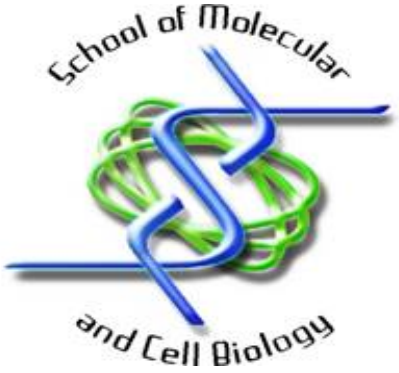
Application of Anti-LRP/LR Specific Antibodies for the Treatment of Alzheimer's Disease

PhD Thesis

Katarina Jovanovic

Student Number:0605417N



Supervisor: Prof. S.F.T Weiss

Co-Supervisor: Prof R. Veale

University of the Witwatersrand

Johannesburg, South Africa

Declaration

I Katarina Jovanovic (Student number: 0605417N) am a student registered for the degree of Doctor of Philosophy (PhD) in the academic year 2013.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this in not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

Signature:  Date: 19 December 2013

Research outputs

Publications:

During the course of this PhD, the following **review articles** have been published:

1. V. Mbazima*, B. Da Costa Dias*, A. Omar, K. Jovanovic, S.F.T. Weiss, Interactions between PrPc and other ligands with the 37-kDa/67-kDa laminin receptor. Frontiers in Bioscience 15 (2010) 1150-1163.

2. A. Omar*, K. Jovanovic*, B. Da Costa Dias*, D. Gonsalves, K. Moodley, R. Caveney, V. Mbazima, S.F.T. Weiss, Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. Expert Opin Ther Pat 21 (2011) 35-53.

3. B. Da Costa Dias, K. Jovanovic, S.F.T. Weiss, Alimentary prion infections: Touchdown in the intestine. Prion 5 (2011) 6-9.

4. B. Da Costa Dias, K. Jovanovic, D. Gonsalves, S.F. Weiss, Structural and mechanistic commonalities of amyloid-beta and the prion protein. Prion 5 (2011) 126 – 137

5. D. Gonsalves*, K. Jovanovic*, B. Da Costa Dias and S.F.T Weiss, Meeting Report: Global Alzheimer's Summit, Present and Future of Alzheimer's Disease Research. Prion 6 (2012) 1-4

During the course of this PhD, the following **original publications** have been submitted/ published:

1. D. Kolodziejczak*, B. Da Costa Dias*, C. Zuber*, K. Jovanovic, A. Omar, J. Beck, K. Vana, V. Mbazima, J. Richt, B. Brenig, S.F. Weiss, Prion interaction with the 37-kDa/67-kDa laminin receptor on enterocytes as a cellular model for intestinal uptake of prions. J Mol Biol 402 (2010) 293-300

2. Katarina Jovanovic, Danielle Gonsalves, Bianca Da Costa Dias, Kiashanee Moodley, Uwe Reusch, Stefan Knackmuss, Clement Penny, Marco Weinberg, Melvyn Little & Stefan F.T. Weiss. Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease. Sci. Rep. 3, 2699; DOI:10.1038/srep02699 (2013)

3. Bianca Da Costa Dias, Katarina Jovanovic, Danielle Gonsalves, Uwe Reusch, Stefan Knackmuss, Clement Penny, Melvyn Little & Stefan Weiss. Anti-LRP/LR specific antibody IgG1-iS18 rescues cells from Aβ42 induced cytotoxicity. Sci. Rep. 3, 2702; DOI:10.1038/srep02702 (2013).

4. Katarina Jovanovic, Ben Loos, Clement Penny and Stefan Weiss. High resolution imaging study of interactions between the 37kDa/67kDa Laminin Receptor and APP, β-secretase and γ-secretase in Alzheimer's disease. PLOS ONE (In revision)

* These authors contributed equally to the work

Patent Application

Patent Title: Compounds for use in the treatment of Alzheimer's disease
Inventors: Stefan Franz Thomas Weiss, Katarina Jovanovic, Danielle Gonsalves, Bianca Da Costa Dias, Stefan Knackmuss, Uwe Reusch, Melvyn Little
Application Date: 19 September 2011
PCT/IB2012/054918 WO 2013/042053A2

Conference Attendance:

Oral Presentations

1. **Oral** presentation at:

Molecular Biology Research Thrust (MBRT) Research Day 2011
7 December 2011
Wits Professional Development Hub, Empire Road, Johannesburg
Title: The 37kDa/67kDa laminin receptor plays a role in amyloid beta shedding in Alzheimer's Disease
Authors: Katarina Jovanovic, Danielle Gonsalves, Bianca Da Costa Dias, Stefan Knackmuss, Uwe Reusch, Melvyn Little and Stefan F.T. Weiss

2. **Oral** presentation at:

SASBMB/FASBMB Congress 2012
29 January – 1 February 2012
Champagne Sports Resort, Drakensberg, KwaZulu-Natal, South Africa
Title: The 37kDa/67kDa laminin receptor specific antibody IgG1-iS18 impedes amyloid beta shedding in Alzheimer's Disease
Authors: Katarina Jovanovic, Danielle Gonsalves, Bianca Da Costa Dias, Stefan Knackmuss, Uwe Reusch, Melvyn Little and Stefan F.T. Weiss

3. **Oral** presentation at:

4th Postgraduate Cross Faculty Symposium
19&22 October 2012
Wits Professional Development Hub, Empire Road, Johannesburg
Title: The 37kDa/67kDa laminin receptor specific antibody IgG1-iS18 impedes amyloid beta shedding in Alzheimer's Disease
Authors: Katarina Jovanovic, Stefan Knackmuss, Uwe Reusch, Melvyn Little and Stefan F.T. Weiss

4. **Oral** presentation at:

Microscopy Society of South Africa Scientific Conference 2013
3 – 5 December 2013
The Farm Inn, Pretoria, South Africa
Title: High resolution study of the possible interactions between the 37kDa/67kDa laminin receptor and Alzheimer's disease proteins
Authors: Katarina Jovanovic, Ben Loos, Clement Penny and Stefan Weiss.

Poster Presentations

1. **Poster** presented at:

Mini Cell and Developmental Biology Symposium at the Wits Medical School (22 May 2011).
Title: The 37kDa/67kDa Laminin receptor specific antibody IgG1-iS18 impedes Aβ shedding in Alzheimer's Disease.
Authors: Katarina Jovanovic, Danielle Gonsalves, Bianca Da Costa Dias and Stefan F.T. Weiss

2. **Poster** presented at:

Global Alzheimer's Summit - Present and Future Alzheimer's Research
September 22-23, 2011
Palacio de Congresos de Madrid, Madrid, Spain
Title: Antibodies targeting the 37kDa/67kDa Laminin receptor impede amyloid beta shedding in Alzheimer's Disease
Authors: Katarina Jovanovic, Danielle Gonsalves, Bianca Da Costa Dias, Stefan Knackmuss, Uwe Reusch, Melvyn Little and Stefan F.T. Weiss

3. **Poster** presentation at:

Molecular Biology Research Thrust (MBRT) Research Day 2012
5 December 2012
Wits Professional Development Hub, Empire Road, Johannesburg
Title: The therapeutic role of the LRP/LR specific antibody IgG1-iS18 in Alzheimer's Disease
Authors: Katarina Jovanovic, Stefan Knackmuss, Uwe Reusch, Melvyn Little and Stefan F.T. Weiss

4. **Poster** presentation at:

Molecular Biology Research Thrust (MBRT) Research Day 2013
4 December 2013
Wits Professional Development Hub, Empire Road, Johannesburg
Title: Application of anti-LRP/LR specific antibodies for the treatment of Alzheimer's Disease
Authors: Katarina Jovanovic, Stefan Knackmuss, Uwe Reusch, Clement Penny, Ben Loos, Melvyn Little and Stefan F.T. Weiss
*Awarded first prize in the Poster presentation category

Acknowlegements

- Financial Aid:
    - National Research Foundation (NRF) Innovation Scholarship (2010 – 2013)
    - Wits Postgraduate Merit Award (2010 – 2013)

- Affimed Therapeutics AG, Germany
- Dr. Bradley T Hyman (Harvard Medical School, Massachusetts, USA) for his generous gift of pEGFPN1-APP770 and pEGFPN1-BACE1.
- Dr. Oksana Berezovska (Massachusetts General Hospital, Massachusetts) for the kind gift of pEGFP-PS1.

- Dr. Ben Loos (University of Stellenbosch)
- Dr. Clement Penny (University of the Witwatersrand)
- Dr. Vusi Mbazima (University of Limpopo)

- Damjan Jovanovic for his constant support over the years
- Guy Swanborough for his love and patience
- Bianca Da Costa Dias for her support, knowledge and guidance
- Aadilah Omar for her amazing friendship and never ending belief in me
- Danielle Gonsalves for sharing in the pain and joy of studying Alzheimer's Disease
- Colleagues for their support and for all the laughs
- My family – my rock and refuge

"I thank You Lord for your strength and guidance in my work; you are the fulfilment of all good things. Fill also my soul with joy and gladness that I may praise you always."

Abstract

Alzheimer's Disease (AD) is the most prevalent neurodegenerative disease. The candidate aetiological agent for this disease is the 4kDa amyloid beta (Aβ) peptide which is derived from the proteolytic cleavage of the amyloid precursor protein (APP) by the β- and γ-secretase, respectively. As cellular prion proteins (PrP^c) both regulate the cleavage of APP and mediate Aβ induced neurotoxicity, a study was undertaken to establish whether the cellular receptor for PrP^c, namely the 37kDa/67kDa laminin receptor (LRP/LR) also played a role in AD pathology. Anti LRP/LR specific antibody (IgG1-iS18) blocking LRP/LR resulted in a significant reduction of both Aβ and sAPPβ levels (the cleavage products of β-secretase), while APP, β- and γ-secretase cell surface levels remained unaltered. LRP/LR was found to co-localise with APP, β- and γ-secretase both on the cell surface and intracellularly. Furthermore, FRET demonstrated that an interaction existed between presenilin 1 (PS1) of the γ-secretase and LRP/LR, while co-immunoprecipitation confirmed that LRP/LR interacted with both β-secretase and PS1. These results indicate that LRP/LR is implicated in the amyloidogenic processing of APP, through an indirect interaction with the β-secretase and a direct interaction with the γ-secretase. These findings also suggest the possibility of utilising IgG1-iS18 as a possible therapeutic for the treatment of AD.

Table of Contents

1. Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is notably the most prevalent form of dementia to affect the aging population. First described in 1906 by the German scientist Alois Alzheimer, the disease is also referred to as Morbus Alzheimer's, Alzheimer's Dementia or simply Alzheimer's (for reviews see[6]. In 2006, over 25 million people were reported to suffer from AD and due to the doubling time of this neurodegenerative disease, it is estimated that by 2040 around 81 million people will be affected [7]; [8]. AD accounts for 50 – 70% of all cases of dementia and its incidence increases significantly with age; 7 in 1000 people are diagnosed while aged 60 – 65, this increases to 70 in 1000 in people aged over 90 [9].

There are difficulties with diagnosing AD as many symptoms appear to be age- or stress-related. It is also difficult to differentiate between AD and other forms of neurodegenerative disorders due to disease progression patterns. For accurate diagnosis, various cognitive and physical examinations as well as behavioural and psychological tests have to be taken into account [10]. Cerebro-spinal fluid biomarkers are fast becoming popular tools for diagnosis and tracking disease progression [11,12]. There are numerous criteria including the Mini Mental State Examination (MMSE) [13] and NINCDS-ADRDA[14] which assess the level of cognitive impairment and assist with diagnosis and prescription of medication. During the initial stage of AD, termed cognitive dysfunction, patients begin to exhibit loss of memory, planning skills and intellectual co-ordination. This stage is also associated with language and speech difficulties. The second stage involves the onset of non-cognitive symptoms, such as behavioural and psychiatric disturbances including aggression, depression, paranoia, hallucinations and delusions. During the final stage, patients experience difficulties in performing complex tasks (such as driving or shopping) and eventually even basic tasks (such as eating and dressing) become problematic. This is followed by loss in bodily functions (due to the degeneration of the brain tissue), whereby patients begin to display ataxia and apraxia, incontinence, loss of co-ordination and extrapyramidal motor disorders. The progressive loss of bodily functions eventually leads to death [15,16].

The aetiology of AD is still not fully understood, however global research efforts have shed some light onto both the causes and molecular mechanisms underlying this neurodegenerative disorder. Numerous factors, both environmental and genetic have been

implicated in contributing to the onset of AD, and it has thus been difficult to find an effective therapy for the treatment of AD. Current therapies are all palliative, alleviating the symptoms of AD but not targeting the actual disease causing mechanism. These include acetylcholine esterase inhibitors such as donepezil, galantamine or rivastigmine [17], non-steroidal anti-inflammatory (NSAIDs) and non-steroidal anti-rheumatics (NSARs) such as Ibuprofen [18], drugs targeting the *N*-Methyl-D-Aspartate (NMDA) receptor such as memantine [19,20,21]. Often patients are prescribed up to 2 of these drugs and upon displaying behavioural symptoms such as aggression, depression and anxiety additional pharmacological drugs are recommended.

1.2 Epidemiology of Alzheimer's Disease

1.2.1 Genetic Epidemiology

AD is classified according to age of onset, the early onset variant accounts for <5%, with the age of onset being ±40 years of age. The more common variant, accounting for >95% of all AD cases is termed late onset AD (LOAD) whereby patients begin to exhibit symptoms at the age of ±60 years [22]. These two variants are clinically identical in terms of symptoms; however, early onset AD is associated with a faster rate of progression and is thought to be the more severe of the two. These two variants exhibit differences in genetic epidemiology. The early-onset AD is inherited as an autosomal dominantly inherited disease and three genes have been implicated. The genes for APP (coding for the Amyloid Precursor Protein) [23,24], PS1 and PS2 (presenilin 1 and 2 - both of which form part of the γ-secretase complex)[25,26] are all involved in amyloid beta production (this will be discussed in detail in section 1.5.2). The late onset variant is linked to mutations in genes related to the lipid transport molecule apolipoprotein ε4 (APOE ε4) [27,28]. The *APOE* gene is a susceptibility and risk gene predisposing the carrier to AD. Genome wide association studies have also revealed a correlation between the clusterin (*CLU)*, phosphotidylinositol-binding clathrin assembly protein (*PICALM)* and complement receptor type 1 (*CR1*) and AD; however no functional effect of the protein products has been found in AD cases [29]. Late onset AD has a heritability of ±60 – 80%.

1.2.2 Environmental Epidemiology

Many environmental factors have been implicated in contributing to the onset or development of AD; however, none have as yet been confirmed as the cause of the disease. Age is the most

obvious factor, while race, gender, economic and social status correlate poorly. It has been found that factors such as bad diet, lack of exercise, excessive alcohol consumption as well as poor brain stimulation and lack of education all increase the risk of developing AD. Other physiological factors which increase the risk AD include obesity and diabetes, strokes and previous head injury. Conversely, it has been found that early education, continuous brain stimulation even in old age, moderate alcohol intake, a healthy balanced diet and exercise seem to decrease the risk of developing this disease [29,30].

1.3 Alzheimer's Disease pathology

The symptoms of AD result from the underlying neurodegeneration which occurs in the regions of the brain affected by the disease. AD results from the loss of synaptic plasticity, neuronal death, dystrophy and apoptosis in the cerebral cortex, hippocampus and subcortical nuclei as well as other regions of the brain [31]. The basis of Alzheimer's disease was first determined from autopsied brain sections of individuals who suffered from the disease. This resulted in the discovery of two abnormalities in the brain tissue that were symptomatic of AD. The first was the formation of extracellular plaques on the neuronal tissue [32,33]. These plaques were composed of a 4kDa protein, which was later identified as Amyloid Beta (Aβ). The second abnormality was the formation of paired helical filaments that form neurofibrillary tangles (NFTs) within neuritic cell bodies [34,35]. These paired helical fragments were formed by the hyperphosphorylation of the cytoskeletal microtubule-associated protein, tau [36,37].

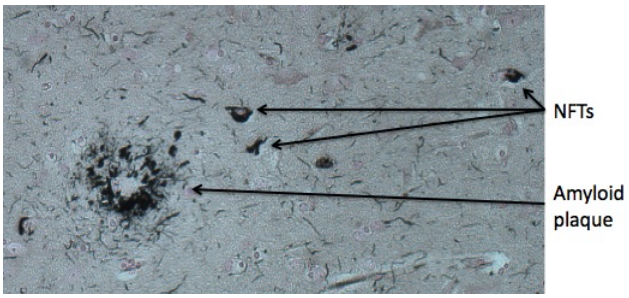


Figure 1.1 Brain section showing neurofibrillary tangles (NFTs) and an amyloid plaque. This brain section was stained with the Gallyas silver impregnation method with visualises the neurofibrillary tangles (4 - 5 stained in black), as well as the dystrophic neurites which form a halo around the amyloid plaque. This method is thus an indirect method of visualising amyloid plaques. [4].

