LRP1 has been implicated to play a crucial role in cell signalling. Several adaptor proteins involved in signalling cascades can bind to the LRP1 tail, some of which seem to play a role in neuronal calcium signalling, platelet derived growth factor (PDGF) signalling or mitogen activated protein kinase (MAPK) signalling (Bacskai, Xia et al. 2000)(Loukinova, Ranganathan et al. 2002)(Lutz, Nimpf et al. 2002)(Qiu, Hyman et al. 2004)(Yang, Huang et al. 2004).

1.6 LRP1 and AD

Several lines of evidence make LRP1 an incriminating player in AD pathogenesis. Firstly, it is the major neuronal receptor for apoE and inheritance of the apoE4 (∈4) allele is a genetic risk factor for late-onset AD (Beisiegel, Weber et al. 1989)(Strittmatter, Saunders et al. 1993)(Saunders, Strittmatter et al. 1993)(Poirier, Davignon et al. 1993)(Mayeux, Stern et al. 1993). LRP1 is also the major α2M receptor in the brain, and α2M may be a genetic risk factor for AD (Kristensen, Moestrup et al. 1990)(Strickland, Ashcom et al. 1990)(Blacker, Wilcox et al. 1998)(Waldron, Jaeger et al. 2006). Secondly, both LRP1 itself and several of its ligands (apoE, α2M*) have been found associated with Aβ deposits in the AD brain (Arelin, Kinoshita et al. 2002)(Qiu, Strickland et al. 2001)(Rebeck, Harr et al. 1995). Thirdly, several studies have reported a link between late-onset AD and two polymorphisms found in the LRP1 gene, i.e. a tetranucleotide repeat in the 5' region and a silent polymorphism in exon 3 (Wavrant-DeVrieze, Perez-Tur et al. 1997)(Kang, Saitoh et al. 1997). Fourthly, LRP1 also has a strong influence over Aβ clearance via its ligands apoE, α2M, and lactoferrin, all of which can bind Aβ and promote its clearance in vitro (Deane, Wu et al. 2004)(Gylys, Fein et al. 2003)(Narita, Holtzman et al. 1997)(Qiu, Strickland et al. 1999)(Kang, Pietrzik et al. 2000). Clearance of Aβ by LRP1 has also been implicated in the transport of Aβ out of the brain parenchyma via the blood-brain barrier.(Deane, Wu et al. 2004)(Shibata, Yamada et al. 2000). LRP1 interacts with isoforms that contain the KPI domain and through adaptor proteins it can also interact with the cytoplasmic domain of APP (Waldron, Jaeger et al. 2006).

1.6.1 LRP1 interacts with APP

LRP1 can interact with APP via extracellular and intracellular interactions (Kounnas, Moir et al. 1995)(Fiore, Zambrano et al. 1995)(Guenette, Chen et al. 1996)(Bressler, Gray et al. 1996)(Trommsdorff, Borg et al. 1998)(Pietrzik, Yoon et al. 2004). Many studies have indicated that APP processing to Aβ is modified by LRP expression. Extracellularly, LRP can bind and internalize secreted APPsα containing the KPI domain. Infact, cell surface APP containing the KPI domain (APP770) complexed with epidermal growth factor binding protein (EGFBP) can be internalized by LRP1. This data indicates that both cell surface APP and APPs are degraded by a similar pathway that requires LRP1 (Kounnas, Moir et al. 1995)(Knauer, Orlando et al. 1996).

An intracellular interaction also exists between LRP and non-KPI containing APP through FE65. FE65 contains a WW domain and two phosphotyrosine binding domains (PTB1 and PTB2). Pull down experiments demonstrated that the N-terminal PTB1 of FE65 binds to LRP1 and the C-terminal PTB2 of FE65 binds APP (Fiore, Zambrano et al. 1995). This suggested that FE65 could act as an adaptor molecule between the two proteins. These interactions between APP and LRP at the cell surface and in the Golgi network have been substantiated in cells overexpressing APP, LRP1, and FE65 (Rebeck, Moir et al. 2001)(Kinoshita, Whelan et al. 2001)(Pietrzik, Busse et al. 2002).

Disruption of an LRP1 and APP interaction extracellularly results in a greater amount of cell surface APP and a reduced secretion of Aβ. This effect could be rescued through restoration of the APP, LRP1 interaction. These data demonstrate that LRP1 expression can influence APP processing to Aβ (Ulery, Beers et al. 2000)(Pietrzik, Busse et al. 2002)(Pietrzik, Yoon et al. 2004).

1.6.2 LRP1 and APP Processing

Multiple steps of APP processing may be modulated by LRP1, most of which can be attributed to its ability to bind and endocytose APP via its cytoplasmic domain (Waldron, Jaeger et al. 2006). In the absence of LRP1, APP internalization rates are reduced to by approximately 50% (Pietrzik, Busse et al. 2002). Such a reduction in APP internalization gives rise to a greater amount of cell surface APP. Since α-secretase has been shown to be most active at the plasma membrane, an increased amount of APPsα can be seen in the media of LRP1-deficient cells (Parvathy, Hussain et al. 1999). Furthermore, in LRP1 deficient cells, the decrease in the internalization rate of APP from the cell surface also gives rise to a reduction in Aβ secretion. The cytoplasmic domain of LRP alone is sufficient for its effect on APP processing (Pietrzik, Busse et al. 2002). These events were later shown to be through the formation of a tripartite complex, where FE65 behaves as a linker protein, allowing LRP1 and APP to form a complex. FE65 has two colinear PID binding domains that bind LRP and APP and both NPxY domains in LRP1 interact with FE65 (Fig 6) (Pietrzik, Yoon et al. 2004). However, it is the distal NPxY domain in LRP1 which is critical in APP processing and the interaction between the second NPxY domain of LRP1 and the first PID domain of FE65 is the bona fide complex combination, or at

least the more important one responsible for the effects seen on APP processing (Pietrzik, Yoon et al. 2004).

Fig. 6 Schematic representation of the trimeric complex consisting of APP, Fe65, and LRP1. Binding of the PID domains of Fe65 to the NPXY motifs of the two receptors accounts for their assembly. Although Fe65 can bind to both NPXY motifs in the C-terminus of LRP1, the more distal one mediates the effects on APP processing. (Waldron, Jaeger et al. 2006)

1.7 The secretory pathway and KKXX motifs

The biosynthetic secretory pathway is the cellular road map guiding proteins to their correct destination. This pathway leads proteins outward from the endoplasmic reticulum (ER) towards the Golgi and cell surface, with a side channel tunnelling certain proteins to lysosomes, while the endocytic pathway guides proteins inward toward endosomes and lysosomes from the plasma membrane (PM). Membrane-bound and cytosolic proteins are

transferred via transport vesicles via cycles of vesicle fusion and budding. Simultaneously with anterograde protein transport, retrograde protein transport occurs within the Golgi complex as well as from the Golgi back to the ER (Nickel and Wieland 1998). Vescular transport within the early secretory pathway is mediated by two types of non-clathrin coated vesicles: COPI- and COPII-coated vesicles. COPI vesicles bud from the Golgi (retrograde), while COPII vesicles bud from the ER (Fig 7) (anterograde) (Scales, Pepperkok et al. 1997).

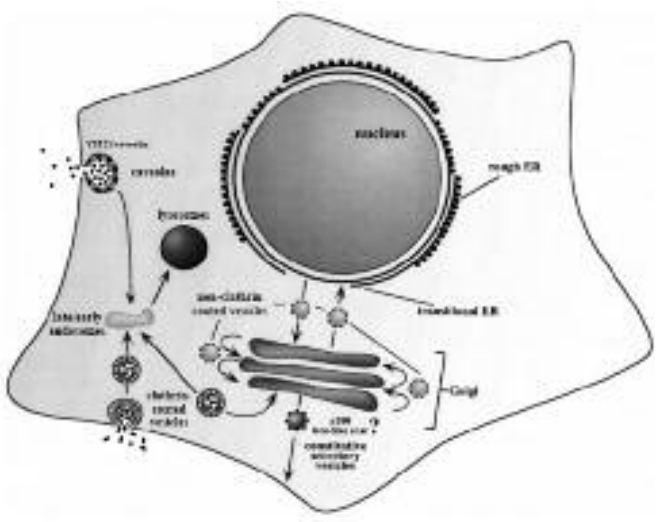


Fig. 7 Schematic representation of the major intracellular transport routes mediated by coated vesicles

While the majority of proteins traffic from the ER to the Golgi, some proteins which are destined for a specific compartmental residence, e.g. Bip, are retained in a particular compartment via unique branches in the secretory system. At each branch, specific protein localization signals mediate the transport of that protein to its correct location within the cell. Soluble ER proteins are retained in the ER by having the KDEL sequence at their C-terminus. Transmembrane ER proteins are retained within the ER by having a C-terminal KKXX sequence, where XX denotes any amino acid. These signal sequences rather than preventing proteins from being packaged and transported, mediate their retrieval from the cis-Golgi back to the ER in a continuous recycling fashion brought about by COPII vesicles (Nickel and Wieland 1998). The KKXX signal is recognised by the COP I complex (Letourneur, Gaynor et al. 1994). The COP I coat is composed of a small GTPase, ARF, and coatamer, a conserved cytosolic ~700 kDa protein complex consisting of seven subunits: α, β-, β'-,γ-, δ-, ε-, and ζ-COP (Rothman and Wieland 1996)(Duden, Kajikawa et al. 1998).

2.1 Reagents, buffers, solutions, media, equipment

2.1.1 Chemicals

Acrylamide 40% (29:1)/Bisacrylamide	Roth, Karlsruhe
Acrylamide 30% (19:1)/Bisacrylamide	Roth, Karlsruhe
Agarose-G-Beads	Invitrogen, Karlsruhe
Ammonium chloride (NH4Cl)	Roth, Karlsruhe
Ammoniumpersulfate (APS)	Sigma, Deisenhofen
Amphotericin (Antimycotic)	Sigma, Deisenhofen
Aprotinin	Sigma, Deisenhofen
β-Mercaptoethanol	Roth, Karlsruhe
Bromphenol blue	Roth, Karlsruhe
BSA (Bovine Serum Albumin)	Pierce, Bonn
Calcium chloride (CaCl2)	Roth, Karlsruhe
Coomassie Brilliant Blue	Merck, Darmstadt
D19 Developer	Kodak, Stuttgart
EDTA	Merck, Darmstadt
Acetic acid	Roth, Karlsruhe
Ethanol	Roth, Karlsruhe
Ethylendiamintetraessigsäure (EDTA)	Merck, Darmstadt
EXPRE 35S Protein Labeling Mix,14mCi (518MBq)	Perkin Elmer, Boston
Fixing solution	Kodak, Stuttgart
Fetal calf serum (FBS)	Gibco Paisley, Schottland
Glycerol	Merck, Darmstadt
Glycine	Roth, Karlsruhe
HEPES	Serva, Heidelberg
Igepal (NP-40)	Sigma, Deisenhofen
Immobilising Agagose-Neutravidin-Beads	Pierce, Rockford, USA
Isopropanol	Roth, Karlsruhe
Calium chloride (KCl)	Merck, Darmstadt
Non fat milk powder	Ralphs, Kalifornien

Magnesium chloride (MgCl2)	Merck/Gibco, Darmstadt
Methanol	Roth, Karlsruhe
Sodium azide (NaN3)	Merck, Darmstadt
Sodium chloride (NaCl)	Merck, Darmstadt
Sodium hydrogen carbonate (NaHCO3)	Merck, Darmstadt
Sodium dihydrogen phosphate (NaH2PO4)	Merck, Darmstadt
Sodium pyruvate	Merck, Darmstadt
Poly-L-Ornithine	Sigma, Deisenhofen
Ponceau S	Roth, Karlsruhe
Sodium dodecylsulfaet (SDS)	BioRad, München
Select Yeast Extract	Gibco Paisley, Schottland
Sulfo-NHS-LC-LC-Biotin	Pierce, Rockford, USA
SuperSignal® Substrate für Western Blot	Pierce, Rockford, USA
TEMED (N,N,N´,N´-Tetramethylethylendiamin)	Merck, Darmstadt
Tris	Merck, Darmstadt
Tris-hydrochlorid	Roth, Karlsruhe
Triton X-100	Sigma, Deisenhofen
Trypsin/EDTA	Gibco, Paisley,Scotland
Tween 20	Sigma, Deisenhofen

2.1.2 Kits

BCA 200 Protein Assay Kit	Pierce, Bonn
Jet Star Plasmid Maxi-Prep Kit	Genomed, Germany
Dual luciferase assay Kit	Promega, Madison, WI, USA

2.1.3 Enzymes used for carbohydrate digestions

Endo H_f	New England Biolabs,
PNGase F	New England Biolabs,

2.1.4 Transfection Reagents

Lipofectamine2000TM	Invitrogen, Karlsruhe
GeneJuiceTM	Novagen, Merck, Darmstadt

2.1.5 Equipment

Acrylamide Gel Electrophoresis System	Biometra, Göttingen
CO2- incubator	New Brunswick, Edison, USA
Conical flask	Schott, Mainz
Shaker	Infors, Boltmingen Schweiz
Freezers and fridges	-80°C Heraeus -20°C Liebherr +4°C Privileg
Wallac 1420 ^{3}Viktor Multilabel Counter	Perkin Elmer, Turku, Finland
Gel dryer (GelAir Dryer)	BioRad, München
Heat block	Eppendorf, Hamburg
Image Master VDS Software	Pharmacia
LAS 3000 FujiFilm	Fuji, Japan
Light microscope	OLYMPUS, Hamburg Zeiss, Göttingen
Plate reader	Thermo Labsystems
Pasteure pipettes	Roth, Karlsruhe
pH-Meter	inoLab, Weilheim
Phosphoimager, IPR 1000	Fuji, Tokio, Japan
Pipettes 1 µl-1 ml	Gilson

Power Pack300	BioRad, München
Protan Nitrocellulose-Membrane	Schleicher & Schuell, Dassel
Developer	Fuji Japan
Trans Blot SD, Semi-dry Blotting Cell	Bio Rad, München
Spectrophotoometer DU800	Beckmann, Krefeld
Laminor flow hood	Nunc, Wiesbaden
UV-Photometer GeneRay	Bioimetra, Göttingen
Vortex-Genie 2™	Bender & Hobein AG, Zürich
Weighing scales 21005	Sartorius BP Mettler PL1200
Water bath GFL1086	GFL, Burgwedel
Xcell II Surelock Electrophoresis Tank	Novex
Centrifuge Hettrich Universal 32	Hettrich, Tuttlingen
Centrifuge Eppendorf 5415D	Eppendorf, Hamburg
Centrifuge Hereaus Fresco	Kendro, Langenselbold
Centrifuge Sorvall RC5B	Kendro, Langenselbold

2.1.6 Standard material

Aluminium foil	Aro, Metro
Cellophane foil	BioRad, München
Clear Blue X-Ray Film	Pierce, Rockford, USA
Gloves	Semperit, Wien
Eppendorf tubes 1,5 ml	TPP, Trasadingen, Schweiz
Filter paper	Schleicher & Schuell, Dassel
Glass pipettes 1 ml-20 ml	VWR, Darmstadt
Cell culture flasks 25 und 75 cm2	TPP, Trasadingen Schweiz

