UNIVERSITY OF NAPLES

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XXXII CYCLE

ANTARIPA BHATTACHARYA

THESIS

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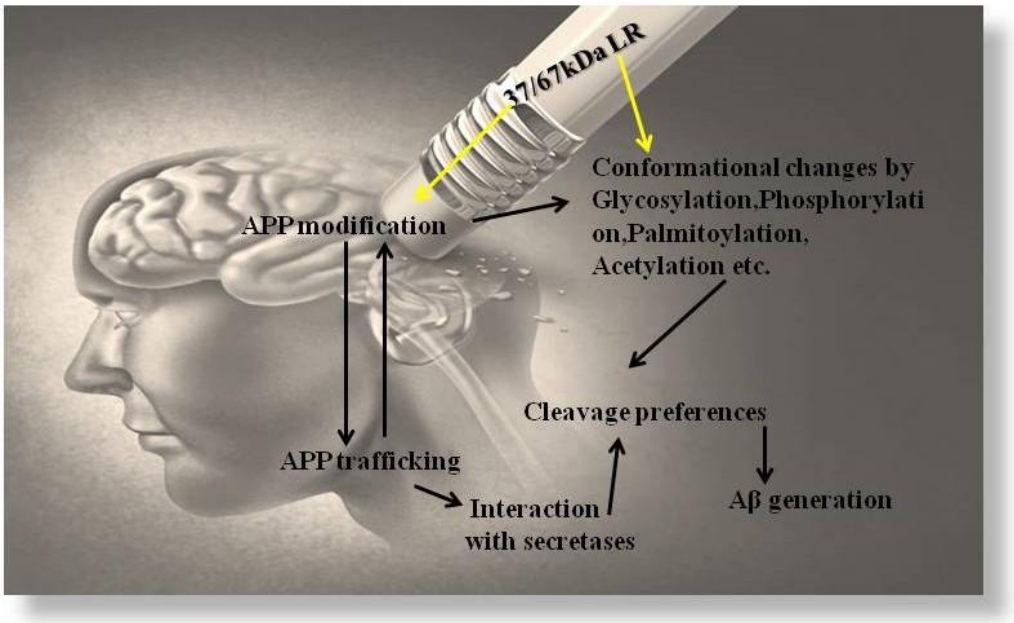

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THESIS TITLE

The role of the 37kDa/67kDa LR inhibitor in Amyloid precursor protein trafficking and Alzheimer's disease

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Abbreviation

aa: Amino Acid

AD: Alzheimer disease

Aβ: Amyloid beta

Aβ40: *40* amino acid proteolytic product from the amyloid precursor protein (APP)

Aβ42: 42-Residue amyloid beta-protein *fragment*

APP: Amlyoid precursor protein

AKT: Protein kinase B

BSE: Bovine spongiform encephalopathy

BACE: β-Site APP-cleaving enzyme

CJD: Creutzfeldt-Jakob disease

CNS: Central nervous system

DC: Dendritic cell

DRM: Detergent resistant membrane

EE: Early Endosome

ER: Endoplasmic reticulum

ERC: Endosomal Recycling Compartment

ERAD: ER-associated degradation system

ERK: Extracellular signal–regulated kinase

fCJD: Familial Creutzfeldt-Jakob disease

FSE: Feline spongiform encephalopathy

GPI: Glycosylphophatidylinositol

GSK3β: Glycogen synthase kinase 3 β

HD: Huntington disease

HS: Heparan sulfate

iCJD: Iatrogenic Creutzfeldt-Jakob disease

kDa: Kilo Dalton

LYS: Lysosomes

Lys: Lysine residue

LiCl: Lithium Chloride

M: Molar

mM: Milimolar

μM: Micromolar

μ: Micron

N-Gly: N-Glycosidase

Neur: Neuramidase

NaOH: Sodium hydroxide

NH_4Cl: Ammonium chloride

nm: Nanometer

NSC48478: 1-((4-methoxyanilino) methyl)-2-naphthol

O-Gly: O-Glycosidase

PFA: Paraformaldehyde

PM: Plasma membrane

Prion: proteinaceous infectious particles

PrP^{C}: Cellular (i.e. wild type) prion protein

PrP^{Sc}: Scrapie (i.e. infectious) prion protein

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulphate

sh: Short hairpin

TGN: Trans-Golgi-network

TME: Transmissible mink encephalopathy

TSE: Transmissible spongiform encephalopathy

TX-100: Triton X-100

UK: United Kingdom

USA: United States of America

WB: Western Blot

37/67kDa LR: 37/67kDa Laminin Receptor

ABSTRACT

Abstract

The vicious circle of spread, seed, and accumulation of misfolded protein aggregates within the central nervous system has been reported for prion protein, but also amyloid beta-peptide (Aβ), α-synuclein and tau, which can form amyloidogenic aggregates that spread through the brain causing distinct neurodegenerative diseases. Amyloid precursor protein (APP) is processed along both the non-amyloidogenic pathway preventing Aβ production and the amyloidogenic pathway, generating Aβ, whose accumulation in the brain is a neuropathological hallmark of Alzheimer's disease. The Aβ is produced by the sequential processing of APP by β- and γ-secretases likely in the endo/lysosomal pathway where APP physiologically traffics and where γ-secretases are preferentially distributed. Items of evidence report that the intracellular trafficking plays a key role in the generation of Aβ and that the 37/67kDa laminin receptor LR, acting as a receptor for Aβ, may mediate Aβ-pathogenicity. Although many reports suggest a critical role for intracellular trafficking of APP in Aβ generation, little is known on the exact site for Aβ production, its localization and the possibility to correct them for proper protein folding and function.

Mutations in APP cleavage sites and in the Aβ sequence can affect the processing of the protein with consequences in the trafficking, folding, posttranslational modifications (PTMs: glycosylation, phosphorylation, acetylation) and amyloidogenic abilities. Therapeutics for misfolding disorders do not exist. Previous reports revealing a critical role for 37/67kDa laminin receptor in APP processing through direct interaction with the γ-secretases and indirect with β-secretase, highlight the importance of identifying therapeutics that point to target the 37/67kDa LR, which has been previously shown by us to be also interactor and regulator of prion protein trafficking.

Starting from these observations, we propose to shed light on the role of 37/67kDa LR in APP processing and trafficking by assaying the effects of receptor inhibitors in neuronal cells and fibroblasts from AD patients challenged with different molecules already tested to be receptor inhibitors.

We show herein that APP and 37/67Kda LR interact in neuronal cells and that the specific 37/67kDa LR inhibitor, NSC48478, can affect this interaction. Moreover, NSC48478 reversibly regulates the maturation of APP, in an NH_4Cl-dependent manner, resulting in the partial accumulation of the immature APP isoforms (unglycosylated/acetylated forms) in both the Endoplasmic Reticulum and Transferrin-positive recycling endosomes, indicating alteration of the APP intracellular trafficking. By shRNA-mediated knockdown of the receptor, we found that these effects were directly mediated by the 37/67kDa LR and accompanied by inactivation of ERK1/2 signaling and activation of Akt, with concomitant inactivation of GSK3β.

Our data reveal NSC48478 inhibitor as a novel small compound to be tested in disease conditions.

Our preliminary data (work in progress, not shown here) reveal a different localization of APP in fibroblasts from AD patients respect to control fibroblasts, and while NSC48478 did not exert any effect on APP localization, the analog small molecule NSC47924 was able to rescue the physiological Golgi distribution of APP in AD-patient fibroblasts.
These data reveal that the small 37/67kDa LR inhibitors are useful tools to be tested against AD.

INTRODUCTION

1. Misfolding diseases, Prion and Prion-like Proteins

The most common neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease, are characterized by the misfolding of a small number of proteins that assemble into ordered aggregates in affected brain cells. For many years, the events leading to aggregate formation were believed to be entirely cell autonomous, with protein misfolding occurring independently in many cells. Recent research has now shown that cell non-autonomous mechanisms are also important for the pathogenesis of neurodegenerative diseases with intracellular filamentous inclusions. The intercellular transfer of inclusions made of tau, a-synuclein, huntingtin and superoxide dismutase 1 has been demonstrated, revealing the existence of mechanisms reminiscent of those by which prions spread through the nervous system[1].

Prion diseases belong to the group of protein misfolding neurodegenerative diseases that are characterized by the abnormal aggregation of cellular Prion Protein (PrP^{C}) after its conformational conversion into the pathological scrapie PrP^{SC}. The misfolded proteins form highly ordered filamentous inclusions with a core region of cross-β conformation. Tau, β-amyloid and α-synuclein are the most commonly misfolded proteins [2].

Radioinactivation studies suggested that the agent of scrapie may replicate without nucleic acid [3, 4] and that its purification led to the identification of the prion protein showing that the scrapie agent was devoid of nucleic acid [5]. To distinguish protein pathogens from viruses, the term "prion" (proteinaceous infectious particle) was introduced [6]. Human prion diseases include Kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) disease, and fatal insomnia. Scrapie, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) are prion diseases in animals. A polymorphism at codon 129 of human *PRNP*, where M or V is encoded, is a susceptibility factor for prion diseases [7]. Cases of variant CJD (vCJD), which humans acquire through the consumption of BSE contaminated food products, have so far almost all been M/M [8]. With the identification of causative mutations in *PRNP*, a possible explanation for dominantly inherited and sporadic cases of CJD emerged [9, 10, 11]. Mutations were surmised to lower the energy barrier for conversion of the cellular form PrP^{C} to its scrapie form PrP^{Sc}. Once a sufficient number of PrP^{Sc} molecules have formed, the conversion of PrP^{C} to PrP^{Sc} occurs readily. Upon introduction into a new host, PrP^{Sc} seeds convert PrP^{C}, which explains how prion diseases can be infectious. More investigations are needed to learn about the sites of prion conversion and the mechanisms of intercellular propagation of prions. The transfer of pathology from one neuron to another appears to occur trans-synaptically [12].

Mutations in *PRNP* account for ~10% of cases of CJD, with 90% being sporadic. This is reminiscent of Alzheimer's disease (AD) and Parkinson (PD),

in which dominantly inherited mutations account for a minority of cases, with the majority being sporadic. Mice heterozygous for human PrP V127 are resistant to Kuru and CJD but not vCJD prions. Remarkably, homozygous mice are resistant to all prion strains [13]. Initially, the prion concept was confined to a small group of diseases, typified by scrapie and CJD, in which PrP, a glycolipid-anchored sialoglycoprotein, adopts a conformation rich in β-sheet[14]. Similar conformational changes have been observed for Aβ, tau, and α-synuclein upon assembly. The prion concept may thus also apply to AD and PD. After injection of misfolded prion protein into the brain, a large increase in the titer of infectivity is observed. It remains to be seen whether this is also the case of assembled Aβ, tau, and α-synuclein. There is reluctance to use the term prion with respect to the inclusions of AD and PD. The main reason is that in contrast to Kuru and CJD, transmission of AD and PD between individuals has not been demonstrated. The infectious cases in humans have occurred under unusual circumstances, such as ritualistic cannibalism, consumption of BSE-contaminated food products, injection of cadaveric growth hormone and gonadotropin, blood transfusions, corneal transplants, and dura mater grafts, as well as implantation of improperly sterilized depth electrodes. It remains to be seen whether the apparent lack of infectivity of AD and PD can be attributed to the more fragile nature of Aβ, tau, and α-synuclein assemblies, as compared with misfolded prion protein, or to other properties. For prion diseases, the molecular species responsible for infectivity and toxicity appear to be different [15, 16, 17]. It has been proposed that neurodegeneration is mediated by a toxic form of PrP, called PrPL, that is distinct from PrP^{Sc}, but whose formation is catalyzed by PrP^{Sc}. Although the molecular species responsible for toxicity are not well defined, they may be crucial for activating one or more downstream pathways. Targeting the unfolded protein response in mice over expressing PrP has been reported to prevent neurodegeneration[18].

Whereas prion diseases, also referred as Transmissible spongiform encephalopathies (TSEs) are rare, AD, PD and frontotemporal dementia (FTD) are common. Like most cases of prion disease, they are largely sporadic, with a small percentage being inherited. Mutations in the genes encoding amyloid precursor protein (APP), tau and α-synuclein cause dominantly inherited forms of AD, FTD and PD [19, 20, 21].

In TSEs prions behave like infectious agents. Though evidence suggests that common misfolding diseases are not transmitted between individuals, investigation of the formation of tau and α-synuclein inclusions as a function of age has shown that they develop in a stereotypical manner in particular brain regions from where they appear to spread [22, 23] [**Fig. 1**]. This is consistent with an intercellular transfer of inclusions, provided one accepts that studies of their temporal appearance describe a single pathophysiological process.

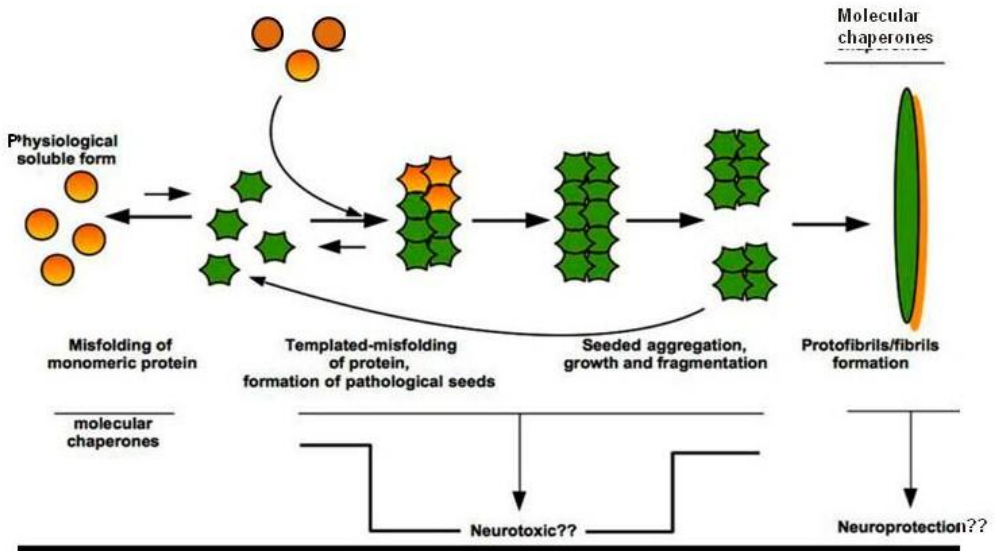


Figure: 1
Prion-like mechanism of misfolded protein aggregation

Misfolding of normal physiological form of a protein and formation of pathological seeds is a rare and energetically unfavourable event, based upon exposition of amide groups and high concentration of a given protein. Genetic mutations or environmental factors (e.g., exposure PrP^{Sc}, pesticides) can induce the conversion from soluble normal form to insoluble pathological oligomers and larger species that aggregate and fibril006Cize. Once a seed has formed, thanks to a template-assisted misfolding, each single molecule can acquire a different shape and add to growing aggregates.
These latter can be fragmented generating new seeds that are able to accelerate the aggregation, giving life to fibrils formation. Question marks (??) indicate open issues. (Adapted from Sarnataro et el. International journal of Molecular Sciences 2018)

The relationship between TSEs and other neurodegenerative diseases has been studied for many years [24, 25, 26, 27]. However, it is the flurry of recent research describing the induction of protein misfolding and spreading between cells [28, 29, 30, 31, 32] that has shown most convincingly that common neurodegenerative diseases can be driven by cell non autonomous mechanisms. It's now clear that characteristics of misfolded prion protein can be shared by other proteins central to neurodegenerative diseases. Proteins exhibiting prion-like properties have also been named 'prionoids' [32].