Together, these two features, i.e. Aβ and NFTs are thought to bring about the symptoms associated with the neurodegeneration. Their presence in the brain triggers processes such as inflammation, cytokine release, microgliosis and astrocytosis which are all thought to play a role in a cascade of events leading to the neurodegeneration and dementia associated with AD [2,29].

1.4 Molecular basis of Alzheimer's Disease

The two hallmark features of AD are the formation of amyloid plaques composed of the 4kDa protein named amyloid beta (Aβ), and neurofibrillary tangles composed of the hyperphosphorylated microtubule associated protein tau. In order to better understand the molecular basis of AD, both will be examined further.

1.4.1 Neurofibrillary tangles

Neurofibrillary tangles are formed by the hyperphosphorylation of the microtubule associated protein tau[35]. Due to the hyperphosphorylation of this protein, it is thought that tau can no longer bind to microtubules and thus self-assembles into paired helical filaments (PHFs) which form the major subunit of the NFTs [38]. The tangles are formed within the cell bodies and apical dendrites of neurons and can also occur as neuropil threads in distal dendrites, as well as in association with amyloid plaques. NFTs are associated with many neurodegenerative side-effects and dementia. There is much debate as to whether the NFTs are the causative agents of degeneration in AD or whether they occur as a result of other processes which are central to the etiopathenogenesis of AD [39,40]. Several observations suggest that the latter may be the more correct assumption. Firstly, mutations in the gene encoding for tau result in the onset of fronto-temporal dementia and are not associated with AD[41]. Secondly, NFTs only form after the deposition of amyloid plaques leading to the third argument, where it has been found that amyloid plaques can induce the formation of NFTs, while the reciprocal induction has not been observed. Due to these reasons, the formation and presence of Aβ peptides and plaques are generally accepted as the central disease causing agents in AD.

1.4.2 Amyloid plaques

Amyloid plaques are composed of the 4kDa Aβ peptide. This peptide can aggregate to form dimers, oligomers and fibrils. The oligomers have been implicated as the pathenogenic

species [42] which elicit their neurotoxic effects, such as inhibition of long term potentiation [43], loss of synapses [44] and cytotoxicity via interacting with specific receptors (such as the NMDA receptors [45]) and cellular proteins such as prion proteins [46,47] and possibly the aforementioned tau [48]. Amyloid plaques cause minimal memory loss [49], while sequestering Aβ oligomers and other Aβ-derived diffusible ligands [50]. It has been shown that an imbalance between the production and clearing of the neurotoxic Aβ peptide (termed Aβ dyshomeostatis [51]) leads to AD progression [1,52,53]. This imbalance is caused by changes in the metabolism of Aβ, whether by an increase in Aβ generation, a shift to the production of the more hydrophobic forms (such as Aβ42) or by a decrease in Aβ degradation and removal. This leads to the amyloid cascade that ultimately results in changes within the intracellular environment and the degenerative damage of neurons, whether direct or indirect (Figure 1.2)[1]. Transgenic mouse models have confirmed these findings by showing that transgenic mice overexpressing Aβ showed correlative memory loss and AD symptoms with age [53,54,55].

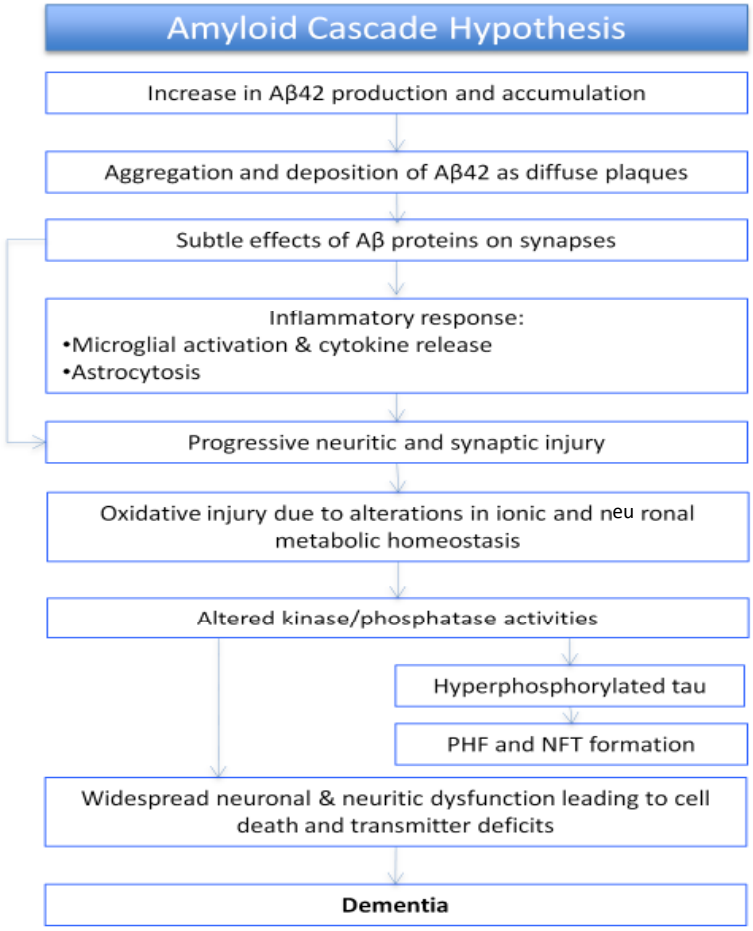


Figure 1.2. Simplified hypothetical sequence of events in the amyloid cascade. An increased level of the hydrophobic variant of Aβ (Aβ42) leads to the formation of amyloid plaques. These plaques affect neuritic and neuronal cells and their intracellular milieu, leading to disturbances such as synaptic injury, neuritic/neuronal dysfunction and ultimately cell death. This cascade of degenerative events results in dementia. Adopted and modified from [1,2]

1.5 The Amyloid Precursor Protein and Amyloid Beta

As Aβ is thought to be the candidate aetiological agent in AD, it is important to understand the underlying processes which lead to its formation. Aβ is proteolytically cleaved from the Amyloid Precursor Protein (APP) and as such, the mechanism of its production and processing will be examined in detail.

1.5.1 Structure and function of the Amyloid Precursor Protein

The gene for the Amyloid Precursor Protein (APP) is localized on chromosome 21, spanning over 3600 base pairs and containing 19 exons [56,57]. Being located on chromosome 21, it presents an elegant explanation for the almost universal appearance of AD-like symptoms in middle-aged Down's Syndrome (trisomy 21) patients [33,58,59]. The APP gene undergoes alternative splicing to release three variants of APP, namely the 695 amino acid (APP695), 751 aa (APP751) or 770 aa (APP770) variants [60]. Neuronal tissue predominantly express the APP695 isoform while non-neuronal cells such as endothelial cells, smooth muscle cells and kidney cells express the other two variants [61].

APP is a type-I transmembrane protein with the majority of the protein, including the N-terminal domain, located extracellularly (Figure 1.3). Part of the protein is located transmembraneously and the relatively small C-terminal is located in the cytosol. Following expression, APP is translocated into the Endoplasmic Reticulum (ER) co-translationally by its signal peptide and thereafter undergoes posttranslational modification (glycosylation, tyrosine sulphation and phosphorylation) and matures via the central secretory pathway [62]. APP achieves intramembranous anchorage (i.e. in the plasmalemma) and intracellular attachment (i.e. to Golgi bodies, ER and endosomes) via a 23 amino acid hydrophobic domain found in its C-terminal [60,62]. The majority of APP resides within the Golgi apparatus and well as the trans-Golgi network (TGN), while a small fraction reaches the cell surface where it either undergoes proteolytic cleavage or is internalised. Internalisation occurs via raft mediated endocytosis or clathrin coated pits, whereby the APP is sorted into the early endosomes and undergoes amyloidogenic processing [63,64].

The amyloid Beta (Aβ) peptide is shed after APP has been cleaved with a subset of secretases (section 1.5.2). The N terminal of the Aβ sequence extends 28 residues into the extracellular

matrix and 11 – 15 residues into the transmembrane domain of APP, depending on the length of the Aβ peptide to be released [60]. 90% of all cleavage of Aβ results in the 40-residue form of the peptide ($A\beta_{40}$), while the other 10% are composed of the 42-residue form ($A\beta_{42}$), which is more hydrophobic and prone to aggregation resulting in the formation of amyloid fibrils due to the additional isoleucine and alanine residues at the C terminus [65,66,67]. Alternative proteolytic processing can result in a 38 residue variant of Aβ ($A\beta_{38}$), however, this shorter form is not implicated in AD pathology[68].

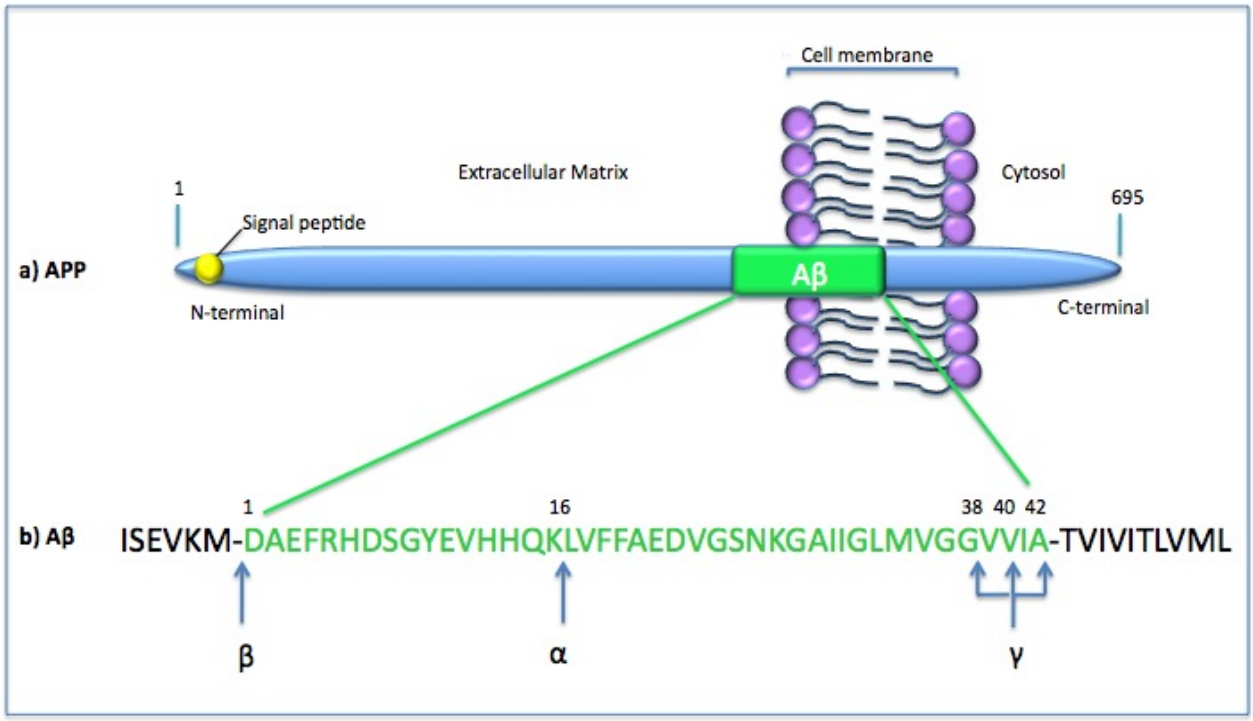


Figure 1.3 Schematic representation of the Amyloid Precursor Protein (APP). The 695 amino acid protein is shown in relation to the cell membrane. The amino acid sequence of Aβ peptide is shown in one letter code. Cleavage sites for the α-, β- and γ-secretase are shown within the Aβ sequence. Adopted from [5].

The function of APP still remains elusive though some theories have been put forward. Notch is a transmembrane protein that is also cleaved proteolytically by γ-secretase and is involved in signal transduction involving the regulation of gene transcription. As notch and APP share structural similarities and both undergo proteolytic processing with the same enzyme (γ-secretase), it is thought that they might have functional similarities [69]. Recent findings have confirmed this hypothesis, as it was found that the post γ-secretase cleavage C-terminal domain of APP, the Amyloid Intracellular Domain (AICD), plays a role in regulating the expression of the cell cycle regulator p53 [70] (see section 1.6.1).

Other reports suggest that APP and the secreted sAPPα (see section 1.2.2) interact and have differential effects on the outgrowth and branching of neurites [71]. sAPPα seems to play a regulatory role in the growth of fibroblasts; this would explain why the APP molecule is so widespread within tissues of the body [60,72]. APP also plays a role in synaptogenesis in early development [73] and is seen to be associated with a loss in synaptic function in AD patients [44,74], which directly correlates with impairment in cognitive function - a major factor contributing to the disease profile of AD [31,75]. Other studies on animal models suggest that it is possible that APP and sAPPα may play a role in enhancing memory [76,77]. Thus, APP appears to be involved in several aspects of neurodevelopment and cognitive functioning; this explains why problems with the processing of this protein result in the neurodegenerative symptoms associated with AD.

1.5.2 The Proteolytic Processing of APP

There are two different pathways by which APP is processed (Figure 1.4). Both pathways occur constitutively in bodily tissues throughout a person's lifetime [2]. The predominant pathway for the processing of APP is the non-amyloidogenic pathway, whereby Aβ is not generated. This pathway involves the cleavage of the extracellular N-terminal of APP between amino acids 105 and 125 (depending on the variant of APP). This cleavage site corresponds to Lys^{16} of the Aβ sequence, thus effectively precluding the formation of Aβ [78,79]. This cleavage is performed by the proteolytic enzyme termed α-secretase, which belongs to the ADAM (a disintegrin and metalloprotease) family and is thought to be composed of ADAM10 and TACE (TNF-α converting enzyme) [80,81]. α-secretase cleavage occurs at the plasma membrane where α-secretase is located. The peptide released from this cleavage is sAPPα, which has been noted to have neuroprotective roles and functions in neuritic outgrowth, as mentioned in section 1.5.1. The remaining 83 residue C-terminal fragment (named α-CTF or C83) is then cleaved by the γ-secretase enzyme to release a 3kDa peptide called p3 (sometimes referred to as $Aβ_{17-42}$) [78]. The function of this 3kDa peptide was previously not well understood [67,82]; however, recent research has shown that these peptides are present in amyloid plaques and can even induce neuronal toxicity; however, the exact mechanism of their pathogenicity still requires elucidation [83].

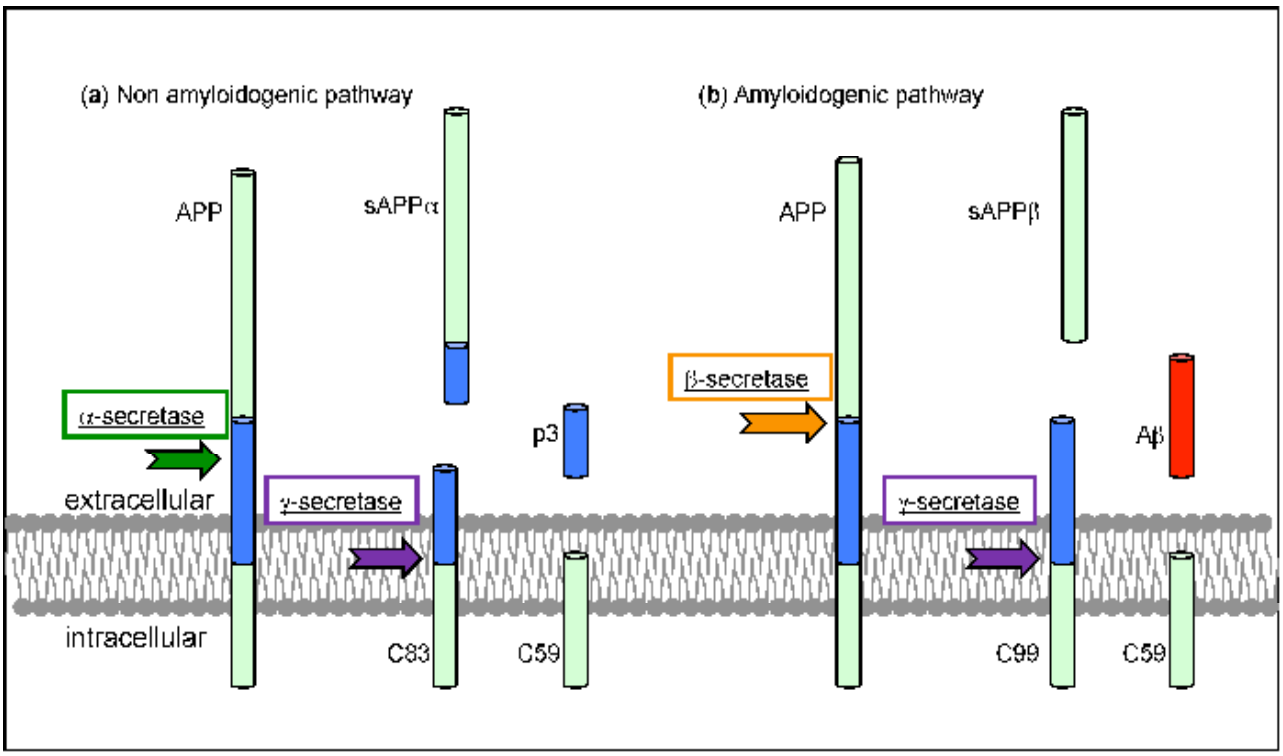


Figure 1.4 Overview of APP cleavage pathways. A) The non-amyloidogenic pathway features the α-secretase which cleaves within the Aβ sequence to release sAPPα into the extracellular space. The remaining C-terminal fragment of APP (C83) is subsequently cleaved intramembraneously by γ-secretase to release the 3kDa peptide, p3. B) The amyloidogenic pathway involves the cleavage of APP by the β-secretase to release sAPPβ. γ-secretase subsequently cleaves C99 and the Aβ peptide is released. The remaining APP C-terminals of both the amyloidogenic and non-amyloidogenic pathways are released into the cytoplasm as the AICD (amyloid intracellular domain) where it plays a role in nuclear signalling [3]. Adopted from [4].