Cell culture plates (Ø 3 cm, 6 cm, 10 cm)	TPP, Trasadingen Schweiz
High performance chemiluminescence film	Amersham Biosciences UK Ltd.
96-well plates	Sarstedt, Nümbrecht
Nitrocellulose-Transfer membrane	Hartenstein, Würzburg
NuPage™ 4-12 %ige Bis-Tris Gradientengele	Invitrogen, Karlsruhe
Parafilm® American National Can™	Neeah, USA

2.1.7 Cell Culture-Media

DMEM	Gibco/BRL, Eggenstein
α-MEM	Cambrex,Verviers, Belgium
OptiMem I Serum-free	Gibco/BRL, Eggenstein
DMEM Methionine- /Cysteine-free	Gibco/BRL, Eggenstein

α-MEM
500 ml DMEM-F12
50 ml FBS, aktive
5 ml 10,000 units/ml Penicillin/10,000 µg/ml Streptomycin
5 ml 100 mM Sodium pyruvate

DMEM
500 ml DMEM
50 ml FBS, aktive
5 ml 10,000 units/ml Penicillin/10,000 µg/ml Streptomycin
5 ml 100 mM Sodium pyruvate

Freezing medium
10 % (v/v) DMSO mit jeweiligem Zellkulturmedium

Trypsin/EDTA:
0,05 % (w/v) Trypsin und 0,02% (w/v)
EDTA

10 x Phosphaete buffered Saline (PBS), pH 7,4:

137 mM NaCl

2,7 mM KCl

10 mM NaH_2PO_4

10 mM KH_2PO_4

H_2Odd

Strarvation medium

DMEM Methionine-/Cysteine-free

FCS, aktive (5 % v/v final)

1 % (v/v) 100mM sodium pyruvate

[^{35}S]-Labeling-Medium:

DMEM Methionine-/Cysteine-free

FCS, aktive (5 % v/v final)

40 mM HEPES

1 % (v/v) 100 mM sodium pyruvate

EXPRE [^{35}S]-Protein Labeling Mix (150 µCi/ml final)

2.1.8 Media and solutions for bacterial culture

Luria-bertani Broth (LB-Medium), pH 7,5:

1,0 % (w/v) Trypton

0,5 % (w/v) Yeast Extrakt

1,0 % (w/v) NaCl

H_2Odd

LB-Medium^{Amp}:

LB-Medium

50 µg/ml Ampicillin (Endkonzentration)

LB-Agar^{Amp}:

1,0 % Trypton

0,5 % yeast extract

1,5 % Agar

1,0 % NaCl

50 µg/ml Ampicillin (Endkonzentration)
H_2Odd

2.1.9 Buffers and solutions for DNA gel electrophoresis

10x TAE-Elektrophorsese buffer:
97,6 g Tris-Base
20 ml Glacial Acetic acid
5,84 g EDTA
2 l H_2Odd

2.1.10 Buffers and solutions for protein biochemistry and polyacrylamide gel electrophoresis

NP40-Cell-Lysis buffer:
50 mM Tris
150 mM NaCl
1 % NP40
0,02 % NaN_3

10x Tris buffered Saline(TBS), pH 7,4:
1,37 M NaCl
27 mM KCl
0,25 M Tris-Base
H_2Odd
Tris buffered Saline with Tween20 (TBS-T):
1x TBS
0,05 % (v/v) Tween20

10 x Western-Blot (wet-blot)Transfer buffer :
250 mM Tris Base
1920 mM Glycin
H_2Odd

Blocking buffer for Nitrocellulose membrane:
1x TBS-T
5 % (w/v) non-fat milk powder

Ponceau S:

0,2 % Ponceau S

3 % Trichloric Acetic Aicd

H_2Odd

20 x MES SDS Elektrophoresis buffer for NuPage™ Bis-Tris-Gels:

1 M (2-(N-Morpholino)-ethansulfonsäure) MES

1 M Tris Base

69,3 mM SDS

20,5 mM EDTA

H_2Odd

4 x Resolving buffer, pH 8,8 for BioRad-Gels:

1,5 M Tris-HCl

0,4 % (w/v) SDS

H_2Odd

4 x Stacking gel buffer, pH 6,8 for BioRad-Gesl:

0,6 M Tris-HCl

0,4 % (w/v) SDS

H_2Odd

10 x Tris-Glycine-Electrophoresise buffer, pH 8,3 for BioRad gels:

0,96 M Glycin

0,125 M Tris-Base

1 % (w/v) SDS

H_2Odd

Fixation buffer für Polyacrylamide-Gels:

20 % (v/v) Methanol

10 % (v/v) Glacial Acetic acid

70 % (v/v) H_2Odd

2.1.11 Buffers and solutions for surface biotinylation

PBS-CM ($CaCl_2$/$MgSO_4$) for surface biotinylation:

1 mM $CaCl_2$

1 mM $MgSO_4$

PBS

Borate buffer for surface biotinylation:

10 mM Sodium borate, pH 9.0

154 mM NaCl

12 mM KCl

2,25 mM $CaCl_2$

0,5 mg/ml Sulfo-NHS-LC-LC-biotin

PBS-CM + NH_4Cl

1 mM $CaCl_2$

1 mM $MgSO_4$

PBS

50 mM NH_4Cl

2.1.12 Buffers and solutions for membrane preparation

Homogenization buffer

10 mM Mops, pH 7.0

10 mM KCl

2.1.13 Buffers and solutions for subcellular fractionation

Cell lysis buffer+protease inhibitor

10 mM HEPES

10 mM NaCl

1 mM KH_2PO_4

5 mM $NaHCO_3$

1 mM $CaCl_2$

0.5 mM $MgCl_2$

5 mM EDTA

Tris-sucrose-EDTA buffer+protease inhibitor

10 mM Tris, pH 7.5

300 mM Sucrose

1mM EDTA

2.1.14 Buffers and solutions for immunocytochemistry

Fixation buffer

3.7 % (v/v) formaldehyde

PBS

Blocking buffer

4 % (w/v) BSA

0.1 % (v/v) Triton-x 100

PBS

PTx buffer

0.1 % (v/v) Triton-x 100

PBS

Mounting media/Anti-fading agent, pH 8.0

10 % (w/v) Elvanol

3.3 % (v/v) Glycerine

0.1 % (w/v) Phenylenediamine

PBS

2.1.15 Software

Adobe Photoshop CS

Adobe Illustrator CS

Microsoft Office 2003

AIDA Imaga Analyzer Version 3.8

2.1.16 Antibodies

Primary antibodies:

Antigen	Species	Type	*Supplier/Reference*
CT15 – **APP**	Rabbit	polyclonal	*Sisodia et al., 1993*
26D6 – **Aβ**	Rabbit	polyclonal	*Pietrzik et al., 2002*
1704 – **LRP**	Rabbit	polyclonal	*Pietrzik et al., 2002*
11H4– **LRP**	Mouse	monoclonal	*ATCC, CRL 1936*
c-*myc*	Mouse	monoclonal	*Hybridoma*
α-Tubulin	Mouse	monoclonal	*Sigma, Deisenhofen*
Bip– LRP	Mouse	monoclonal	*BD Transduction laboratories*
GM130	Mouse	monoclonal	*BD Transduction laboratories*
EEA1	Mouse	monoclonal	*BD Transduction laboratories*
Flag	Mouse	monoclonal	*Sigma, Deisenhofen*

Secondary antibodies

Antigen	Conjungate	*Supplier*
Mouse IgG	HRP	*Jackson ImmunoResearch lab. Inc.*
Rabbit IgG	HRP	*Jackson ImmunoResearch lab. Inc.*
Mouse Alexa Fluor 546	Fluorscein	*Molecular probes*
Rabbit Alexa Fluor 488	Fluorscein	*Molecular probes*
Rabbit Alexa Fluor 546	Fluorscein	*Molecular probes*
Mouse Alexa Fluor 488	Fluorscein	*Molecular probes*

In studies aimed at assessing the effect of LRP on APP processing in early secretory compartments, the polyclonal antibody CT15 descibed previously (Sisodia, Koo et al. 1990), which reacts with the cytoplasmic domain of APP was used for the detection of full length APP and APP CTFs. When investigating the effect of LRP retention over APP, the monoclonal LRP1 antibody (11H4) (ATCC, CRL 1936), which recognizes the LRP C-terminus, was used to detect LRP-CT, LRP-CT KKAA, and LRP-CT KKFF to ensure equal expression. The polyclonal LRP antibody (1704) recognizes the LRP C-terminus as described previously and was used in combination with the monoclonal organelle antibody markers, i.e. Bip, GM130, EEA1 in microscopic analysis to detect LRP-CT, LRP-CT KKAA, and LRP-CT KKFF when expressed alone in CHO 13-5-1 cells (Pietrzik, Busse et al. 2002). While 11H4 was used to detect the truncated LRP constructs, LRP-CT, LRP-CT

KKAA, and LRP-CT KKFF when expressed in combination with APP_{695} in CHO 13-5-1 695 cells and the polyclonal antibody, CT15 was used to detect APP. The monoclonal antibody 26D6, which reacts with the ectodomain of APP, was used to detect secreted Aβ and has been described previously (Kang, Pietrzik et al. 2000).

In studies aimed at assessing the influence of NH_4Cl over AICD levels, the anti-APP antibody CT15 was used in immunoblots for the detection of full length APP, full length APPsw, APPsw CTFs, full length APP Gal4, APP CTFs, APP Gal4 CTFs, AICD and AICD Gal4. In addition 26D6 antibody directed against the Aβ domain was used for the detection of secreted APPsα and C99. The monoclonal Flag antibody against the flag epitope sequence was used to immuoprecipitate Flag tagged Fe65. In studies investigating the influence of an alkaline environment over Notch process, the cell line stably expressing a truncated Notch construct, which was N-terminally flag-tagged and C-terminally myc-tagged allowed for the detection of the secreted Nβ peptide and the intracellularly released NICD using the monoclonal 9E10 antibody against the myc epitope sequence.

2.2 Cell Culture

2.2.1 Cell lines

Cell Line	Cell Type	*Referenence*
CHO K1	Chinese Hamster Ovary	*ATCC , CCL 61*
CHO 13-5-1 LRP ko	Chinese Hamster Ovary	*(FitzGerald, Fryling et al. 1995)*
CHO 13-5-1 APP 695	Human Embryonic Kidney Cells	*(Pietrzik, Busse et al. 2002)*
CHO 7WML	Chinese Hamster Ovary	*(Weggen, Eriksen et al. 2001)*
CHO 7WD10	Chinese Hamster Ovary	*(Weggen, Eriksen et al. 2001)*
HEK 293T	Human Embryonic Kidney Cells	*ATCC, CRL-11268*
HEK FNEXT	Human Embryonic Kidney Cells	*(Okochi, Steiner et al. 2002)*

LRP1-deficient CHO cell line (13-5-1) and the LRP1-deficient CHO cell line stably expressing human APP_{695} (13-5-1 695) have been described previously (Pietrzik, Busse et al. 2002). CHO K1 cells which were a kind gift from Dr. S.Leppla. CHO cells either stably over-expressing wild type human APP_{751} (7WD10) alone or stably co-expressing human mutant PS1 (M146L) (7WML) were a kind gift from S. Weggen. CHO cells stably expressing C99 was obtained by insertion of C99 in to the pLHCX retroviral expression vector and transferral in to the HEK GP2 packaging cell line. After infection with recombinant viruses, CHO K1 cells were selected with hygromycin. All CHO cells were

cultured in α-Dulbecco's modified medium containing 10% fetal bovine serum, 1mM MEM sodium pyruvate and 1x antibiotics/antimycotics (penicillin G, sodium, streptomycin sulphate, amphotericin B) in a humidified 5% CO_2 incubator at 37°C. HEK 293 cells stably co-expressing an N-terminally flag-tagged and a C-terminally myc-tagged mNotchΔE construct and APP Swedish (FNEXTsw) were also a gift from S.Weggen and were maintained in Dulbecco's modified medium supplemented with 10% FCS, 1mM MEM sodium pyruvate, and 1x antibiotics/antimycotics (penicillin G, sodium, streptomycin sulphate, amphotericin B) in a humidified 5% CO_2 incubator at 37°C.

2.2.2 Transfection

Transfections were carried out when the cells had reached 90% confluency with equal amounts of DNA using Lipofectamine 2000 according to manufacturer's protocol. The cells were used for experiments 24 h to 48 h post-transfection.

2.2.3 Treatments

To investigate the effect of a pH neutralized environment of APP metabolites, media from cells was replaced with fresh media containing either vehicle (H_2O) or 10mM NH_4Cl and incubated for 16 hours. To control for AICD generation by γ-secretase, media from cells was removed and replaced with fresh media containing either vehicle (DMSO) or 2.5μM L685, 458. In order to examine the effect of Aβ on AICD levels and/or generation, media from transfected cells was replaced with media containing either vehicle (DMSO) or 10ng/ml $Aβ_{40}$ and incubated for a further 16 hours.

2.3 cDNA constructs

The cytoplasmic tail of the LRP1 β-subunit has been described previously (9). The cDNA encoding LRP-CT was modified by standard PCR techniques using DNAzyme-DNA-polymerase (Finnzymes). KKAA and KKFF motifs were added between the last amino acid and the stop codon of the previously described LRP-CT construct, and were verified by sequencing (Andrea Schweizer, Dipoloma, 2005).

pCDNA3-Fe65 has been described previously (Pietrzik, Yoon et al. 2004). Plasmids encoding the Gal4 DNA-binding domain (pMST), the Gal4 DNA-binding domain engineered into the APP695 cytoplasmic tail (pMST-APP), the APP Gal 4 fusion protein with a mutation in the NPTY motif of the APP cytoplasmic tail 8pMST-APPΔ, and a Gal4 reporter plasmid encoding firefly luciferase (pG5E1B-luc) have been described and were a kind gift from Dr. T. Sudhof (Dallas, Texas). Plasmid pRL-TK encoding Renilla luciferase was from Promega (Madison, WI, USA), and pCDNA3 was purchased from Invitrogen (Carlsbad, CA, USA). p12-C99 has been described previously and was a kind gift from Dr. S.F Lichtenthaler (Lichtenthaler, Multhaup et al. 1999). The C99 fragment of this construct was cloned by PCR in to the pLHCX vector that was digested with Hind III/Cla I. The identity of the construct obtained by PCR was confirmed by DNA sequencing (Sebastian Jaeger, PhD work in progress).

2.4 Immunoprecipitation

Equal amounts of lysates were rotated with the antibodies and protein G-agarose overnight at 4°C. The samples were then washed four times with lysis buffer (NP40), after which the agarose beads were pelleted and the proteins were eluted into SDS sample buffer by heating at 95^{o}C for 10 min. To detect secreted Aβ or Nβ peptides conditioned media was collected 24 hr post-transfection, centrifuged to remove dead cells and immunoprecipitated using appropriate antibodies overnight at 4°C with rotation. The samples were washed with chilled PBS three times and the proteins were eluted into SDS sample buffer by heating at 55^{o}C for 10 min.

2.5 Membrane preparation

The cells were collected and resuspended in 500µl hypotonic buffer containing 1x protease inhibitor mixture and dounce-homogenized fifty times on ice. To prepare a postnuclear supernatant, the homogenate was centrifuged at 1000x g for 15 minutes at 4^{o}C. The membranes were then isolated from the supernatant by centrifugation at 20 000x g for 60 minutes at 4^{o}C. The membranes were resuspended in RIPA buffer and 50% of the total crude fraction was separated by SDS-PAGE.