A typical prion-like protein is tau. Tau misfolding causes several human neurodegenerative diseases including AD, some cases of GSS, familial British and Danish dementias, Pick's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), Guam Parkinsonism-dementia complex, tangle-only dementia, white matter tauopathy with globular glial inclusions and frontotemporal dementia and Parkinsonism

linked to chromosome 17 (FTDP-17T) [33]. In these diseases, the normally soluble tau protein is hyperphosphorylated and filamentous. Whereas the hyperphosphorylated sites in tau are similar between diseases, filament morphologies vary widely [34]. Intraneuronal tau inclusions coexist with extracellular deposits of Aβ in AD. These findings are consistent with an intercellular transfer of tau aggregates. The presence of inclusions made of distinct sets of tau isoforms in different diseases is consistent with the existence of tau strains, like to the prion strains made of distinct conformers of PrP^{Sc}. In some of these diseases, tau inclusions are also found in glial cells.

A growing body of research supports the concept that misfolding and aggregation of the endogenous protein fragment Aβ initiates and sustains the pathogenesis of AD, which is characterized by the presence of Aβ plaques and neurofibrillary tangles (NFTs), these latter consisting of intracellular bundles of hyperphosphorylated tau protein [35, 36]. Both Aβ and tau, as well as PrP^{C}, assume a tertiary structure (or fold) rich in β-sheets which in turn promotes the self-assembly of monomers into small oligomeric species, with neurotoxic properties, and fibrillary assemblies [37, 38].

Several years ago, it was shown that the injection of brain extract from AD patients into the brain of mice transgenic for human mutant APP promotes the aggregation and deposition of β-amyloid [39].

Recently, eight hormone recipients in Great Britain who died of CJD at ages ranging from 36 to 51 yr were examined for the co-presence of AD-type lesions. Four of them had significant Aβ accumulation in the form of Aβ plaques, and two others had sparse Aβ deposits [40]. These findings raise the possibility that some batches of growth hormone were contaminated with Aβ seeds that originated from pituitary glands collected from patients who had died with AD (or incipient AD)[41]. Because the growth hormone recipients had died of CJD, it is unknown whether they ultimately would have developed AD (none of them displayed signs of tauopathy). It will be informative to follow the surviving recipients who did not manifest CJD to assess their relative risk of AD and other neurodegenerative proteopathies. Also important will be to examine any remaining batches of cadaveric growth hormone for the presence of Aβ and other types of prion-like seeds. The risk of AD is increased by mutations and polymorphisms that promote Aβ misfolding and aggregation. Whether and how physiological and environmental factors influence the Aβ cascade and how the aggregation of Aβ is ultimately linked to dementia remain open questions, but it now seems likely that Aβ aggregation is a central mechanism on which risk factors converge to facilitate the development of AD.

2. Amyloid beta and Alzheimer's disease

Aβ peptides, cleavage products of APP, were discovered as the major component of amyloid plaques and cerebral vascular inclusions, whereas tau protein was identified as the major component of the neurofibrillary lesions in AD. In 1991, a missense mutation in *APP* gene was shown to cause AD [42], and in 1998, mutations in *MAPT*, the tau gene, were reported to cause frontotemporal dementia and Parkinsonism [43, 44, 45]. More recently, it has become apparent that AD is caused by protein assemblies that adopt alternative conformations and become self-propagating, like prions [46, 47].

Among all neurodegenerative diseases, AD is the most common one and defined clinically by a progressive decline in memory, other cognitive functions, and neuropathologically by brain atrophy**,** the accumulation of abundant extracellular Aβ plaques and intraneuronal neurofibrillary tau lesions. Electron microscopy images showed that plaques and neurofibrillary lesions are made of abnormal filaments with the fine structure of amyloid [48]. Each amyloid filament exhibits characteristic dye-binding properties and consists of several proto filaments with β-sheet structures that are stabilized through hydrogen bonding. Parallel β-sheet organization predominates in amyloid filaments. The crystal structures of amyloidogenic peptides have shown that amino acid side-chains complement each other across the sheet-sheet interface and that the space between sheets is devoid of water (dry steric-zipper)[49, 50]. Besides that, non filamentous aggregates are also present [2, 51] which are β-sheet–rich but transient and are objects of intense investigation [52].

Age is the strongest risk factor for the development of AD. Moreover, the age of 65 is used to arbitrarily classify AD. Patients with early-onset AD (EOAD) start to manifest symptoms before age 65, while patients with the late-onset form of AD (LOAD) manifest symptoms after this age. The most common form of AD is LOAD, with only 10% of cases being diagnosed with EOAD, usually between 45 and 60 years [53]. The first genetic studies of AD were based on genetic linkage analyses of AD families and proved the involvement of genetic factors in this disease, as well as its genetic heterogeneity with differences between EOAD and LOAD forms. EOAD includes sporadic cases and the majority of the autosomal-dominant forms of the disease (AD EOAD), also often referred to as familial AD. This autosomal dominant form is classified as a rare disease corresponding to less than 1% of all cases of AD [54]. Three genes harboring mostly heterozygous mutations are associated with EOAD: *APP*, *PSEN1* (Presenilin1), and *PSEN2* (Presenilin 2). Differently from EOAD, LOAD is a multi factorial and complex disease with no consistent pattern of inheritance and estimated heritability of 60–80%. Genetic factors play a very important role in LOAD development and age of onset [55]. Most cases of LOAD are sporadic with no clear familial pattern of disease. The application of genome-wide association studies (GWAS) to LOAD contributed to the identification of several loci associated with increased or reduced AD

risk. Interestingly, some of the variants identified are located in or near genes known to be involved in specific biologic pathways: cholesterol metabolism, immune response, and endocytosis/vesicle-mediated transport.

2.1. Amyloid precursor protein: posttranslational modifications, trafficking and processing

APP encodes a widely expressed type 1 transmembrane glycoprotein that is alternatively spliced to produce three major transcripts: APP695, APP751, and APP770 [56, 57], with APP695 being the major isoform in neurons. APP posttranslational modifications including N-glycosylation, O-glycosylation, phosphorylation and acetylation[58] are directly correlated to intracellular localization and protein trafficking, which are of fundamental importance for the correct protein processing that in turn plays a key role in the production of Aβ as well as phosphorylated tau in AD. Specifically, APP goes through broad post-translational modifications, including *N*-glycosylation (N-Gly), *O*-glycosylation (O-Gly) and phosphorylation. The residue numbering corresponds to the APP695 [59].

When the nascent APP translocates into the ER, it is glycosylated by the oligosaccharyltransferase complex with the addition of a precursor oligosaccharide to the luminal side of a polypeptide chain, forming the immature APP. Two sites, Asn467 and Asn496, are predicted to be *N*-glycosylated[60].The *O*-glycosylation occurs in the Golgi apparatus to form the mature APP. Numerous *O*-Gly sites of APP have been identified by both *in vitro* and *in vivo* studies. Thr291, Thr292, Thr576, and Thr353 (numbering of APP770) are found to be *O*-Glycosylated in cell cultures [61]. The *O*-Gly of Ser597, Ser606, Ser611, Thr616, Thr634, Thr635, Ser662 and Ser680 (numbering of APP770) has also been identified in human CSF. Alteration of *O*-GlcNAcylation in APP plays an important role in regulating APP processing and Aβ generation [62, 63].

Several reports showed that APP *O*-GlcNAcylation acts as a vital key substance in APP processing. Jacobsen *et* el. stated that a high amount of *O*-GlcNAcylated APP by *O*-GlcNAcase (OGN) inhibitor stimulated α-cleavage of APP and reduction in Aβ generation [64]. Similarly, the Chun group in 2015 showed that the OGN inhibitor increased α-cleavage and inhibited the β-cleavage of APP [65]. This indicates that glycosylation regulates APP processing and Aβ generation, which is associated with the alteration of APP trafficking. The fully glycosylated (*N*- and *O*-glycosylated) APP is prone to phosphorylation [66, 67, 68]. Till now ten phosphorylated sites of APP have been identified, where two of them are in the ectodomain (Ser198 and Ser206) and eight sites are in the cytoplasmic domain (Tyr653, Tyr682, Tyr687, Ser655, Ser675, Thr654, Thr668, and Thr686) [69, 66] Phosphorylation of Ser655 is

mainly detected in the mature APP; on the contrary, Thr668 is the common phosphorylated site in the immature APP [67]. The phosphorylation of APP and Aβ generation is related to the dysregulation of multiple kinases and phosphatases including, GSK3, PKC, DYRK1A, PP1, PP2A [70] in AD brains. Even enhancement of Thr668 phosphorylation has been detected in AD brains and resulted from the imbalance between kinases and phosphatases. Numerous studies have suggested that Thr668 phosphorylation increases Aβ generation both *in vitro* and *in vivo* [71, 72] and that phosphorylated Tyr682 and Tyr687 have been detected in AD brains but not in healthy controls [73, 74].

Protein acetylation is another major PTM step for proteins, in which the acetyl group from acetylcoenzyme A (Ac-CoA) is transferred to a specific site on a polypeptide chain. The N-terminal acetyltransferases interacts with the cytoplasmic domain of the APP. The acetylated protein is able to proceed along the secretory pathway and achieve conformational maturation, whereas the non-acetylated intermediates are localized in the ER-Golgi intermediate compartment (ERGIC) [75]. Two Endoplasmic Reticulum (ER)/ER Golgi Intermediate Compartment-based Lysine Acetyltransferases post-translationally regulate BACE1 levels [76]. Importantly, lack of acetylation is associated with several neurodegenerative disorders including AD, PD etc [77].

APP is rapidly processed, undergoing two cleavage events that require the presence of α- or β- and γ-secretases. This process can occur in all cells by two different pathways. In the nonamyloidogenic or constitutive pathway α-secretase cleaves APP in the extracellular domain and γ-secretase in the transmembrane region, leading to the formation of sAPPα and the α-C-terminal fragment (C83), both classified as non pathogenic fragments. The amyloidogenic pathway involves the proteolytic cleavage by β-secretase at the N-terminus of the Aβ domain and by γ-secretase, resulting in the formation of the Aβ peptide, sAPPβ and the amyloid precursor protein intracellular domain (AICD). Since the site of γ-secretase cleavage can vary, several Aβ peptides with different C terminals and lengths may be formed. Typically, these peptides have between 39 and 43 residues. Aβ1–40 is the most abundant alloform (90%), followed by Aβ1–42 (10%) [78, 79, 80]. The Aβ1–42 form is considered to be the most responsible for AD development, since the conformational changes suffered by this alloform result in its aggregation, promoting the neurotoxicity and neuronal loss characteristic of this disease [**Fig. 2**] [78]. The first APP mutation associated with AD was the p.Val717lle identified in a family with an age of onset of 57 years [81].

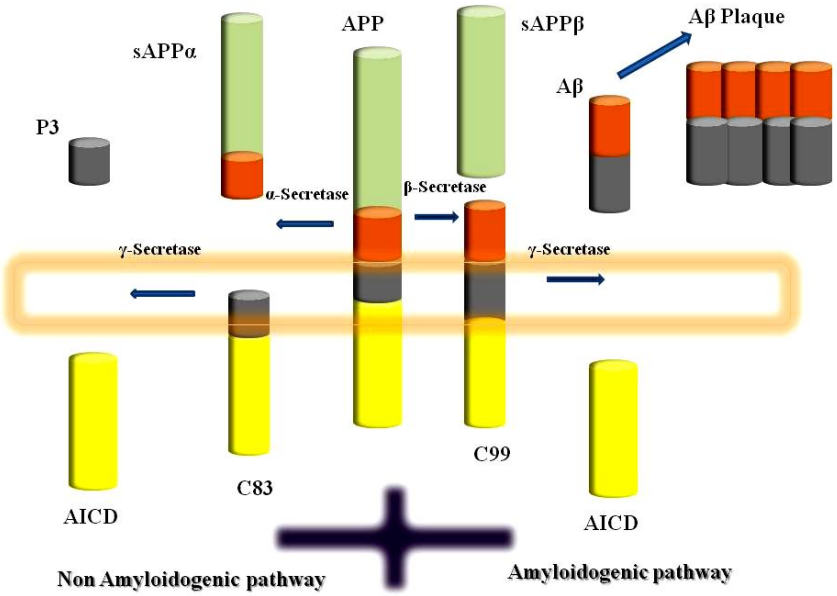


Figure: 2
Processing of APP
Cleavage of APP through two different pathways: amyloidogenic pathway and non-amyloidogenic pathway. Sequential cleavage of APP by β-secretase (BACE1) and γ-secretase generates N-terminal ectodomain (sAPPβ) and Aβ*(Modified. Strooper T. *et* el., 2011)
C83 = 83 amino acid C-terminal fragment,
CTF = C terminal fragment, C99 = 99-amino acid C terminal fragment, BACE1 = β-secretase, AICD = the amyloid intracellular domain

Missense mutations are all located within or immediately flanking the Aβ sequence. A double mutation affecting codons 670 and 671 is located just N-terminal to the Aβ domain. The p.Lys670Asn/ Met671Leu mutation was predicted to increase the total production of Aβ, including the Aβ1–42 form [82]. Other mutations have been found within the Aβ domain, disturbing APP processing through different mechanisms. An increase of Aβ formation, changes in β-secretase local cleavage, and enhanced propensity to form protofibrils increasing the insolubility of Aβ, are some of the described mutational mechanisms causative of AD [83, 84]. Mutations located C-terminal to the Aβ domain can also cause EOAD. In fact, in addition to the first APP AD mutation (p.Val717lle), other mutations in the same or neighboring residues have been shown to affect γ-secretase cleavage, causing an increase in Aβ1–42 over Aβ1–40 [85]. In contrast to other APP mutations, the p.Ala673Val mutation was found to be pathogenic only in the homozygous state, in an Italian family. Heterozygous individuals did not manifest the disease, suggesting a recessive model of inheritance for AD. In addition to point mutations, genomic duplications of APP have been shown to cause EOAD [86

[87, 88, 89]. In these patients the duplication of APP leads to the overexpression of the gene and consequent increased formation and accumulation of Aβ.

2.1.1. Trafficking of APP

Along the endocytic trafficking pathway, APP and BACE1 (Beta-site amyloid precursor protein cleaving enzyme-1) have been shown to undergo internalization from the plasma membrane through clathrin-mediated endocytosis [90, 91]. Internalized APP and BACE1 both traffic to the early endosomes, an acidic compartment whereby BACE1 activity is favourable and Aβ production has been detected [92, 93]. From the early endosomes, APP is transported to the late endosomes/lysosomes for degradation [92]. APP may also be recycled back to the TGN [94], which is likely dependent on the phosphorylation state of APP [95]. However, work from the Gleeson laboratory has shown that internalized APP predominantly traffics along the lysosomal pathway [92]. On the other hand, BACE1 has been shown to traffic from the early endosomes to the recycling endosomes and back to the PM with very little BACE1 detected at the Golgi using an antibody internalization assay [92, 96] [**Fig. 3A**]. BACE1 may also be targeted to the lysosomes for turnover [97]. Collectively, these studies indicate that APP and BACE1 are likely to be segregated along the endocytic pathway at the early endosomes. Both APP and BACE1 are synthesized in the ER and undergo posttranslational modifications within the Golgi as described [**Fig. 3B**]. The co-transport of newly synthesized APP and BACE1 is likely to provide an opportunity for APP processing and subsequently, Aβ production along the biosynthetic/secretory pathway [98, 99, 100] .The transport and sorting of APP and BACE1 in the Golgi is currently under investigation in a number of laboratories. The secretory pathway has been shown to also contribute to the generation and release of Aβ in cultured CHO cells stably expressing either the APP751wt or APP751 (K670N/ M671L) Swedish mutation (APPSwe) [100]. However, it remains unclear whether the endocytic or secretory pathway plays a more significant role in Aβ production [99]. APP appears to be cleaved by α-, β-, and γ-secretases during its transport through the Golgi apparatus after *O*-glycosylation of APP, or at the TGN [98]. Moreover, given the acidic nature of the TGN [101], this site may serve as a favourable location for BACE1 activity. From the TGN, early studies proposed that newly synthesized APP is transported directly to the plasma membrane [102, 103]. However, more recent work suggests that APP is transported from the TGN directly to early endosomes, a transport process regulated by AP-4 [104, 105]. The sorting and trafficking of APP and BACE1 is regulated by sorting signals present on their cytosolic tails.