In the amyloidogenic pathway, whereby Aβ peptides are produced, cleavage is initiated by the β-secretase instead of the α-secretase. β-secretase (also referred to as BACE1: beta-site APP cleaving enzyme) is a membrane-bound enzyme displaying high homology to the pepsin family of transmembrane aspartyl proteases [84]. β-secretase is distributed throughout various cellular locations, but is most notably present in the Golgi apparatus, TGN, endosomes and on the cell surface [85]. The slightly acidic pH of endosomes provides the optimal environment for β-secretase cleavage thus amyloidogenic processing is higher at this site compared to the cell surface [84]. β-secretase cleaves APP at the N-terminal of the Aβ peptide, to release sAPPβ and leaves the 99 residue C-terminal fragment of APP (named β-CTF or C99) [86]. This membrane bound protein subsequently forms the substrate for γ-secretase, which cleaves C99 at residue 38, 40 or 42 to release $A\beta_{38}$, $A\beta_{40}$ or $A\beta_{42}$, respectively [79,86]. γ-secretase cleavage results in the release of the remaining C-terminal of

APP (C59), termed the AICD (amyloid intracellular domain), into the cytoplasm where it plays a role in nuclear signalling and gene transcription [3]. The γ-secretase is membrane bound and is unusual as it performs intramembranous proteolysis of its substrate [87]. γ-secretase is an aspartyl protease composed of four subunits, of which the enzymatically active subunit is either presenilin 1 or 2 (PS1 or PS2), which result in familial Alzheimer's disease if the gene for presenilin is mutated [26,87]. The remaining three subunits essential for functional γ-secretase activity are presenilin enhancer 2 (PEN-2), nicastrin and anterior pharynx defective 1 (APH-1) [87,88]. A 'spatial paradox' has been described with regard to the cellular locations of γ-secretase as it is seen to function at numerous cellular sites including cholesterol- and sphingolipid-rich lipid raft microdomains of the plasma membrane, endosomes and TGN [89].

APP is expressed in highest levels in neuronal tissue, although it is also found in most other tissues. The concomitant overexpression of the otherwise ubiquitously expressed β-secretase in the brain makes this site the principal tissue for elevated Aβ production and explains why AD pathology is localised to the brain as opposed to other tissues.

1.6 Prion Proteins

Prion proteins (PrP) are infectious proteins that occur naturally in the body as the cellular prion proteins, PrP^{c}, and are found predominantly in the brain tissue [90,91,92,93]. PrP^{c} is encoded by the *PRNP* gene found on chromosome 20, and the gene is found within a single open reading frame [94]. Prion proteins function predominantly in the brain, and it is speculated that PrP^{c} has a neuroprotective function against oxidative and apoptotic stress as well as in the generation and maintenance of synapses, transmembrane signalling and extracellular matrix adhesion [95]. However, if PrP^{c} undergoes a conformational alteration to become PrP^{Sc}, it can become an infectious agent that leads to the onset of neurodegenerative diseases [96]. In humans, PrP^{Sc} is responsible for Creutzfeldt-Jakob disease (CJD) as well as Familial Fatal Insomnia (FFI), Gerstmann-Sträussler-Sheinker disease (GSS) and Kuru. Infectious PrP^{Sc} also affects a wide range of species, causing Transmissible Spongiform Encephalopathies (TSEs) such as Bovine Spongiform Encephalopathies (BSE) in cattle, Scrapie in sheep and Chronic Wasting disease in ungulates [97,98,99].

PrP is translated in the endoplasmic reticulum and undergoes modification at the Golgi apparatus, whereby it is transported via secretory vesicles to the cell surface and coupled to a glycosylphosphatidyl inositol (GPI) anchor. PrP^c is predominantly localised to the cell membrane, although it has been found that it also can undergo cleavage by phosphatidyl inositol-specific phospholipase C, thus being released from the membrane [100,101]. The cleavage pathways to which PrP^c can be subjected are outlined a recent article by Ostapchenko et al[102]. PrP^c is internalised via clathrin coated pits [103] or alternatively by caveolae-like domains [104] and recently it has been found that the ADP Ribosylation factor 6 (ARF6) (a GTPase) is involved in clathrin independent internalisation of PrP^c[105]. Interestingly, ARF6 is also responsible for endosomal sorting of the β-secretase[106]. The 37kDa/67kDa laminin receptor precursor/ laminin receptor (LRP/LR) is the cellular receptor for both cellular and infectious prion proteins and thus also plays an important role in their internalisation [93]. The conversion of PrP^c into PrP^{Sc} is thought to occur in the endosomes during the endocytic pathway, however, recent studies have also showed that the phenomena occurs on the cell membrane and within vesicles, and that the presence of certain RNA and lipids are required for the conversion to occur in vitro [107,108,109,110]. The conformational changes involve the conversion of α-helices (found in PrP^c) into β-sheets, which are predominantly found in PrP^{Sc} [111] . There are now many therapeutic approaches targeting both PrP^{Sc} and its receptor, LRP/LR, for the treatment of prion diseases [4,5,92,96,112,113,114,115]

1.6.1 The Role of Prion Proteins in Alzheimer's disease

Several factors have led to the belief that cellular prion proteins play a role in AD pathology. As mentioned in section 1.6, they are believed to have neuroprotective properties against oxidative stress [116,117], and play a controversial role in the prevention of apoptosis [118,119]. Initial reports suggested that PrP^c may play a role in preventing or delaying the onset of Alzheimer's disease [120,121]. The link between prion proteins and AD was confirmed by *in vitro* assays, as; when the human neuroblastoma cell line (SH-SY5Y) over-expressed PrP^c a significant decrease in Aβ shedding was observed [122]. The converse was also confirmed whereby the downregulation of PrP^c expression with the use of siRNA resulted in a significant increase in Aβ shedding [122]. This implies that the prion protein is directly involved in the regulation of APP processing and the production of Aβ. Co-immunoprecipitation studies revealed that this was due to a direct interaction between the β-

secretase (BACE1) and PrP^c [122,123].

A feedback model was proposed between Aβ processing and PrP^c (Figure 1.5) [124]. As mentioned, PrP^c regulates β-secretase activity thus reducing the amount of Aβ produced by the proteolytic processing of APP [122]. In 2009, a study revealed that the remaining C59 fragment of APP, the AICD (that is formed by γ-secretase cleavage) is translocated to the nucleus (in association with Tip60 and Fe65) where it behaves like a transcription factor and regulates the expression of p53, which in turn acts as a promoter for the *PRNP* gene for the expression of PrP^c [70]. PrP^c then downregulates Aβ shedding and thus production of the AICD is also reduced, hence reducing the expression of prion proteins. Another study showed that prion proteins can also act as receptors for Aβ oligomers and thereby mediate the neuronal toxicity of the Aβ fibres [125]. Any disruption in this feedback loop, as would be expected in Alzheimer's Disease patients, would result in the excessive production of Aβ peptides which would in turn bind to prion proteins and effectively prevent the prion proteins from regulating β-secretase cleavage [124]. Thus increased levels of Aβ would also result in an increase in AICD production which would in turn upregulate PrP^c production, allowing for the attachment of more Aβ peptides. This would in turn accelerate neurotoxicity.

Recent reports have highlighted the role of PrP^c in regulating Aβ induced neurotoxicity and contributing to neurodegeneration. PrP^c has been implicated in mediating toxic signalling of not only Aβ oligomers but also other β-sheet rich proteins [46]. It has also been found to play an important physiological role in inhibiting the NMDA receptor from excessive activity that may lead to the onset of neuronal injury [45]. Aβ mediates neuronal and synaptic injury by disrupting the normal PrP^c dependent regulation of NMDAR activity [45]. PrP^c was also shown to be essential for neuronal death induced by Aβ as $Prnp^{-/-}$ mice were completely resistant to the effects of Aβ oligomers [126,127]. Furthermore, PrP^c is responsible for regulating Aβ-induced inhibition of long term potentiation (LTP) – a feature associated with AD [128]. The interaction between PrP^c and Aβ also recruits Fyn kinase which has been shown to impair neurons [47,129].

These findings all suggest that PrP^c plays a significant role in not only regulating the processes responsible for the production of Aβ, but also the neurotoxic effects thereof.

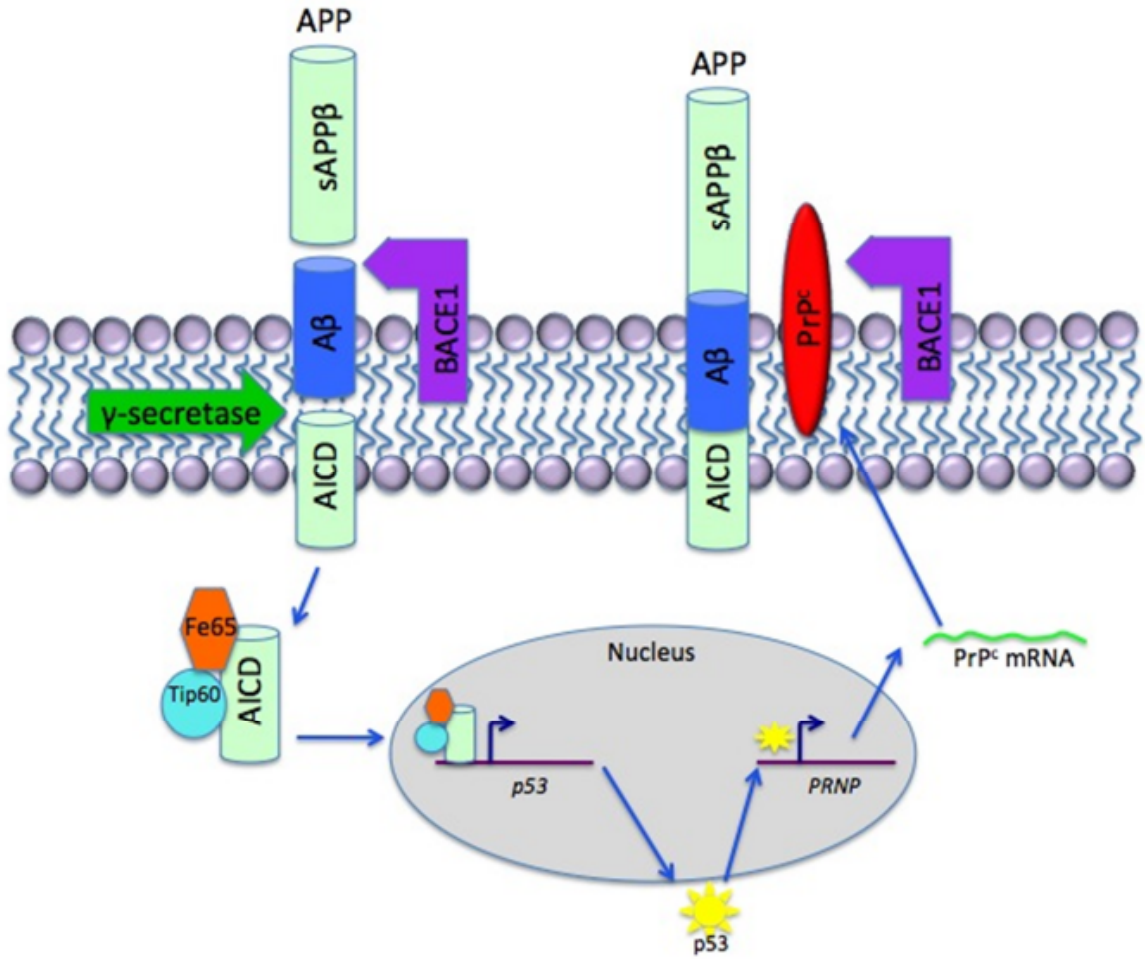


Fig 1.5 Proposed feedback mechanism for regulation of PrP^c expression, β-secretase activity and Aβ production. On the left, Aβ is produced and the remaining C-terminus becomes the Amyloid Intracellular Domain (AICD). This AICD forms a complex with Fe65 and Tip60 and is translocated to the nucleus where it interacts with the *p53* promoter to regulate expression of this gene. The p53 protein then acts a regulator of the *PRNP* gene for the expression of PrP^c which is subsequently transported to lipid rafts in the cell membrane where it can mitigate the action of the β-secretase, BACE1. This results in less Aβ being produced, resulting in less AICD which thus downregulates the production of PrP^c. Adopted and modified from [5]

1.7 The 37kDa/67kDa Laminin Receptor Precursor/Laminin Receptor

The 37kDa/67kDa Laminin receptor precursor/Laminin receptor (LRP/LR) was first isolated from tumour cells in 1983 [130]. This type II membrane receptor has an intracellular N-terminal and its C-terminal is located extracellularly (Figure 1.6). Two main isoforms exist, namely the 37kDa laminin receptor precursor (LRP) and the 67kDa laminin receptor (LR); both are found in plasma membrane fractions. It has been found that the 67kDa LR is formed from the 37kDa LRP, however the exact mechanism whereby the 37kDa LRP forms the 67kDa LR remains elusive [4,96,113].

The significance of LRP/LR in cancer was based on its ability to bind laminin. It is also a receptor for various viruses such as Venezuelan Equine Encephalitis virus (VEE) [131], Sindbis virus [132], Dengue virus [133] and Adeno-Associated Viruses (AAV). In 2001, it was confirmed that LRP/LR was the cellular receptor for both cellular prion proteins (PrP^{c}) and infectious prions (PrP^{Sc}) (section 1.6) [93]. It has important functions in not only the life cycle and propagation of PrP^{c} [134,135] but also in the endocytosis of PrP after which both PrP^{c} and LRP/LR are found to co-localise within the endosomes[136]. Subsequent studies have also described the role of LRP/LR within the cytosol where it functions as part of the translation machinery [137] and is also found to be a one of the p40 ribosome associated proteins [138]. LRP/LR is also found in the nucleus where it is tightly associated with DNA through interactions with the histone proteins [139,140]. This multifunctional protein has became a target for many therapeutic strategies because of its important role in the metastasis of cancers as well as its receptor function for prions and viruses [4,5,92,96,113,114]

1.7.1. LRP/LR as a therapeutic target

Therapies targeting LRP/LR have focused on the use of antibodies that prevent the action of LRP/LR as a receptor. Initially the W3 antibody was used to cure Scrapie infected neuronal N2a cells [135] and was also found to reduce the binding between infectious prions and mammalian cells [141]. Employing W3 also showed that internalisation of the BSE prion protein (PrP^{BSE}) in human intestinal cells was impeded [136]. The antibody might also interfere with the binding of PrP^{BSE} to cervid and ovine intestinal cells [99,142]. Further studies on LRP/LR blocking strategies led to the discovery of the scFv S18 antibody by

phage display [112]; after which, another variant of the antibody, scFv iS18 was created by chain shuffling [113,143]. scFv iS18 was found to be more efficient than scFv S18 as not only did it have a 10 fold higher binding affinity, but it also has a longer half life (21 days) [113,143].