2.6 Cell surface biotinylation

Confluent cell cultures in 6cm dishes transfected for 24 hrs with LRP-CT chimeras were washed three times with PBS-CM. Cells were biotinylated with 0.5mg/ml Sulfo-NHS-LC-Biotin dissolved in ice-cold borate buffer for 30 minutes at 4^{o}C. Biotin was changed twice during the 30 minute incubation period. Biotinylation was quenched with three 50mM NH_4Cl-PBS-CM washes followed by two PBS washes. Cells were harvested and lysed in NP-40 buffer and protein concentration was determined via BCA. Equal amounts of protein were immunoprecipitated using Neutravidin agarose beads overnight at 4^{o}C. Proteins were eluted from the Neutravidin agarose beads by heating at 95^{o}C for 10 min.

2.7 Endo H and PNGaseF Digestions

The deglycosylation of LRP chimera- transfected cells with Endo H and PNGase F was performed according to the supplier's instructions. Briefly 20µg of lysate was denatured in 1x glycoprotein denaturing buffer (supplied with enzymes) at 100^{o}C for 10 min. 1/10 volume of 10x G5 reaction buffer (supplied with EndoH) and 10x G7 reaction buffer [(+ 1/10 volume of 10%NP40), supplied with PNGaseF] and 2µl EndoH and PNGaseF,respectively was added to the appropriate samples. Samples were then incubated at 37^{o}C for 1h. Samples were then made up to a volume of 600µl using NP40 buffer + PI and immunoprecipitated overnight with rotation at 4^{o}C using LRP 1704 and protein G agarose beads.

2.8 Confocal Microscopy

Immunofluoresence was performed using standard protocols. Briefly, cells were fixed for 5 minutes at room temperature with 4% paraformaldehyde in PBS. Cells were washed with PBS and cell membranes were permeabilised using 0.1% Triton-X-100 in PBS (PTx). Non-specific binding was blocked using 4% BSA for 1 hr at room temperature. Primary and secondary antibodies were diluted in blocking buffer and incubation periods were conducted for 1 hr at room temperature, followed by 3 washes with PTx buffer. Alexa 546 and 488-labeled secondary antibodies were used subsequently. Microscopy was performed using an LSM 510 Meta (Carl Zeiss GmbH; Oberkochen, Germany) equipped with a 63x objective. Images were taken in the line-averaging mode and at a setting for the confocal pinhole of one Airy unit. Micrographs were arranged and exported using LSM Image

Browser, Version 2.30.011 (Carl Zeiss Jena GmbH) and Photoshop 7 (Adobe, San Jose, CA, USA).

2.9 Western Blotting

For detection of intracellular proteins, cells were lysed in an NP-40 buffer. Protein concentration was determined by the BCA method and equal amounts of lysate were separated on a 14% polyacrylamide gel.Whole cell lysates from APP Gal4 transfected cells were lysed in 1x Passive lysis buffer supplied with the dual luciferase assay reporter kit. Whole cell lysates from CHO 7WD10 and 7WML or HEK FNEXTsw cells were lysed in an NP-40 buffer. Protein concentration was determined by the BCA method and equal amounts of lysate were separated by SDS-PAGE. For the detection of Aβ, equal amounts of conditioned media was loaded on pre-cast 12% Bis-Tris polyacrylamide gels. Proteins were transferred onto PVDF and membranes were boiled at 95^{o}C for five minutes. For the detection of AICD derived from over-expressing CHO cell lines, proteins were also separated on 12% Bis-Tris polyacrylamide gels transferred onto PVDF membranes. Immunoblotting was carried out with the indicated antibodies and detected by enhanced chemiluminescence.

2.10 Metabolic labelling

HEK FNEXTsw cells were plated on poly-D-lysine coated 6 cm dishes. After 24 hrs cells were starved in DME without cysteine/methionine, 5% sodium pyruvate for 1 hour. Cells were labeled for 16 hours with 150μCi/ml [^{35}S] Cys/Met free DMEM, 5% FCS, 40mM HEPES, 5% sodium pyruvate, 1x antibiotics/antimycotics and 10mM NH_4Cl. Conditioned media was collected and centrifuged at 5 000x g for 5 minutes to remove dead cells. For the detection of Nβ media was immunoprecipitated using anti-flag and protein G agarose beads. Immunoprecipitates were separated on 12% Bis-Tris polyacrylamide gels.

2.11 AICD Reporter assay

Confluent CHO K1 cells were transiently transfected in 12 well plates (0.25μg of each plasmid). 16.67ng pRL-TK was added to each plasmid mix to control for transfection efficiency. 24 hours post-transfection, media from cells was removed and replaced with

fresh media containing NH_4Cl. After an additional 16 hours, the cells were lysed, and firefly and renilla luciferase activities were quantified using a dual luciferase assay reporter system (Promega) and a dual injector laminator. Firefly luciferase values were standardized to the corresponding Renilla luciferase values.

2.12 Subcellular fractionation

Cells were lysed in cell lysis buffer containing a protease inhibitor cocktail and allowed to swell on ice for 5 minutes. Following Dounce homogenization, nuclei plus debris were pelleted at 800 X g and resuspended in TSE buffer containing a protease inhibitor cocktail and dounce homogenized. Pure nuclei were pelleted at 600 X g and washed twice in the TSE buffer. The cytosolic fraction from the first centrifugation step was saved and centrifuged at 16000 X g for 1 hour at 4^oC. The clear supernatant was then subjected to immunoprecipitation with either CT15 or Flag for the detection of the AICD gal4 fragment. The pellet was lysed directly resuspended in 2x loading buffer.

3.1 Results: LRP1 modulates APP trafficking and metabolism within compartments of the secretory pathway

3.1.1 LRP-CT and LRP-CT chimeras are glycsosylated in a manner indicative of their compartmental residence

LRP1 and APP have the potential to interact during transport through all compartments of the secretory pathway. In addition, LRP1 imparts a strong influence over APP processing and Aβ production due to its effects on APP internalization and trafficking through the endosomal system. To verify the hypothesis that LRP1 regulates APP trafficking prior to the cell surface; truncated LRP1 mutants targeted to different organelles were generated. The amino acid sequence KKAA, previously described as mediating permanent ER retention of transmembrane protein was fused to the C-terminus of the well documented LRP-CT construct, while the amino acid sequence KKFF, described as an ER exit determinant, was fused to the C-terminus of LRP-CT (Fig. 1A) (Andersson, Kappeler et al. 1999; Pietrzik, Busse et al. 2002).

3.1 Results: LRP1 modulates APP trafficking and metabolism within compartments of the secretory pathway

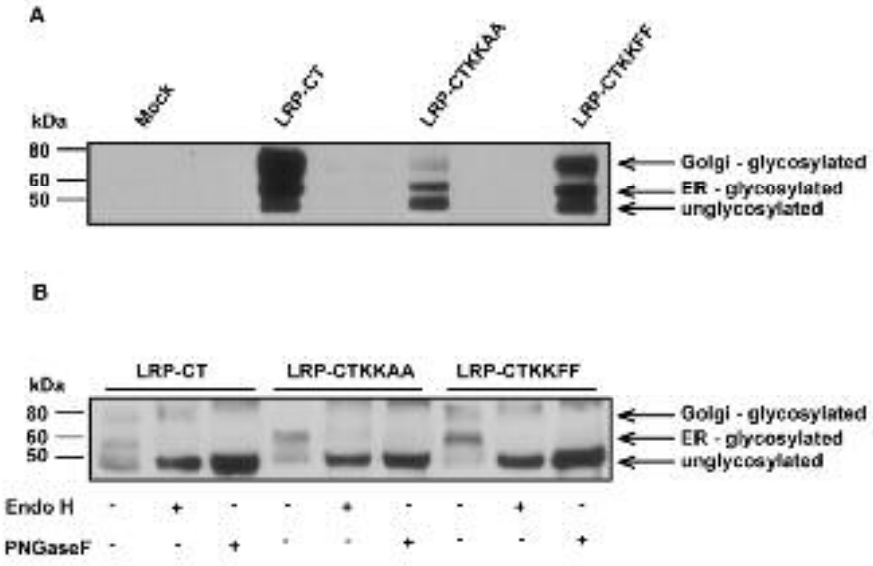


Fig. 1 Glycosylation pattern of LRP-CT, LRP-CT KKAA, and LRP-CT KKFF. (A) LRP1-deficient CHO cells were transiently transfected with vectors encoding LRP-CT with the wild type C-terminus of LRP1 (LRP-CT), or with vectors coding for a construct with a C-terminus containing the ER retention motif LRP-CT KKAA or the Golgi retention motif LRP-CT KKFF. Cells were harvested 24hr post-transfection and subjected to a crude membrane isolation and western blotting using an LRP1 antibody specific for the last fifteen residues of the LRP1 C-terminus (11H4) (A). Immunoblots show the expression levels of LRP-CT, LRP-CT KKAA, and LRP-CT KKFF and arrows indicate the predicted glcosylation pattern for all constructs. (B) Cells transfected with LRP-CT, LRP-CT KKAA, and LRP-CT KKFF were subjected to EndoH and PNGase F digestion. After SDS-PAGE and transfer to nitrocellulose, blots were probed with 11H4 antibody. Note that upon EndoH digestion LRP-CT migrates as a set of two bands, indicating that the ~58 kDa band was EndoH sensitive while the slower migrating band of ~75kDa remained EndoH resistant indicating post-ER glycosylation. (B). After PNGase F digestion, both, the 58 and the 75kDa bands were absent from all LRP-CT chimeras, indicating PNGase F sensitivity. Experiments were performed in triplicate and the results of one representative experiment are shown.

To assess efficiency of the ER- and Golgi-retention signals, we characterized the glycosylation patterns of the constructs, LRP-CT KKAA and LRP-CT KKFF in relation to glycosylation of the LRP-CT construct. LRP1-deficient CHO cells (CHO 13-5-1) were transiently transfected with each construct and expression levels were determined using immunoblotting (Fig 1A). The LRP-CT construct migrated as a set of three bands, while LRP-CT KKAA migrated as two bands. We hypothesized that the lack of the slower

migrating, i.e. upper third band, was due to the altered glycosylation pattern caused by ER retention of LRP-CT KKAA. This notion was supported by the observation that the LRP-CT KKFF construct displayed a similar migration pattern, i.e. identical glycosylation pattern to that of the LRP-CT construct, indicative of its ER-exit and transport to the Golgi compartment (Fig 1A). In order to further investigate the carbohydrate modifications of the constructs, cells lysates of LRP-CT, LRP-CT KKAA, and LRP-CT KKFF expressing cells were subjected to Endoglycosidase H (EndoH) and Peptide-N-glycosidase F (PNGaseF) digestion (Fig 1B). EndoH is known to cleave oligomannose and most hybrid types of glycans which are transferred onto proteins in the ER. When further modified in the Golgi, these moieties become EndoH resistant. On the other hand, PNGase F can remove all N-linked carbohydrate moieties attached to proteins in the ER and further modified within the Golgi apparatus. Thus, EndoH resistance/PNGaseF sensitivity is a hallmark of proteins that are properly folded and able to traffic from the ER to the trans-Golgi network. As predicted, the slower migrating band of LRP-CT was EndoH resistant but PNGase F sensitive, demonstrating that it reached trans-Golgi compartments. EndoH sensitivity was reflected by the absence of the ~57 kDa form of LRP-CT, LRP-CT KKAA, and LRP-CT KKFF and the resultant accumulation of their unglycosylated counterparts (~ 48 kDa) (Fig 1B). PNGase sensitivity removed all N-linked moieties reducing the ~78 kDa form of LRP-CT, LRP-CT KKFF and the ~57 kDa form of LRP-CT KKAA to their unglycosylated states (Fig 1B). Taken together with the expression patterns (Fig. 1A), we conclude that the LRP-CT KKAA construct was mainly retained in the ER, while the LRP-CT KKFF construct was able to exit the ER and acquired EndoH-resistance due to its transport to the Golgi.

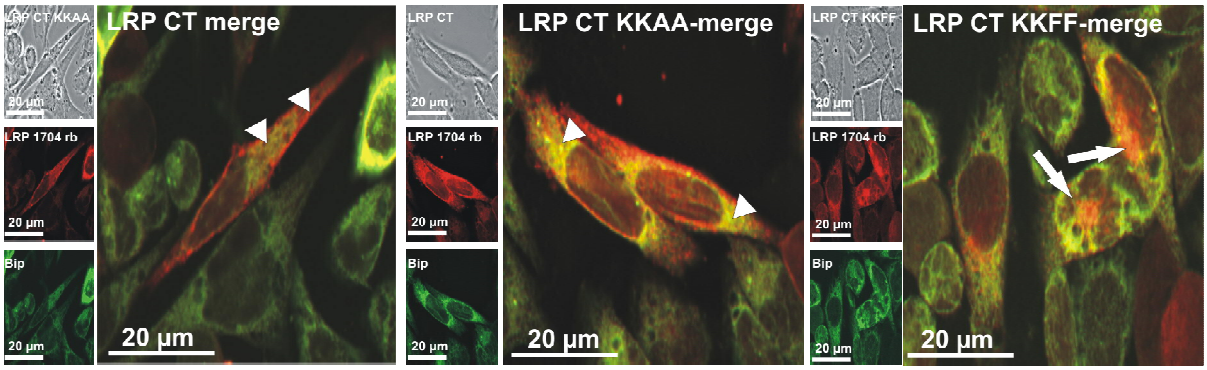
3.1.2 Subcellular localization of LRP-CT, LRP-CT KKAA, and LRP-CT KKFF

Previous studies have shown that in addition to the dilysine signal, the two C-terminal phenylalanines of the KKFF-motif operate as an ER exit determinant, while the dilysine signal combined with two alanines in the KKAA sequence mediates a direct ER retention of transmembrane proteins (Andersson, Kappeler et al. 1999). Due to the nature of the dilysine motifs used in these studies, we predicted ER-retention for LRP-CT KKAA and Golgi-retention for LRP-CT KKFF, which was supported by the patterns of glycosylation (see Fig. 1A and B). As an alternative to the carbohydrate digestions and to further