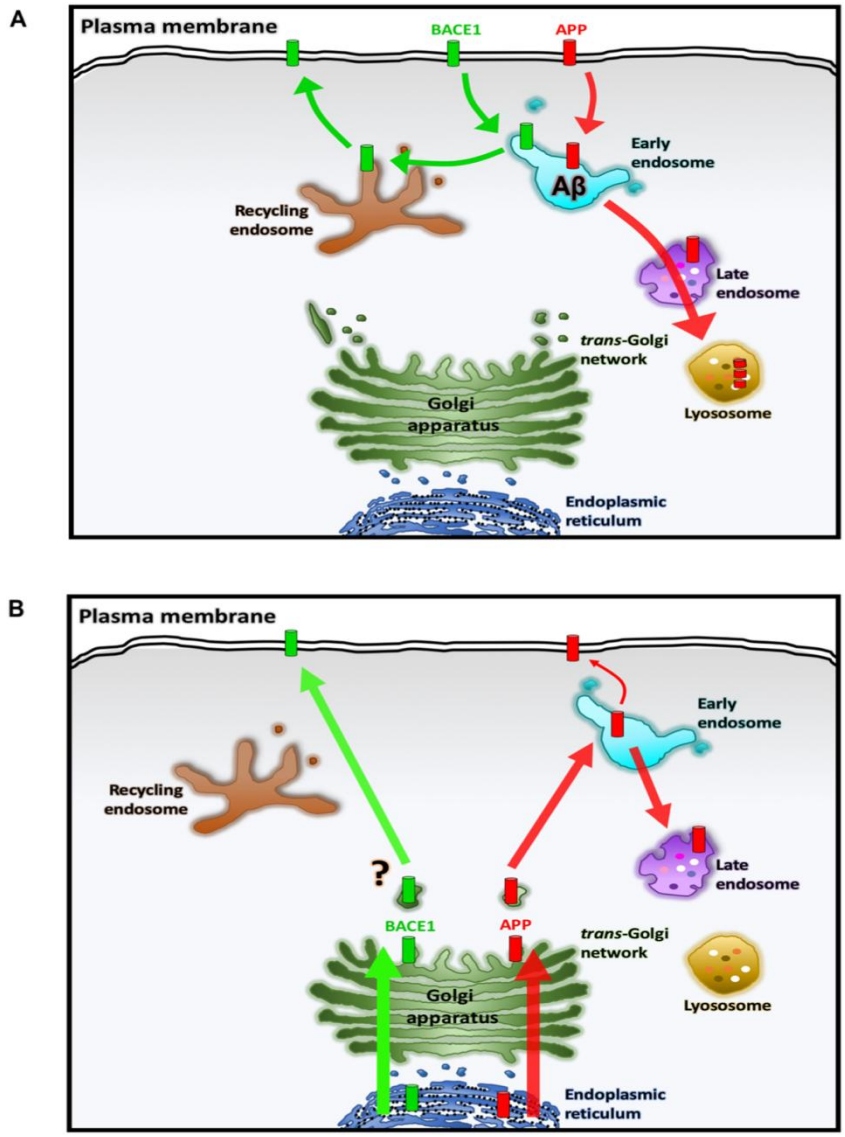


Figure: 3 Intracellular trafficking of APP

(A) Endocytic pathway of APP (red arrows) and BACE1. Both APP and BACE1 (green arrows) are internalized from the PM to the early endosomes. The early endosome is one of the major organelles for Aβ production. From the early endosomes, APP traffics to the late endosomes/lysosomes for degradation while BACE1 traffics to the recycling endosomes and recycles back to the PM.

(B) APP and BACE1 are both synthesized at the endoplasmic reticulum and posttranslationally modified along the Golgi apparatus. APP is directly transported to the early endosomes, whereas BACE1 may be transported directly to the PM. This

model proposes that APP and BACE1 are sorted into distinct transport carriers at the TGN. (Adapted: Jing et el; BBA-Biomembranes, 2019)

3. 37/67kDa laminin receptor and Neurodegenerative diseases

3.1. Structure and Function of the 37/67kDa LR

The 37/67kDa LR, also referred to as the 37kDa laminin binding protein (37LBP), laminin receptor (LamR) [106], laminin-binding protein precursor p40 (LBP-p40) [107], and the ribosomal protein SA (RPSA) [108] is a multifunctional protein. The 295aa, type II receptor protein has a theoretical molecular mass of 32.854 kDa [109] but has been detected via western blotting at apparent molecular masses of both 37kDa and 67kDa [109, 110]. Although both isoforms of the protein are encoded by the same gene sequence, the mechanism through which the 37kDa LRP (precursor) forms the 67kDa LR (mature) is elusive. Various isoforms of 37/67kDa LR, corresponding to different maturation states (44kDa, 60/67kDa and 220 kDa) all of which retain PrP^C binding affinities, have been identified in the murine brain [111].

This protein is divided into two domains, namely an intracellular N-terminal domain (1-209 aa), which contains a palindromic LMWWML sequence which has been conserved in all metazoans [109] and an extracellular C-terminal domain (210-295aa), which is highly conserved amongst vertebrates [109, 110] [**Fig. 4**].

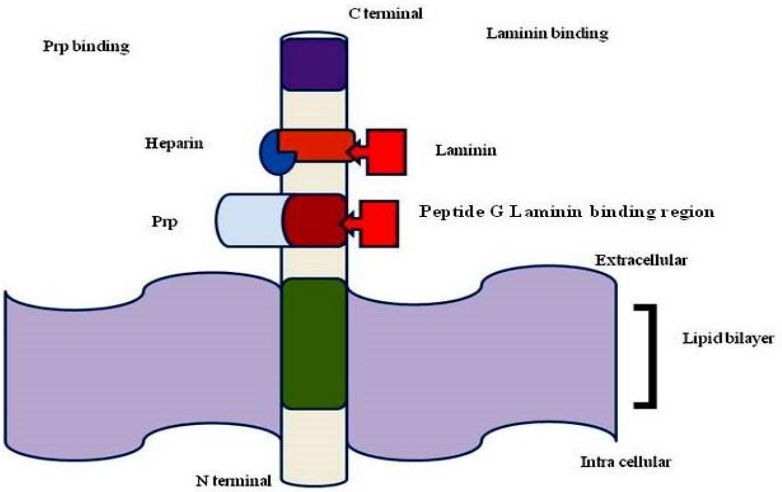


Figure 4: Structure of 37/67 kDa LR

The 37-kDa LRP is made of 295 amino acids and located on the cell surface encompassing four functional domains.
A tran membrane domain located between amino acids 86-101 which docks the receptor to the cell membrane. (Modified; Nelson et al., 2008).
Two laminin binding domains localized between amino acid 161-180 (peptide G lamimin binding domain) and 205-229 (direct laminin binding domain) which also serves as attachment sites for PrP (direct PrP binding site) and heparin(Heparin dependent PrP binding domain), respectively.
The third binding domain is localized towards the C terminal of the receptor between amino acids 272-280 and serves as an attachment site for IgG antibodies.

The amino acid sequence of this receptor is 98% identical in all mammals, thereby implying that the receptor occupies a central physiological role. It must be noted that 37/67kDa LR is post-translationally modified by phosphorylation at multiple sites [112]. The phosphorylation status of the 37/67kDa LR is regulated in part by TGF-β inhibited membrane associated protein (TIMAP) and protein phosphatase-1 (PP-1) [113], both of which interact with the intracellular N-terminus of the protein. TIMAP phosphorylation may be induced by GSK-3β [114] and 37/67kDa LR phosphorylation as a consequence of its association with TIMAP may be involved in filopodia formation [113]. The consequence of the alterable phosphorylation status of 37/67kDa LR requires further investigation but it may be suggested that this feature may be of particular significance with regards to the signal transduction pathways triggered by the protein. 37/67kDa LR is located within the cholesterol-rich lipid raft domains of the plasma membrane, in the cytoplasm, as well as in the nucleus [115, 116]. Within the nucleus, the receptor has been identified in association with DNA and histones H2A, H2B and H4 [117], and has been suggested to play a role in the maintenance of nuclear structures. In the cytosol 37/67kDa LR has been implicated in the maturation and assembly of the ribosome (through its associations with 18s rRNA and the S21 ribosomal protein) [118]. Moreover, 37/67kDa LR has been shown to associate with cytoskeletal proteins including actin and α-tubulin, a major component of microtubules (to which tau similarly binds as discussed in above) [119].

At the cell surface [**Fig. 4**], this protein serves as a multifunctional receptor exhibiting binding sites for heparin and heparan sulphate proteoglycans (HSPGs)[115]; viruses including Sindbis202, Dengue203, Venezuelan equine encephalitis (VEE) and Adeno-associated virus 168 as well as the cellular (PrP^{C}) [120] and infectious (PrP^{Sc}) prion proteins[121]. Many of the binding sites for different ligands overlap, namely 161-180aa (termed the peptide G region) has been reported to serve as a binding site for both laminin-1 and heparin, whilst the region 205-229 may bind laminin-1 in addition to PrP^{C} [116, 122]. The conformation of laminin-1 is altered upon binding to the peptide G region (containing the palindromic sequence) of receptor. This association has been

implicated in the induction of several signal transduction cascades and thereby largely underlies the receptor's physiological roles in cellular proliferation, growth, differentiation, migration [123] and the remodelling of the extracellular matrix [124] through the induction of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 9 (MMP-9) 209 activity. Cell surface associated 37/67kDa LR also serves as a receptor for midkine (MK) [125], a growth factor which promotes gene expression as well as cellular growth, survival and migration [126].

3.2. The 37/67 kDa LR and neurodegenerative disease

As a transmembrane receptor, 37/67kDa LR serves several functions such as cell migration, cell-matrix adhesion, cell viability proliferation and angiogenesis [127, 128, 129, 130]. However, increasing evidence suggests that the receptor could be implicated in neurodegenerative diseases.

37/67kDa LR has been shown to bind with high affinity both the cellular and infectious prion protein isoforms, PrP^{C} [120, 116] and PrP^{Sc} [120, 131]. It is important to note that heparin sulphate proteoglycans (HSPGs) serve as co-receptors, facilitating the 37/67kDa LR-PrP^{C} association [116]. Most importantly, the receptor is central in mediating the internalization of both PrP^{C} and PrP^{Sc} isoforms [120, 130, 114]. As the conversion of PrP^{C} to PrP^{Sc} is proposed to occur both at the cell surface and within endocytic vesicles [120, 132], and intracellular accumulation of the aggregated isoform underlies neuronal death, the fact that the 37/67kDa LR is involved in prion uptake mechanism [133] suggests that it is central in mediating the progression and pathogenesis of prion disorders. This has been confirmed by the fact that targeting the receptor, through the use of antibodies and decoy mutants, significantly hampers PrP^{Sc} propagation both *in vitro* [121, 134] and *in vivo* [119, 132]. Recent reports have demonstrated that 37/67kDa LR is crucial for alimentary PrP^{Sc} uptake [135] across different species (between livestock species as well as between livestock and humans).

Laminin receptor could be implicated in Alzheimer's disease too. The cleavage of APP producing Aβ occurs through sequential cleavage by β-secretase and γ-secretase possibly at the plasma membrane or within endosomes [102]. The resultant Aβ may consequently be shed into the extracellular space, be exocytosed or accumulated intracellularly. Aβ (particularly Aβ42 which binds PrP^{C}) shares the same binding partners and a common cellular location of 37/67kDa LR, thus it has been proposed a link between 37/67kDa LR receptor and the pathogenesis of AD **[Fig. 5]**.

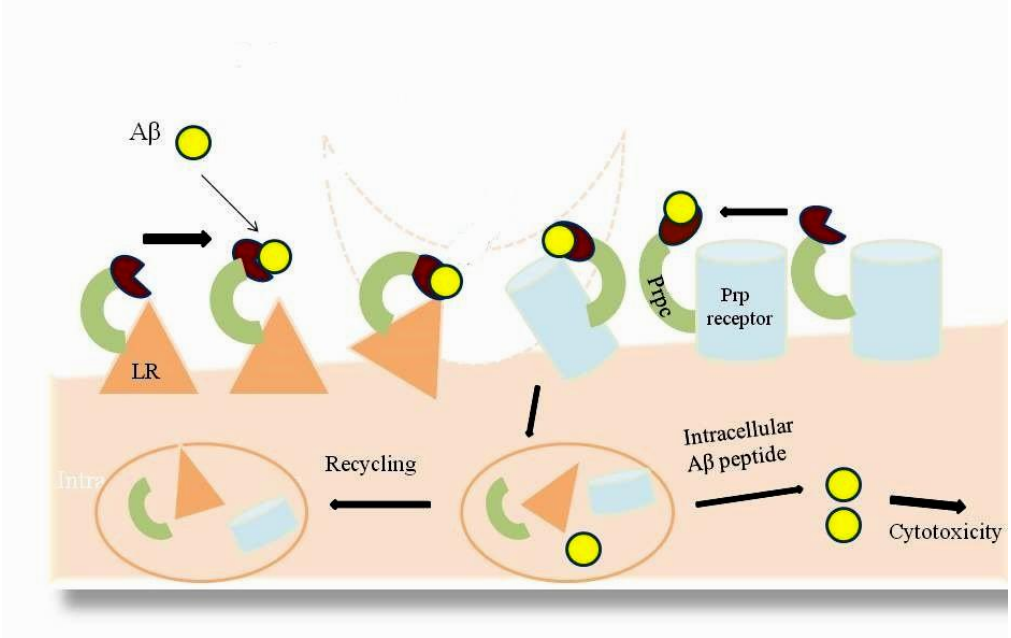


Figure 5: 37/67LR plays a role in Aβ internalization, presumably by facilitating the endocytosis of the toxic PrP^{C}-Aβ complex. (Modified; *Jovanovic, K. et al.2015*)

37/67kDa LR has been reported to co-localize with APP, β- and γ-secretase both on the cell surface and intracellularly within the cytoplasm [136] revealing a role for 37/67kDa LR in the regulation of APP processing. Moreover, its modulation could affect Aβ cytotoxicity and its release from cells [137, 138 , 139, 136]. This co-localization is limited to 200 nm when observed for BACE1 and 37/67kDa LR, to 80 nm between APP and 37/67kDa LR and less than 10 nm for 37/67kDa LR and PS1 in 2D cytofluorogram. These results reveal a novel interaction between 37/67kDa LR and the PSEN1 catalytic subunit of the γ-secretase and suggest that the previously observed interaction between 37/67kDa LR and BACE1 is likely an indirect connection only [136].

Interaction between these proteins, be it direct or indirect, seems plausible and thus the possibility of such an interaction and the influence thereof on Aβ42-mediated cell loss warranted investigation.