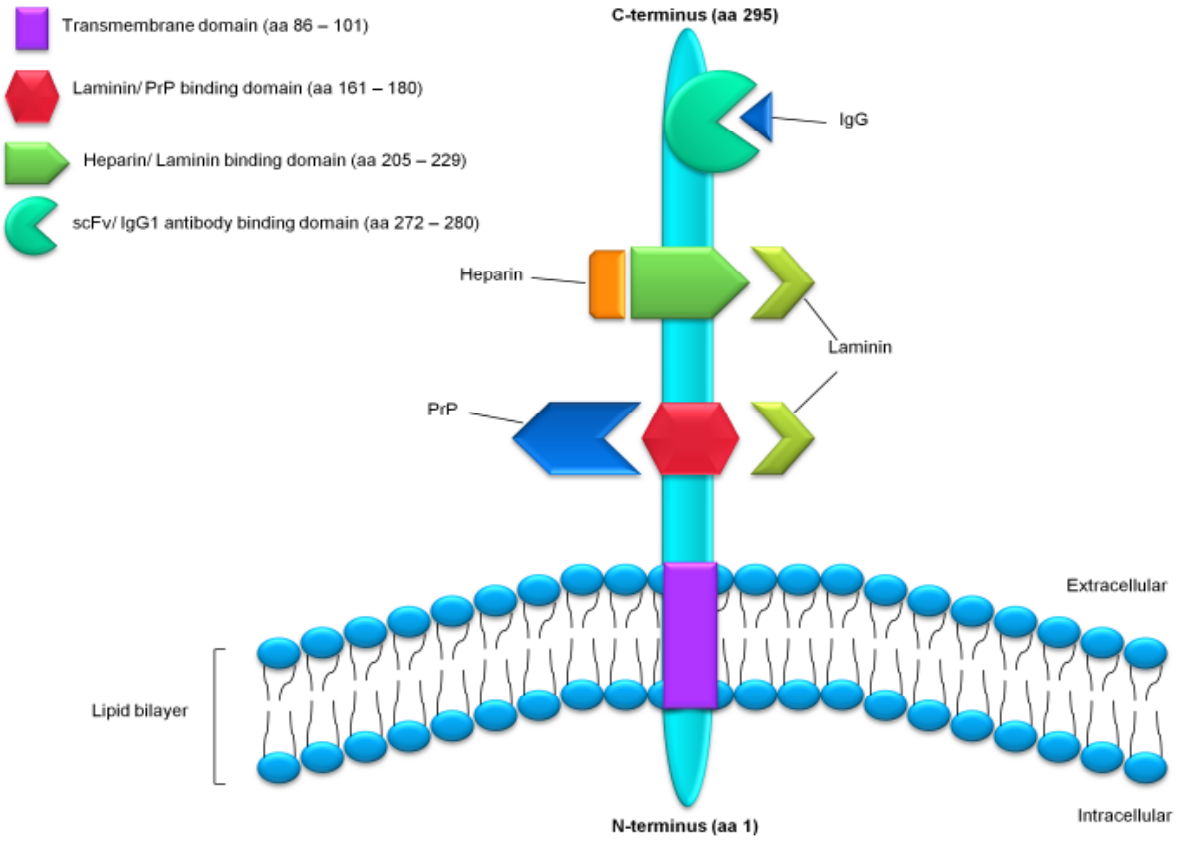


Figure 1.6 Schematic representation of the 37kDa/67kDa LRP/LR. LRP/LR is 295aa long and has 4 functional domains, as shown above interacting with their ligands. PrP^c binds at the Laminin/PrP binding domain between aa 161 – 180. This receptor also binds antibodies, heparin and laminin. Adopted from [4].

The interaction between laminin and LRP/LR stimulates collagenase type IV which has the ability to break down the basement membrane, allowing cells to metastasise. Blockage of LRP/LR using the IgG1-iS18 antibody resulted in a significant decrease in both adhesion and invasion of various neoplastic cell lines [143,144,145]. Furthermore, when the W3 antibody

was applied to human umbelical vein endothelial cells (HUVECs), angiogenesis was significantly reduced [146]. Downregulation of LRP/LR with anti-LRP siRNA resulted in a significant reduction in cell viability in malignant cell lines suggesting that LRP/LR is involved in apoptosis evasion – one of the key features of cancer [147].

The findings show that antibodies directed against LRP/LR can be used in the treatment of not only prion infections but metastatic cancers too [143]. The full-length humanised anti-LRP/LR antibody, IgG1-iS18, is now the antibody of choice for investigations into prion and cancer therapies.

1.7.2 Implications of LRP/LR in Alzheimer's disease

Since LRP/LR is the receptor for both infectious and non-infectious prion proteins, it plays a key role in the internalisation of prions [141] and also functions in the life cycle[148]. LRP/LR has also been implicated in the propagation of prions in neuronal cells [135]. As was discussed in section 1.6.1, prion proteins play an important regulatory role in both the processing of APP and Aβ induced neurotoxicity. Thus, PrP^{c} plays a pivotal role in Alzheimer's disease onset and progression. It is thus plausible to consider that LRP/LR as the cellular receptor for the prion protein may indeed also play a role in the processes involved in the etiopathenogenesis of AD.

This PhD project was an investigation into whether LRP/LR played a role in the molecular mechanisms of Alzheimer's disease and to investigate whether the IgG1-iS18 antibody could potentially be used as a therapeutic for the treatment of AD in the future.

2. Hypothesis

LRP/LR plays a significant role in Alzheimer's disease through an effect on the amyloidogenic processing of the Amyloid Precursor Protein.

3. Aim

The aim of this research is (i) to determine the role of the 37kDa/67kDa laminin receptor in the proteolytic processing of the Amyloid Precursor Protein and the shedding of the amyloid beta peptide; (ii) to determine whether the LRP/LR specific antibody IgG1-iS18 is a possible therapeutic antibody for the treatment of AD.

3.1 Objectives

1. To determine cell surface levels of LRP/LR, APP β- and γ-secretases via Flow cytometric analysis.
2. To determine the effects of the LRP/LR specific antibody IgG1-iS18 on the shedding of Aβ by means of an Aβ ELISA.
3. To perform dose-dependency assays for the IgG1-iS18 treatment of cells to determine the effects on Aβ shedding by quantifying Aβ levels using an Aβ specific ELISA
4. To determine the molecular mechanisms underlying this reaction, that is, the interaction of LRP/LR with APP and the β- and γ-secretases. This will be done via detection of sAPPβ (the result of β-secretase cleavage) after the cells have been treated with the anti-LRP/LR antibody IgG1-iS18.
5. To determine the levels of APP, β- and γ-secretase present on the cell surface after IgG1-iS18 treatment.
6. To perform co-localisation studies between LRP/LR and the AD relevant proteins using Immunofluorescence Microscopy for cell surface co-localisation and confocal microscopy for intracellular co-localisation.
7. To determine whether LRP/LR interacts with APP, β- or γ-secretase by means of pull down assays.
8. To obtain, (using a confocal microscope) Z-stacks of images of the co-localisation between LRP/LR and APP, β- and γ-secretase in order to determine the sub-cellular location of the interactions between these proteins.
9. To perform FRET (Försters Resonance Energy Transfer) in order to further ascertain whether interactions exist between LRP/LR and APP, β- and γ-secretase.

4. Review Articles

During the course of this PhD project, numerous review articles have been published, in collaboration with other members of Prof. S.F.T Weiss research group. Some of these articles focus on LRP/LR and its role in Prion and neurodegenerative disorders as well as cancers. Others are more specific to Alzheimer's disease and prion disorders.

Articles are arranged according to date of publication:

1. Interactions between PrP^c and other ligands with the 37-kDa/67-kDa laminin receptor (2010)
2. Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor (2010)
3. Alimentary Prion Infections: Touchdown in the intestine (2011)
4. Structural and mechanistic commonalities of amyloid-β and the prion protein (2011)
5. Global Alzheimer Research Summit: Basic and clinical research; Present and future Alzheimer research (2012)

Interactions between PrP^c and other ligands with the 37-kDa/67-kDa laminin receptor

V. Mbazima*, B. Da Costa Dias*, A. Omar, K. Jovanovic, S.F.T. Weiss

Frontiers in Bioscience 15 (2010) 1150-1163.

*These authors contributed equally to the work

In this review article, both the physiological and pathological roles of LRP/LR are discussed in detail. Insights are given into the various diseases and disorders in which LRP/LR plays a fundamental role and therapeutic options for the treatment thereof are explored. These options include antibodies and siRNA targeting LRP/LR as well as pentosan polysulfate and truncated forms of LRP/LR (LRP 102 – 295) as decoy mutants.

[Frontiers in Bioscience 15, 1150-1163, June 1, 2010]

Interactions between PrP^c and other ligands with the 37-kDa/67-kDa laminin receptor

Vusi Mbazima, Bianca Da Costa Dias, Aadilah Omar, Katarina Jovanovic, Stefan F T Weiss

School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa (RSA)

TABLE OF CONTENTS

1. ABSTRACT

The 37-kDa/67 kDa laminin receptor (LRP/LR) represents a multifunctional protein. It is a receptor for viruses such as Dengue Viruses, Alphaviruses and Adeno-associated-Viruses (AAV), as well as the cellular prion protein (PrP^c) and infectious prions (PrP^{Sc}). The 37-kDa/67-kDa LRP/LR plays furthermore fundamental roles in basic cell biological processes such as cell adhesion and cell growth and acts as a key player in metastatic cancer, affecting invasion, adhesion and apoptotic processes. This review gives fundamental insights into basic cellular processes affected by LRP/LR including signal transduction and cell cycle progression and focuses on pathophysiological implications of the interaction of prion proteins, laminin, viruses and other ligands with LRP/LR affecting the development of highly-prevalent diseases such as cancer, neurodegenerative diseases such as prion disorders and Alzheimer's Disease as well as viral infections. Molecular tools such as LRP/LR specific antibodies and siRNAs targeting LRP expression as possible alternative therapeutics for the treatment of neurodegenerative diseases, metastatic cancer and viral infections are emphasized.

2. THE 37-kDa/67-kDa LRP/LR REVEALS A MULTIFUNCTIONAL PROTEIN

The 67-kDa laminin receptor (LR) is a non-integrin cell surface receptor with high affinity binding for its corresponding ligand, laminin, an extracellular matrix glycoprotein involved in cell growth, movement, attachment and differentiation (for review: (1-4)). Laminin interacts with the 67-kDa LR via two binding domains; the first domain is known as the peptide G sequence which is located between amino acids 161-180 and the second laminin binding domain is located between amino acids 205-229 at the C-terminal of the 37-kDa LRP (5). Besides having high affinity for laminin, 67-kDa LR also acts as a receptor for other extracellular matrix molecules such as elastin and carbohydrates (1). Furthermore, 67-kDa LR is reported to mediate laminin-induced tumor cell growth, migration, invasiveness and metastasis (6, 7) . This is due to the high laminin receptor levels found in a number of cancer types which correlates with an increase in the invasiveness and metastasis of tumor cells (8, 9). The relationship between the 67 kDa high affinity receptor (LR) and the 37-kDa laminin receptor precursor (LRP), is poorly understood. Several reports suggest that 67-kDa LR

might be formed through homo/heterodimerization reactions involving 37 kDa LRP and fatty acid acylation (10, 11). The fact that recombinant LRP is monomeric and fails to interact with itself in yeast two hybrid system (12), however, strongly suggests that 67 kDa LR is not the dimer of 37 kDa LRP. It is reported that both the 37-kDa LRP and the 67-kDa LR forms of the protein are found on the cell surface and also function as receptors for cellular prion proteins (PrP) (13) and infectious prions (14). The interaction between the 37-kDa/67-kDa LRP/LR and the prion proteins (PrP) was demonstrated by using several cell culture models as well as the yeast two-hybrid system. Furthermore, data from yeast two-hybrid system demonstrated the presence of two binding domains for 37-kDa/67-kDa LRP/LR on PrP that are of importance in the PrP-37-kDa/67-kDa LRP/LR interaction. These include a direct (PrPLRPbd1, aa144-179) and an indirect (PrPLRPbd2, aa53-93) binding domains. The interaction between PrP and 37-kDa/67-kDa LRP/LR through the indirect binding domain is reported to be dependent on heparan sulfate proteoglycans (HSPGs) that function as co-factors or co-receptors for the binding of PrP^c to the 37-kDa/67-kDa LRP/LR (12). HSPGs are multifunctional macromolecules comprising of a core polypeptide covalently bound to glycosaminoglycans (GAGs) which act as initial attachment receptors for several extracellular molecules (15, 16) Additionally, HSPGs are known to be associated with Amyloid-beta (A-beta) deposits in Alzheimer's disease (AD) (17). Experimental investigations using the yeast two-hybrid system demonstrated that the direct PrP-binding domain on 37-kDa/67-kDa LRP/LR is located between amino acid residues 161 and 180 (12) (Figure 1). On the other hand, a HSPG-dependent binding site has so far not been identified on LRP/LR but it is presumed to be located between amino acid 180 and 285 (17). The 37-kDa/67-kDa LRP/LR has also been shown to function as a receptor for a number of viruses such as Dengue virus (18), Sindbis virus (19), Venezuelan equine encephalitis (20), Adeno-associated viruses serotypes 2, 3, 8 and 9 (21). Recently, 37-kDa/67-kDa LRP/LR has been demonstrated to play a role as a receptor and mediator of epigallocatechin-3-gallate (EGCG)-induced anti-cancer and anti-allergic activity in human colon cancer Caco2 cells (22). In another study, the down-regulation of 37-kDa LRP expression using siRNAs in Hep3B cells was shown to lead to the induction of apoptosis suggesting a role of 37-kDa LRP in tumor cell viability (23).

In addition to its role as a cell surface receptor, the 37-kDa LRP is also reported to be present in the cytoplasm where it exists as p40 ribosome-associated protein and is involved in the maturation of the 40S subunit of the ribosomes (24, 25). Moreover, the 37-kDa LRP is localized in the perinuclear compartment and nucleus where it interacts with PrP and histones, respectively. (24, 26, 27). The presence of the 37-kDa LRP in various parts of the cell suggests that it is a multifunctional protein that plays an important role in a variety of cellular processes including proliferation, viability and protein synthesis and could be a potential target for the treatment of various human diseases.

3. THE 37-kDa\67-kDa LRP\LR AND ITS INFLUENCE ON PRION AND ZOONOTIC DISEASES

The transmission of prions amongst individuals of different species (interspecies) is characterized by prolonged incubation and survival periods, a phenomenon termed the "species barrier" (28). Factors influencing this phenomenon are numerous and may include: variations in the prion protein (29) and laminin receptor sequences in the donor and recipient species; the strain of PrP^{Sc} employed for experimentation and the route of infection. Although intraspecies transmission efficiencies vary (high amongst captive deer suffering from Chronic Wasting Disease (CWD) and low in cattle and sheep infected with Bovine Spongiform Encephalopathy (BSE) and Scrapie respectively, (Figure 2) (30, 31), interspecies transmissibility is still lower and may fail completely. BSE is the only TSE that has been transmitted from animals to humans and thereby caused the zoonotic disease variant Creutzfeldt-Jakob disease (vCJD) (32). The consumption of infected meat is the most common mode of infection and thus further investigations to study the mode of oral prion uptake were undertaken (33). The 37-kDa/67-kDa LRP/LR is expressed on the surface of enterocytes (the major cell population of the intestinal epithelium) as well as in the apical brush border of the small intestine and Paneth cell secretory granules (34). Thus, it was hypothesized that the 37-kDa/67-kDa LRP/LR may influence the susceptibility to oral prion infection and the transmissibility of Transmissible Spongiform Encephalopathies (TSEs) across the species barrier. Recent research suggests that the transmissibility of TSEs is dependent on the strain of the infectious PrP as PrP^{BSE} (Cattle strain of the infectious prion) agents were internalized via 37-kDa/67-kDa LRP/LR receptors on human enterocytes (Caco-2/TC7 cells) whereas PrP^{Sc} (Sheep strain of the infectious prion) failed to do so (34). The mode of transmission is a further factor that must be taken into consideration. Sheep, Goats (35), minks and mice (36) are susceptible to oral infection with PrP^{BSE} in contrast to pigs which are only successfully infected upon parenteral inoculation (37). To investigate whether other prion diseases in livestock and game, such as sheep scrapie and CWD in deer, may cause zoonotic diseases further binding studies investigating the interactions between bovine, ovine, cervid and porcine prions and human enterocytes, and possibly the 37-kDa/67-kDa LRP\LR receptor, are being undertaken.

4. THE ROLE OF PRION PROTEINS AND THE 37-kDa\67-kDa LRP\LR IN SIGNAL TRANSDUCTION AND CELL CYCLE

Data from studies performed by (38) demonstrated that the activation, via phosphorylation, of Fyn (a tyrosine kinase) is PrP^c dependent. This activation is supposedly related to neuronal cell maturation, morphology and functions associated with neurotransmitters (38). Fyn is an intracellular protein and PrP^C is bound to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor thereby suggesting that intermediate proteins function in the signaling cascade. Caveolin has been identified as one

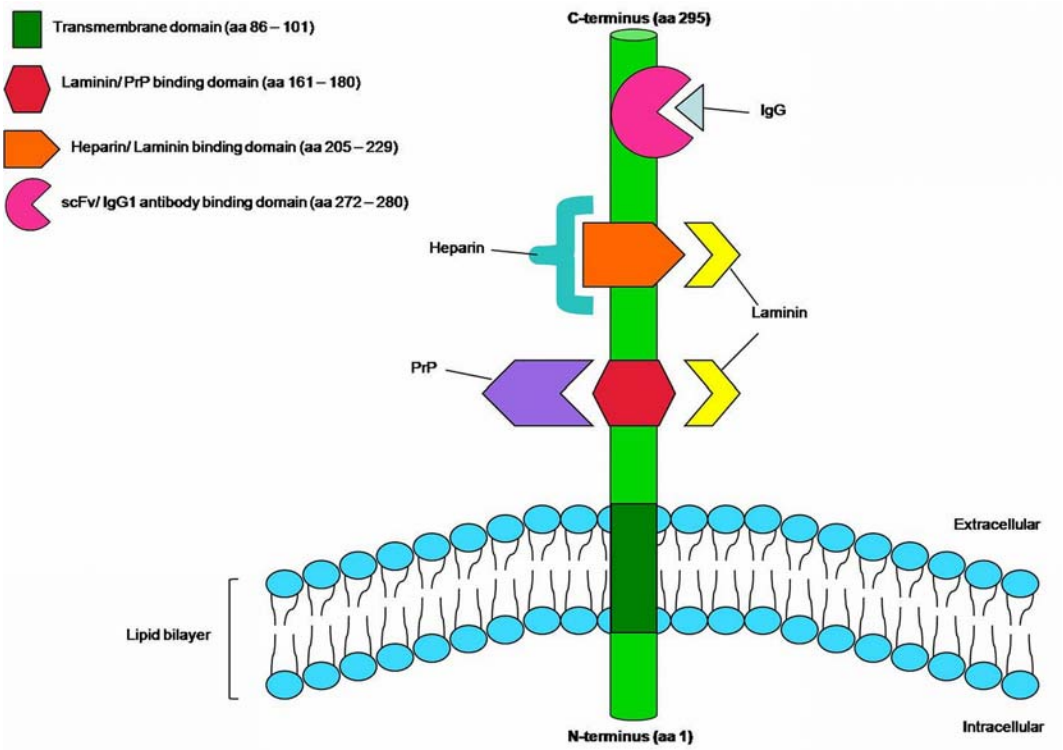


Figure 1. Schematic representation of the 37-kDa laminin receptor precursor (LRP). The 37-kDa LRP is 295 amino acids in length located on the cell surface encompassing four functional domains. A transmembrane domain located between amino acids 86-101 which docks the receptor to the cell membrane. Two laminin binding domains localized between amino 161-180 and 205-229 which also serves as attachment sites for PrP and heparin, respectively. The third binding domain is localized towards the C-terminus of the receptor between amino acids 272-280 and serves as an attachment site for IgG antibodies. Figure modified from (4).

such intermediary protein (38). The C-terminus of the GPI anchor and the dimer formation by the PrP^{c}'s hydrophobic domain confer stress-protection to the cell. The infectious prion protein (PrP^{Sc}) is suggested to induce apoptosis by promoting the phosphorylation of the mitogen-activated protein kinase (MAPK) termed Jun-N-terminal kinase (JNK) (39). However, this PrP^{Sc} activation of JNK is dependent on the presence of the GPI-anchored PrP^{c} (39).