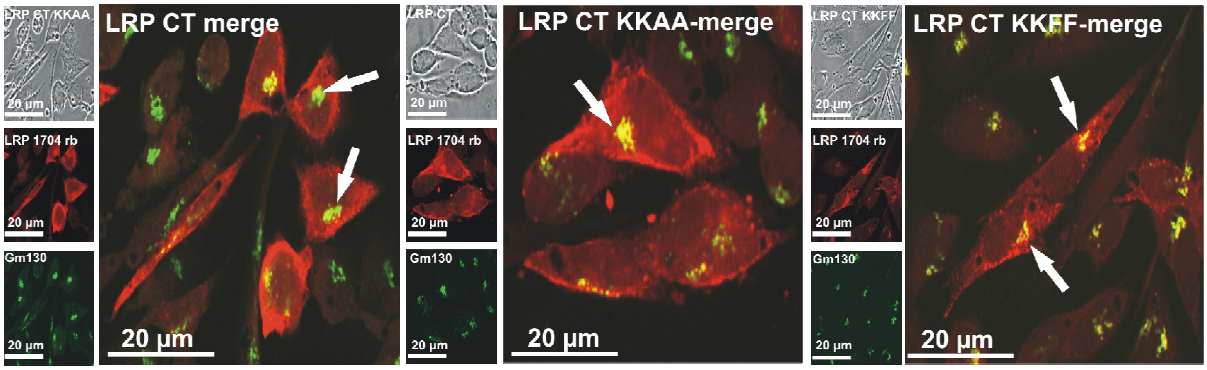
investigate the subcellular distribution patterns for proof of correct targeting of LRP-CT constructs to either ER or Golgi, we performed colocalization studies and compared the subcellular localization of LRP-CT, LRP-CT KKAA, and LRP-CT KKFF with the distribution of an ER marker protein, GRP 78 (Fig 2A), a cis-Golgi marker, GM 130 (Fig 2B), and an endosomal marker, EEA1 (Fig 2C) by immunofluoresence labeling and confocal LSM. LRP-CT was detected in a reticular and vesicular pattern and mainly concentrated within the perinuclear region (Fig. 2). It was co-localized with the ER marker, GRP 78 (Fig 2A, yellow). As LRP-CT is a protein traveling along the secretory route, it displayed reticular ER, perinuclear and cisternal Golgi-like stainings in addition to its presence within vesicles scattered throughout the cytoplasm, which most likely represented secretory vesicles (Fig 2A and B). Since LRP1 is responsible for internalization of bound ligands into early endosomes, we predicted a similar fate for LRP-CT as it still bears the internalization motif of its full length counterpart. LRP-CT was found to colocalize with the endosomal marker, EEA1 (Fig 2C). In contrast, LRP-CT KKAA was localized predominantly in a reticular network throughout the cytoplasm of expressing cells (Fig. 2A, B, and C). This expression pattern was identical to that of the ER-marker GRP 78 (Fig. 2A, yellow), demonstrating co-localization and thereby highlighting retention of LRP-CT KKAA in the ER (Fig 2C). Moreover, the expressed LRP-CT KKAA chimera displayed no staining resembling Golgi-cisternae, which further indicated its ER retention (Fig 2B). However, LRP-CT KKAA did show some over lap with GM130, indicating this construct was capable of reaching the cis-Golgi (Fig 2B). LRP-CT KKAA showed limited colocalization with EEA1 as expected since only proteins which have reached the trans-Golgi or cell surface can be internalized or transferred, respectively to the endosomal system (Fig 2C). The cells expressing LRP-CT KKFF displayed more Golgi-like staining together with a weak reticular, i.e. ER-like distribution (Fig. 2B). LRP-CT KKFF was observed in complete co-localization with the cis-Golgi marker, GM 130 (Fig 2B). Such a distribution pattern was expected, since its dilysine motif combined with the two phenylalanine residues are essential for ER exit and Golgi retrieval. However, this construct could also be found colocalized with EEA1, which may indicate that this construct reaches the cell surface where it may be internalized via endosomes or alternatively upon reaching the trans-Golgi it may be transferred to the endosomal system (Fig 2C).

3.1 Results: LRP1 modulates APP trafficking and metabolism within compartments of the secretory pathway

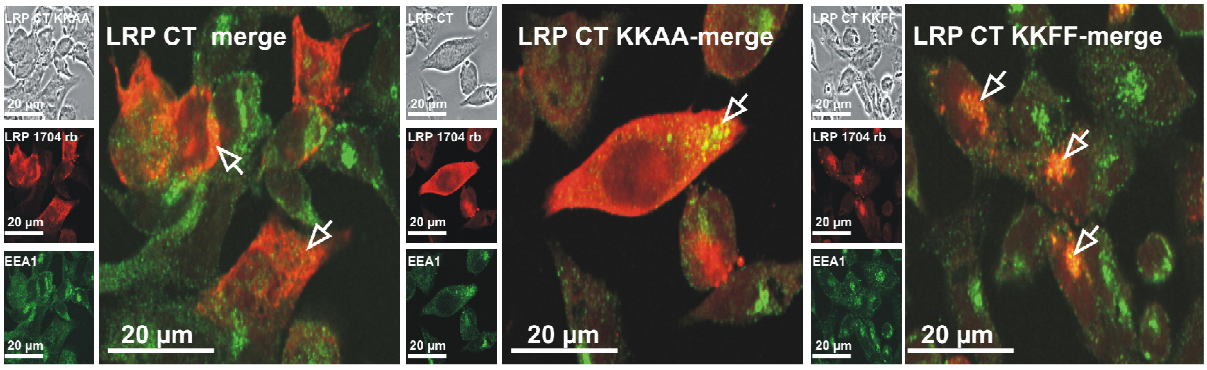
A Bip



B Gm130



C EEA1



3.1 Results: LRP1 modulates APP trafficking and metabolism within compartments of the secretory pathway

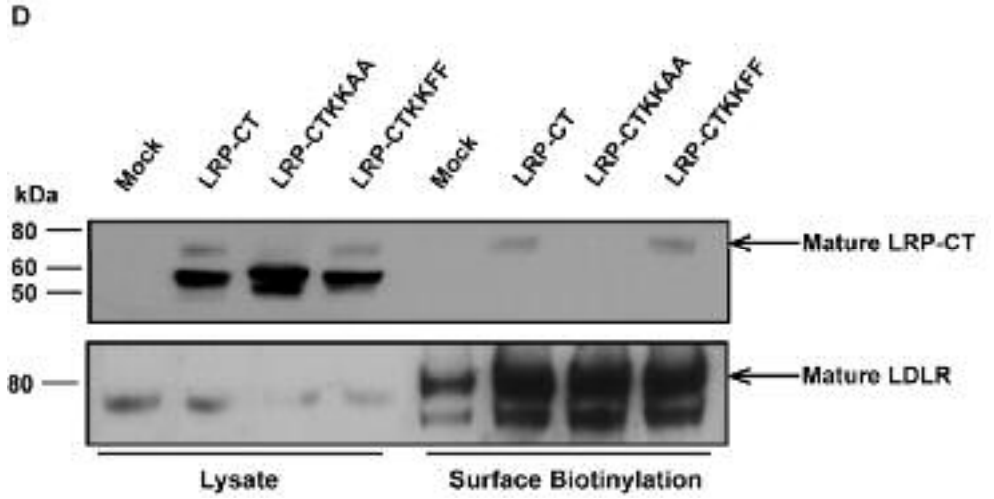


Fig 2. Immunofluoresence localization of LRP-CT chimeras with Bip, GM130, and EEA1 and cell surface distribution of LRP-CT, LRP-CT KKAA, LRP-CT KKFF. LRP1-deficient cells were transfected with LRP-CT chimeras as indicated and fixed 24 hr post-transfection. Localization of LRP-CT proteins was determined using polyclonal (1704) followed by Alexa Fluor 546-conjugated goat anti-rabbit IgGs (red) (A, B, C). Cells were further incubated with an affinity-purified mouse monoclonal antibody against Bip/GRP 78 (A), GM 130 (B), and EEA1 (C) followed by appropriate Alexa Fluor-conjugated goat anti-mouse IgGs to immunostain the ER, Golgi, and endosomes (green) (A, B, C) respectively. Filled-in arrow heads indicate ER staining, tailed arrows are indicative of Golgi staining, and hollow arrows indicate endosomal staining. (D) LRP1-deficient cells were rescued with LRP-CT chimeras. Cell surface proteins were biotinylated 24 hr post-transfection and immunoprecipitated using neutravidin beads. Cell surface LRP1 was immuno-identified using the 1704 antibody. On the left half of the panel, equal aliquots of cell lysates were loaded and only the mature form of the constructs could be recovered from biotinylated samples. Note that LRP-CT KKAA is completely absent from the cell surface and the transport of another protein, LDLR was not affected by the LRP-CT chimeras. Experiments were performed in triplicate and one representative experiment is shown.

To further assess the transport of the LRP-CT chimeras and to ensure only LRP-CT was capable of reaching the cell surface, we performed cell surface biotinylation experiments 8Fig 2D). LRP-CT in its fully mature state was capable of reaching the cell surface as the EndoH sensitive form present in the lysate was absent at the cell surface (Fig 2D). While LRP-CT KKAA detected in the lysate was not present at the cell surface, indicating its full retention in early secretory compartments, LRP-CT KKFF could be found at the cell surface to the same extent as mature LRP-CT, indicating the leakiness of this construct (Fig 2D). To ensure the LRP-CT chimeric constructs did not alter the trafficking of other

proteins we next assessed the cell surface distribution of the low density lipoprotein receptor (LDLR). The LDLR was found in high abundance at the cell surface, an effect observable due to its slow internalization rate. LRP-CT chimeras, specifically LRP-CT KKAA had no influence over LDLR cell surface transport (Fig 2D).

3.1.3 The cellular localization of LRP1 affects subcellular APP trafficking

Previous studies have indicated LRP1 as a modulator of APP trafficking between the cell surface and compartments of the endocytic pathway (Ulery, Beers et al. 2000; Pietrzik, Busse et al. 2002; Pietrzik, Yoon et al. 2004). By immunoprecipitation, it was shown that APP co-precipitates with full length LRP1, indicating an LRP1-APP interaction prior to furin cleavage of LRP1, i.e. early in the secretory pathway (Pietrzik, Yoon et al. 2004). Since LRP1 and APP both traffic along the secretory route and because LRP1 is a critical protein mediating multiple APP processing steps, we decided to compare the subcellular distribution patterns of the LRP-CT constructs with localization of APP. In light of the early APP-LRP1 interaction, we postulated that APP may be retained in compartments along the secretory pathway in which LRP1 retention occurred. Therefore, the distribution of stably overexpressed APP_{695} in the presence of the LRP1 constructs, LRP-CT, LRP-CT KKAA, and LRP-CT KKFF was investigated (Fig 3). LRP1 constructs were detected using the monoclonal anti-LRP1 antibody 11H4, which recognizes the C-terminus of LRP1. The specificity of the 11H4 antibody can be appreciated from control experiments (Fig 3A), and from the observation that only expressing cells were stained (Fig 3B, C, and D). In cells co-expressing LRP-CT and APP_{695}, APP was mainly concentrated in Golgi-like compartments, where it partially co-localized with LRP-CT (Fig 3B, yellow). A more profound co-localization of both proteins was observed in vesicles scattered throughout the cytoplasm (Fig 3B, yellow). In contrast, expression of APP_{695} in the presence of LRP-CT KKAA (Fig 3C) revealed retention of APP_{695} entirely in the compartments that were LRP-CT KKAA-positive (Fig. 3C, yellow). Based on the retentiveness of LRP-CT KKAA, as shown in Fig 2A, the results highlight its capacity to hold APP back in the ER. This retention of APP in the ER is further emphasized by the absence of Golgi-like staining (Fig. 3C, cf. Fig 3B). In the presence of LRP-CT KKFF, which was mainly localized to the Golgi (Fig. 2B, red), the staining pattern of APP_{695} appeared colocalized with LRP-CT KKFF (Fig. 3D, yellow). In addition, a colocalization of APP_{695} and LRP-CT KKFF was

also detectable in some vesicles, which may implicate an incomplete retention of APP due to incomplete Golgi-retention of LRP-CT KKFF, or due to an inadequate capacity of LRP-CT KKFF to retard APP_{695} transport out of the Golgi apparatus.

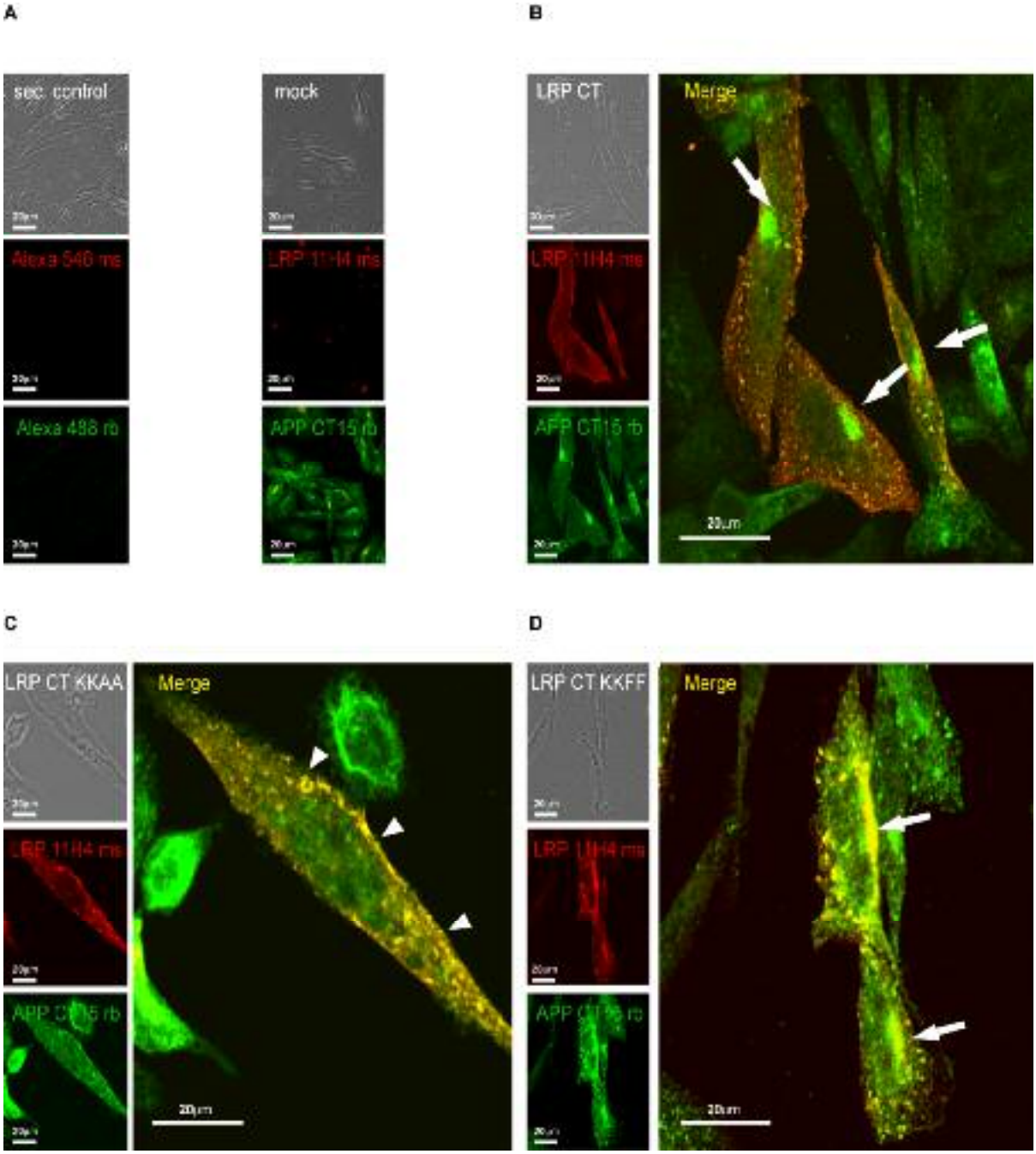


Fig. 3 Immunofluoresence localization of LRP-CT constructs in relation to APP_{695}. LRP1-deficient cells, stably expressing $APP_{695,}$ were rescued with LRP-CT chimeras and fixed 24 hr post-transfection. Secondary and primary antibody controls are depicted in panel A. The distribution of APP was determined with a polyclonal antibody against the last fifteen residues of its C-terminus

(CT15), followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (green). LRP-CT proteins were visualized using mouse-anti LRP1 C-terminus antibodies (11H4) followed by Alexa Fluor 546-conjugated goat anti-mouse IgG (red). Filled-in arrow heads indicate ER staining, tailed arrows are indicative of Golgi staining .