3.3. Inhibition of 37/67kDa LR and consequences for neurodegenerative disease-related proteins

As the receptor exhibits binding affinities for both PrP^{C} and the infectious PrP^{Sc} isoforms, 37/67kDa LR may be implicated in either direct or indirect PrP^{Sc} uptake or consequently the establishment of prion disorders [115]. Thus, understanding the role of 37/67kDa LR in prion pathogenesis and targeting this association has become ever more critical. The importance of the 37/67kDa LR

in prion disorders has been verified by the observation that therapeutics targeting the receptor or impedance of the 37/67kDa LR-PrP^C cell surface association significantly reduced PrP^{Sc} accumulation *in vitro* [121, 140] and prolonged the preclinical (before symptom onset) phase *in vivo* [132]. Such strategies included the use of an 37/67kDa LR decoy mutant (102-295aa, thereby lacking a transmembrane domain required for cell surface anchorage) [134], as well as use of siRNA-mediated downregulation of 37/67kDa LR [141], heparin mimetics [121] and pentosane polysulfates (which interfere with the cell surface pathogenic association, thereby hindering PrP endocytosis)[126] as well as antibodies directed against 37/67kDa LR.

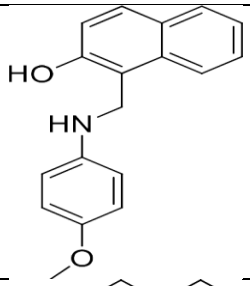
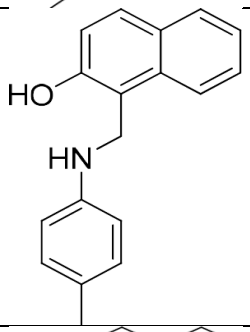
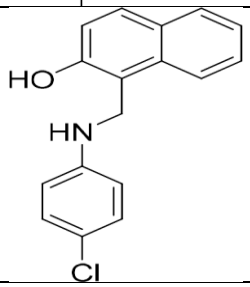
Numerous approaches have been developed to target 37/67kDa LR [142, 143, 144] including i) anti-37/67 kDa LR antibodies, ii) small interfering RNAs directed against the LRP mRNA[145]. Though anti-37/67 kDa LR antibody (polyclonal antibody) has shown effect in prion formation in scrapie-infected N2a cells [146] this antibody doesn't bind effectively with infectious prions in mammalian cells [121].
Since a polyclonal antibody format is not suitable for therapy in animals or humans, single-chain antibodies directed against 37/67kDa LR have been developed. Different studies characterized two single-chain antibodies (scFvs) N3 and S18 that are directed against 37/67kDa LR. The single-chain antibody S18 shows inhibitory effects on prion replication *in vivo*, indeed, peripheral prion propagation levels are reduced. However, the S18 mediated reduction of the peripheral PrP^{Sc} propagation was not concomitant with a significant prolongation of the incubation and survival times in scrapie-infected mice treated with scFv S18 [147].
37/67kDa LR blockage by IgG1-iS18 has been reported to effectively impede Aβ shedding. This was further confirmed by shRNA-mediated downregulation of the receptor. These results taken together suggest that 37/67kDa LR plays a pivotal role in the amyloidogenic processing of APP.
sAPPβ is the initial cleavage product of APP by β-secretase and is released into the extracellular space. The administration of IgG1-iS18 at increasing concentrations resulted in a dose dependent decrease in sAPPβ levels, suggesting that blocking 37/67kDa LR impedes β-secretase activity. Similar results were seen when 37/67kDa LR was downregulated, thus further corroborating the possibility of an interaction between these two proteins. These results implicate 37/67kDa LR in the amyloidogenic process, specifically via promoting β-secretase activity [137].

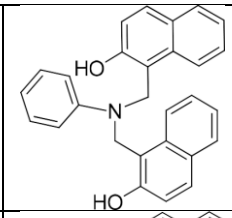
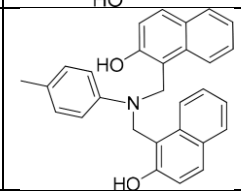
3.4. NSC small molecule inhibitors of 37/67kDa LR

A structure-based virtual screening (SB-VS) was employed to search for 67LR inhibitory small molecules, by focusing on a 37LRP sequence, the peptide G, able to specifically bind laminin-1 (LM).
An in silico screening approach was undertaken to identify compounds from the NCI Diversity library with a potential to bind at the putative cavity on the

peptide G of 67LR and hamper laminin binding. 3D structures of compounds from the NCI's chemical libraries were downloaded from the NCI Developmental Therapeutics Program web site (http://dtp.nci.nih.gov/branches/dscb/repo_open. html/) and processed with LigPrep software to produce 3D structures for the Diversity Set. Among the 46 candidates, only five compounds (Table 1) were able to inhibit 37/67kDa LR cell adhesion to LM [148].

Table 1: Experimentally Determined IC_{50} values and properties predicted by QikProp of the five NSC compounds which are able to inhibit LM binding by interacting with 37/67 kDa LR receptor.

cpd	Structure	NCI No.	MW[a]	IC_{50} (μM)
13		47924	279.3	19.3
50		47923	263.3	1.9
52		**48478**	**283.8**	**1.8**

60		48869	405.5	4.0
37		48861	419.5	3.4

(For detailed description of the compounds see Pesapane et al., 2015).

The fact that 37/67kDa LR may play a role in Alzheimer's disease [142,139 ,149] and that modulation of 37/67kDa LR could affect Aβ cytotoxicity [137,150], highlights the importance of NSC molecules along with its capacity to amend 37/67kDa LR trafficking and degradation. This prompted us to investigate the effect of NSC molecules on post-translational modification of APP and traffickig.

Sarnataro research group (Sarnataro *et* al., 2016) demonstrated that the small molecule inhibitor (NSC47924) treatment affects 37/67kDa LR-PrP^C interaction both *in vitro* and stabilizes PrP^C on the cell surface by inducing 37/67kDa LR internalization. The presence of 37/67kDa LR from the cell surface is decreased along with increasing times of NSC treatment, resulting in its partial early endosomes accumulation and progressive late endo-lysosomal localization with subsequent degradation. This phenomenon suggests that the intracellular trafficking and the normal endocytic pathway of 37/67kDa LR are regulated by NSC47924 [151]. These findings are fundamental if we consider the effect of 37/67kDa LR inhibitor on prion and prion-like proteins. All these data provide a logical anticipation to test a laminin receptor inhibitor against APP.

4. 37/67kDa LR and Cellular Signalling

It has been proposed that the 37/67kDa LR occupies a central role in mediating the vital physiological cellular processes listed above largely as a result of its binding to laminin-1, and the subsequent induction of the MAPK signal transduction pathway. The 37/67kDa LR has been shown to regulate expression levels of MKP1 and PAC1, MAPK phosphatases [152], which dephosphorylate and deactivate ERK, p38 and JNK.

Interestingly, it has recently been reported that the 37/67kDa LR-laminin-1 interaction, via MAPK signal transduction cascades, increases the phosphorylation status of c-Myc and thereby induces the expression of the Fas ligand (FasL) [153]. Moreover, the expression of the 37/67kDa LR may in turn

be regulated by the ERK and JNK MAPK signal transduction cascades, upon stimulation of these cascades by hypoxia. This is of particular relevance to cancer biology as hypoxia promotes tumour metastasis.

4.1. MAPK in Alzheimer's disease

Mammalian MAPK subgroups are differentially activated during the course of the Alzheimer's disease [154]. According to reports by Zhu et al., [154], non demented patients lacking AD-associated pathology exhibit either ERK or JNK activation whilst the activation of both ERK and JNK was observed in patients displaying pathological features of AD but not yet demonstrating dementia. However, in patients suffering from mild to severe Alzheimer's disease, all three pathways (ERK, JNK and p38) were activated [154]. And augmented JNK signalling activity has been implicated in the phosphorylation of tau. In addition, this pathway has been implicated in Aβ- induced cytotoxicity as inhibition hereof significantly hindered apoptosis induced by Aβ [155, 156]. The p38 MAPK pathway occupies a central role in AD pathogenesis and is implicated in neuroinflammation, cytotoxicity, reduced synaptic plasticity, tau hyperphosphorylation and apoptosis (through increased expression of Bax proteins) [157].

ERK activation, although classically associated with cell survival, has been reported to be increased in neurons treated with Aβ peptides [158, 159] [**Fig. 6**]. ERK signalling has been shown to play a pro-apoptotic role, in a caspase-independent manner, in the event where external stimulus is plasma membrane damage, which is a consequence of Aβ insertion into the plasma membrane [157].

It must be stated that altered phosphatase activity cannot be excluded as a contributor to the MAPK signalling modulation (ERK, JNK and p38 levels and activities) observed in AD [154].

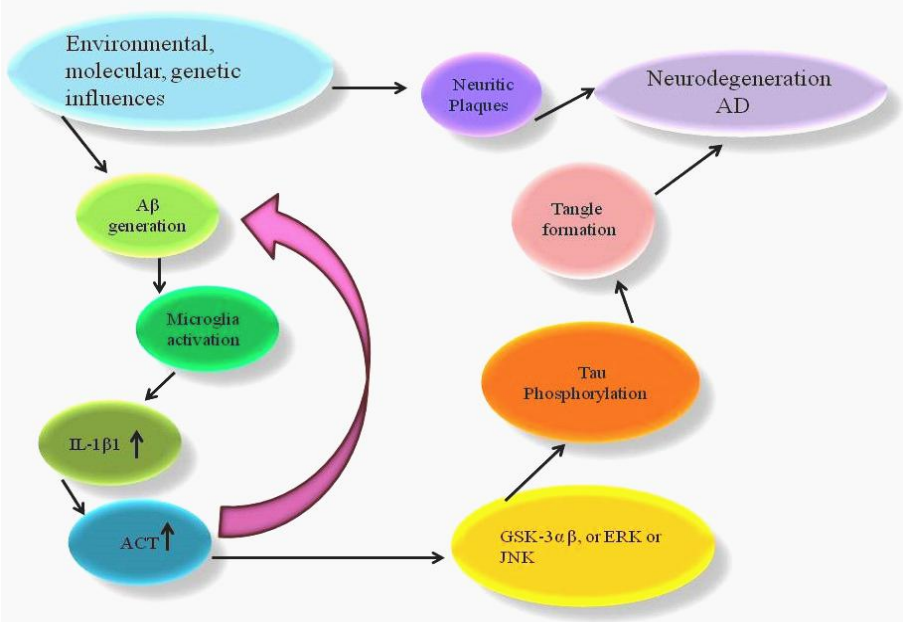


Figure 6: Steps leading to neurodegeneration in AD

Several factors including environmental, brain injury, enhanced inflammation, and genetic deformation induce Aβ generation as well as microglial activation which lead to increased generation of IL-1β (cytokine) which in turn has been shown to enhance alpha-1-antichymotrypsin (ACT) expression. ACT has been shown to induce tau hyperphosphorylation through activation of GSK-3α β, or ERK or JNK. This may lead to AD and neurodegeneration. ACT already has been shown to enhance Aβ aggregation and accelerated plaque formation.

4.2. GSK-3 in AD

GSK3β (Glycogen synthase kinase-3) is a negative regulator of the growth factor-mediated PI3K-Akt survival pathway; indeed, active GSK3β can lead to a loss of survival pathway [160].

It has been reported that GSK3β phosphorylates APP, resulting in increased mature APP production [161] and that GSK3β activation is a critical step in brain aging and the cascade of detrimental events in AD, preceding neuronal death pathways.

GSK3β is intimately linked to the amyloid plaque pathology of AD through its interactions with both the APP and Aβ. An aberrant production of Aβ in AD brain could amplify its neurotoxic effects through activation of GSK3β [**Fig. 6**]. In fact, the neurotoxic Aβ peptide has been shown to activate GSK3β in hippocampal neurons and to cause cell death, an event that is blocked by introduction of antisense oligonucleotides to GSK3β [162].

Furthermore, Aβ-induced activation of GSK3β leads to increased phosphorylation of tau [163, 164].

AIM

Aim of the study

Previously proposed drugs against misfolding diseases range from small organic compounds to antibodies. Various therapeutic strategies have been proposed, including blocking the conversion of normal to misfolded protein, increasing clearance of amyloid aggregates, and/or stabilizing amyloid fibrils. While several compounds have been effective *in vitro* and animal models, none have been proven effective in clinical studies to date mostly because, for many of them, it has not been discovered or described both the mechanism of action and the eventual molecular target. Such lack of *in vivo* efficacy is attributable to high compound toxicity and the lack of permeability of the selected compounds across the blood-brain barrier. Our proposed small molecules (naphthol derivatives) have the advantages to be not cytotoxic and to be small enough to possibly cross the blood-brain barrier. Moreover, these compounds are known to act on a specific cellular target: the 37/67kDa LR.
The overarching objective of our study is to shed light on the role of 37/67kDa LR in the trafficking, PTMs, and processing of APP in neuronal cells by assaying the effects of small molecules known to be its specific inhibitors.

My PhD research has been focused on:

- **The effects of a 37/67 laminin receptor (LR) inhibitor (NSC48478) on the post-translational modification and trafficking of amyloid precursor protein (APP)**

MATERIALS & METHODS

Materials and methods

Reagents and antibodies

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY). The SAF32 anti-PrP antibody was from Cayman Chemical (USA). The recombinant His-tagged 37LRP polypeptide (r37LRP) was made in bacteria and Nickel affinity purified, as previously described (Pesapane et.al, 2015). The polyclonal 4290 Ab was made against a C-terminal peptide derived from LR and was a kind gift from Dr. Mark E. Sobel (Bethesda, MD); the NSC48478 inhibitor has been already described (Pesapane et.al, 2015). Protein-A-Sepharose was from Pharmacia Diagnostics AB (Uppsala, Sweden). Transferrin Alexa-594- conjugated (Tfr), Alexa-488-, Alexa-546-conjugated secondary Abs and Lysotracker Red DND-99 were from Invitrogen (Molecular Probes). The anti- KDEL, anti-Giantin, antibodies were from StressGen Biotechnologies Corp (Victoria, BC, Canada). Anti-β-tubulin antibody was from Abcam. DAPI was purchased from Cell Signal Technology. Biotin-LC was from Pierce and all other reagents were from Sigma Chemical Co. (St Louis, MO).

Cell culture and drug treatment

GT1 (mouse hypothalamic neuronal cell line) and HeLa were grown in Dulbecco's modified Eagle's medium (DMEM), with 4500 mg/glucose/L, 110 mg sodium pyruvate and L-glutamine (SIGMA D6429), supplemented with 10% fetal bovine serum. For inhibitor NSC48478 treatment, the cells were washed in serum free medium, incubated for 30 min at room temperature in Areal medium (13.5 g/l of Dulbecco's modified eagle's medium with glutamine SIGMA-D-7777 without NaHCO3, 0.2% BSA and 20 mM HEPES, final pH 7.5) and for further indicated times at 37 °C under 5% CO2 in the presence of 20 μM inhibitor in DMEM supplemented with 1% serum. NH4Cl (20 mM in culture medium) was used for 24 h.

shRNA interfering

Short hairpin RNA sequence used

"TGCTGTTGACAGTGAGCGAGCATTGCAGAAGTGAAGGATTTAGTG
AAGCCACAGATGTAAATCCTTCACTTCTGCAATGCCTGCCTACTGCC
TCGGA" against mouse 37/67kDa LR (m 2170)(from Open Biosystems library, Euroclone) was cloned in pSHAG-MAGIC vector. GT1 cells at 65% confluence were transfected in Optimem with shRNA by Lipofectamine LTX (Invitrogen see manufacturer's protocol). Puromycin (Sigma Aldrich p8833) 2μg/ml for 5 days was used to select cells with integrated plasmid.