The 37-kDa/67-kDa LRP/LR is suggested to have an influence on cell signaling. The is attributed to its involvement in the progression of the cell cycle, namely from Gap phase (G1) to synthesis phase (S phase) and the suppression of the receptor results in transient cell cycle arrest (24). It has been illustrated that the receptor may suppress the expression of cyclin dependent kinase inhibitors such as p21, tumor suppressors and Survivin (an anti-apoptotic protein) (24). Thus, the receptor may function in anti-apoptotic signaling. Furthermore, evidence showed that 37-kDa/67-kDa LRP\LR induces MMP-2 and enhances the expression of the enzyme MKP-1 which promotes the dephosphorylation of MAKs (40). The 37-kDa/67-kDa LRP\LR inhibits the granulocyte macrophage colony stimulating factor (GM-CSF) receptor (GMR) by interacting with the beta-subunit of the receptor complex (41). GM-CSF is a cytokine and functions in inducing the phosphorylation of MAPK (Erk-1 and Erk-2) and Stat5 (signal transducer and activator of transcription) and promotes tumor necrosis factor-alpha (TNF-alpha) synthesis by neutrophils (41). Therefore, since the 37-kDa/67-kDa LRP\LR inhibits GM-CSF signaling, the aforementioned processes will be hindered.

5. EMPLOYING ANTIBODIES DIRECTED AGAINST 37-kDa/67-kDa LRP/LR AS THERAPEUTIC TOOLS FOR THE TREATMENT OF PRION DISEASES

Antibodies are becoming increasingly favourable as potential therapeutic tools in the treatment of multiple human diseases. This is evident as numerous antibodies, in particular monoclonal antibodies, are being used in clinical trials and have obtained U.S. food and Drug Administration (FDA) approval for therapeutic applications (for example Herceptin™, Avastin ™, Erbitux ™ and Vectibix ™ targeting receptor tyrosine kinases for cancer therapy).

Prion diseases, fatal neurodegenerative diseases, are caused by the modification of the cellular prion protein (PrP^{c}) that results in increased beta-sheet content thereby forming the infectious prion protein (PrP^{Sc}). The 37-kDa/67-kDa laminin receptor has been identified as the receptor for both the aforementioned proteins (13, 14). Thus, antibodies directed against the receptor have been developed as

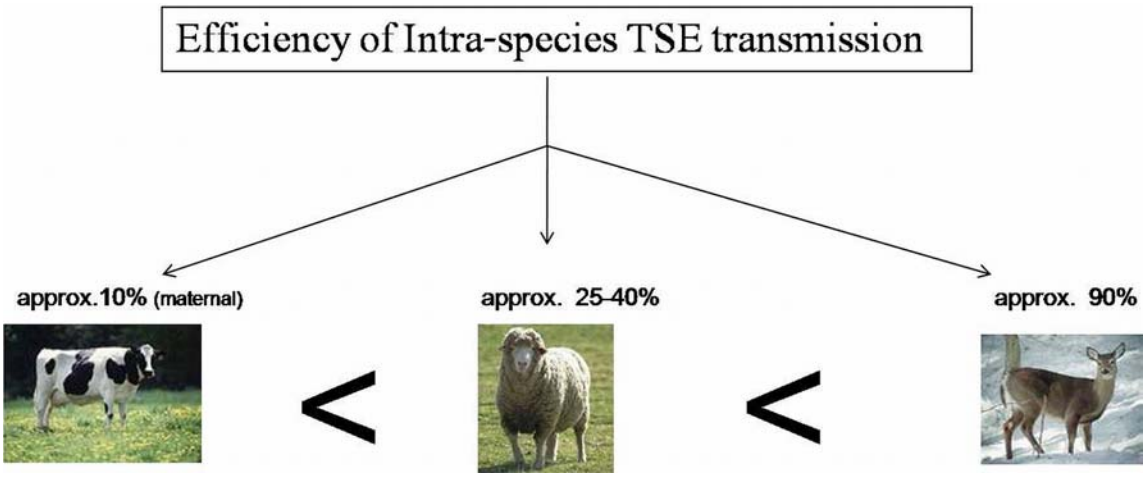


Figure 2. The efficiency of intraspecies transmision of transmissible spongiform encephalopathies (TSEs). Studies in cattle demonstrated that the maternal transmission of BSE to offspring, although at the rate of 10 %, is of statistical significance. The calculated rate of scrapie transmission 25-40% (32) amongst sheep, although greater than that amongst cattle, is still 50-65% lower than those reported in captive deer suffering from CWD (101).

potential therapeutic and prophylactic tools against prion infections and diseases (42-44). W3, a limited polyclonal antibody, represents such an antibody (Figure 3). This antibody has reduced PrP^{Sc} propagation in cell culture (45) and hampered the binding of PrP^{BSE} and PrP27-30 to enterocytes (34). Furthermore, upon passive immune-transfer of the antibodies into PrP^{Sc}- infected mice, a 66% reduction in peripheral propagation of the infectious protein and a 1.8 fold enhancement in the survival of the mice were observed (46).

Single chain anti-LRP antibodies (scFv), referred to as S18, have also been employed in this regard. The size of the antibody, a mere 30-kDa, renders it more effective in tissue penetration and enhances the probability of the antibody passing the Blood Brain barrier (BBB). Upon passive immunotransfer of these antibodies into PrP^{Sc}-infected mice, a 40% reduction of peripheral PrP^{Sc} propagation in the spleen was observed (47). However, the antibodies failed to extend incubation times or survival and this may be attributed to their instability and short half-life (approximately 12 hours). For permanent expression of the single chain antibodies, recombinant Adeno-associated-viruses encoding scFvs directed against LRP/LR were constructed and delivered to the brain of Scrapie infected mice by microinjection (48). Surprisingly, a significant reduction of peripheral PrP^{Sc} propagation was observed in the spleen, which was due to the presence of recombinant AAV in the spleen. This was the first proof for trafficking of recombinant AAVs from the brain to the spleen (48) Unfortunately, also here no significant prolongation of incubation times and survival was recorded, which might be due to the short half-life times of scFvs in blood. As a result thereof, an improved full-length antibody termed IgG1-iS18 was engineered (5). Due to the nature of full-length antibodies with regard to enhanced stability and an approximately 21-day half-life, we proposed that the antibody might be more successful in the survival prolongation of scrapie infected mice.

Epitope mapping has demonstrated that the LRP/LR antibody-binding site (scFvS18) is located at the C-terminus of the receptor (aa 272-280) (47), and not at the direct PrP/LRP-LR binding site located between aa161-180 of LRP (12). This suggests that the antibody prevents the PrP-37-kDa/67-kDa LRP/LR interaction through sterical hindrance as opposed to direct competition.

6. PENTOSAN POLYSULFATE IN THE TREATMENT OF PRION DISEASES

Pentosan polysulfates (PPS), sulfated polyanions (49) which imitate the physiological roles of heparans (50), are amongst the most widely tested and effective drugs used in the treatment of prion diseases. These compounds have been demonstrated to hinder PrP^{Sc} propagation in cell culture (51); significantly prolong incubation times and survival of infected mice (49, 52-55) as well as reduce infectious prion protein deposition and the associated neurodegenerative cerebral alterations (53). It is suggested that PPS compete with endogenous heparan sulphate proteoglycans (HSPGs) (co-receptors for the prion proteins) (12). They thereby inhibit the binding of PrP^{Sc} to 37-kDa/67-kDa LRP/LR (14) (Figure 3) by binding to the receptor's heparan binding site and thus potentially alter the physiological activities of 37-kDa/67-kDa LRP/LR (50). The combined treatment of PPS and Fe (III) meso-tetra (4-sulfonatophenyl)porphine has been shown to further enhance the survival of infected mice (56). However, the efficacy of an oral or intra-peritoneal administration of PPS in the treatment of TSEs is questionable as the compound is unstable and unable to penetrate the blood-brain barrier (54). PPS has been administered via chronic intraventricular infusion in patients diagnosed with vCJD (50, 57) with no clinical improvement. However, recent findings indicate that PPS therapy over a period of 6 months may significantly prolong the mean survival of humans but further in vivo animal experiments are required to assess the efficacy of this treatment.

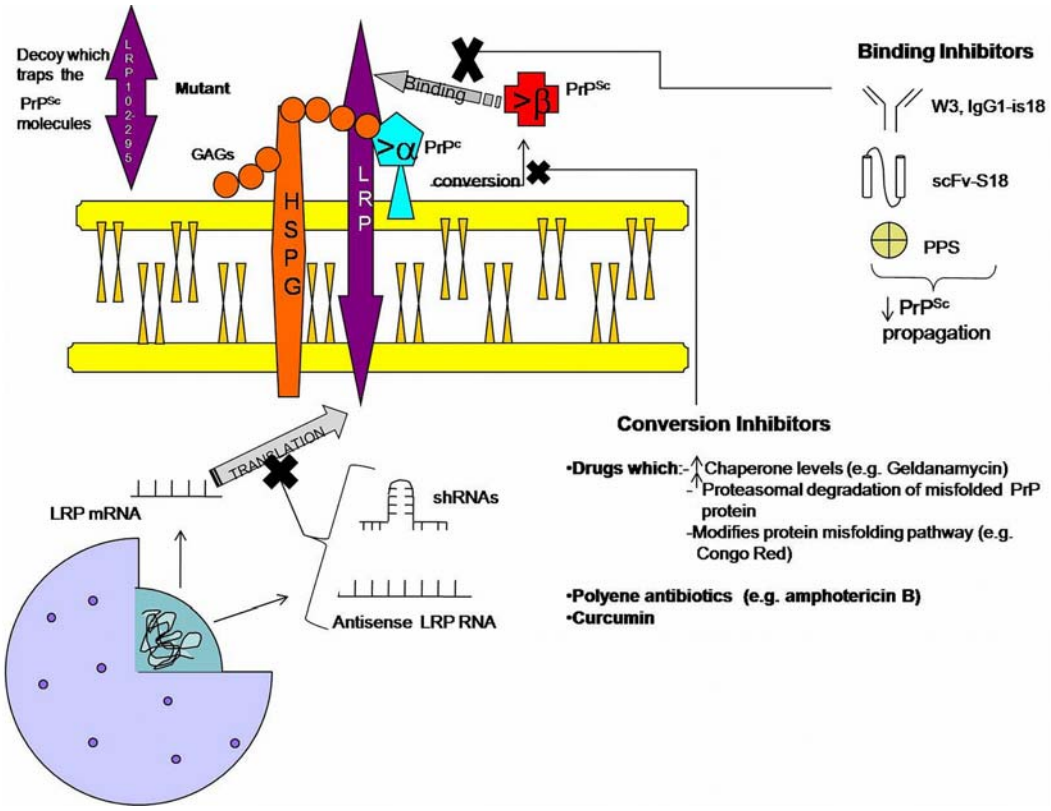


Figure 3. Therapeutic strategies for prion disorders. LRP/LR has been identfied as the receptor for both the cellular (PrP^c) and the infectious prion protein (PrP^{Sc}). Several therapeutic strategies have been developed to inhibit (i) the binding of the PrP^{Sc} to LRP/LR, (ii) conversion of PrP^c to PrP^{Sc} and (iii) the expression LRP/LR mRNA. Antisense RNA and short hairpin RNA have been employed to down-regulate LRP/LR expression and thereby inhibit PrP^{Sc} propagation in scrapie infected cells (45). Lentiviral vector delivery of siRNAs downregulating LRP expression, resulted in a prolonged pre-clinical phase in Scrapie infected mice (61). Blocking the 37-kDa/67-kDa LRP/LR receptor serves as an alternative strategy. Anti-LRP/LR antibodies (full length such as W3 and IgG-is18 and single chain scFv s18) as well as the drug pentosan polysulfate (PPS) have been used in this regard (14, 45-47). Experiments with neuroblastoma cells infected with scrapie illustrate that the LPR/LR mutant (LRP102-295::FLAG) sequesters PrP^{Sc} thus reducing PrP^{Sc} accumulation (62, 63). Curcumin (65) and Polyene antibiotics (66) are compounds that hamper the conversion of PrP^c to PrP^{Sc}. Furthermore, compounds that enhance chaperone activities or the proteosomal degradation of the misfolded protein as well as interfere with the misfolding pathway (67) may be employed as potential tools in prion therapy.

7. THERAPEUTIC INTERVENTIONS UTILIZING RNA INTERFERENCE FOR PRION DISEASE TREATMENT

The presence of cellular prion proteins, PrP^c, is imperative for the propagation of infectious, prion disease causing, PrP^{Sc}. It is a known fact that transgenic mice in which the Prnp gene has been knocked out ($Prp^{0/0}$ mice) are resistant to prion diseases (28) and those in which the protein's expression is reduced exhibit slowed disease progression (58). RNA interference (RNAi) strategies, such as those employing lentiviral PrP specific short hairpin RNAs (shRNAs) have been successful in suppressing the prion protein expression and thus presents an approach for prion disease treatment (58, 59). Similarly, suppression of 37-kDa/67-kDa LRP\LR expression in scrapie infected neuronal cells (ScN2a and ScGTI) (45) inhibits PrP^{Sc} accumulation and has been achieved upon the transfection of the aforementioned cell lines with antisense LRP mRNA and small interfering RNAs (siRNAs) directed against the LRP mRNA (45). In addition, transgenic mice (tgN(NSEasLRP) with reduced 37-kDa/67-kDa LRP\LR levels in the hippocampal and cerebellar brain regions did not exhibit abnormal behaviour (60).

Lentiviral siRNA delivery allows down-regulation of disease associated genes in dividing and embryonic stem cells (58). Lentivector-mediated RNAi was employed to generate chimeric mice expressing lower levels of PrP. In highly chimeric Scrapie infected mice, survival was significantly prolonged (58). Microinjection of lentiviral vectors expressing small interfering RNAs directed against LRP mRNA significantly prolonged the pre-clinical phase in Scrapie infected mice (61). Both reports confirm the cellular prion protein and its 37-kDa/67-kDa laminin receptor as important players in prion pathogenesis and recommend the lentiviral mediated gene transfer system for RNAi expression.

8. LRP102-295 MUTANT AS A DECOY RECEPTOR FOR THE TREATMENT OF PRION DISORDERS

Investigations employing Baby Hamster Kidney (BHK) cells which express and secrete an LRP-mutant which contains merely the extracellular domain of 37-kDa/67-kDa LRP/LR (LRP102-295::FLAG) demonstrate that these cells exhibit reduced PrP27-30 binding (62). The mutant receptor lacks the transmembrane binding domain (aa86-101) and is thereby secreted into the extracellular matrix. However, the mutant still contains the direct PrP binding domain (aa161-180) (12) and is therefore still able to bind to infectious prion proteins (62). The transdominant negative decoy mutant traps PrP^{Sc} molecules and reduces PrP^{Sc} propagation in Scrapie infected neuronal cells (62). Due to the promising results in cell culture, hemizygous transgenic mice were generated expressing the LRP102-295::FLAG mutant in the brain. After Scrapie infection the transgenic mice revealed a significant prolongation of the incubation time compared to wild-type mice (63). Interestingly, at the terminal stage of disease the transgenic mice revealed significantly reduced PrPres levels compared to wild-type mice (63). The results recommend the laminin receptor decoy mutant as an alternative therapeutic vehicle for treatment of prion disorders.