3.1.4 LRP1 retention in the ER strongly influences APP transport to the cell surface and its processing

To further demonstrate the retentive influence of LRP-CT KKAA and LRP-CT KKFF over APP in the ER and the Golgi, respectively, we next assessed the amounts of APP that reached the cell surface. Cell surface biotinylation experiments of LRP1-deficient CHO 13-5-1 cells stably overexpressing APP_{695}, and transfected with the LRP-CT constructs showed strikingly reduced levels of APP that reached the plasma membrane in the presence of LRP-CT KKAA as compared to all other LRP-CT constructs (Fig 4). To investigate the influence of compartment-specific retention on APP processing, we specifically immuno-recovered APP-CTFs from the cell surface. The results indicated that the amounts of APP-CTFs in the presence of LRP-CT were larger than those seen in mock-transfected cells. This was previously shown and described to be the result of an LRP1-stabilizing influence onto APP-CTFs (9). However, APP-CTFs were greatly reduced in the presence of LRP-CT KKAA compared to that seen in the presence of LRP-CT (Fig 4). Interestingly, LRP-CT KKFF-mediated Golgi-retention resulted in a much less pronounced reduction of APP-CTFs at the plasma membrane when compared to LRP-CT KKAA-mediated ER-retention of APP_{695}. This might be due to the incomplete retention of KKFF-motifs in the Golgi network. Therefore, some APP may still be capable of traveling to the surface, where it can be processed by the respective secretases (Itin, Kappeler et al. 1995; Parvathy, Hussain et al. 1999; Carey, Balcz et al. 2005; Kaether, Schmitt et al. 2006).

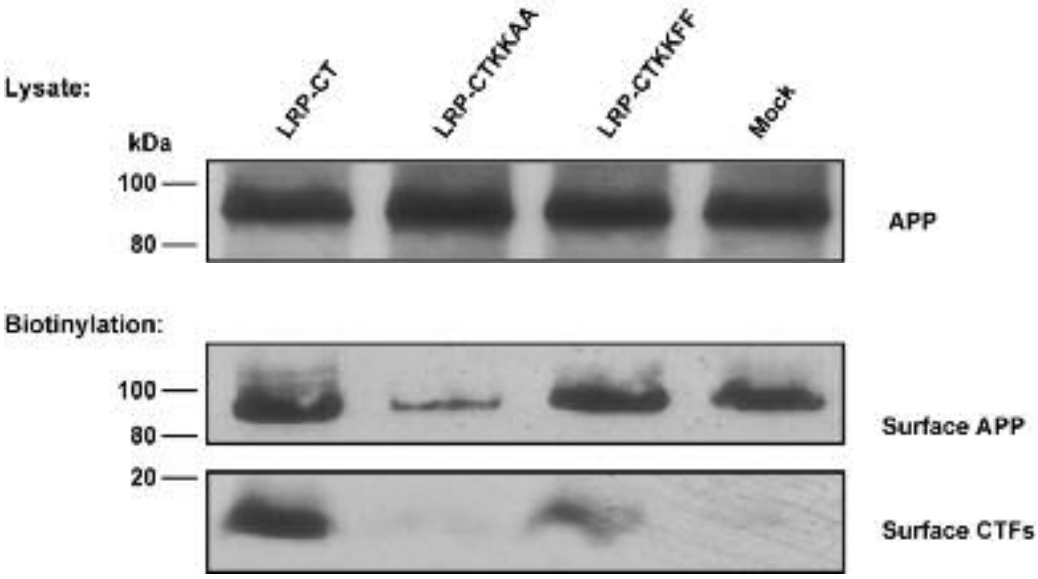


Fig. 4 Addition of an ER retention signal to LRP-CT reduces APP transport to the plasma membrane. LRP1-deficient cells stably expressing APP_{695} were rescued with LRP-CT chimeras. Cell surface proteins were biotinylated 24 hr post-transfection and immunoprecipitated using neutravidin beads. Cell surface APP was immuno-identified using the CT15 antibody. In the top panel, equal aliquots of cell lysates were loaded and reacted with the CT15 antibody, verifying that APP_{695} expression levels were comparable in all conditions. Immunoblots depicting cell surface APP and APP-CTFs showed that the CTFs were reduced in cells expressing LRP-CT KKAA as compared with cells expressing LRP-CT and LRP-CT KKFF. Experiments were performed in triplicate and one representative experiment is shown.

Due to the apparent differences in APP-CTF levels at the cell surface, in particular, upon APP retention in the ER via LRP-CT KKAA, we next wanted to investigate the total amount of APP-CTFs that were generated in transfected cells (Fig 5). Therefore, LRP1-deficient CHO 13-5-1 cells stably overexpressing APP_{695} were transfected with different LRP-CT constructs and subjected to a crude membrane preparation (Fig 5). Expression levels of each LRP-CT targeting mutant were determined to be comparable in all transfectants (Fig 5, lower panel). As was shown previously, expression of LRP-CT gives rise to an increase in APP-CTFs when compared to mock-transfected LRP1-deficient CHO 13-5-1 cells stably overexpressing APP_{695} (Fig 5). However, we found that the total amount of APP-CTFs that were recovered in membrane preparations in the presence of LRP-CT KKAA was similarly reduced in comparison to that recovered in the presence of

LRP-CT or LRP-CT KKFF (Fig 5). Under these conditions, the APP-CTF levels recovered in the presence of LRP-CT KKAA were in fact closer to those seen in mock-transfected cells. This provides evidence for the notion that LRP1 retention within the ER affects either APP-CTF generation or the stability of CTFs. Complementary to the biotinylation experiment (see Fig. 5) LRP-CT KKFF demonstrated no strong influence on cellular generation of APP-CTFs.

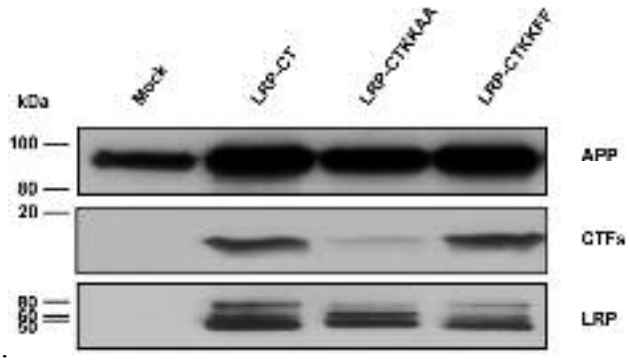


Fig. 5 Addition of an ER retention signal to LRP-CT reduces APP CTFs. LRP1-deficient cells stably expressing APP_{695} were rescued with LRP-CT chimeras. Cells were harvested 24 hr post-transfection and subjected to crude membrane isolation. Immunoblotting demonstrated comparable expression levels of LRP-CT, LRP-CT KKAA, and LRP-CT KKFF. Standard ECL was used for the detection of bound IgGs. Immunoblotting for APP CTFs showed that the CTF amounts were reduced in cells expressing LRP-CT KKAA as compared with cells expressing LRP-CT. Experiments were performed in triplicate and one representative experiment is shown.

3.1.5 The ER-retention of APP by LRP-CT KKAA causes a decrease in Aβ secretion

It has been demonstrated that LRP1 imparts its influence on Aß production through an enhancement of the rate of APP internalization, which in turn gives rise to an increase in Aβ generation (7, 9). We therefore speculated that the retention of APP in ER or Golgi compartments via LRP-CT KKAA and LRP-CT KKFF, respectively, might also affect Aβ production. As previously published, LRP1-deficient cells expressing LRP-CT showed an elevation in the amount of secreted Aβ when compared to mock-transfected LRP1-deficient cells (Fig 6) (Pietrzik, Busse et al. 2002). However, LRP1-deficient cells expressing LRP-CT KKAA showed a striking decrease in the levels of Aβ released in to the culture medium as compared to that secreted in the presence of normally transported LRP-CT (Fig 6A and B). On the other hand, the LRP1-deficient cells transfected with LRP-CT KKFF showed an increase in the amount of Aβ secretion compared to that

secreted in the presence of LRP-CT KKAA, but KKFF-expressing cells secreted slightly less Aß than cells expressing normally transported LRP-CT. These results clearly demonstrate that LRP1 and APP traffic not only along the same route in the cell but rather interact during this journey. Furthermore, the reduction in APP-CTF levels in the presence of LRP-CT KKAA (see Fig 6) is most likely not the result of inefficient stability conferral by LRP1, but rather the result of a reduction in APP-CTF generation. We conclude that LRP1 affects transport and processing of APP as early as in the secretory pathway, since retention of both proteins within the ER resulted in reduced Aß secretion.

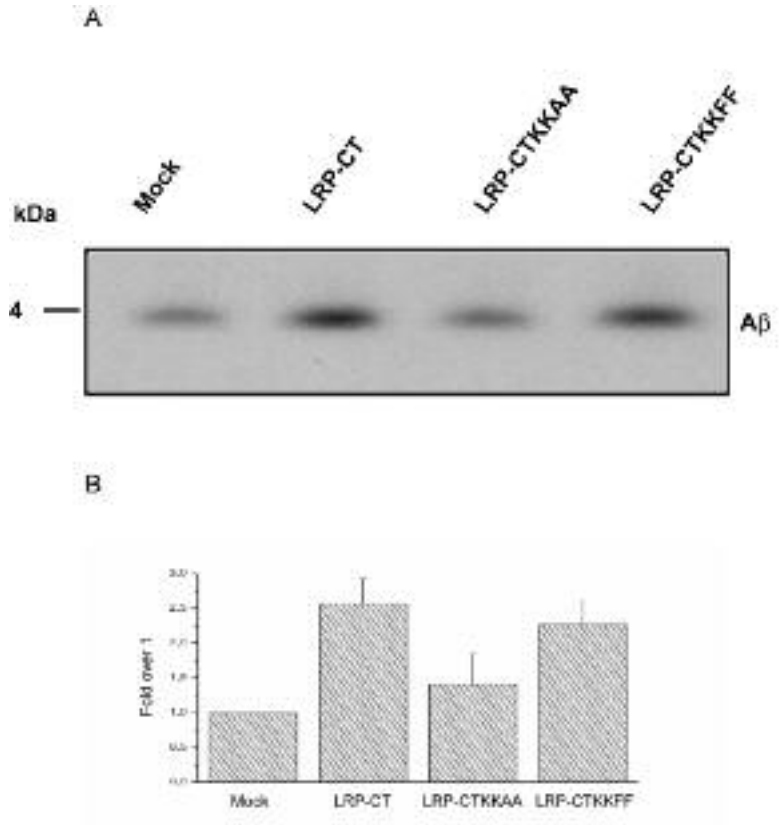


Fig. 6 ER retention of LRP1 reduces Aβ production destined for secretion. (A) LRP1-deficient cells stably expressing APP_{695} were rescued with LRP-CT chimeras. Aliquots of conditioned media were subjected to immunoprecipitation using the Aβ - specific antibody 26D6, followed by western blotting to determine the levels of total Aβ generation. Experiments were performed in triplicate and one representative experiment is shown. (B) For the quantification of secreted Aβ in the presence of LRP-CT chimeras, equal amounts of media were immunoprecipitated using 26D6 and blots were analyzed by densitometry. Error bars indicate standard error of the mean (S.E., n=3).

3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

3.2.1 APP but not NOTCH cleavage events are abrogated under pH neutralized conditions

The endosomal/lysosomal pathway constitutes the main degradation route of membrane bound proteins (Winchester 2005; van der Goot and Gruenberg 2006). Raising the intracellular pH appears to impede this function, such that degradation is partially blocked (Seglen and Gordon 1980; Yoshimori, Yamamoto et al. 1991). As shown in several studies the presence of NH_4Cl, which is a known alkalizing agent of endosomes and lysosomes, an accumulation of APP-CTFs can be detected due to a blockade in their degradation(Caporaso, Gandy et al. 1992; Golde, Estus et al. 1992; Lahiri 1994; Schrader-Fischer and Paganetti 1996). For our studies we employed two CHO cell lines stably over-expressing APP751, one overexpressing a familial presenilin 1 mutation (7WML) while the other expresses endogenous wild type presenilin 1 (7WD10). APP-CTFs are the substrate for γ-secretase and hence under pH neutralizing conditions, more substrate might be available for γ- and ε-cleavage. Under similar conditions in our lab, we also observed an accumulation in APP-CTFs and AICD using the polyclonal anti-APP antibody CT15 and a concomitant reduction in Aβ secretion using the monoclonal antibody 26D6 (Fig 1a). In most circumstances, an increase in APP-CTFs would imply a corresponding increase in APPs secretion since both catabolites are generated by a single cleavage event. However, α-secretase has been shown to be most active at the cell surface and therefore should not be heavily influenced by an intracellular alkaline environment (Parvathy, Hussain et al. 1999).Therefore, we investigated whether α-secretase cleavage resulting in APPsα secretion was affected under these conditions. Using the monoclonal antibody 26D6 we were unable to show any change in the amount of APPsα secreted in to the media under pH neutralizing conditions compared to that secreted under normal conditions (Fig 1a).

3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

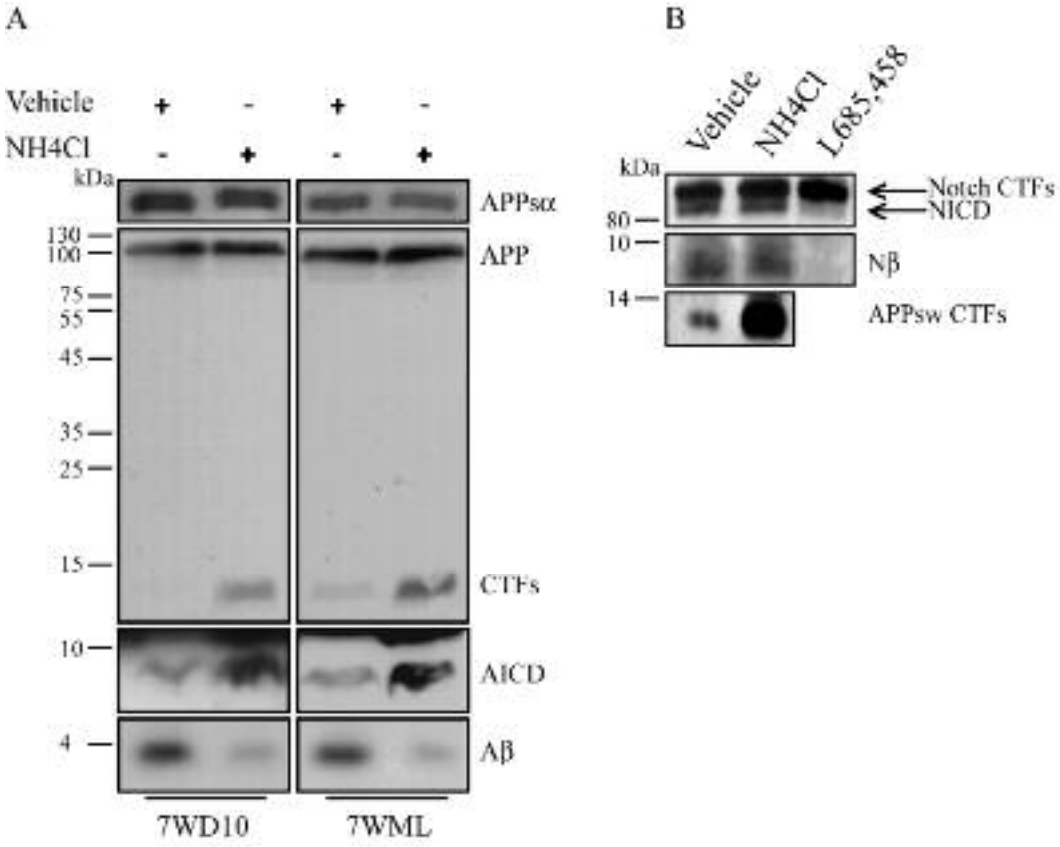


Fig. 7 γ-secretase cleavage of APP but not Notch is altered under pH neutralizing conditions. (A) CHO cells stably expressing APP 751 (7WD10) and APP 751 with a presenilin mutation (7WML) were treated with water (vehicle) and ammonium chloride (NH_4Cl) where indicated for 16 hours. For the detection of AICD, 70% of the cell harvest was subjected to crude membrane preparation as described in materials and methods and loaded on a 13% SDS-PAGE. APP, APP-CTFs, and AICD were detected using the C-terminal anti APP antibody, CT15. Aβ and APPsα were detected using the monoclonal anti APP antibody 26D6. No change in APPsα secretion was detected while C83 and AICD accumulated under NH_4Cl treated conditions. (B) For the detection of Nβ, HEK 293 cells stably co-expressing an N-terminally flag-tagged and a C-terminally myc-tagged mNotchΔE construct and APP Swedish (APPsw) were labelled with [^{35}S] methionine/[^{35}S] cysteine for 16 hours while been co-treated with NH_4Cl and L685,458. Proteins of cell media were immunoprecipitated with a monoclonal anti-flag antibody and resolved on a 12% Bis/Tris SDS-PAGE. For the detection of NICD, equal amounts of cell lysate were loaded on a 6% SDS-PAGE and immunoblotted with monoclonal anti-myc 9E10 antibody. The same cell lysates were also analysed on a 13% SDS-PAGE using CT15 for the detection of APPsw CTFs. The Notch ΔE catabolites, NICD and Nβ did not respond in an accumulative fashion under NH_4Cl treated conditions while APPsw CTFs did. Experiments were performed in triplicate and one representative experiment is shown.