Deglycosylation assays

PNGaseF, Endo-H, and O-glycosidase/Neuraminidase digestions were performed as follows: for PNGaseF treatment, protein extracts were denatured at 100°C in denaturing buffer for 10 min (as indicated in manufacturer instruction, code 1365169, Sigma-Aldrich), then treated with the enzyme in Glyco buffer with NP40 for 1h at 37°C. For Endo-H digestion (code 1088726, Roche), samples were first denatured for 3 min at 100°C in 0.1M sodium citrate, 0.1% SDS, then incubated with the enzyme 16h at 37°C. For O-glycosidase/Neuraminidase treatment, samples were denatured as reported in manufacturer instruction (code P0733S/P0720S New England BioLabs) and digested with the enzymes 1h at 37°C. All samples were analysed by SDS-PAGE and western blotting.

Indirect immunofluorescence and confocal microscopy

GT1 cells were cultured to 50–70% confluence in growth medium for three days on coverslips, washed in PBS, fixed in 4% paraformaldehyde (PFA), permeabilized or not with 0.1% TX-100 for 30 min (where indicated) and processed for indirect immunofluorescence using specific antibodies 30 min in PBS/BSA 0.1%. The cells were incubated with rabbit anti-APP (A8718) antibody and markers of intracellular organelles, followed by incubation with fluorophore-conjugated secondary antibodies. For lysosomal staining, cells were incubated for 1 h with Lysotracker (1:10000) in complete medium before fixing. For Tfr-Alexa 594 staining, the cells were incubated 30 min in complete medium before proceeding with immunofluorescence. Nuclei were stained by using DAPI (1:1000) in PBS**.**

Pearson's Correlation Coefficient (PCC) was employed to quantify co-localization (Bolte et.al.2006) between APP and KDEL (as well as other intracellular markers), and was determined in at least 25 cells from four different experiments. PCC was calculated in regions of APP and reference protein co-presence (Bolte et.al. 2006). In brief, the Otsu algorithm was applied to segment APP and KDEL (as well as other intracellular markers) images, in order to define co-localization regions of the reference proteins. The PCC was then calculated in the defined regions for the images of interest.

Immunofluorescences were analyzed by the confocal microscope Zeiss META 510 equipped with an oil immersion 63 × 1.4 NA Plan Apochromat objective, and a pinhole size of one airy unit. We collected twelve-bit confocal image stacks of 10–15 slices at 0.4 μm Z-step sizes from dual-labeled cells using the following settings: green channel for detecting Alexa-488, excitation 488 nm Argon laser, emission bandpass filter 505– 550 nm; red channel for detecting Alexa-546, excitation 543 nm Helium/Neon laser, emission bandpass filter

560– 700 nm (by using the meta monochromator); blue channel for detecting DAPI, excitation 405 nm blue diode laser, and emission bandpass 420–480 nm.

Measurements of fluorescence intensity were taken on a minimum of three confocal stacks per condition, from a single experiment (~50 cells), using LSM 510 Zeiss software. The background values raised by fluorescent secondary antibodies alone were subtracted from all samples.

37LRP-His-tag protein and 37LRP conjugated agarose beads preparation

To investigate the interaction of APP with 37/67kDa LR, a 37LRP-His-tag fusion protein was generated. To this end, wild-type 37LRP cDNA [165] was cloned into the pTrc-His B expression vector (Invitrogen, San Diego, CA, USA) and expressed in TOP-10 bacteria (Invitrogen) and the resulting plasmid was named pPLR2-1. According to the procedures specified by Invitrogen, TOP-10 bacteria were transformed with pPLR2-1 and pTrc-His B alone, as a control, and lysed in a denaturing lysis buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) containing 6M guanidium. Both bacterial lysates were bound to nickel-NTA agarose beads, through their His-tagged N-terminus, in the same denaturing buffer containing 8M urea. Beads were washed several times at pH 6.0 and 5.3, to dissociate contaminating proteins and His tagged proteins were eluted at pH 4.0. His tagged recombinant 37LRP (r37LRP) purity was than 90% pure, as assessed by SDS-PAGE and Coomassie stain, as compared to pTrc-His B eluate.

r37LRP conjugated beads and pTrc-His B-conjugated control beads, produced as described above, were washed in 50 mmol/l Tris (pH 7.5)-0.1% Triton X-100, to remove urea, and resuspended in the same buffer for pull-down assays.

Binding of soluble r37LRP to immobilized GT1 and HeLa cell lysates

High binding plates with 96 flat-bottomed wells (Corning, Amsterdam, ND) were coated with GT1 and Hela cell lysates, or BSA as a negative control, and incubated at 4°C overnight. After a wash in PBS, residual binding sites were blocked for 1 h at 37°C with 200 µl of blocking buffer (2% FCS, 1 mg/ml BSA, in PBS). Wells were incubated with 2 µg of pTrc-His B control eluate (diluted in PBS, 1 mg/ml BSA), or 2 µg of r37LRP (diluted in PBS, 1 mg/ml BSA), which both contained a 6 × His-tag, for 1 h at 37°C. Each well was washed three times with wash buffer (0.5% Tween in PBS). Penta-His HRP conjugate (1:500) (Qiagen) was added for 2 h at room temperature. After washing, substrate solution was added and absorbance was detected at 490 nm on an ELISA plate reader (Bio-Rad). Binding affinity was determined by subtracting background absorbance (BSA wells).

For inhibition experiments, wells pre-coated with GT1 cell lysates were incubated with 2 μg of pTrc-His B, as control, and 2 μg of r37LRP, alone and in the presence of APP antibody (1:1000), or NSC48478 compound (20 μM).

Pull-Down Assay

Neuronal GT1 cell line was harvested into 500 μl of magnesium lysis buffer (MLB, 125mM HEPES, pH 7.5, 750mM NaCl, 5% Igepal CA-630, 50mM MgCl2, 5mM EDTA and 10% glycerol), containing protease and phosphatase inhibitors. Total cell lysates were pre-cleared with 50 μl of nickel-NTA agarose beads (Invitrogen) overnight at 4°C.After precleaning, cell lysates were incubated in the presence of 50 μl of agarose-bound r37LRP (approximately 2 μg) or in the presence of 50 μl of agarose-bound His pTrc-His B, as a control, for 2h at 4°C. Beads were washed five times with MLB, and then resuspended in Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl) followed by boiling for 5 minutes and centrifugation at 25,000g for 3 min. Supernatants were analysed by SDS-PAGE and blotted with anti-APP antibody. Separately, 50 μg of total cell lysate were immunoblotted for APP.

Biotinylation assay

GT1 cells grown on dishes were cooled on ice and biotinylated with NHS-LC-Biotin at 4°C (as previously indicated Pepe A 2017, Caputo A BJ 2010). Cells were lysed for 20 min using buffer 1 (25 mM Tris-HCl pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% TX-100). Biotinylated cell surface proteins were immunoprecipitated with streptavidin beads (40 μl/sample, Pierce n. 20349). APP was specifically immunorevealed with the A8717 antibody. In the case of NSC48478 treatment, the cells were first incubated with the inhibitor (or not, control) for 24 h, then biotinylated on ice, following the protocol described above.

Statistical analysis: Statistical significance of samples against untreated cells was determined by One-way analysis of Variance (ANOVA), followed by the Dunnett's test. Each value represents the mean ± SEM of at least three independent experiments performed in triplicate (*$P < 0.05$).

RESULTS

Results

1. Inhibitor of 37/67kDa LR, NSC48478, affects maturation of APP in neuronal cells

Newly synthesized APP can undergo a series of posttranslational modifications during its transit from the ER to the Golgi apparatus, *N*- and *O*-linked glycosylation, phosphorylation, sulfation[69, 166] and acetylation [167]
To evaluate the possible effects of NSC48478 on APP posttranslational modifications, as well as on APP levels, we employed neuronal GT1 cells that we had previously used to analyze 37/67kDa LR trafficking [151]. As previously observed, APP migrated on SDS-PAGE gel as a typical glycosylated protein, showing different bands ranging from ~110 to ~135 kDa [168].
Starting from the finding that for affecting the binding between 37/67kDa LR and laminin-1 the calculated IC_{50} value was 19,35 μM[148], we found that, NSC48478 exerted a strong effect on the maturation of APP at 20 μM concentration, with accumulation of the immature APP **[Fig.1A]**, which has a faster electrophoretic mobility respect to mature (mAPP) [169].

We found that APP was not affected by incubation with this enzyme both in the cell extracts from control (untreated cells, -) and treated with NSC48478 [**Figure 1B, Endo-H, lanes +].**
To further characterize APP glycosylation, we used PNGaseF, an enzyme which hydrolyzes nearly all types of *N*-linked sugar chains from glycoproteins, unless there is a α(1-3) Fucose on the core GlcNAc of the protein [170].
We found that APP was again largely unaffected both in untreated cells (without NSC48478) and in drug-treated cells [**Figure 1B**, **PNGaseF]**, suggesting two possibilities: 1) the endogenous APP is mainly *O*-glycosylated in GT1 cells, or 2) PNGaseF is not able to cleave because an α(1-3) Fucose is on the core GlcNAc [170].
It has been reported that APP is a substrate for *O*-glycosylation modifications [171].
We thus performed a digestion assay by using *O*-glycosidase which removes *O*-linked galactose-*N*-acetylgalactosamine disaccharides after cleavage of the terminal sialic acids by Neuraminidase [172]. A clear shift in the electrophoretic mobility of APP was found after incubation with Neuraminidase, especially when coincubated with *O*-glycosidase **[Fig. 1B**, **lanes 3 and 4]**. Under NSC48478 (bands indicated with #, lane 5), in contrast, immature APP accumulated and seemed to be not affected by digestion with these two enzymes **[Fig. 1B**, **lanes 6 and 7 compared to 5].** Conceivably, the APP immature band (asterisk) is the unglycosylated form, while the intermediate bands (indicated with #) are not *O*-glycosylated APP isoforms.

To check whether the mature APP (m) and the intermediate bands (#) derived from *N*-glycosylation events and APP was resistant to PNGaseF digestion, we employed tunicamycin **[Fig. S1],** a drug known to prevent *N*-glycosylation events. Treatment of GT1 cells with tunicamycin for 16 h [**Fig. S1]**, in agreement with previous observations [173], revealed that the mature APP results from *N*-glycosylation **[**and *O*-glycosylation, as shown in **Fig. 1B**] modifications and that after NSC48478 treatment **[Fig. S1]** the resulting immature APP isoforms (* and #) were not *N*-glycosylated. PrP, a typically *N*-glycosylated protein, was carried as control of the procedure.

Our finding that APP was affected by tunicamycin treatment but not by PNGaseF, suggests that APP can be differently glycosylated in GT1 cells respect to other cell lines (such as SHSY5Y cells) where APP sugar modifications can be digested by PNGaseF (not shown). Further analyses will be necessary to validate this assumption.

We hypothesized that the band of immature APP is the unglycosylated form (*) and the upper bands (#) could be constituted by other post-translational modifications, presumably acetylation or phosphorylation which occur in the ER [174, 167].

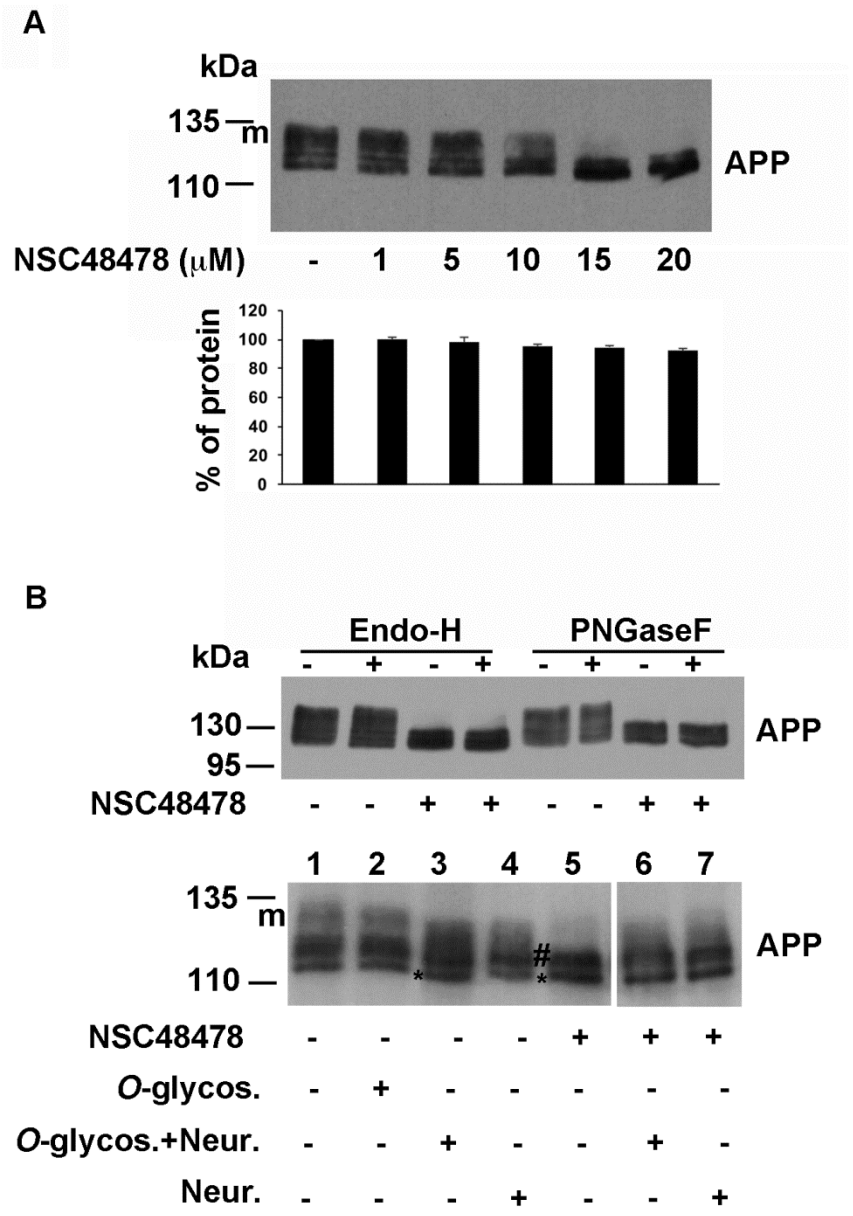


Figure 1: NSC48478 inhibitor affects maturation of APP in neuronal cells

(A) GT1 cells grown in DMEM supplemented with 10% fetal bovine serum were scraped in lysis buffer and 40 μg of total proteins were subjected to SDS-PAGE. APP was revealed by Western blotting on PVDF and hybridization with the A8717 antibody. NSC48478 was used at different indicated concentrations for 24 h. Protein levels of APP were calculated by densitometric analysis with Image J software and expressed as a percentage. The plot shows the percentage of APP after indicated concentration of NSC48478 treatment, using as 100% the expression value in control

conditions (NSC48478 -). Data are expressed as the means ± SEM of three independent experiments (*$P < 0.05$).

(B) GT1 cells were either untreated or digested for 16 h with PGNaseF, EndoH, Neuraminidase and/or *O*-glycosidase. After lysis in buffer 1, cell extracts were incubated with the specific buffers (see materials and methods for details) and APP was revealed by SDS-PAGE and Western blotting. Asterisk (*) points to the immature unglycosylated APP; # indicates intermediate, not *O*-glycosylated APP isoform; "m" points to mature glycosylated APP.

Since this phenomenon has not been observed for other glycosylated proteins, such as the prion protein PrP and the placental alkaline phosphatase PLAP, [**Fig. S1**], we can assume that the effect of the drug is specific for APP.

Different glycosidases, followed by western blot analysis with anti-APP antibody, were employed to test if the accumulation of immature APP, by NSC48478 treatment, corresponded to immaturely glycosylated APP.