9. OTHER STRATEGIES FOR PRION DISEASE THERAPY

Numerous substances have been investigated as potential tools in prion therapy, and yet despite promising results in vivo commercial therapeutics is non-existent (for review (1-4). Many such substances share a common strategy: hamper the conversion of PrP^{c} to PrP^{Sc} (64). These include: Curcumin, a non-toxic component of turmeric which has the ability to penetrate the BBB (65), polyene antibiotics such as Amphotericin B which indirectly hinder the conversion process by altering the properties of membrane micro-domains (66), Porphyrins, polyamines and anthracyclines (64). Drugs such as geldanamycin, which augments chaperone activity and may thereby reduce levels of the misfolded PrP^{Sc} and Congo Red, binds to β-sheets and interferes with the misfolding pathway, have also been investigated in this regard (67). However, the efficacy of these compounds is low or questionable.

Researchers have also tried to stimulate innate immunity in their attempts to develop effective therapeutics for prion diseases. (68) employed cytidyl guanosyl oligodeoxynucleotides (CpG-ODNs) to stimulate innate immunity. This treatment was effective in prolonging incubation times but the side effects were severe and may have resulted in the destruction of Follicular dendritic cells (FDCs) (64).

10. THE ROLE OF THE PRION PROTEIN AND ITS RECEPTOR IN OTHER NEURODENERATIVE DISEASES

Alzheimer's disease (AD) is the most prevalent type of dementia to affect the aging population. In 2006 this neurodegenerative disease affected more than 25 million people worldwide and it is thought that this number will quadruple within the next 40 years (69). The incidence of AD increases with age, hence, the majority of patients are over 65 years old when disease onset begins. Initial symptoms include memory loss (especially short term) and within a number of years, disease progression leads to dementia that involves both behavioural and cognitive spheres of the brain. This leads to extrapyramidal symptoms, slowed and involuntary movements, uncontrollable speech and loss of bodily functions ultimately leading to death (70).

The molecular mechanisms underlying this neurodegenerative disease involve the formation of neurofibrillary tangles and amyloid plaques (also referred to as senile plaques) on neuronal tissue in the brain, which leads to the degeneration of this tissue. Two main factors have been associated with AD, namely the amyloid beta (A-beta) peptide and tau proteins. Tau is a phospho-protein that functions in the stabilization of microtubules. However, hyperphosphorylation of tau proteins leads to neurofibrillary tangles within neurons and these tangles are implicated in the onset of AD (Figure 4) (71). The amyloid plaques associated with this neurodegeneration are composed of the A-beta peptide that is released during the cleavage of the amyloid precursor protein (APP). There are two different cleavage pathways for membrane bound APP, namely; the non-amyloidogenic and amyloidogenic pathways. In the non-amyloidogenic pathway, the N-terminal of APP is cleaved by alpha-secretase to release sAPP-alpha into the extracellular matrix. The remaining membrane bound C-terminal is subsequently cleaved by gamma-secretase and the cleaved fragment, the soluble p3 peptide, is shed into the extracellular matrix. In the amyloidogenic pathway, the beta-secretase acts instead of the alpha-secretase to cleave the N-terminal of the APP at a different site. The fragment released is thus sAPP-beta. Once the remaining c-terminal is cleaved by the gamma-secretase, a 4-kDa insoluble peptide, A-beta, is released into the extracellular matrix instead of p3 (Figure 5) (72). These A-beta peptides then aggregate to form the amyloid plaques on neuronal tissue that are characteristic of Alzheimer's disease.

Prion proteins (PrP) are proteins that are found in the brain. Though their function is not completely understood, misfolded forms of cellular prion proteins (PrP^{c}) lead to TSEs such as scrapie in sheep, BSE (mad cow disease) in cattle and Creutzfeldt-Jacob disease among others in humans (14, 34). A connection has also been established between prion proteins and AD. Several factors suggest that prions have a protective role against AD. Firstly, it has been found that mutations within the gene for PrP^{c}, such as the $Y125^{stop}$ and $Q160^{stop}$ mutations, result in early onset AD, suggesting the PrP^{c} has a role in preventing or delaying the onset of this disease (73, 74). Subsequent in vitro studies further confirmed this, as, when PrP^{c} was over expressed in neuronal cell lines, the amount of A-beta released by neuronal cells was significantly reduced. Conversely when PrP^{c} was down-regulated with the use of siRNA, the levels of A-beta increased significantly (75). Co-immunoprecipitation studies revealed that this is due to

Figure 4. Paired helical fragments (PHF)-tau containing dystrophic neurites (black) and between 8 and 10 neurofibrillary tangles (NFTs; black). The Alzheimer brain is characterized by two types of lesions, amyloid-beta-containing plaques and neurofibrillary tangles (NFTs). The Gallyas silver impregnation method visualizes the flame-shaped NFTs. In addition it picks up the dystrophic neurites which form a halo around the amyloid plaques. Hence, this method is an indirect means to visualize an amyloid plaque such as the one shown in the center of the micrograph. The micrograph is the courtesy of Prof Juergen Goetz, Sydney, Australia.

an interaction between the beta-secretase and PrP^c, whereby the beta-secretase is in some way inhibited by the presence of PrP^c. The exact mechanism of this inhibition remains elusive as the catalytic ability of the beta-secretase is not reduced and the amount of this enzyme expressed on the surface of the cell remains the same. It is thus probable that PrP^c somehow interferes with the initial steps in the proteolytic processing of APP. This inhibition was found to be most successful when PrP^c is localized in cholesterol-rich lipid rafts and interacting with glycosaminoglycans (75).

The receptor for both infectious and non-infectious prion proteins is 37-kDa/67-kDa LRP/LR. This receptor interacts with PrP^c directly and indirectly via heparan sulfate proteoglycans (12). Since PrP^c plays an important role in the regulation of A-beta secretion, it is possible that the receptor for PrP^c (37-kDa/67-kDa LRP/LR) may also be involved in regulating the pathways involved in the development of Alzheimer's disease.

11. 37-kDa/67-kDa LRP/LR AND CANCER

Cancer is one of the leading causes of mortality worldwide and was found to be responsible for approximately 13% of all deaths worldwide in 2007 (http://www.who.int/cancer/en). Predictions have shown an increased mortality rate resulting from this disease with an estimated 12 million deaths by 2030 (http://www.who.int/cancer/en). The most striking feature of this disease is that it affects people of all ages, races, genders and economic status. Several types of cancer have been identified with the most common in the Western World being lung, breast, prostate and colon cancers (http://info.cancerresearchuk.org). However, this differs to developing countries where the prominent cancer types include cervix and stomach cancer (Figure 6). This variance is due to progress in diagnostic techniques, which is often unaffordable to those in poorer regions. Early diagnosis is possible with the help of magnetic resonance imaging (MRI) and leads to an overall decrease in mortality. Another aspect that plays an influential role in reducing the number of fatalities includes the discovery and consequently the spread of awareness regarding risk factors for cancer. Currently several modes of treatment are available such as surgery, chemotherapy, radiation therapy, immunotherapy and monoclonal antibody therapy. In cases when the tumor becomes metastatic, treatment becomes challenging since various tissues are affected.

Cancer is a disease whereby mutations result in uncontrolled cell growth by either unregulated cell proliferation or the lack of apoptosis. These cells then continue to grow and mutate with the aid of oncogenes or tumor suppressor genes that act by altering cell cycle checkpoint regulators and the DNA repair system (76). It has been shown that changes in the general apoptotic process could have a significant effect on the progression of tumors, suggesting the inactivation of apoptosis at the time of tumor progression (76). The acquired capability of evading apoptosis can be attained through various strategies, with the most common being that of mutations caused by the p53 tumor-suppressing gene (76). Loss of pro-apoptotic regulators occurs as a result of this mutation. It is suspected that most cancer types are able to induce changes that allow for the evasion of apoptosis (Figure 7). A report has hinted that with the use of small interfering RNAs 37-kDa/67-kDa LRP/LR can be inhibited and consequently result in the induction of apoptosis in various cells (23). It was shown that silencing of 37-kDa/67-kDa LRP/LR induces apoptosis in Hep3B cells (23). Thus LRP/LR supports cancer by inhibiting apoptosis.

The degree of metastasis of neoplastic cells is highly dependent on the interaction with 37-kDa/67-kDa LRP/LR, this in turn affects the general laminin mediated basement attachment and consequently local degradation and cell movement. Expression of the mature 67-kDa form of the receptor is found on most normal cells, the immature 37-kDa form had been noted as being an oncofetal antigen. A direct link between the over-expression of this form and an increased invasiveness has been identified (23).

This trend was found particularly in gastric cancer (77), colon carcinoma (78), colorectal carcinoma (79), cervical (80), breast (81), lung (82), ovary (83), pancreatic (84) and prostate cancer (85). This discovery has lead to the suggestion that the expression level of 67-kDa laminin receptors could be a reliable method of prognosis for metastatic tumors (84, 85). Furthermore the over-expression of this receptor could provide an opportunity for cancer therapy directed at this site.

Human fibrosarcoma cells that were pre-treated with an anti-LRP antibody showed significantly reduced

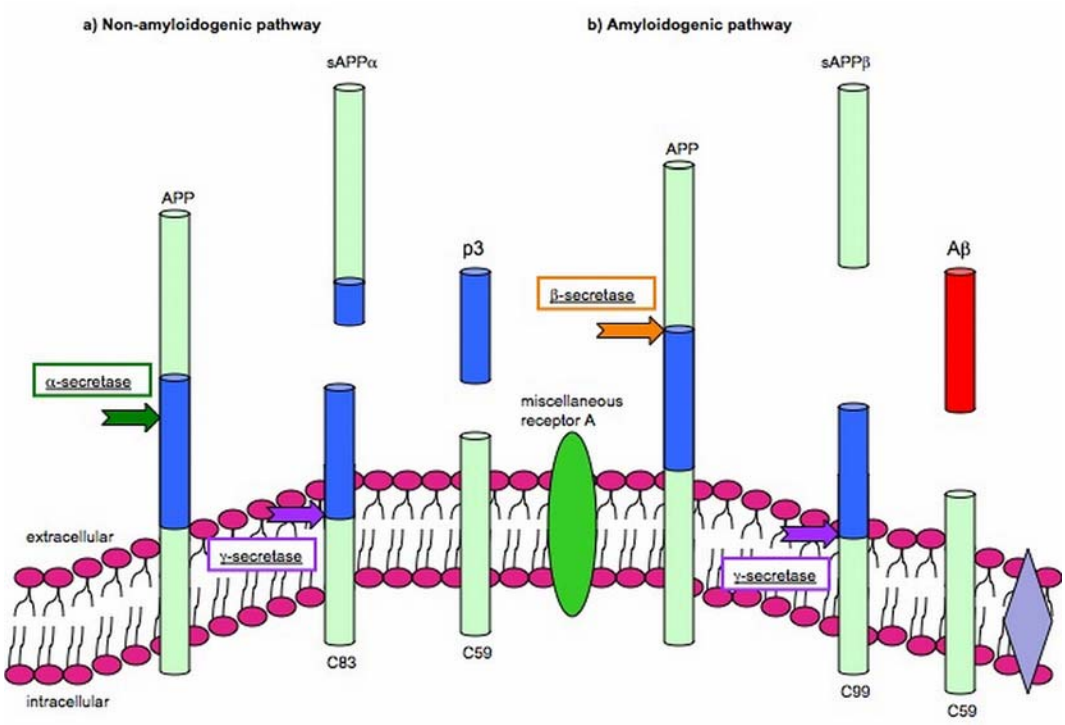


Figure 5. Overview of APP cleavage pathways. **(**a) The non-amyloidogenic pathway is shown. This pathway makes use of the alpha-secretase enzyme belonging to the ADAM (a disintegrin and metalloprotease) family. Alpha-secretase proteolytically cleaves the APP (amyloid precursor protein) to release sAPP-alpha, which is shed into the extracellular matrix. The remaining fragment of the APP (the C-terminal) remains membrane bound and is subsequently cleaved by the gamma-secretase. The gamma-secretase is composed of four sub-units: the catalytic domain is composed of the presenilins (aspartyl proteases) and the remaining subunits are composed of anterior-pharynx defective 1 (APH1), nicastrin and presenilin enhancer (PEN-2). The cleavage of C–terminal of APP by gamma-secretase results in the shedding of p3, a soluble 3-kDa peptide. (b) The amyloidogenic pathway is shown, where beta-secretase cleaves APP to release sAPP-beta. Beta-secretase (beta-site APP cleaving enzyme, BACE1) cleaves at a different site to alpha-secretase, so that when the gamma-secretase cleaves the remaining C-terminal, the fragment that is shed is the insoluble A-beta peptide. The A-beta fragment is 4-kDa in size and accumulates to form amyloid plaques on neuronal tissue which causes the neurodegeneration associated with Alzheimer's disease.

lung metastases compared to untreated cells in a murine model (86). In vivo studies demonstrated that suppression of 37-kDa LRP expression lead to a reduction in the proliferation and tumor formation of lung cancer cells (6).

Most forms of prion disease therapy directed at 37-kDa/67-kDa LRP/LR have the potential to be utilized in tumor intervening therapy. Studies done using human fibrosarcoma cells (HT1080) have illustrated that the blocking or down-regulation of the 37-kDa/67-kDa LRP/LR receptor minimized the invasive potential of these cells (5). Interference of the laminin-37-kDa LRP interaction results in a hindered invasion. Ways in which this can be achieved include anti-37-kDa/67-kDa LRP/LR scFv antibodies, full length format IgG, as well as heparan mimetics and pentosan polysulfate (5). The success of this brings forward the possibility of developing alternative methods capable of reducing the metastatic nature of cancer. Interestingly, current research has shown a definite link between 37-kDa/67-kDa LRP/LR and the maintenance of cell viability (23). An increased level of 37-kDa/67-kDa LRP/LR found on the neoplastic cell line HT1080 (5) in conjunction with the fact that 37-kDa/67-kDa LRP/LR inhibits apoptotic processes (23) indicates that the receptor plays a critical role in the progression of cancer and the development of metastatic tumors.

12. 37-kDa/67-kDa LRP/LR AND VIRAL DISEASES

Viral entry into host's cells is dependent on its interaction cell surface receptors. Indeed, various distinct protein receptors have been identified as initial virus attachment sites for several viruses. For example, the CD4 receptor together with its chemokine co-receptors, CCR5 and CXCR4, have been shown to act as attachment sites for the human immunodeficiency virus (HIV) (87). This AIDS causing retrovirus plays a major role in the worldwide and in South Africa and the virus host cell interaction represents a promising target for therapeutic interventions (88) and might supplement conservative targets such as reverse transcriptase (89-91) (for review: (92)), integrase (93), protease (94) and the budding event (95) (for review: (96, 97)). The complement receptor CR2 acts as a receptor for the Epstein-Barr virus that is associated with infectious mononucleosis and the development of cancer (98). Wang et al. (19) demonstrated that Hamster cells with up-

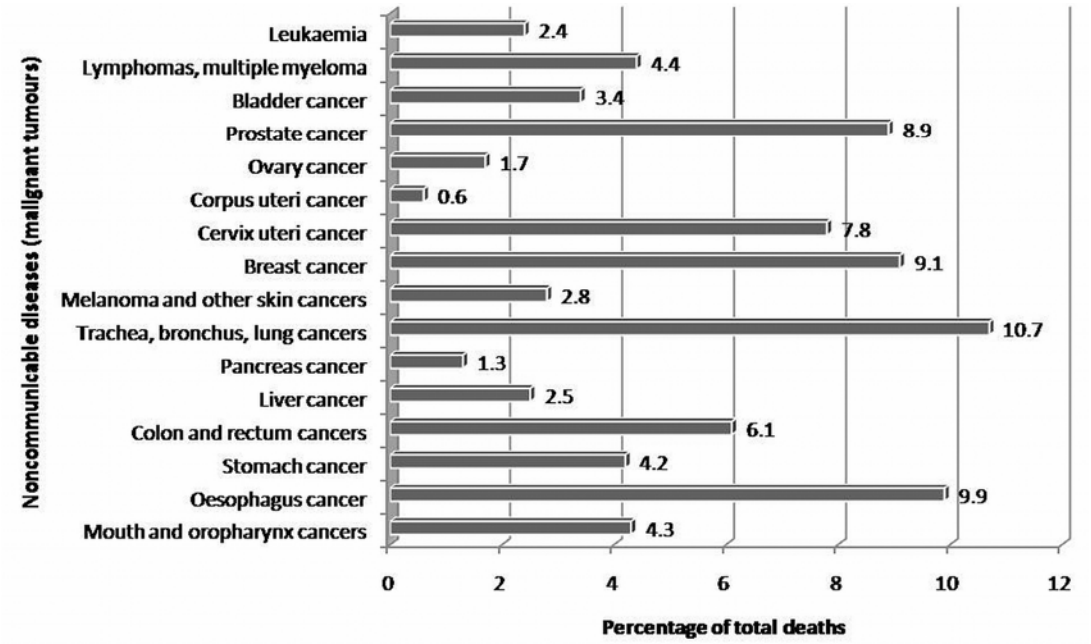


Figure 6. Estimated cancer deaths per 100,000 people by cause, and member state, 2004, Republic of South Africa (RSA) (http://www.who.int/cancer/en/).