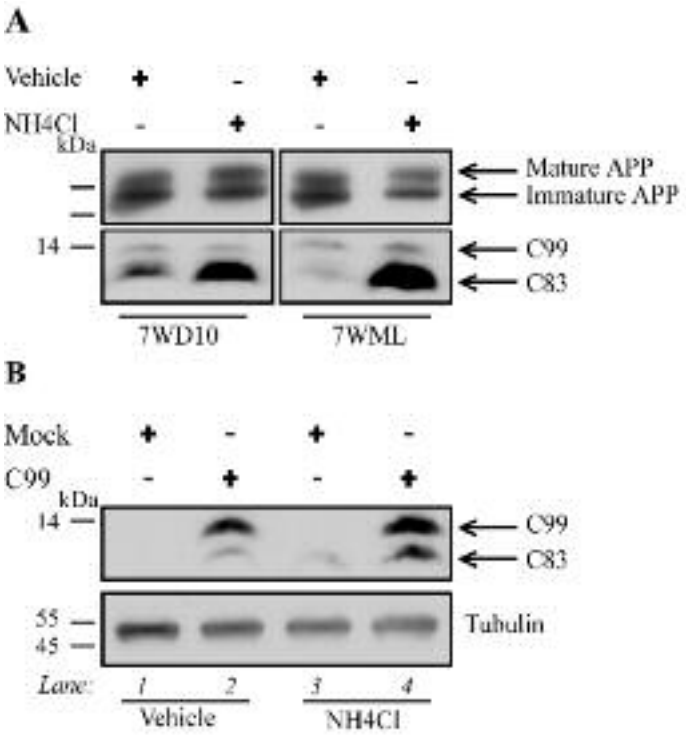
3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

It has become clear that both Notch and APP share a similar cleavage fate, therefore we wanted to determine whether Notch cleavage is affected in a pH neutralized environment(De Strooper, Annaert et al. 1999; Selkoe and Kopan 2003; Iwatsubo 2004; Barrick and Kopan 2006). Notch cleavage by γ-secretase is essential for the release of its ICD (NICD) so that it may participate in nuclear translocation and target gene activation, therefore it is crucial that its cleavage remains unaltered(Lecourtois and Schweisguth 1998; Schroeter, Kisslinger et al. 1998; Struhl and Adachi 1998; Barrick and Kopan 2006). Cells stably co-expressing C-terminally Myc-tagged and N-terminally Flag- tagged Notch ΔE together with APP Swedish (APPsw) were treated with NH_4Cl, at which point the cells were harvested and lysed. While the NICD and Notch ΔE proteins were detected using anti-myc antibody 9E10, Nβ was detected using an ani-flag antibody. In order to measure Nβ, the proteins were labelled overnight with Meth/Cys $^{[35]}$ S while being co-treated with vehicle or NH_4Cl. As shown by Vingtdeux and colleagues we were able to confirm that the NICD was unaffected under alkalizing conditions (Vingtdeux, Hamdane et al. 2007). In order to assess the effectiveness of the treatment we simultaneously monitored APPsw-CTFα levels, which did indeed increase in the presence of NH_4Cl. Furthermore, we also wanted to investigate if a pH neutralized environment affects Nβ cleavage. Like the cleavage of NICD, the cleavage of Nβ was also unaffected in the presence of NH_4Cl. This data suggests that the sequential cleavage of Notch ΔE liberating NICD and Nβ is not altered under alkaline conditions which stands in contrast to APP processing.

3.2.2 C83 but not C99 accumulates under alkalizing conditions

We next wanted to investigate if the observed reduction in Aβ secretion was due to a reduction in the γ-secretase substrate, C99, generated through BACE1 cleavage. Under pH neutralizing conditions the C99 protein level was unchanged compared to control and treatment had no effect on mature or immature APP levels (fig 2a). However, C83 protein levels were dramatically increased upon NH_4Cl stimulation (fig 2a). To further investigate the effect of a pH neutralized environment on C99 levels we transfected C99 itself in wild type CHO cells (fig 2b). These cells transiently transfected with C99 were incubated with NH_4Cl, harvested and processed as above. The C99 levels failed again to respond to their alkaline environment unlike endogenous C83 levels which accumulated only in treated

samples (fig 2b). This thus rules out a failure of treatment as being responsible for the non-effect on C99 protein amount. In vehicle treated C99 transfected cells, a portion of C99 was degraded to C83 and upon NH_4Cl treatment C83 further accumulated due to degradation inhibition. Under the conditions employed here, C83 protein levels are more responsive to an alkaline environment compared to C99 protein levels, which might be due to the further processing of C99 to C83 by α-secretase. To further determine if the increase in C83 was also due to the partial degradation of C99 to C83, we employed stably expressing C99 cells and analyzed C83 and C99 protein amount, in addition to AICD under pH neutralizing conditions (Fig 2C). Upon NH_4Cl treatment, C99 levels were reduced compared to nontreated while C83 accumulated. Furthermore, AICD was only detectable in NH_4Cl treated conditions (Fig 2C). Therefore, the accumulation in C83 can be explained by both an inhibition in its degradation, in addition to the partial degradation of C99 to C83. Thus, the AICD which accumulates under alkaline conditions is most likely that generated from C83.



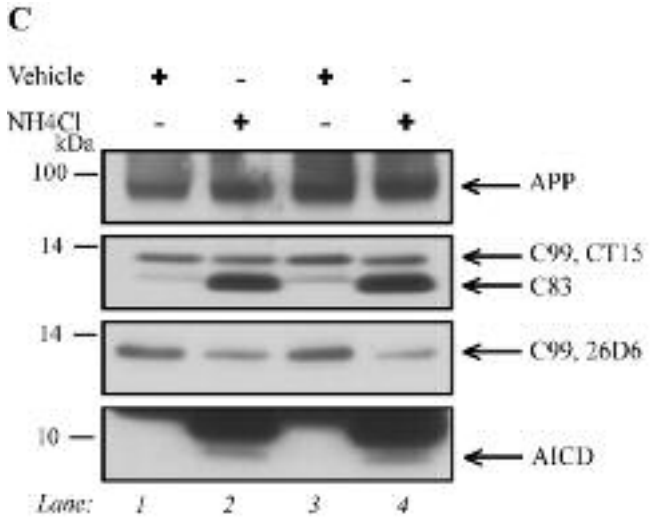


Fig. 8 C83 but not C99 accumulates under pH neutralizing conditions. (A) CHO 7WD10 and 7WML cells were treated with NH_4Cl for 16 hours. Equal amounts of lysate were loaded on a 12% Bis/Tris SDS-PAGE gel, blots were probed for APP and APP-CTFs using the anti APP antibody CT15 for the detection of all APP metabolites. Mature and immature APP are unaffected by the intracellular alkalinity while C83 levels respond accumulatively. (B) Wild type CHO K1 cells were transiently transfected with pLPCX as mock or C99 pLPCX constructs. 24 hours post transfection, the cells were treated with NH_4Cl for a further 16 hours and analysed as in Fig 2 (A). A portion of C99 is degraded to C83 and C83 accumulates additively upon NH_4Cl treatment. (C) Wild type CHO K1 cells stably expressing C99 were treated as above and subjected to a crude membrane preparation. Immunoblots were probed with CT15 for the detection of all APP CTFs and reprobed with a monoclonal anti-Aβ antibody IC16 recognizing the first 12 amino acids of the Aβ sequence for the detection of C99 specifically. C99 is partially degraded to C83 under alkaline conditions while C83 accumulates additively as does the AICD fragment. Experiments were performed in triplicate and one representative experiment is shown.

3.2.3 The APP Gal4 system resembles the mechanistic situation, by which APP is metabolized in the absence of a Gal4 binding domain

The AICD fragment was first shown to play an active role in transcriptional transactivation through a complex formation with Fe65 and Tip60 (Cao and Sudhof 2001). The authors used the DNA binding domains Gal4 and LexA, which were engineered, into the intracellular tail of APP_{695} at the cytoplasmic boundary of the transmembrane region (TMR). This well documented transactivation assay allows measurement of transcriptional

transactivation through a co-operation with either a Gal4- or LexA-dependent reporter plasmid expressing luciferase(Cao and Sudhof 2001). However, unlike previous assays conducted, which rely solely on the luciferase read-out, here we sought out to analyse AICD production using standard biochemical readouts. In addition, to ascertain no alteration in transcriptional activation occurred under the conditions used, we also monitored the luciferase activity. To first identify the secretase metabolites derived from APPGal4, we performed western blot analysis using the polyclonal anti-APP antibody CT15. In the presence of Fe65, the AICDGal4 fragment becomes stabilized and detectable using our C-terminal anti-APP antibody (Fig 3a). To verify that the AICD fragment derived from the APPGal4 construct was indeed a γ-secretase metabolite, we used the γ-secretase inhibitor, L685, 458. In the presence of L685, 458, the characteristic increase in endogenous CTFs can be observed, as can the increase in the CTFs derived from APP Gal4 construct (APPGal4 CTFs), which verifies their identity as a γ-secretase substrate in addition to the similarity in their behaviour to that of the endogenous system (Fig 3b). Furthermore, the APPGal4 AICD fragment is no longer generated following γ-secretase inhibition and so proving it as the γ-secretase generated by-product comparable to the mechanistic system in the absence of a Gal4 domain (Fig 3b).

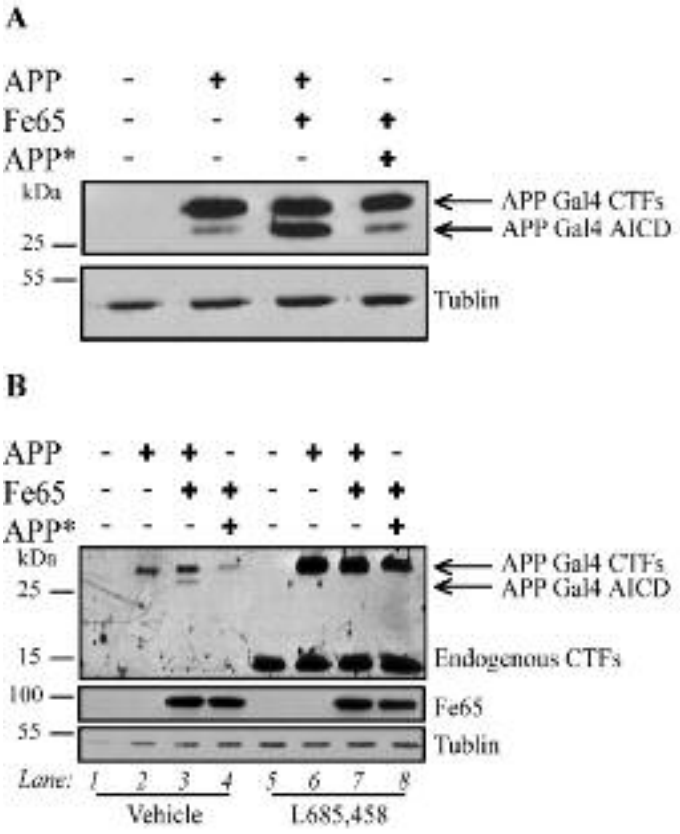


Fig. 9 The AICD fragment generated from APP_{695} with a Gal4 domain is stabilized and detectable in the presence of Fe65. Wild type CHO K1 cells were transiently transfected as depicted. (A) 24 hours post-transfection, the cells were harvested and lysed using the 1x passive lysis buffer supplied with the Dual luciferase reporter assay kit. Equal amounts of lysate were loaded on a 12% SDS-PAGE and analysed for APP-CTFs and tubulin to ensure equal protein loading. The AICD fragment generated from APP Gal4 is stabilized in the presence of Fe65 and so detectable via western blotting (B) Wild type CHO K1 cells were transfected as in Fig 3 (A), treated overnight in the presence of DMSO (vehicle) and 2.5μM L685,458. The cells were harvested and processed as in Fig 3 (A). The AICD Gal4 fragment is generated via γ-secretase as its production in the presence of L685, 458 is inhibited. Experiments were performed in triplicate and one representative experiment is shown.

3.2.4 Neutralization of Acidic Compartments increases the AICD Gal4

We next wanted to investigate whether the APPGal4 AICD fragment responds in a similar fashion as an untagged AICD fragment without a Gal4 domain under pH neutralizing conditions. To analyse the metabolites of the APPGal4 assay in an alkaline environment, wild type CHO K1 cells were transiently transfected and subsequently treated with NH_4Cl.

3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

As demonstrated previously with untagged APP (Fig 1a), APPsα derived from the APP Gal4 construct showed no response to its alkaline environment compared to control samples.

In the presence of NH_4Cl, the endogenous APP-CTFs become clearly visible due to a suppression of their degradation (compare lanes 1-4 with lanes 5-8), as do the APPGal4-CTFs (compare lanes 2-4 with lanes 6-8) and thus the assay in question, closely resembles the endogenous state of the cell (Fig 4a). We could further demonstrate that this assay closely resembles the situation of untagged transfected APP shown in Fig 1a, as the AICD derived from untagged APP accumulates in a similar fashion as the AICD Gal4 fragment under pH neutralizing conditions (compare lane 3 and 7).