Immature glycoproteins that traffic through the early secretory pathway between the ER and the first cisternae of the Golgi complex can be digested by Endoglycosidase-H (Endo-H), which cleaves N-linked mannose-rich oligosaccharides. In the late Golgi cisternae, highly processed complex oligosaccharides are added to glycoproteins and cannot be digested by Endo-H.

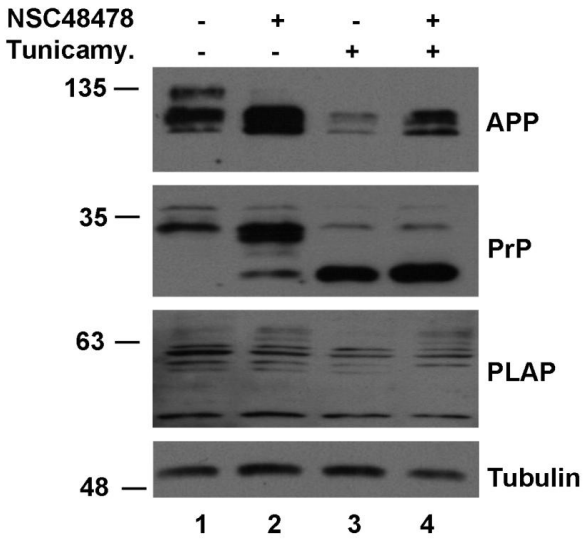


Figure S1: Inhibition of *N*-glycosylation by tunicamycin and specific effects of NSC48478 on APP maturation

GT1 cells, treated or not with NSC48478 for 24h and/or tunicamycin for 16h at 37°C, were processed for western blotting analysis to reveal indicated proteins (APP, PrP, PLAP) in the total (35 µg) cell extract. Anti-tubulin antibody was used to reveal the amount of tubulin in the lysates, as loading control.

The finding by Jonas M.C. et. al., [167] reporting that APP is a substrate of the ER-based acetylation machinery, prompted us to verify this possibility. APP was immunoprecipitated from total cell lysates of GT1 cells treated (+) or not (-) with NSC48478, and the presence of acetylated isoforms were tested by an antibody against acetylated lysine. We found that only the immature forms of APP were acetylated **[Fig. 2, arrowheads]** and the upper bands, the mature mAPP, were not acetylated. The possibility that the upper APP bands (#) derived from APP sulfation was ruled out since reaction protein sulfation occurs in the late Golgi compartments from which APP is excluded under NSC48478 treatment (see figure IF with Giantin).

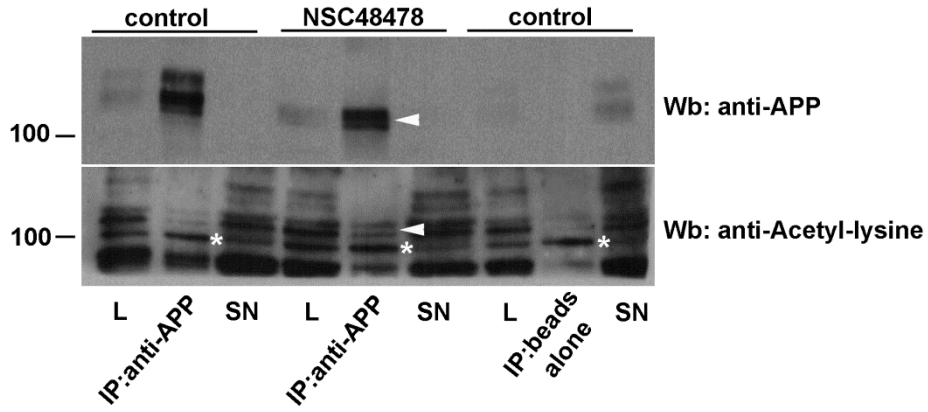


Figure 2: The immature APP isoforms accumulating under NSC48478 are acetylated

Total cell lysates were immunoprecipitated with an anti-APP antibody and then analysed for both APP (upper panel) and anti-acetylated lysine (lower panel) antibodies. Only the immature APP isoforms are acetylated (arrowheads). Beads alone indicate the negative control of the IP.

Asterisks * (around 100 kDa) point to aspecific band deriving from anti-acetylated lysine antibody hybridization.

Moreover, to further analyze APP post-translational modifications, as shown in [**Fig. 3]**, we decided to digest immunoprecipitated APP by alkaline phosphatase enzyme to cleave phosphate groups from protein. The treatment produced a shift on SDS-PAGE gel APP migration (compare IP lane –, black arrow with lane +, white arrowheads) indicating the presence of phosphorylated APP in GT1 cells. Interestingly, after NSC48478 incubation of GT1 cells, we found that [**Fig. 3**, **right panel]** alkaline phosphatase digestion did not affect APP migration, concluding that APP was not phosphorylated under inhibitor exposure.

These results, altogether indicate that NSC48478 prevents both N- and O-glycosylation of APP, as well as phosphorylation, presumably affecting its intracellular localization.

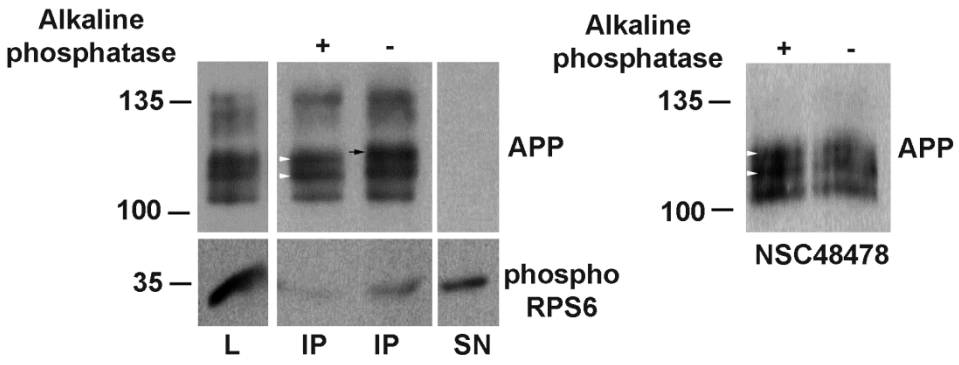


Figure 3: NSC48478 affects APP phosphorylation

Immunoprecipitated APP from untreated (left panel) or NSC48478 treated GT1 cells (right), was digested with alkaline phosphatase for 1h at 37°C and run on SDS-PAGE followed by western blotting analysis and hybridization of PVDF membranes with the anti-APP antibody. Black arrow indicates phosphorylated APP; white arrowheads point to non-phosphorylated APP. Phospho-ribosomal protein RPS6 was carried as control of the procedure. Note to the disappearance of phosphor-RPS6 from the gel after alkaline phosphatase treatment (+). L: cell lysate; IP: immunoprecipitate; SN: supernatant.

2. APP is partially retained both in the ER and TfR-positive recycling endosomes under NSC48478 inhibitor incubation

In order to analyze the effects of NSC48478 on the intracellular localization of APP, we employed confocal fluorescence microscopy. Respect to control conditions (without drug), where APP was mainly localized to Golgi complex and lysosomes [**Fig. 4]**, the treatment with NSC48478 revealed a deep change in the intracellular localization of APP, which was distributed in puncta scattered in the cytoplasm and in a pattern that resembled the typical ER structures **[Fig. 5A]**. However, biotinylation-based assays indicated that the presence of APP on the cell surface was not prevented by NSC48478 **[Fig. 5B]**. The colocalization analysis of APP with the ER-resident KDEL was measured by Pearson's Correlation Coefficient (PCC), which produced a value of 0.68, [**Fig. 5A**, **panel NSC48478+],** reflecting the good degree of co-localization between the two proteins and confirming the partial ER localization of APP.

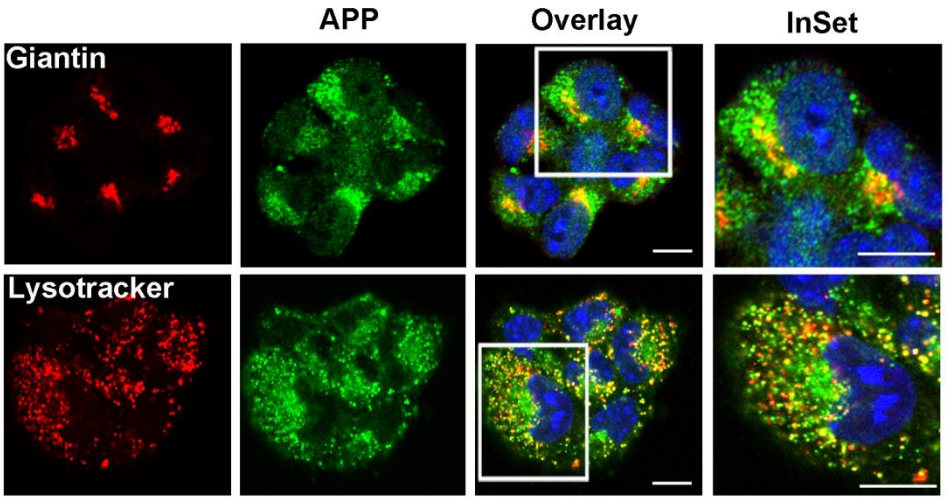


Figure 4: APP is localized in the Golgi apparatus and in the endolysosomal compartment

GT1 cells were grown on coverslips, fixed in PFA 4% and permeabilized in 0.1% TX-100 for 30 min, then they were stained with A8717 rabbit Ab (1:500) and Giantin (1:50) to label APP and Golgi, respectively. Lysotracker, was used 1 h in cell culture medium before fixation to label endolysosmes. Images are representative of at least 100 cells analysed. Scale bars, 10 μm.

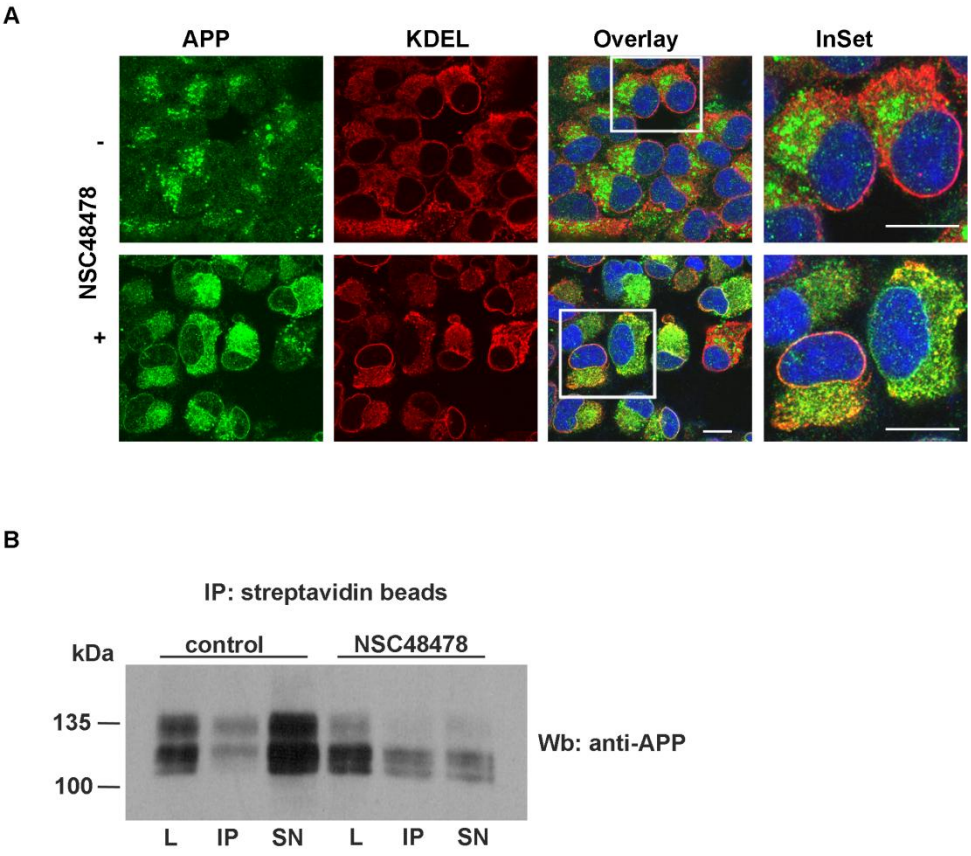


Figure 5: NSC48478 inhibitor induces partial ER retention of APP but does not affect APP cell surface distribution

(A) GT1 cells grown on coverslips, either untreated or treated with NSC48478 for 24 h, were subjected to immunofluorescence analysis by using anti-KDEL and anti-APP antibodies. Co-localization between APP and KDEL was then measured as indicated in the methods section. Scale bars, 10 μm.

(B) Cell surface proteins were biotinylated at 4°C in control (without inhibitor) or after treatment with NSC48478 and were recovered from cell lysates by immunoprecipitation with streptavidin-beads. Total (40 μg of total cell lysates) and cell surface proteins (IP from streptavidin beads), were loaded on the gel and processed for SDS-PAGE and ECL. APP was immunodetected by blotting with A8717 Ab. L: cell lysate; IP: immunoprecipitated biotinylated proteins by streptavidin beads; SN: supernatant.

The PCC close to zero produced by co-localization analysis of APP puncta scattered in the cytoplasm and KDEL (PCC close to zero) suggested that these structures are not part of the ER structure. Next, by using different markers of intracellular organelles, after NSC48478 incubation of GT1 cells, we found a significant co-localization of APP with the marker of the endosomal recycling compartment Transferrin (Tfr) **[Fig. 6**, **PCC 0.89]**, with a concomitant loss of APP localization in the Golgi **[Fig. 6**, **compare control *versus* NSC48478, APP/Giantin]**.

These results strongly suggest that NSC48478 triggers the entry of APP in the ER-associated structures that favor the sorting of APP in the endosomal recycling-dependent pathway against the physiological ER to the Golgi secretory pathway, thus affecting regular APP maturation.

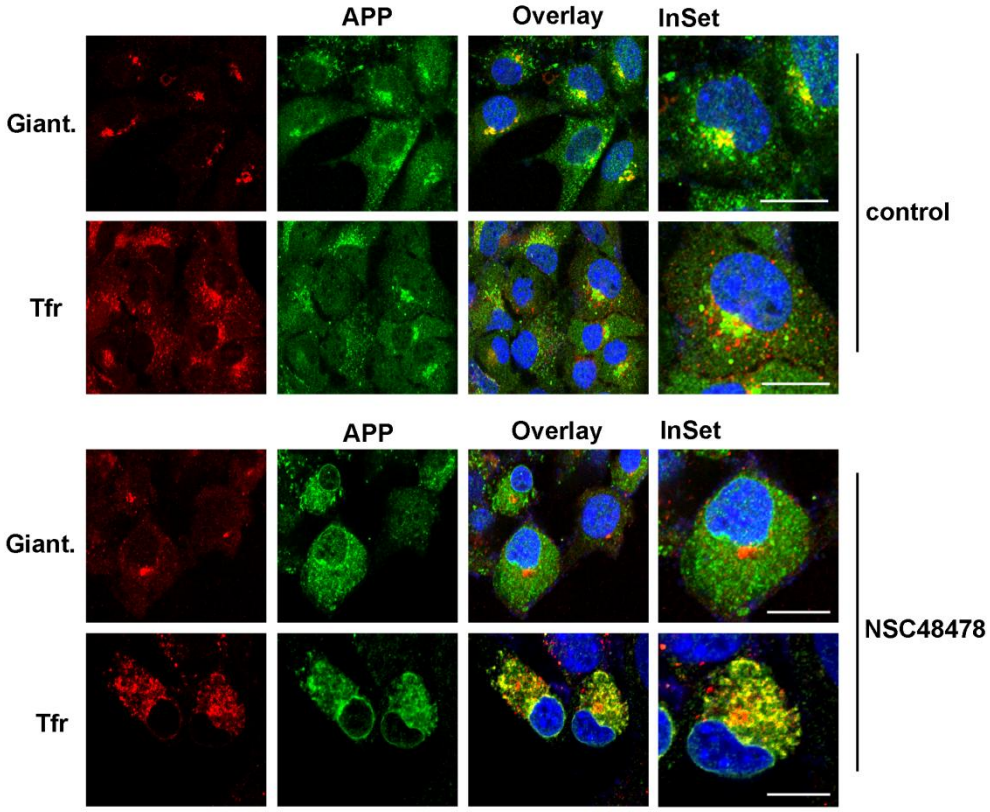


Figure 6: Localization of APP in the Golgi apparatus was lost, under NSC48478, in favor of Tfr-enriched endosomal compartment redistribution

GT1 cells grown on coverslips, either untreated or treated with NSC48478 for 24 h, were subjected to immunofluorescence analysis by using anti-Giantin and anti-APP antibodies. TfrAlexa-594 in the cell culture media was used to label recycling endosomes. Colocalization between APP and the different markers was then measured as indicated in the methods section. Scale bars, 10 μm.