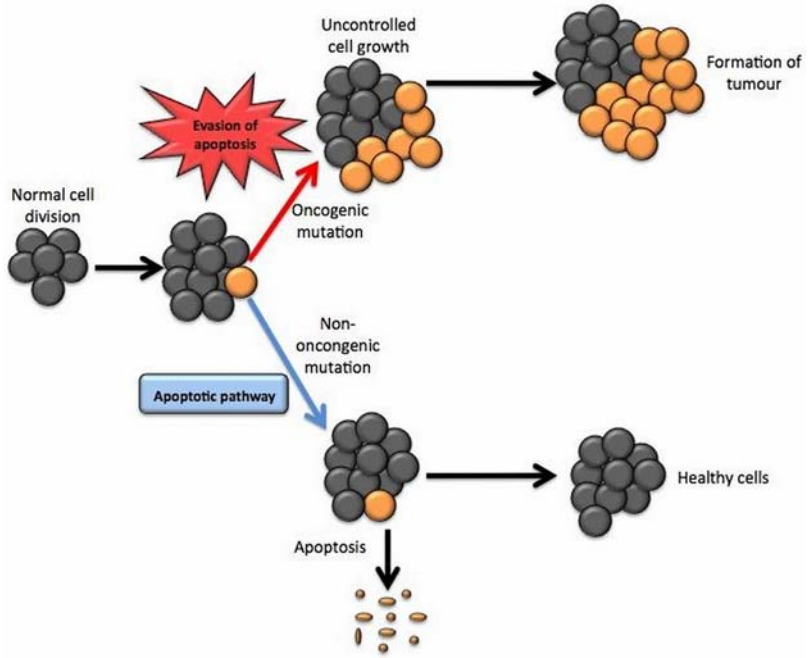


Figure 7. Comparison of normal (lower panel) and tumor (upper panel) cell division/proliferation. Healthy cells are displayed in grey and mutated cells in yellow. With the activation of oncogenes apoptosis is evaded and the tumor progresses. Non-oncogenic mutations induce apoptosis and allow for the continued proliferation of healthy cells.

regulated levels of the laminin receptor were more prone to infection by the Sindbis virus as compared to cells that were expressing lower amounts of the receptor. This led to the identification of the laminin receptor as a receptor for the Sindbis virus. The 37-kDa/67-kDa LRP/LR was also identified as a receptor for (i) dengue virus, which is the causative agent of dengue fever and dengue hemorrhagic fever, in human liver cells (18), porcine kidney cells (99) and mosquito cells (100), respectively, and (ii) for several AAV subtypes (21). The ability of 37-kDa/67-kDa LRP/LR to act as a receptor for various viruses and other pathogens suggests that LRP might also be involved in the attachment and internalization of other viruses as a receptor or co-receptor. Thus, it is imperative to investigate the potential

interaction of the 37-kDa/67-kDa LRP/LR with other viruses that cause fatal human diseases.

13. PERSPECTIVE

The 37-kDa/67-kDa LRP/LR is a key player in major cellular processes and is involved in a many diseases such as metastatic cancer, neurodegenerative diseases and viral infections. Recent findings describing a major role of the receptor in signal transduction processes, apoptosis and cell cycle progression together with the proven finding that 37-kDa/67-kDa LRP/LR plays critical roles in invasion and cell adhesion, two key components in metastatic tumors recommends the receptor as an alternative target for therapeutic intervention in many important metastatic cancer types worldwide. The 37-kDa/67-kDa LRP/LR functions moreover as a receptor for prions and recent research indicates that blocking or down-regulating the receptor results in prolonged incubation times (pre-clinical phases) and/or survival. Since 37-kDa/67-kDa LRP/LR interacts with the misfolded prion protein, which has a proposed protective effect in Alzheimer's disease, a possible role of the receptor in other neurodegenerative protein misfolding diseases such as Morbus Alzheimer might be conceivable. Since the receptor acts as a cell surface receptor for a series of different viruses (e.g. Sindbis, Dengue and Adeno-associated virus), targeting the receptor might represent an alternative therapeutic intervention for the treatment of viral diseases. Especially antibodies targeting 37-kDa/67-kDa LRP/LR might become effective alternative tools for the treatment of all kind of diseases associated with or caused by the non-integrin laminin receptor 37-kDa/67-kDa LRP/LR.

14. ACKNOWLEDGEMENTS

Vusi Mbazima, and Bianca Da Costa Dias contributed equally to this work. We thank the Deutsche Forschungsgemeinschaft (DFG) grant WE 2664/2-1, Germany and the National Research Foundation (NRF), Republic of South Africa (RSA) for financial support. We thank Prof Juergen Goetz, Director, Alzheimer's and Parkinson's Disease Laboratory, Brain and Mind Research Institute, University of Sidney, Australia, for providing a micrograph of neurofibrillary tangles.

15. REFERENCES

1. K. Vana, C. Zuber, D. Nikles and S. Weiss: Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy. *Cell Mol Neurobiol*, 27(1), 107-28 (2007)

2. C. Zuber, H. Ludewigs and S. Weiss: Therapeutic approaches targeting the prion receptor LRP/LR. *Vet Microbiol*, 123(4), 387-93 (2007)

3. H. Ludewigs, C. Zuber, K. Vana, D. Nikles, I. Zerr and S. Weiss: Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther*, 5(4), 613-30 (2007)

4. K. Vana, C. Zuber, H. Pflanz, D. Kolodziejczak, G. Zemora, A. K. Bergmann and S. Weiss: LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. *Infect Disord Drug Target*s, 9(1), 69-80 (2009)

5. C. Zuber, S. Knackmuss, G. Zemora, U. Reusch, E. Vlasova, D. Diehl, V. Mick, K. Hoffmann, D. Nikles, T. Frohlich, G. J. Arnold, B. Brenig, E. Wolf, H. Lahm, M. Little and S. Weiss: Invasion of Tumorigenic HT1080 Cells Is Impeded by Blocking or Downregulating the 37-kDa/67-kDa Laminin Receptor. *J Mol Biol*, 378(3), 530-539 (2008)

6. S. Menard, E. Tagliabue and M. I. Colnaghi: The 67 kDa laminin receptor as a prognostic factor in human cancer. *Breast Cancer Res Treat*, 52(1-3), 137-45 (1998)

7. K. Satoh, K. Narumi, T. Abe, T. Sakai, T. Kikuchi, M. Tanaka, T. Shimo-Oka, M. Uchida, F. Tezuka, M. Isemura and T. Nukiwa: Diminution of 37-kDa laminin binding protein expression reduces tumour formation of murine lung cancer cells. *Br J Cancer*, 80(8), 1115-22 (1999)

8. D. Umeda, S. Yano, K. Yamada and H. Tachibana: Green tea polyphenol epigallocatechin-3-gallate signaling pathway through 67-kDa laminin receptor. *J Biol Chem*, 283(6), 3050-8 (2008)

9. E. Ardini, G. Pesole, E. Tagliabue, A. Magnifico, V. Castronovo, M. E. Sobel, M. I. Colnaghi and S. Menard: The 67-kDa laminin receptor originated from a ribosomal protein that acquired a dual function during evolution. *Mol Biol Evol*, 15(8), 1017-25 (1998)

10. T. H. Landowski, E. A. Dratz and J. R. Starkey: Studies of the structure of the metastasis-associated 67 kDa laminin binding protein: fatty acid acylation and evidence supporting dimerization of the 32 kDa gene product to form the mature protein. *Biochemistry*, 34(35), 11276-87 (1995)

11. S. Buto, E. Tagliabue, E. Ardini, A. Magnifico, C. Ghirelli, F. van den Brule, V. Castronovo, M. I. Colnaghi, M. E. Sobel and S. Menard: Formation of the 67-kDa laminin receptor by acylation of the precursor. *J Cell Biochem*, 69(3), 244-51 (1998)

12. C. Hundt, J. M. Peyrin, S. Haik, S. Gauczynski, C. Leucht, R. Rieger, M. L. Riley, J. P. Deslys, D. Dormont, C. I. Lasmezas and S. Weiss: Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *Embo J*, 20(21), 5876-86 (2001)

13. S. Gauczynski, J. M. Peyrin, S. Haik, C. Leucht, C. Hundt, R. Rieger, S. Krasemann, J. P. Deslys, D. Dormont, C. I. Lasmezas and S. Weiss: The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *Embo J*, 20(21), 5863-75 (2001)

14. S. Gauczynski, D. Nikles, S. El-Gogo, D. Papy-Garcia, C. Rey, S. Alban, D. Barritault, C. I. Lasmezas

and S. Weiss: The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis*, 194(5), 702-9 (2006)

15. C. A. Kirkpatrick and S. B. Selleck: Heparan sulfate proteoglycans at a glance. *J Cell Sci*, 120(Pt 11), 1829-32 (2007)

16. C. Urbinati, S. Nicoli, M. Giacca, G. David, S. Fiorentini, A. Caruso, M. Alfano, L. Cassetta, M. Presta and M. Rusnati: HIV-1 Tat and heparan sulfate proteoglycan interaction: a novel mechanism of lymphocyte adhesion and migration across the endothelium. *Blood*, 114(15), 3335-42 (2009)

17. A. D. Snow, R. T. Sekiguchi, D. Nochlin, R. N. Kalaria and K. Kimata: Heparan sulfate proteoglycan in diffuse plaques of hippocampus but not of cerebellum in Alzheimer's disease brain. *Am J Pathol*, 144(2), 337-47 (1994)

18. C. Thepparit and D. R. Smith: Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. *J Virol*, 78(22), 12647-56 (2004)

19. K. S. Wang, R. J. Kuhn, E. G. Strauss, S. Ou and J. H. Strauss: High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. *J Virol*, 66(8), 4992-5001 (1992)

20. G. V. Ludwig, J. P. Kondig and J. F. Smith: A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. *J Virol*, 70(8), 5592-9 (1996)

21. B. Akache, D. Grimm, K. Pandey, S. R. Yant, H. Xu and M. A. Kay: The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *J Virol*, 80(19), 9831-6 (2006)

22. H. Tachibana, K. Koga, Y. Fujimura and K. Yamada: A receptor for green tea polyphenol EGCG. *Nat Struct Mol Biol*, 11(4), 380-1 (2004)

23. T. Susantad and D. R. Smith: siRNA-Mediated silencing of the 37/67-kDa high affinity laminin receptor in Hep3B cells induces apoptosis. *Cell Mol Biol Lett*, 13(3), 452-464 (2008)

24. J. Scheiman, J. C. Tseng, Y. Zheng and D. Meruelo: Multiple functions of the 37/67-kd laminin receptor make it a suitable target for novel cancer gene therapy. *Mol Ther*, 18(1), 63-74 (2010)

25. D. Auth and G. Brawerman: A 33-kDa polypeptide with homology to the laminin receptor: component of translation machinery. *Proc Natl Acad Sci U S A*, 89(10), 4368-72 (1992)

26. M. Sato, K. Kinoshita, Y. Kaneda, Y. Saeki, A. Iwamatsu and K. Tanaka: Analysis of nuclear localization of laminin binding protein precursor p40 (LBP/p40). *Biochem Biophys Res Commun*, 229(3), 896-901 (1996)

27. D. Nikles, K. Vana, S. Gauczynski, H. Knetsch, H. Ludewigs and S. Weiss: Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins. *Biochim Biophys Acta*, 1782(5), 335-40 (2008)

28. H. Bueler, A. Aguzzi, A. Sailer, R. A. Greiner, P. Autenried, M. Aguet and C. Weissmann: Mice devoid of PrP are resistant to scrapie. *Cell*, 73(7), 1339-47 (1993)

29. J. W. Wilesmith, G. A. Wells, J. B. Ryan, D. Gavier-Widen and M. M. Simmons: A cohort study to examine maternally-associated risk factors for bovine spongiform encephalopathy. *Vet Rec*, 141(10), 239-43 (1997)

30. I. H. Pattison: Scrapie in the welsh mountain breed of sheep and its experimental transmission to goats. *Vet Rec*, 77(47), 1388-90 (1965)

31. W. Kremer, N. Kachel, K. Kuwata, K. Akasaka and H. R. Kalbitzer: Species-specific differences in the intermediate states of human and Syrian hamster prion protein detected by high pressure NMR spectroscopy. *J Biol Chem*, 282(31), 22689-98 (2007)

32. J. L. Hourrigan, A. Klingsporn, W. W. Clark and M. deCamp: Epidemiology of scrapie in the Untited States. In: S.B Prusiner, W.J. Hadlow (eds) Slow transmissible diseases of the nervous system: clinical, epidemiological, genetic and pathological aspects of the spongiform encephalopathies. Academic Press, New York, 1, 331-356 (1979)

33. B. Da Costa Dias and S. F. T. Weiss: A kiss of a prion: New implications for oral transmissibility. *J infectious Diseases* (in press)

34. E. Morel, T. Andrieu, F. Casagrande, S. Gauczynski, S. Weiss, J. Grassi, M. Rousset, D. Dormont and J. Chambaz: Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. *Am J Pathol*, 167(4), 1033-42 (2005)

35. A. Aguzzi, M. Heikenwalder and M. Polymenidou: Insights into prion strains and neurotoxicity. *Nat Rev Mol Cell Biol*, 8(7), 552-61 (2007)

36. R. Bradley and J. W. Wilesmith: Epidemiology and control of bovine spongiform encephalopathy (BSE). *Br Med Bull*, 49(4), 932-59 (1993)

37. M. M. Robinson, W. J. Hadlow, T. P. Huff, G. A. Wells, M. Dawson, R. F. Marsh and J. R. Gorham: Experimental infection of mink with bovine spongiform encephalopathy. *J Gen Virol*, 75 (Pt 9), 2151-5 (1994)

38. S. Mouillet-Richard, M. Ermonval, C. Chebassier, J. L. Laplanche, S. Lehmann, J. M. Launay and O. Kellermann: Signal transduction through prion protein. *Science*, 289(5486), 1925-8 (2000)

39. A. S. Rambold, V. Muller, U. Ron, N. Ben-Tal, K. F. Winklhofer and J. Tatzelt: Stress-protective signalling of prion protein is corrupted by scrapie prions. *Embo J*, 27(14), 1974-84 (2008)

40. D. Givant-Horwitz, B and Reich, R: Laminin-induced signaling in tumor cells: the role of the M_r 67,000 laminin receptor. *Cancer Res*, 64, 3572-3579 (2004)

41. J. Chen, J. M. Carcamo, O. Borquez-Ojeda, H. Erdjument-Bromage, P. Tempst and D. W. Golde: The laminin receptor modulates granulocyte-macrophage colony-stimulating factor receptor complex formation and modulates its signaling. *Proc Natl Acad Sci U S A*, 100(24), 14000-5 (2003)

42. A. R. White, P. Enever, M. Tayebi, R. Mushens, J. Linehan, S. Brandner, D. Anstee, J. Collinge and S. Hawke: Monoclonal antibodies inhibit prion replication and delay the development of prion disease. *Nature*, 422(6927), 80-3 (2003)

43. N. M. Hooper: Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). *Mol Membr Biol*, 16(2), 145-56 (1999)

44. V. Perrier, J. Solassol, C. Crozet, Y. Frobert, C. Mourton-Gilles, J. Grassi and S. Lehmann: Anti-PrP antibodies block PrPSc replication in prion-infected cell cultures by accelerating PrPC degradation. *J Neurochem*, 89(2), 454-63 (2004)

45. C. Leucht, S. Simoneau, C. Rey, K. Vana, R. Rieger, C. I. Lasmezas and S. Weiss: The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. *EMBO Rep*, 4(3), 290-5 (2003)

46. C. Zuber, Mitteregger, G., Pace, C., Zerr, I., Kretzschmar, H.A., Weiss, S.: Anti-LRP/LR Antibody W3 hampers peripheral PrPSc propagation in scrapie infected mice. *Prion*, 1(3), 207-212 (2007)

47. C. Zuber, S. Knackmuss, C. Rey, U. Reusch, P. Rottgen, T. Frohlich, G. J. Arnold, C. Pace, G. Mitteregger, H. A. Kretzschmar, M. Little and S. Weiss: Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases. *Mol Immuno*l, 45(1), 144-51 (2008)

48. C. Zuber, Mitteregger, G., Schuhmann, N., Rey, C., Knackmuss, S., Rupprecht, W., Reusch, U., Pace, C., Little, M., Kretzschmar, H.A., Hallek, M., Büning, H., Weiss, S.: Delivery of single chain antibodies scFvs directed against the 37 kDa/67 kDa laminin receptor into mice via recombinant Adeno-associated viral vectors for prion disease gene therapy. *J Gen Virol*, 89, 2054-2060 (2008)

49. C. Larramendy-Gozalo, A. Barret, E. Daudigeos, E. Mathieu, L. Antonangeli, C. Riffet, E. Petit, D. Papy-Garcia, D. Barritault, P. Brown and J. P. Deslys: Comparison of CR36, a new heparan mimetic, and pentosan polysulfate in the treatment of prion diseases. *J Gen Virol*, 88(Pt 3), 1062-7 (2007)