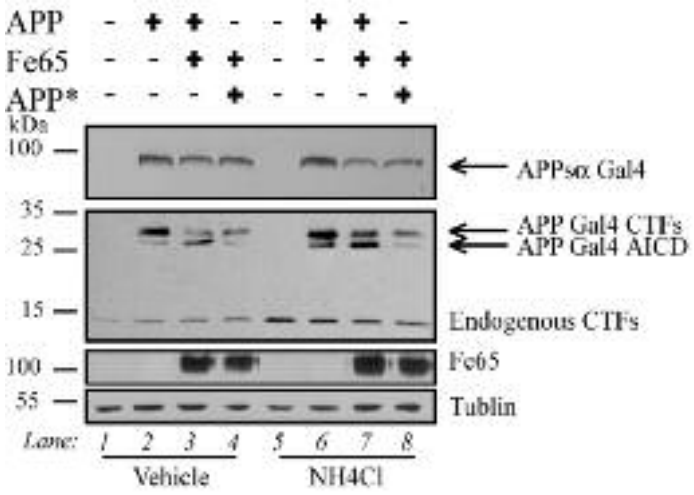


Fig. 10 The AICD fragments generated from APP-Gal4-CTFs accumulate in the presence of NH_4Cl. Wild type CHO K1 cells were transfected as depicted in Fig 3 (A). 24 hours post-transfection, the cells were treated with vehicle and NH_4Cl for a further 16 hours, followed by cell harvest and lysis using the 1x passive lysis buffer. Equal amounts of lysate and conditioned media were loaded on a 12% SDS-PAGE and analysed for APP full length (APPGal4) and its metabolites (APPsα Gal4, APPGal4-CTFs, APPGal4-AICD), Fe65 and tubulin. Both endogenous APP CTFs and APP Gal4 CTFs, in addition to the AICD Gal4 fragment respond in an accumulative fashion under pH neutralizing conditions. Furthermore we failed to detect any difference in the amount of APPsα secreted between NH_4Cl and control conditions. Experiments were performed in triplicate and one representative experiment is shown.

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3.2.5 The reduction in Aβ secretion in a neutralized environment is not a result of an increase in its degradation

As it has been recently published that AICD mediates neprilysin induction and thereafter Aβ degradation, it might well be that the increase in AICD under pH neutralized conditions is responsible for an increase in neprilysin expression and hence could explain the reduction in Aβ secretion seen here (Pardossi-Piquard, Petit et al. 2005). If this were a regulated feedback mechanism, then AICD would be generated on a need-to-have basis to degrade Aβ. This would therefore make Aβ the preceding influence governing AICD levels. If this were a genuine system then it should also work in reverse, meaning an increase in Aβ should give rise to an increase in AICD. Based on the above theory, we sought to reverse the cellular parameters by adding synthetic Aβ to the APP-Gal4 system described above. Cells were transiently transfected with APP in the presence of Fe65, in addition to a mutant APP (APP*), which is incapable of binding Fe65. To induce a postulated AICD accumulation the media was replaced with fresh media containing 10ng/ml $A\beta_{40}$ or DMSO (vehicle) for a further 16 h, at which point the cells were harvested and processed as before. We saw no upregulation in AICD production in the APP Gal4 assay upon Aβ treatment compared to vehicle treated (Fig 5). Treatment had no effect on neither Fe65 nor tubulin levels. This data suggests that the reduction in Aβ secretion is not a consequence of the increased AICD production and hence not an increase in neprilysin expression.

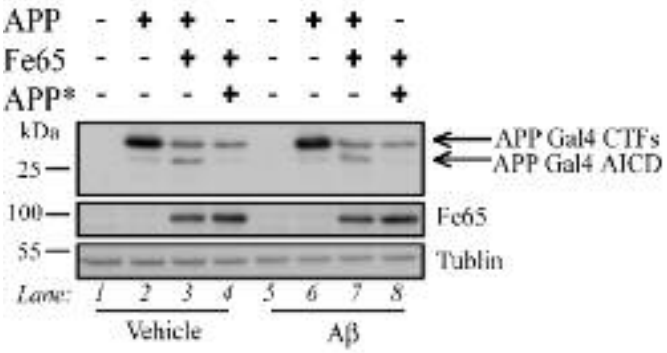


Fig. 11 The observed accumulation in AICD fragments is not responsible for the decrease in Aβ. Wild type CHO K1 cells were transfected as depicted in Fig 5. 24 hours post-transfection, cells were treated for a further 16 hours with DMSO (vehicle) and $A\beta_{40}$, at which point the cells were harvested and processed as previously described above. No change in AICD Gal4 protein levels was detectable between treated and non treated conditions. Experiments were performed in triplicate and one representative experiment is shown.

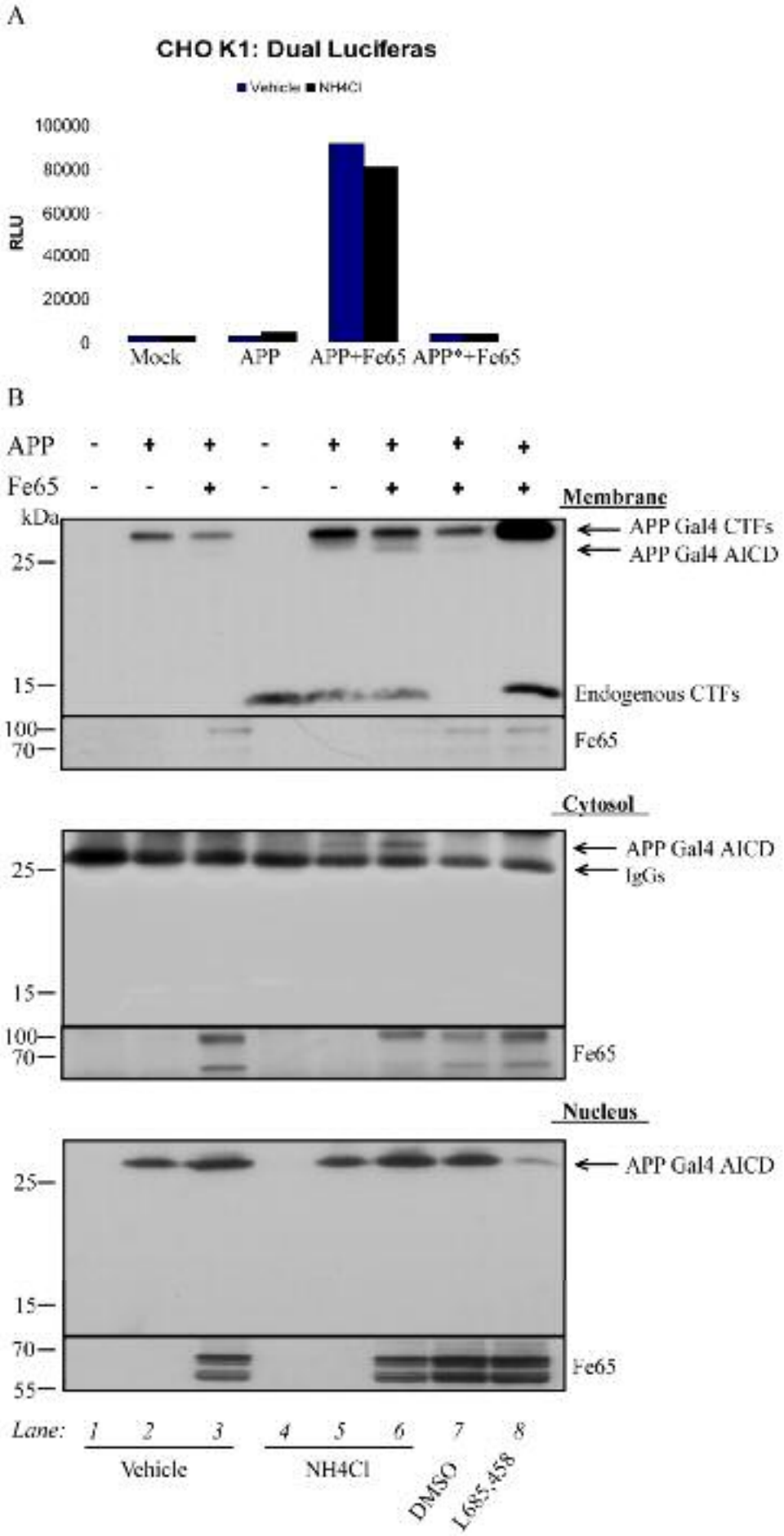
3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

3.2.6 The AICD surplus is transcriptional inactive because it remains membrane tethered and free in the cytosol where it interacts with Fe65

We next wanted to investigate if the AICD surplus was functionally active in transcriptional activation under alkaline conditions. Unexpectedly, we found no significant difference in luciferase activity between vehicle and NH_4Cl treated cells (Fig 6a). We therefore hypothesized that the system was already at a maximum so that no further increase in reporter activity can be detected or that the AICD fragment generated might not reach its compartment where it is functional. To further substantiate this, we decided to investigate the cellular location of the surplus AICD produced under pH neutralizing conditions. Transfected cells were treated as before and subjected to a nuclear fractionation technique. As shown in Fig 6b, top panel, both endogenous APP-CTFs and APPGal4-CTFs exclusively localized to the membrane fraction as expected. The characteristic increase in both endogenous APP-CTFs and APPGal4-CTFs can also be seen upon NH_4Cl treatment (lanes 4-6). Moreover, under pH neutralizing conditions and in the presence of Fe65, the APPGal4-AICD fragment is also clearly localized to the membrane fraction (lane 6). This finding alone already suggests that a portion of the AICD surplus is still tethered to the membrane. However, after immunoprecipitation with a polyclonal C-terminal APP antibody, we also found a portion of the AICD surplus to be present in the cytosolic fraction (Fig 6B, lanes 5 and 6). Moreover, the cytosolic AICD Gal4 fragment was able to co-immunoprecipitate Fe65 (Fig 6B, lane 6). Despite having also co-immunoprecipitated Fe65 in the other conditions, the level of AICD-Gal4 was below the detection threshold (Fig 6B, lanes 3 and 7-8). To further demonstrate that the cytosolic AICD Gal4 surplus interacts with Fe65 and is not a consequence of fraction contamination, we reversed the immunoprecipitation procedure (Fig 6C). Here we demonstrate the interaction between the AICD cleaved from the APPGal4-CTF and Fe65 under pH neutralizing conditions in reverse via co-immunoprecipitation of cytosolic Fe65 with AICD-Gal4 (Fig 6C, lane 6). This data suggests that the AICD surplus, which is free in the cytosol interacts with Fe65. Furthermore, we observed no change in nuclear AICD levels between non-treated and treated conditions (Fig 6B, lower panel, compare lanes 3 and 6). The purity of the fractions can be seen by the absence of endogenous APP-CTFs in the cytosolic and nuclear fractions (Fig 6B middle and lower panel). In addition, we further controlled for the identity of the AICD-Gal4 in this assay by using the γ-secretase inhibitor, L685, 458 Fig 6B and C lane 8). In the presence of L685, 458 a small portion of AICD Gal4 fragment was detected in

the nuclear fraction (Fig 6B, lane 8). Since the half life of the AICD Gal4 is much greater than that of its untagged counterpart (data not shown), the AICD Gal4 fragment seen in the nucleus is most likely that which has been generated in the first 24hours of transfection prior to L685, 458 addition.

3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation



3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

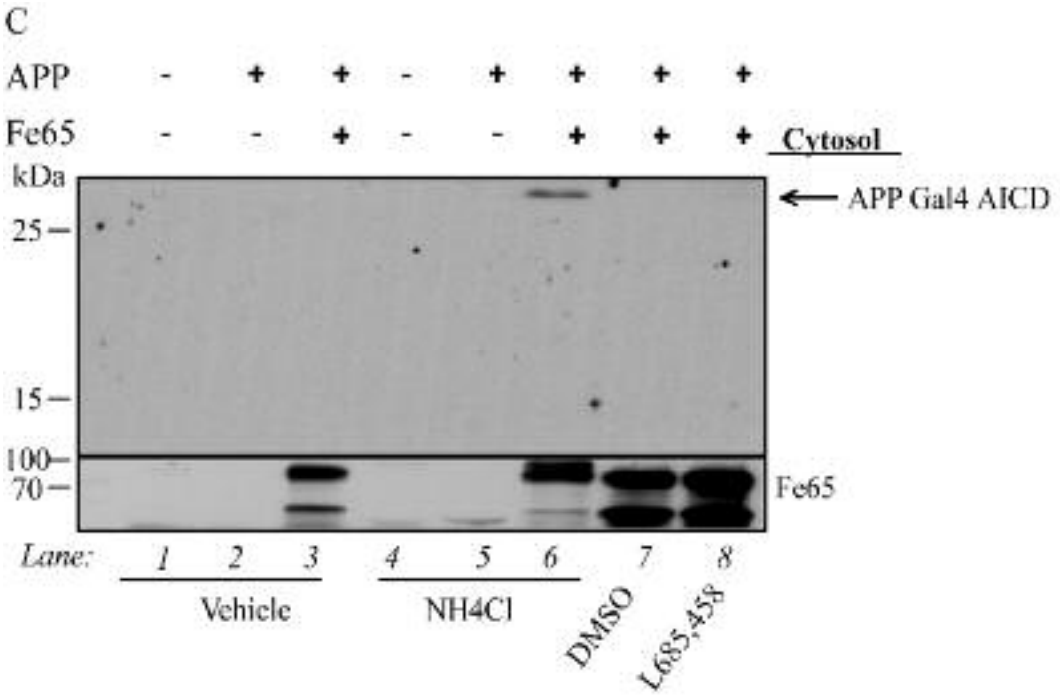


Fig. 12 The AICD surplus is not functionally active in transcription because it remains membrane tethered and free in the cytosol. (A) Wild type CHO K1 cells were co-transfected with expression plasmids encoding APP Gal4, Fe65, and APPΔ Gal4. 24 hours post-transfection media was exchanged for fresh media containing NH_4Cl and incubated for a further 16 hours. Expression of the APP Gal4 fusion protein induced only minor tranactivation of the Gal4 dependent reporter gene. Co-expression of Fe65 together with APP Gal4 induced a strong increase in reporter transactivation, which could be abolished by overexpressing APPΔ Gal4 constructs with a mutation in NPTY motif (APPΔ Gal4), preventing Fe65 binding. No obvious difference in reporter transactivation between vehicle and NH_4Cl was detected. (B) To examine the cellular localization of the APP Gal4 metabolites wild type CHO K1 cells were co-transfected as described above. 24 hours post-transfection media was exchanged for fresh media containing NH_4Cl and incubated for a further 16 hours. Cells were subjected to a crude subcellular fractionation assay. The cytosolic fraction was immunoprecipitated using the anti APP antibody CT15. Fractions were run on a 12% SDS-PAGE and analyzed for APP metabolites using the anti APP antibody CT15. The AICD Gal4 surplus was found still membrane bound and free in the cytosol where it interacts with Fe65. No change in the nuclear AICD Gal4 amount was observed between treated and non-treated. (C) Cells were treated as in (B) but the cytosolic fraction was immunoprecipitated using anti-flag and immunoblots were probed using CT15 to detect the AICD Gal4. Immunoprecipitation of the flag-tagged Fe65 was successful as demonstrated in the lower panel. Experiments were performed in triplicate and one representative experiment is shown.