3. Correct APP maturation and intracellular localization are rescued by regulation of endolysosomal activity

Previous findings reporting the involvement of a pH-sensitive compartment for APP sorting to the plasma membrane [102], prompted us to check the consequences of inhibition of vesicular acidification by NH_4Cl when NSC48478 was administrated to GT1 cells.
In agreement with previous findings reporting APP transport from the Golgi to lysosomes for processing and degradation [175], we found that treatment with NH4Cl increased APP level respect to basal conditions [**Fig. 7].** Interestingly, maturation of APP was completely rescued by NH4Cl without perturbing total APP and tubulin levels, under NSC48478 treatment [**Fig. 7, bottom panel].** These results indicate that the drug is perturbing the acidic-dependent compartments.

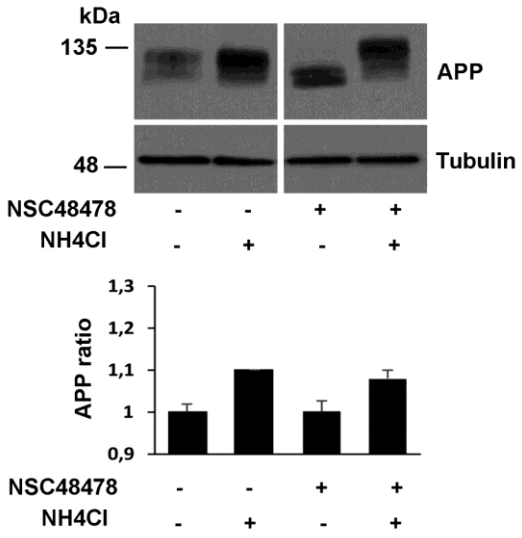


Figure 7: NH_4Cl-induced acidification rescues drug effects on APP maturation

GT1 cells grown on dishes were treated or not with the inhibitor NSC48478 in the presence or absence of NH_4Cl (method section). The cells were scraped in lysis buffer 1 and 40 μg of total proteins were subjected to SDS-PAGE. APP and tubulin (as a loading control) were revealed by Western blotting on PVDF and hybridization with A8717 and anti-tubulin Ab, respectively. Protein levels of APP were calculated by densitometric analysis with Image J software and expressed as a ratio, which was determined by imposing as 100% (ratio 1) the signal of APP in the untreated cells (lane 1, NSC48478-, NH_4Cl-). Mean ± SEM of three experiments were considered ($P < 0.05$). All data were statistically significant.

These data were strengthened by results from fluorescence microscopy **[Fig. 8 and 9]**. Here we show that after NSC48478 incubation, the partial ER

localization of APP **[Fig. 8, compare upper and bottom panels]** was completely rescued by using NH_4Cl.
Similarly, the same experiment performed by analyzing the fluorescence signal deriving from APP and lysotracker (a marker of endolysosomes), revealed that NH_4Cl rescued the endolysosomal localization of APP which was lost under NSC48478 treatment [**Fig. 9**].

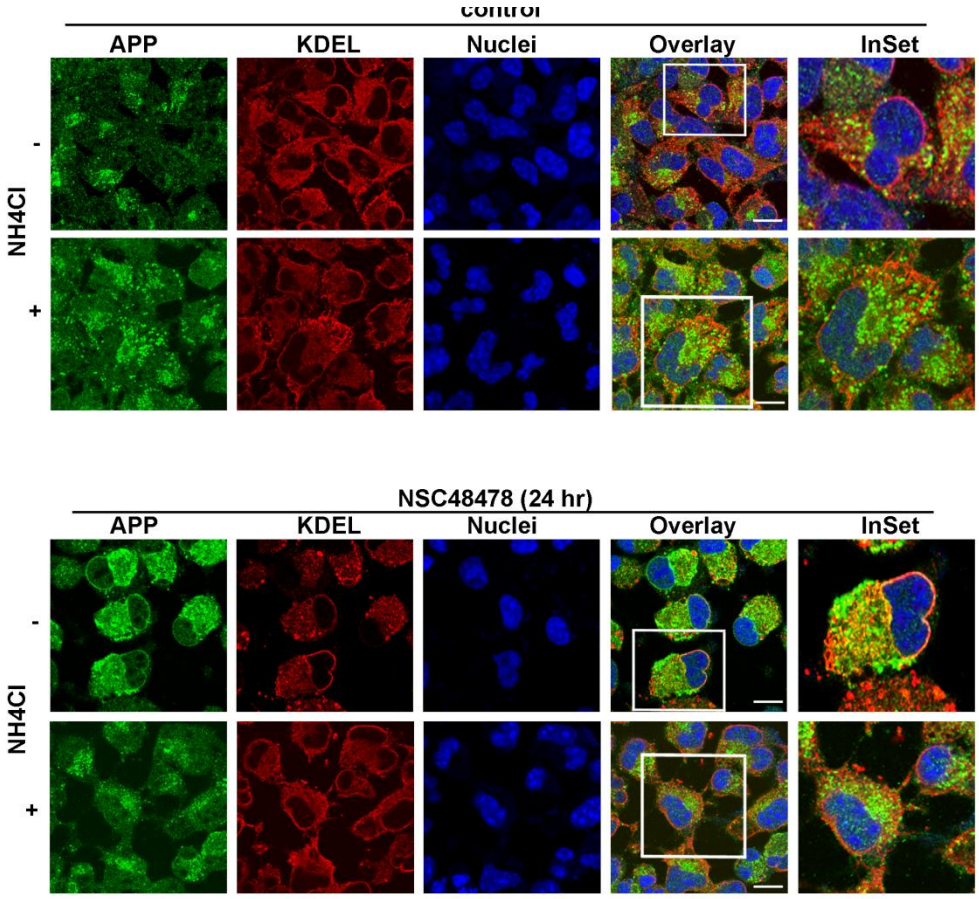


Figure 8: NH_4Cl-induced acidification rescues drug effects on APP subcellular localization

GT1 cells grown on coverslips, were treated or not with the inhibitor NSC48478 in the presence or absence of NH_4Cl. GT1 cells were subjected to immunofluorescence analysis by using anti-KDEL and anti-APP antibodies. Co-localization between APP and KDEL was then measured as indicated in the methods section. Scale bars, 10 μm.

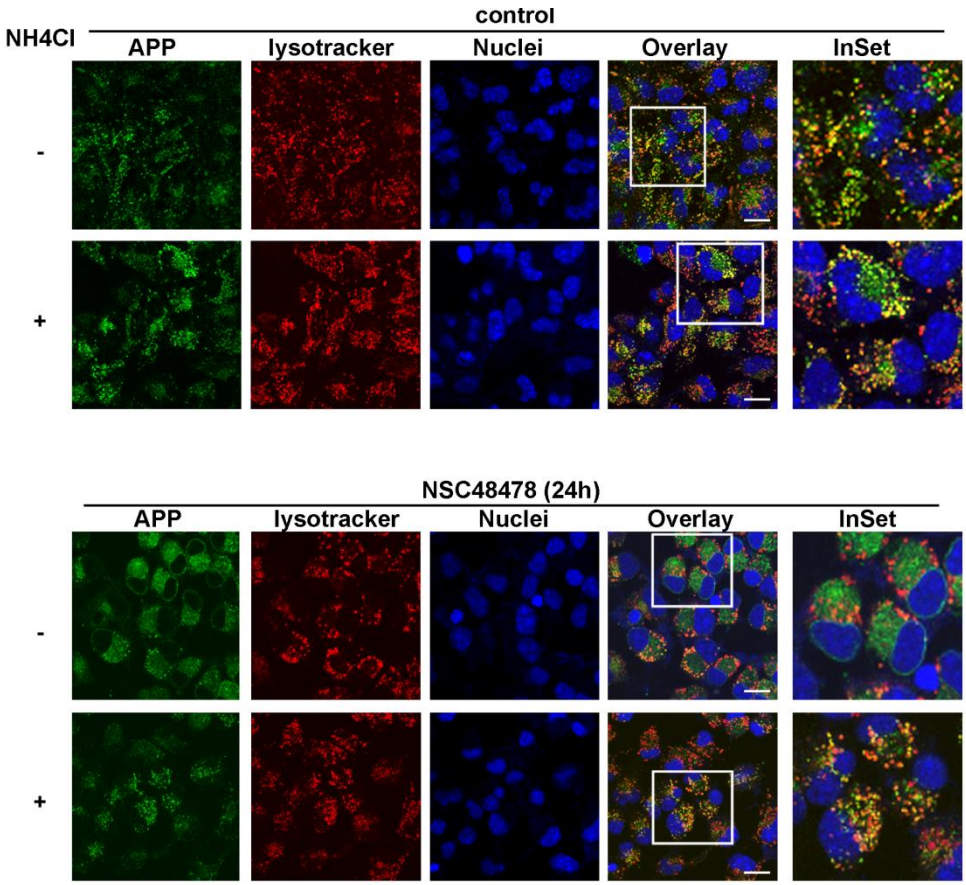


Figure 9: NH_4Cl-induced acidification rescues drug effects on APP subcellular localization

The cells were treated as above (Figure 8), with the exception that here lysotracker was added *in vivo* before fixation and immunofluorescence analysis. Scale bars, 10 μm.

4. NSC48478 affects the interaction between APP and 37/67kDa LR

In recent years, it has been demonstrated that direct or indirect interaction between 37/67kDa LR and APP was possible [115, 116, 137, 149] . Thus we analyzed the structural interaction between these proteins in neuronal GT1 cells both *in vitro* and *ex vivo*.

To assess whether APP directly interacts with 37/67kDa LR, the binding of human recombinant soluble 37LRP (r37LRP) to APP was evaluated *in vitro* by ELISA assays on cell lysates from neuronal GT1 cells. Purified His-tagged r37LRP and pTrc-His B, as a control, were incubated on wells pre-coated with

cell lysates and binding was detected by anti-His HRP. As a control for the specificity of binding, r37LRP binding to BSA-coated wells was also evaluated in parallel and the absorbance readings subtracted. As shown in **[Fig. 10A]**, r37LRP binding to cell lysate-coated wells was significant.
These initial data provide the first evidence that APP could interact with 37/67kDa LR.
The ability of NSC48478 to inhibit the binding of r37LRP to APP was first evaluated by ELISA assays **[Fig. 10B]**. Purified His-tagged r37LRP and pTrc-His B were incubated on wells pre-coated with GT1 cell lysate and binding was detected by anti-His HRP. r37LRP binding to BSA-coated wells was evaluated in parallel, as control, and the absorbance readings subtracted.
r37LRP binding to the cell lysate was significantly higher than pTrc-His B and specifically inhibited by both anti-APP antibody, as the control for binding specificity, and by NSC48478.
A pull-down assay with r37LRP coupled to nickel-NTA agarose on GT1 cell lysates was performed to confirm the data obtained from ELISA, in both control condition and after addition of 20 µM NSC48478. As shown in **[Fig. 10C]**, no APP was detectable using pTrc-His B bound agarose whereas r37LRP specifically and directly interacted with APP and this interaction could be strongly inhibited by NSC48478. Thus, NSC48478 is a specific inhibitor of 37/67kDa LR binding to APP.

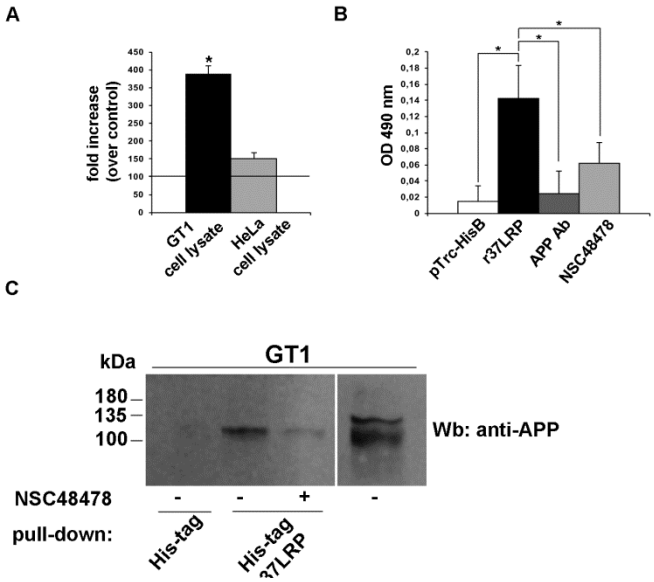


Figure 10: NSC48478 inhibitor affects the interaction between APP and 37/67kDa LR in neuronal cells

(A) Purified human His-tagged recombinant 37LRP (r37LRP) and control

pTrc-His B were placed for 1 hour at 37 °C on wells coated with 2 μg of GT1 cell lysates. Bound r37LRP was revealed by anti-His-HRP and OPD staining; the absorbance at 490 nm was measured. r37LRP binding to BSA-coated wells was subtracted to obtain a specific binding. Results are expressed as a percent increase of absorbance value over pTrc-His control. Values represent the mean ± SEM of three experiments carried out in triplicate; (*$P < 0.05$).

(B) Purified human His-tagged recombinant 37LRP (r37LRP) and control pTrc-His B were placed for 1 hour at 37 °C on wells coated with 2 μg of GT1 cell lysates in the presence of anti-APP antibody, or NSC48478, or DMSO as vehicle control. Bound r37LRP was revealed by anti-His-HRP and OPD staining; the absorbance at 490 nm was measured. r37LRP binding to BSA-coated wells was subtracted to obtain a specific binding. Values represent the mean ± SEM of three experiments carried out in triplicate; (*$P < 0.05$).

(C) Lysates from GT1 cells were incubated with agarose-bound recombinant His-tagged 37LRP (His-tag 37LRP) or with agarose bound His-tag (His-tag), as a negative control. His-tag 37LRP conjugated beads were washed, resuspended in Laemmli sample buffer, boiled and supernatants were analyzed by 15% SDS-PAGE and blotting with the anti-APP antibody. Separately, 50 μg of total GT1 lysate were immunoblotted with the anti-APP antibody.

5. Effects of NSC48478 inhibitor are dependent on 37/67kDa LR expression

To analyse the role of the 37/67kDa LR in the regulation of APP intracellular localization and maturation, the receptor was downregulated by short hairpin RNAs (shRNAs) (material and methods) **[Figure 11A]**. Downregulation of 37/67kDa LR (by shRNA 37/67kDa LR 2170), compared to the shRNA scrambled (shRNA GFP), impeded the effects of inhibitor on APP subcellular localization. The co-localization between APP and KDEL in shRNA GFP cells treated with NSC48478 **[Figure 11B panels a,b, PCC 0.65]** was lost in cells interfered for receptor (shRNA 37/67kDa LR) **[Figure 11B, panels c,d, PCC close to zero]**.
These results indicate that the presence of the receptor in GT1 cells mediates the effects of inhibitor on APP.

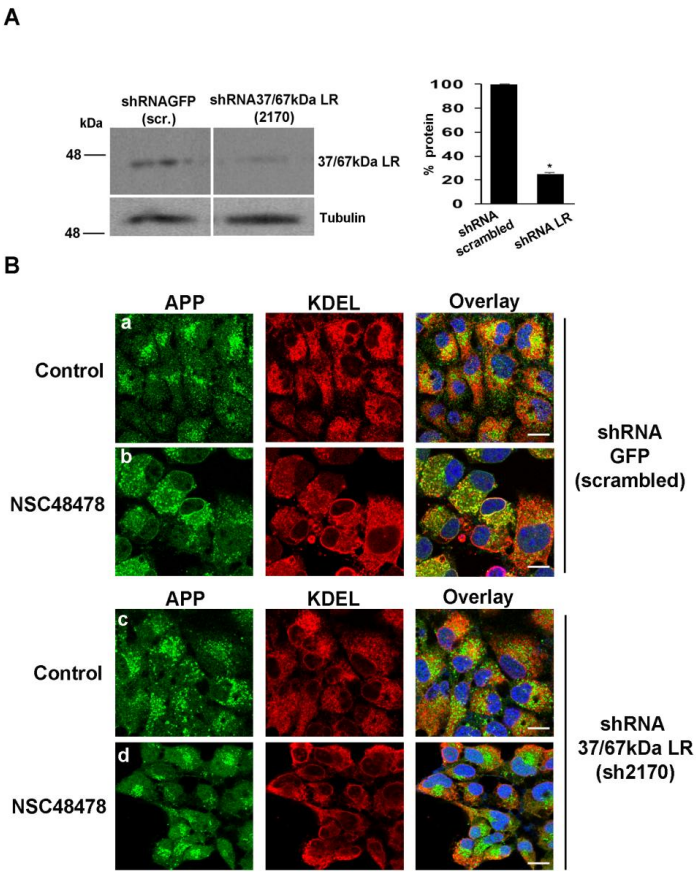


Figure 11: Downregulation of 37/67kDa LR by short hairpin RNA hampers the effects exerted by the inhibitor

(A) 37/67kDa LR was silenced using a specific shRNA (mouse shRNA 2170) for 48h. In comparison to shRNA 37/67kDa LR (mouse shRNA 2170), 35 mg of total cell lysate from control cells (nontargeting RNA, shRNA GFP, scrambled) were loaded for reference. Membranes were probed with an anti-37/67kDa LR antibody to reveal the receptor. The same membranes were probed with an anti-tubulin antibody followed by ECL. The amount of silenced receptor was quantified from three independent experiments (*$P < 0.05$).

(B) Immunofluorescence analysis of scrambled or receptor silenced cells was conducted to analyze the effects of NSC48478 on APP localization. The cells were processed as in Figure 4. Colocalization analysis has been described in the methods section. Scale bars, 10 μm.

6. Inhibitor treatment inactivates the MAPK-ERK1/2 axis and activates Akt negatively regulating GSK3β pathway in neuronal cells

A link between APP and activation of Aβ-dependent Ras-MAPK signalling pathway has been previously reported. Ras-MAPK activation can induce APP hyperphosphorylation. Indeed, APP phosphorylation is high in Alzheimer's disease brains [176].

According to previous reports[177], we found that, under control steady-state conditions, GT1 cells show activation of ERK1/2, indicative of activation of Ras-MAPK signalling axis **[Fig.12A]**. The administration of NSC48478 inhibitor induced a clear inactivation of the ERK1/2 signalling axis **[Fig. 12A]**, without affecting total ERK1/2 levels.

In the same conditions, the analysis of Akt activation by western blot under NSC48478 treatment, revealed that pAkt was increased respect to control conditions **[Fig.12B]**, indicating that the inhibitor is acting through two parallel signalling pathways.

The enhancement of APP phosphorylation at Thr668 detected in AD brains, where dysregulation of multiple kinases and phosphatases including, GSK3, PKC, DYRK1A, PP1, PP2A has been reported [178], prompted us to test the effect of NSC48478 on GSK3β activation, which is known to be controlled by Akt pathway [160]. Thus, to analyse GSK3β phosphorylation in total cell lysates, treated or not with the inhibitor, we employed an anti-GSK3β antibody, which specifically recognizes the GSK3β isoform phosphorylated in Serine 9 (phosphor-GSK3β-Ser9 antibody). The presence of this isoform is indicative of inactivation of the GSK3β pathway [160]. Results from western blotting analysis revealed that under NSC48478, pGSK3β was increased by about 10% respect to control conditions and this effect was amplified by using LiCl, known to inhibit the GSK pathway [179, 180]. These data suggest that the inhibitor is acting in an additive manner with LiCl on the GSK3β.

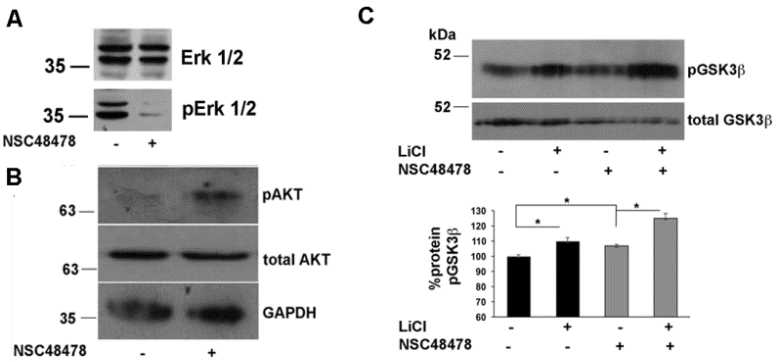


Figure 12: NSC48478 induces inactivation of ERK signaling and activation of Akt with consequent inactivation of GSK3β

Total cell lysates (40 μg) were loaded on gels and expression levels of ERK,Akt, and GSK3b were analyzed by SDS-PAGE followed by western blotting and hybridization of PVDF membranes by respective antibodies. The same membranes were probed with anti-GAPDH and anti-Tubulin antibodies as loading controls.

DISCUSSION

Discussion

Recent evidence indicates that prion-like mechanisms underlie the pathological spreading of misfolded proteins observed in various neurodegenerative diseases, such as Alzheimer's. Misfolded forms of these disease-associated proteins can be transmitted to recipient healthy cells, where they act as 'seeds' to recruit endogenous proteins into aggregates [1]. In prion diseases, the vectors are believed to be "prions" which refers to transmissible, pathogenic agents that induce abnormal folding of normal cellular prion protein (PrP^{C}) into the so-called scrapie-PrP^{Sc}. We contend that the conversion of PrP^{C} to the pathogenic PrP^{Sc} is strongly influenced by the normal trafficking/processing of prion protein in the cells. In Alzheimer's disease, aggregates of amyloid-beta and hyperphosphorylated tau are transmitted in a prion-like manner and the spreading of these aggregates, which appears to occur along connected neurons throughout the brain, is linked to neurodegeneration [39, 48].

The finding that the 37/67kDa LR may play a key role in Alzheimer's disease [136, 139] and that it could act as a receptor mediating Aβ cytotoxicity [138, 150], prompted us to verify the effects of a specific 37/67kDa LR inhibitor on the expression levels and posttranslational APP modifications, which are known to have a critical role in Aβ generation [136, 149]. In addition, the correct localization and trafficking of proteins are fundamental for their correct function [181].

Firstly, after verifying the canonical APP localization in the Golgi, as well as endolysosomal and plasma membrane distribution in neuronal GT1 cells, we tested the effects of NSC48478 on APP levels, posttranslational modifications, and intracellular localization. Interestingly, we found that *N*- and *O*-glycosylations of APP as well as phosphorylation, were both inhibited by the NSC48478 compound and that these effects were due to a different intracellular localization of APP in GT1 drug-treated cells. We found that APP was partially retained into the ER, co-localizing with the ER marker KDEL, and into the transferrin-positive recycling compartment, suggesting that the drug was affecting the physiological subcellular trafficking of APP.

Moreover, the drug seemed to specifically affect APP and not other glycoproteins, such as PrP or PLAP. Concurrently, the inhibitor was inactive in another neuronal cell type, such as the human neuroblastoma SHSY5Y cells (data not shown). Although we can speculate that the drug effects are cell type-specific, this evidence deserves further investigation.

In GT1 cells, NSC48478 prevented APP from being transported from the ER to the Golgi complex. Indeed, under drug treatment, APP does not co-localize with Giantin, a typical Golgi marker, but partially localizes with both the ER and the recycling compartment **[Fig.6].** Interestingly, the recycling compartment has been already described to have a critical role in the regulation of peptides metabolism and protein function [182]. Altogether these results strongly indicate that the receptor inhibitor can control APP maturation through regulation of its intracellular trafficking and subsequent localization.

Since by ELISA and pull-down assays we found that APP interacts with 37/67kDa LR and that their interaction was significantly reduced by the inhibitor, we decided to check whether NSC48478 action was mediated by 37/67kDa LR. Interestingly, we found that the inhibitory effects were hampered by the 37/67kDa LR knockdown and that its effects were exerted through a pH-dependent compartment [102].

In addition, studies from [183], report that APP shows a pH-dependent conformational switch in its E1 domain which can influence its trafficking and processing. Specifically, at neutral pH 7.4 (representing the cell surface) APP may adopt a more open overall conformation than at low pH 5.7 (representing endosomal pH) [102].

From this evidence and our data herein shown, we can envisage the following scenarios: 1) APP and 37/67kDa LR are able to interact, 2) their interaction is affected by the inhibitor NSC48478 (see ELISA and pull-down assays) which diverts the "regular" APP secretory pathway (ER to Golgi to endolysosomes and plasma membrane) towards a "non-canonical" pathway (ER to recycling to plasma membrane), 3) inhibition of vesicular acidification by NH_4Cl prevents the receptor inhibitor from acting on APP localization and maturation. This latter finding suggests that NSC48478 acts by a mechanism that, according to the previous report [102], involves a pH-sensitive compartment through which APP traffics and where, based upon specific pH value, APP becomes more or less able to interact with other molecular partners because of its specific conformation. The ability of NSC48478 to divert the normal traffic of APP towards an alternative pathway (ER to endosomal recycling compartment to the cell surface) can be critical for APP processing and reveal NSC48478 a useful drug to be tested in disease conditions for switching APP processing (possibly from amyloidogenic to non-amyloidogenic) and/or against Aβ production.

Indeed, an aberrant subcellular localization of mutant Swedish APP (APPSwe) respect to APP wt processing has been reported, and that pathological APPSwe is more prone to generate Aβ via a major activation of the cleaving enzymes involved in the amyloidogenic pathway [184]. Thus, 37/67kDa LR inhibitors represent useful small molecules to be tested in diseased cells by checking their ability to control and rescue the physiological localization/trafficking of APP and to possibly prevent the amyloidogenic pathway and/or switch it towards the non-amyloidogenic one.

To analyze the molecular signaling connected to drug treatment, and starting from previous observation of ERK1/2 pathway activation in AD patients with mild to severe pathology [154], together with previous reports showing 37/67kDa LR-dependent regulation of MAPK phosphatases activity, which control ERK signaling [153, 157], we analyzed pERK levels in inhibitor-treated GT1 cells compared to untreated control cells. Inactivation of the ERK1/2 pathway in NSC48478 treated-cells was accompanied by increased Akt phosphorylation and consequent pGSKβ-Ser9 production **[Fig.12]**. Since the presence in the cells of this latter isoform of GSK is indicative of Akt-GSK

pathway inactivation [160,162], from our results showing decrement of APP phosphorylation in drug-treated cells, we can conclude that NSC48478 inhibitor can negatively regulate GSK signaling with reduction of phosphorylated APP isoform.

Our preliminary results (not shown) obtained in fibroblasts from an AD patient (specifically a genetic form of AD with *PSEN2* mutation) show that endogenous APP is distributed in recycling endosomes rather than in the Golgi apparatus, which represents the main organelle where APP is physiologically localized in control cells and fibroblasts from healthy patients.

Interestingly, NSC48478 inhibitor did not exhibit any effect on APP maturation or localization in diseased fibroblasts, whilst one of its analog, NSC47924 (which we found active in the case of PrP^C-37/67kDa LR interaction in GT1 cells [151], was able to rescue the normal APP localization in the Golgi apparatus, compared to untreated fibroblasts. These data suggest that NSC47924 is a useful tool to be tested for Aβ generation in diseased cells.

CONCLUSIONS

Conclusions and future perspective

The current applications for the proposed small molecule inhibitors of 37/67kDa LR are based either on the unique properties of the molecules or on their specific inhibitor element able to bind the peptide G domain of the receptor. However, the enormous potential use of these naphtol derived drugs in modifying the trafficking and processing of cellular proteins involved in neurodegenerative diseases, has not been generally recognized. Hence, our study suggests challenging these compounds with the trafficking/clearance of amyloid intra/extracellular protein aggregates and eventually with the misfolding mechanism.

Once established the potential use of the proposed drugs in a cell culture model of neurodegenerative diseases, we can test, in a long-term project, their activity in induced pluripotent stem cells (IPSCs), reprogrammed from human skin cells to become brain cells. It will be important to investigate APP and tau PTMs, as well as their processing in drug-treated *versus* untreated cells to highlight the role of 37/67kDa LR as a specific target to treat diseases and possibly use these drugs to regulate/control the protein trafficking and the consequent core cellular events.

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"Science is the poetry of reality"-Richard Dawkins

LIST OF CONGRESS ATTAINED

AND PUBLICATION

Research Output: Original article

"APP Maturation and Intracellular Localization Are Controlled by a Specific Inhibitor of 37/67 kDa Laminin-1 Receptor in Neuronal Cells"

Authors: *Antaripa Bhattacharya*, Adriana Limone, Filomena Napolitano, Carmen Cerchia, Silvia Parisi, Giuseppina Minopoli, Nunzia Montuori, Antonio Lavecchia , Daniela Sarnataro*

(Published: Int. J. Mol. Sci. 2020*)*

Peer-reviewed article

"Alzheimer puzzle: Reviewing the last decade finding on amyloid-β precursor protein and Alzheimer's disease"

Antaripa Bhattacharya, Daniela Sarnataro*

(*In preparation)*

Poster Presentation at conferences:

1. **Federation of European Neuroscience Societies (FENS) 2018:** 11th FENS Forum of Neuroscience, Berlin. Germany; July 2018

Title: Effect of small synthetic compound on Biochemical properties of prion and prion-like proteins

Authors: Bhattacharya A, Pepe A, Pesapane A, Nitsch L, Montuori N, Lavecchia A, Zurzolo C, Sarnataro D

2. **Federation of European Neuroscience Societies regional meeting 2019 (FRM 2019):** FRM2019 (FENS Regional meeting), Belgrade,Serbia; July 2019

Title: Effect of small synthetic compound on Biochemical properties of Prion-like proteins (Amyloid precursor protein)

Authors: Bhattacharya A, Pepe A, Pesapane A, Nitsch L, Montuori N, Lavecchia A, Zurzolo C, Sarnataro D

3. **XI NEAPOLITAN BRAIN GROUP MEETING:** Telethon Institute of Genetics and Medicine, Naples, Italy; Dec 2019

Title: 37/67 kDa laminin receptor LR as potential therapeutic target for Alzheimer's disease

Authors: Bhattacharya A, Limone A, Minopoli G, Parisi S, Napolitano F, Montuori N, Lavecchia A, and Sarnataro D

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