3.2 Results: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

We also found the AICD-Gal4 fragment to be present in the nucleus even in the absence of Fe65 (Fig 6B, lower panel, lanes 2 and 5). However, the amount of AICD-Gal4 fragment present in the nucleus was less than that which was present in combination with Fe65 (Fig 6B, lower panel, compare lane 2 with 3, and lane 5 with 6). This might suggest that Fe65 is more required for transcriptional activation as opposed to translocation of the AICD to the nucleus. In agreement with previously published data, we found Fe65 to be present in all three subcellular fractions (Fig 6B and C). Fe65 was present least at the membrane and predominately in the cytosol and nucleus (Fig 6B and C). In the cytosol and membrane fractions, Fe65 migrated as a set of two bands, mature Fe65 and what has been described as the endoproteolytically cleaved form (Hu, Wang et al. 2005).

4.1 Discussion and conclusion: LRP1 modulates APP trafficking and metabolism within compartments of the secretory pathway

Despite its role in lipid transport and metabolism, LRP1 has been shown to be capable of mediating APP endocytosis, and, in doing so, it may influence APP processing at multiple stages. LRP1 can contribute to APP trafficking and metabolism through its ability to increase the rate of APP internalization from the cell surface, an effect which is mediated by an indirect interaction of APP with LRP1 through the adapter protein FE65 (Kinoshita, Whelan et al. 2001; Pietrzik, Yoon et al. 2004). In the absence of LRP1, a decreased rate of APP internalization increases the amount of APP available for α-secretase cleavage at the cell surface, and, hence a greater amount of APPsα can be found in the media of LRP1-expressing cells. Due to the decrease in the APP internalization rate, a reduction in Aβ secretion can also be observed (Ulery, Beers et al. 2000; Pietrzik, Busse et al. 2002). Since LRP1 can modulate both, APP trafficking and its processing concurrently at the cell surface, we wanted to test whether LRP1 could modulate APP trafficking in early secretory compartments, and, if so, whether this would also influence APP metabolism. The first part of this study was designed to test whether LRP1 could be effectively retained in the ER- and Golgi-compartments in a manner indicative by alterations in LRP1 glycosylation patterns. We began by adding a dilysine ER retention-motif KKAA to the C-terminus of the previously described LRP-CT construct. The KKAA-motif has been described to mediate a direct ER retention of transmembrane proteins, as opposed to being recycled between the ER- and early Golgi-compartments (Itin, Kappeler et al. 1995; Andersson, Kappeler et al. 1999). In addition to the KKAA signal, we also added a dilysine signal with the amino acid sequence KKFF, where the two phenylalanines were described to operate as an ER exit signal (Itin, Kappeler et al. 1995; Andersson, Kappeler et al. 1999). All investigations were performed in an LRP1 null background, whereby LRP1-deficient CHO cells were rescued by the transient expression of LRP1 chimeras. Retention of LRP-CT KKAA could be demonstrated by comparison with the localization of the normally transported LRP-CT construct. LRP-CT KKAA did not acquire Golgi-dependent glycosylation, and it did not display any Golgi-like staining. While LRP-CT KKFF migrated identical to LRP-CT (Fig 2A), which indicated that it did indeed traffic past the ER, it was not clear alone whether LRP-CT KKFF is solely retained in the Golgi apparatus. Since it displayed the same glycosylation pattern as LRP-CT, which traffics along the entire secretory pathway up to the cell surface, and, thereafter, is internalized, the same could have been true for LRP-CT KKFF. However, confocal microscopy analysis showed a co-localization of the LRP-CT KKFF chimera with the cis-Golgi marker, GM 130, indicating that it was retained to a certain extent within the Golgi compartment. In

addition, LRP-CT KKAA showed a distribution pattern indicative of the reticular compartment where it was retained, i.e. no Golgi-like stainings were detectable like those seen for the LRP-CT construct, highlighting ER-retention of LRP-CT KKAA.

Indeed, LRP1 may traffic with APP along the secretory pathway, which could be demonstrated by its capacity to impede APP exit out of the ER- and Golgi-compartments via LRP1's retentive influence in the aforementioned secretory compartments. In fact, the truncated LRP1 construct LRP-CT KKAA seemed to have a greater retentive capacity over APP compared to LRP-CT KKFF as could be deduced from the amount of APP that reached the cell surface. It has been previously demonstrated that some proteins can undergo an alternative route in cellular trafficking, more specifically some proteins, after reaching the trans-Golgi network, are subsequently routed to the endosomal system, e.g. lysosomal enzymes follow such a pathway, from where they are transported to the cell surface, a notion which could explain the lack of a retentive influence of LRP-CT KKFF over APP (Futter, Connolly et al. 1995; Itin, Kappeler et al. 1995; Orzech, Cohen et al. 2000; Kim, Lampert et al. 2005 Gravotta, 2007 #3; Lock and Stow 2005; Kim, Deng et al. 2007).

The retentive influence of LRP-CT KKAA and LRP-CT KKFF was also seen in the amounts of cell surface and total cellular APP-CTFs. The reduction of APP-CTFs, most notably in the presence of LRP-CT KKAA, may be due to their non-production or alternatively, APP-CTF production may still take place, but LRP1 might be unable to stabilize generated CTFs as it does after cell surface transport.

Although, activity of all secretases has been described in early and late secretory compartments, no study has anticipated the influence of other secretase substrates in combination with APP in these compartments (Lammich, Kojro et al. 1999; Parvathy, Hussain et al. 1999; Huse, Pijak et al. 2000; Huse, Liu et al. 2002; Baulac, LaVoie et al. 2003). On the other hand, since LRP1 has been described as being a BACE substrate in addition to being a competitive substrate for γ-secretase, it may well be that substrate affinity is dependent on the compartment of its residence, and, if so, perhaps LRP1 is the preferred substrate of choice in early secretory compartments (Lleo, Waldron et al. 2005; von Arnim, Kinoshita et al. 2005).

4.1 Discussion and conclusion: LRP1 modulates APP trafficking and metabolism within compartments of the secretory pathway

Measurements of Aβ secretion in to the conditioned media of cells expressing LRP-CT KKAA showed an almost complete abolishment in the secretion of total Aβ. This result is in agreement with an earlier study that showed the direct retention of C99 in the ER, which caused an almost complete inhibition in the secretion of Aβ and which was not the result of its intracellular accumulation (Maltese, Wilson et al. 2001). Although we are performing rescue experiments of LRP1-deficient cells, one could argue that we induce a loss of function of the LRP-CT KKAA construct compared to the LRP-CT construct. However, overexpression of the LRP-CT KKAA construct in cells expressing endogenous LRP1 would result in a complete blockage of γ-secretase due to its competitive effect on the substrate APP as shown before (32). In addition to the endosomal compartments, the trans-Golgi network has been strongly linked to Aβ production (Peraus, Masters et al. 1997; Huse, Liu et al. 2002; Baulac, LaVoie et al. 2003). The amount of Aβ secreted in the presence of LRP-CT KKFF was more than that detected in the presence of LRP-CT KKAA but less than in the presence of normally transported LRP-CT. Golgi residence of LRP1 may therefore not be able to impart a strong inhibitory influence over Aβ production, or alternatively, due to an incomplete APP retention, APP may still travel to the cell surface, where it can be subsequently cleaved by the secretases there.

In conclusion, the results described herein demonstrate the potential of LRP1 to modulate APP trafficking in early secretory compartments. This modulation is also accompanied by a secondary influence on APP processing, further demonstrating the importance of LRP1 in general regulation of APP processing steps. Together with previously published data, our results provide new insights into APP trafficking and metabolism, and demonstrate that APP processing should not be seen as an uncoupled process in the cellular metabolism. Our findings suggest that other binding partners should be rather studied in combination as opposed to being assessed singly, in order to broaden the currently narrow picture of APP trafficking and metabolism so that another avenue in AD therapeutics may be anticipated.

4.2 Discussion and conclusion: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

One of the main sought after therapeutic targets for AD intervention is the inhibition of the enzymes which govern Aβ production i.e. BACE1 and γ-secretase. Inhibiting either one of these enzymes would reduce the Aβ load and hence plaque formation seen in AD brains (Pietrzik and Behl 2005). However, inhibition of BACE1 or γ-secretase activity would also result in reduced processing of other secretase substrates e.g. Notch, LRP1, Sorla and most importantly prevent the release of γ-secretase generated ICDs. To date, only a well defined functional role exists for the ICD released after Notch cleavage. After its release, the NICD translocates to the nucleus where it activates transcription of genes critical in early development (Schroeter, Kisslinger et al. 1998; Struhl and Adachi 1998). Through the use of a gene reporter assay similar to Notch expression, translocation and activation of gene transcription has also been reported for the AICD (Cao and Sudhof 2001). Since the discovery that AICD together with Fe65 can activate transcription, many putative target genes have been documented, many of which are currently under dispute (Baek, Ohgi et al. 2002; von Rotz, Kohli et al. 2004; Pardossi-Piquard, Petit et al. 2005; Hebert, Serneels et al. 2006). Altogether, this assay has been widely used and is heavily relied on, to study the effects of compounds and other proteins on AICD transcriptional activity in relation to Aβ production (Weggen, Eriksen et al. 2003; Khvotchev and Sudhof 2004; Hass and Yankner 2005; Lleo, Waldron et al. 2005; von Arnim, Spoelgen et al. 2006).

Although the aforementioned assay allows for the assessment of general AICD transcriptional activity, much basic information regarding the processing of the construct used in this assay, in addition to the cellular location of its metabolites, are missing. Here we describe for the first time, the processing and location of the APP construct used in this assay. Furthermore, the factors which might necessitate AICD release are unknown. It was recently published that a rise in intracellular pH leads to an accumulation in AICD together with a concomitant decrease in Aβ (Vingtdeux, Hamdane et al. 2007). Moreover, the authors have shown that APP together with its catabolites i.e. APP-CTFs and AICD are associated with exosomes (Vingtdeux, Hamdane et al. 2007). This represents an interesting finding since Aβ was also found to be associated with exosomes, although the amount released via exosomes was insignificant (Rajendran, Honsho et al. 2006). We sought to elaborate on this paradigm by addressing the mechanistic surrounding AICD accumulation under pH neutralizing conditions in addition to exploring a possible functional significance for the increase in AICD.

4.2 Discussion and conclusion: Increased AICD generation is ineffective in nuclear translocation and transcriptional activation

As shown previously increasing the intracellular pH gives rise to an increase in AICD but a decrease in Aβ (Vingtdeux, Hamdane et al. 2007). The two latter metabolites are the products of a sequential cleavage step executed by γ-secretase such that an equal ratio between the two might prevail (Kakuda, Funamoto et al. 2006). If the elevated pH would abrogate enzyme activity, then the same effect should be observable for other γ-secretase substrates. However this cleavage ratio did not extend to another γ-secretase substrate, Notch. Both NICD and Nβ were unaffected by a pH neutralized environment. This data might suggest that γ-secretase is enzymatically sensitive to its substrate depending on environmental factors. In other words, cleavage of one substrate over another might be preferred depending on an environmental stimulus which favours substrate accumulation. Alternatively, γ-secretase may metabolize its substrates in different cellular compartments. In our study, we measured diverse APP processing parameters and found no effect on APPsα secretion which suggests that the abrogated cleavage is further downstream from the plasma membrane where α-secretase is most active. In compliance with the latter, the increase in C83 is not met with a likewise increase in APPsα secretion because the increase in C83 is a result of an inhibition in its degradation and not an increase in its generation. Therefore the proceeding cleavages after α-secretase are likely carried out on the APP-CTFs, which are currently undergoing degradation along the endosomal to lysosomal degradation route.

BACE1 has been suggested to be active along the endosomal system, a point which could explain the reduction in Aβ secretion seen under these conditions (Koo and Squazzo 1994; Huse, Pijak et al. 2000; Daugherty and Green 2001; Koh, von Arnim et al. 2005; Zou, Wang et al. 2007). If BACE was rendered inactive by the rise in intracellular pH, this would give rise to less of its product being generated, C99 and hence less substrate for γ-secretase and subsequent Aβ production. However, C99 levels did not reflect any strong inactivation of BACE1 under alkaline conditions but was rather partially degraded to C83. Therefore, the reduction in Aβ under alkaline conditions is rather due the partial degradation of C99 to C83, which thereby reduces the amount of C99 substrate available for γ-secretase cleavage. This led us to postulate that the increase in AICD was that cleaved from C83, which would complement previously published data showing that the AICD is in fact produced from C83 (Kume, Maruyama et al. 2004). While the authors

employed a cell free assay, we substantiated this claim in a complete intact cellular system via the above mentioned gene reporter assay (Kume, Maruyama et al. 2004). As shown here for the first time, the reporter gene constructs of APP resembles the mechanistic situation in a cell by which APP is processed, i.e. L685, 458 prevented AICD release and gave rise to a concomitant rise in APP-CTFs. In addition, the assay mimics the endogenous situation under pH neutralizing conditions, demonstrating increased APP Gal4-CTF levels due to suppression in their degradation and the AICD Gal4 accumulates due to a rise in the intracellular pH. Alternatively, previous authors have suggested that the increase in AICD might be a result of an inhibition in its degradation (Vingtdeux, Hamdane et al. 2007). If the accumulation in AICD was merely due to a degradation blockade then one would expect to see an increase in C99 levels as well. However, C99 did not respond in an accumulative fashion to NH_4Cl treatment but was rather partially degraded to C83. Therefore, the increase in AICD upon NH_4Cl treatment is likely that cleaved from C83 as a result of its accumulation. Furthermore, we could next demonstrate that the decrease in Aβ was not attributable to the increase in AICD and the expression of one of its putative target genes, neprilysin, a well documented Aβ degrading protein as the proposed feed back mechanism, was not reversible under the conditions used in these studies. For example, we found the addition of Aβ to transfected cells had no influence over AICD levels.

The accumulation of the AICD in an alkaline environment did not give rise to a corresponding increase in transcriptional activity as we would have expected. We later showed that this was likely a result of assay saturation as the same amount of AICD Gal4 fragment was present in the nucleus under NH_4Cl and vehicle treated conditions. Furthermore the AICD Gal4 surplus was still tethered to the membrane and free in the cytosol where it interacted with Fe65. The latter also suggests that the AICD still has the potential to be functional in transcriptional activation. Unlike other studies which show an interaction between AICD and Fe65 which employ a pre-generated AICD fragment, here we show that the AICD cleaved from full length APP interacts with Fe65 and translocates to the nucleus(Kimberly, Zheng et al. 2001). Furthermore, the AICD Gal4 fragment was capable of translocating to the nucleus in the absence of Fe65 but was not capable of initiating transcription of the luciferase reporter gene. As previously suggested Fe65 has a more dominant role in transcriptional activation as opposed to translocating with the AICD to the nucleus (Cao and Sudhof 2004). Interestingly, we observed alternative migration patterns for Fe65 in the different subcellular fractions. More of the endoproteolytically

cleaved form was present in the cytosol. This may point to a functional specificity for Fe65, which depends on its cellular location.

In conclusion, the data documented here sheds new light on the mechanistic players involved in AICD production, in that, an alkaline environment favours AICD production but not NICD generation. The majority of the AICD produced is likely that cleaved from C83 and not C99, which might suggest a more specialized role for AICD and hence help elucidate the physiological function(s) of APP. A more in depth understanding in to the mechanisms governing AICD production, in addition to other ICDs, can offer new routes which allow for the prevention of Aβ accumulation without interfering with AICD generation.

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