50. S. Dealler and N. G. Rainov: Pentosan polysulfate as a prophylactic and therapeutic agent against prion disease. *IDrugs*, 6(5), 470-8 (2003)

51. S. L. Shyng, S. Lehmann, K. L. Moulder and D. A. Harris: Sulfated glycans stimulate endocytosis of the cellular isoform of the prion protein, PrPC, in cultured cells. *J Biol Chem*, 270(50), 30221-9 (1995)

52. H. Diringer and B. Ehlers: Chemoprophylaxis of scrapie in mice. *J Gen Virol*, 72 (Pt 2), 457-60 (1991)

53. K. Doh-ura, K. Ishikawa, I. Murakami-Kubo, K. Sasaki, S. Mohri, R. Race and T. Iwaki: Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. *J Virol*, 78(10), 4999-5006 (2004)

54. C. Farquhar, A. Dickinson and M. Bruce: Prophylactic potential of pentosan polysulphate in transmissible spongiform encephalopathies. *Lancet*, 353(9147), 117 (1999)

55. A. Ladogana, P. Casaccia, L. Ingrosso, M. Cibati, M. Salvatore, Y. G. Xi, C. Masullo and M. Pocchiari: Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. *J Gen Viro*l, 73 (Pt 3), 661-5 (1992)

56. B. Caughey and G. J. Raymond: Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J Virol*, 67(2), 643-50 (1993)

57. D. A. Kocisko, B. Caughey, J. D. Morrey and R. E. Race: Enhanced antiscrapie effect using combination drug treatment. *Antimicrob Agents Chemother*, 50(10), 3447-9 (2006)

58. A. Pfeifer, S. Eigenbrod, S. Al-Khadra, A. Hofmann, G. Mitteregger, M. Moser, U. Bertsch and H. Kretzschmar: Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. *J Clin Invest*, 116(12), 3204-10 (2006)

59. M. C. Golding, C. R. Long, M. A. Carmell, G. J. Hannon and M. E. Westhusin: Suppression of prion protein in livestock by RNA interference. *Proc Natl Acad Sci U S A*, 103(14), 5285-90 (2006)

60. C. Leucht, K. Vana, I. Renner-Muller, D. Dormont, C. I. Lasmezas, E. Wolf and S. Weiss: Knock-down of the 37-kDa/67-kDa laminin receptor in mouse brain by transgenic expression of specific antisense LRP RNA. *Transgenic Res*, 13(1), 81-5 (2004)

61. H. Pflanz, K. Vana, G. Mitteregger, C. Pace, D. Messow, C. Sedlaczek, D. Nikles, H. A. Kretzschmar and S. F. Weiss: Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor

precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. *J Gen Virol*, 90(Pt 1), 269-74 (2009)

62. K. Vana and S. Weiss: A Trans-dominant Negative 37kDa/67kDa Laminin Receptor Mutant Impairs PrP(Sc) Propagation in scrapie-infected Neuronal Cells. *J Mol Biol*, 358, 57-66 (2006)

63. H. Pflanz, K. Vana, G. Mitteregger, I. Renner-Muller, C. Pace, H. Kuchenhoff, H. A. Kretzschmar, E. Wolf and S. Weiss: Scrapie-infected transgenic mice expressing a laminin receptor decoy mutant reveal a prolonged incubation time associated with low levels of PrPres. *J Mol Biol*, 388(4), 721-9 (2009)

64. A. Aguzzi, M. Heikenwalder and G. Miele: Progress and problems in the biology, diagnostics, and therapeutics of prion diseases. *J Clin Invest*, 114(2), 153-60 (2004)

65. B. Caughey, L. D. Raymond, G. J. Raymond, L. Maxson, J. Silveira and G. S. Baron: Inhibition of protease-resistant prion protein accumulation in vitro by curcumin. *J Virol*, 77(9), 5499-502 (2003)

66. A. Mange, N. Nishida, O. Milhavet, H. E. McMahon, D. Casanova and S. Lehmann: Amphotericin B inhibits the generation of the scrapie isoform of the prion protein in infected cultures. *J Virol*, 74(7), 3135-40 (2000)

67. C. A. Ross and M. A. Poirier: Protein aggregation and neurodegenerative disease. *Nat Med*, 10 Suppl, S10-7 (2004)

68. M. Heikenwalder, M. Polymenidou, T. Junt, C. Sigurdson, H. Wagner, S. Akira, R. Zinkernagel and A. Aguzzi: Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat Med*, 10(2), 187-92 (2004)

69. C. Qiu, D. De Ronchi and L. Fratiglioni: The epidemiology of the dementias: an update. *Curr Opin Psychiatry*, 20(4), 380-5 (2007)

70. D. M. Walsh and D. J. Selkoe: Deciphering the molecular basis of memory failure in Alzheimer's disease. *Neuron*, 44(1), 181-93 (2004)

71. C. Ballatore, V. M. Lee and J. Q. Trojanowski: Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci*, 8(9), 663-72 (2007)

72. S. S. Sisodia and D. L. Price: Role of the beta-amyloid protein in Alzheimer's disease. *Faseb J*, 9(5), 366-70 (1995)

73. U. Finckh, T. Muller-Thomsen, U. Mann, C. Eggers, J. Marksteiner, W. Meins, G. Binetti, A. Alberici, C. Hock, R. M. Nitsch and A. Gal: High prevalence of pathogenic mutations in patients with early-onset dementia detected by sequence analyses of four different genes. *Am J Hum Genet*, 66(1), 110-7 (2000)

74. T. Kitamoto, R. Iizuka and J. Tateishi: An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques. *Biochem Biophys Res Commun*, 192(2), 525-31 (1993)

75. E. T. Parkin, N. T. Watt, I. Hussain, E. A. Eckman, C. B. Eckman, J. C. Manson, H. N. Baybutt, A. J. Turner and N. M. Hooper: Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U S A*, 104(26), 11062-7 (2007)

76. D. Hanahan and R. A. Weinberg: The hallmarks of cancer. *Cell*, 100(1), 57-70 (2000)

77. G. de Manzoni, A. Guglielmi, G. Verlato, A. Tomezzoli, G. Pelosi, I. Schiavon and C. Cordiano: Prognostic significance of 67-kDa laminin receptor expression in advanced gastric cancer. *Oncology*, 55(5), 456-60 (1998)

78. V. Cioce, V. Castronovo, B. M. Shmookler, S. Garbisa, W. F. Grigioni, L. A. Liotta and M. E. Sobel: Increased expression of the laminin receptor in human colon cancer. *J Natl Cancer Inst*, 83(1), 29-36 (1991)

79. X. Sanjuan, P. L. Fernandez, R. Miquel, J. Munoz, V. Castronovo, S. Menard, A. Palacin, A. Cardesa and E. Campo: Overexpression of the 67-kD laminin receptor correlates with tumour progression in human colorectal carcinoma. *J Pathol*, 179(4), 376-80 (1996)

80. W. al-Saleh, P. Delvenne, F. A. van den Brule, S. Menard, J. Boniver and V. Castronovo: Expression of the 67 KD laminin receptor in human cervical preneoplastic and neoplastic squamous epithelial lesions: an immunohistochemical study. *J Pathol*, 181(3), 287-93 (1997)

81. M. Nadji, M. Nassiri, M. Fresno, E. Terzian and A. R. Morales: Laminin receptor in lymph node negative breast carcinoma. *Cancer*, 85(2), 432-6 (1999)

82. G. Fontanini, S. Vignati, S. Chine, M. Lucchi, A. Mussi, C. A. Angeletti, S. Menard, V. Castronovo and G. Bevilacqua: 67-Kilodalton laminin receptor expression correlates with worse prognostic indicators in non-small cell lung carcinomas. *Clin Cancer Res*, 3(2), 227-31 (1997)

83. F. A. van den Brule, A. Berchuck, R. C. Bast, F. T. Liu, C. Gillet, M. E. Sobel and V. Castronovo: Differential expression of the 67-kD laminin receptor and 31-kD human laminin-binding protein in human ovarian carcinomas. *Eur J Cancer*, 30A(8), 1096-9 (1994)

84. G. Pelosi, F. Pasini, E. Bresaola, G. Bogina, P. Pederzoli, S. Biolo, S. Menard and G. Zamboni: High-

affinity monomeric 67-kD laminin receptors and prognosis in pancreatic endocrine tumours. *J Pathol*, 183(1), 62-9 (1997)

85. D. Waltregny, L. de Leval, S. Menard, J. de Leval and V. Castronovo: Independent prognostic value of the 67-kd laminin receptor in human prostate cancer. *J Natl Cancer Inst*, 89(16), 1224-7 (1997)

86. G. Gasparini, M. Barbareschi, P. Boracchi, P. Bevilacqua, P. Verderio, P. Dalla Palma and S. Menard: 67-kDa laminin-receptor expression adds prognostic information to intra-tumoral microvessel density in node-negative breast cancer. *Int J Cancer*, 60(5), 604-10 (1995)

87. G. Alkhatib and E. A. Berger: HIV coreceptors: from discovery and designation to new paradigms and promise. *Eur J Med Res*, 12(9), 375-84 (2007)

88. A. E. Proudfoot, C. A. Power and M. K. Schwarz: Anti-chemokine small molecule drugs: a promising future? *Expert Opin Investig Drugs*, 19(3), 345-55 (2010)

89. S. Weiss, B. Konig, Y. Morikawa and I. Jones: Recombinant HIV-1 nucleocapsid protein p15 produced as a fusion protein with glutathione S-transferase in Escherichia coli mediates dimerization and enhances reverse transcription of retroviral RNA. *Gene*, 121(2), 203-12 (1992)

90. S. Weiss, B. Konig, H. J. Muller, H. Seidel and R. S. Goody: Synthetic human tRNA(UUULys3) and natural bovine tRNA(UUULys3) interact with HIV-1 reverse transcriptase and serve as specific primers for retroviral cDNA synthesis. *Gene*, 111(2), 183-97 (1992)

91. S. Weiss, G. Hausl, M. Famulok and B. Konig: The multimerization state of retroviral RNA is modulated by ammonium ions and affects HIV-1 full-length cDNA synthesis in vitro. *Nucleic Acids Res*, 21(21), 4879-85 (1993)

92. S. G. Sarafianos, B. Marchand, K. Das, D. M. Himmel, M. A. Parniak, S. H. Hughes and E. Arnold: Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. *J Mol Biol*, 385(3), 693-713 (2009)

93. N. J. Anthony: HIV-1 integrase: a target for new AIDS chemotherapeutics. *Curr Top Med Chem*, 4(9), 979-90 (2004)

94. A. M. Wensing, N. M. van Maarseveen and M. Nijhuis: Fifteen years of HIV Protease Inhibitors: raising the barrier to resistance. *Antiviral Res*, 85(1), 59-74

95. H. Chen, X. Liu, Z. Li, P. Zhan and E. De Clercq: TSG101: a novel anti-HIV-1 drug target. *Curr Med Chem*, 17(8), 750-8 (2010)

96. W. Coley, K. Kehn-Hall, R. Van Duyne and F. Kashanchi: Novel HIV-1 therapeutics through targeting altered host cell pathways. *Expert Opin Biol Ther*, 9(11), 1369-82 (2009)

97. S. Bhattacharya and H. Osman: Novel targets for anti-retroviral therapy. *J Infect*, 59(6), 377-86 (2009)

98. N. R. Cooper, B. M. Bradt, J. S. Rhim and G. R. Nemerow: CR2 complement receptor. *J Invest Dermatol*, 94(6 Suppl), 112S-117S (1990)

99. P. H. Tio, W. W. Jong and M. J. Cardosa: Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3. *Virol J*, 2(1), 25 (2005)

100. P. Sakoonwatanyoo, V. Boonsanay and D. R. Smith: Growth and production of the dengue virus in C6/36 cells and identification of a laminin-binding protein as a candidate serotype 3 and 4 receptor protein. *Intervirology*, 49(3), 161-72 (2006)

101. C. J. Sigurdson and M. W. Miller: Other animal prion diseases. *Br Med Bull*, 66, 199-212 (2003)

Abbreviations: aa: amino acid; A-beta: amyloid beta protein; AD: Alzheimer's diseases; APP: amyloid precursor protein; AAV: Adeno-associated virus, BACE: beta-site APP cleaving enzyme; BBB: blood brain barrier; BSE: Bovine Spongiform Encephalopathy; CJD: Creutzfeldt-Jakob Disease; CWD: Chronic Wasting Disease; GAG: Glycosaminoglycan; LRP: laminin receptor precursor; LR: laminin receptor; MRI: magnetic resonance imaging; LRP/LR: Laminin receptor precursor/laminin receptor; PHF: paired helical fragments; PrP: prion protein; PrP^{c}: cellular prion protein; PrP^{Sc}: scrapie form of prion protein; PrP27-30: protease resistant prion protein; GPI: glycosylphosphatidylinositol; TSE: Transmissible Spongiform Encephalopathy; PPS: pentosan polysulfate; WHO: World Health Organisation.

Key Words: AAV, Adeno-associated virus, alphaviridae, adhesion, Alzheimer's disease, Apoptosis, Cancer, Cell Cycle, Dengue virus, ECM, HIV, human immunodeficiency virus, laminin receptor, lentiviral vectors, LRP/LR, metastasis, prion, PrP, signal transduction, Sindbis virus, Venezuelean Equine Encephalitis virus, Zoonosis, Cell Adhesion, Amyloid, Review

Send correspondence to: Prof Stefan F T Weiss, School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, South Africa, Tel: 2711-7176346, Fax: 2711-7176351, E-mail: stefan.weiss@wits.ac.za

http://www.bioscience.org/current/vol15.htm

Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor

Aadilah Omar*, Katarina Jovanovic*, Bianca Da Costa Dias*, Danielle Gonsalves, Kiashanee Moodley, Robert Caveney, Vusi Mbazima & Stefan FT Weiss

Expert Opinion on Therapeutic Patents 21 (2011) 35-53.

This article reviewed some of patents focused on targeting LRP/LR for therapeutic intervention in diseases such cancer, prion disorders, Alzheimer's disease as well as viral, bacterial and yeast infections. Patents for anti-LRP/LR antibodies as well as a vaccine formulated to target immature LRP are discussed in the context of cancer treatments. Patents for anti-LRP siRNA, LRP mutant receptor decoys, polysulfated polyanions and antibodies targeting LRP/LR are all reviewed as treatments for Prion disorders. Patents for the treatment of Alzheimer's disease include vaccines with laminin and Aβ binding fragments of laminin since this extracellular matrix protein exhibits an Aβ binding site and inhibits Aβ fibrillation. Alternate therapies for the treatment of bacterial diseases such as meningitis (where bacteria use LRP/LR as a receptor) and viral infection (caused by Sindbis and Dengue viruses which also use LRP/LR as a receptor) are also briefly examined. Overall, this paper provides an easily accessible overview of the current patented therapeutic options targeting LRP/LR in various diseases.

* These authors contributed equally to this work

Expert Opinion

informa
healthcare

Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor

Aadilah Omar, Katarina Jovanovic, Bianca Da Costa Dias, Danielle Gonsalves, Kiashanee Moodley, Robert Caveney, Vusi Mbazima & Stefan FT Weiss[†]

[†]*University of the Witwatersrand, School of Molecular and Cell Biology, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa (RSA)*

***Importance of the field*:** The 37/67 kDa laminin receptor precursor/laminin receptor (LRP/LR) represents a multifunctional protein located on the cell surface, in the cytosol and the nucleus. The receptor acts as a mediator for cell adhesion, cell proliferation and cell differentiation. It is a key player in invasion and adhesion, major functions of several important metastatic cancer types. The receptor hampers apoptosis thereby favoring cancer progression. LRP/LR plays a major role as a cell surface receptor in prion disorders and may be of considerable importance for other neurodegenerative diseases such as Alzheimer's disease. A series of viruses including Sindbis virus, Dengue virus and Adeno-associated virus use LRP/LR as attachment receptors. Bacteria and *Candida albicans* use the receptor for pathogenesis.

Areas covered in this review: Background and patented biological approaches for therapeutic modulation of LRP/LR in neurodegenerative diseases, cancer, viral disorders, bacterial and yeast infections.

What the reader will gain: A comprehensive review of the role of LRP/LR in infectious and non-infectious diseases and an insightful assessment of published or patented biological approaches for the therapeutic modulation of LRP/LR.

Take home message: Molecular tools such as antibodies directed against LRP/LR have the potential to act as promising alternative therapeutics for the treatment of various diseases.

Keywords: Alzheimer's disease, antibody, apoptosis, cancer, dengue virus, laminin receptor, LRP/LR, metastasis, prion, therapy

Expert Opin. Ther. Patents (2011) ***21****(1):35-53*

1. Introduction

Laminin, an extracellular matrix (ECM) glycoprotein, is a main component of the basement membrane which plays a role in several cellular processes, namely cell growth, movement, adhesion, migration and differentiation [1]. The above-mentioned laminin influenced cellular processes are mediated by several integrin and non-integrin cell surface laminin receptors (for review: [2]), including the 37/67 kDa laminin receptor precursor/laminin receptor (37/67 kDa LRP/LR) (for review: [3]). The 37/67 kDa LRP/LR is a non-integrin cell surface receptor

10.1517/13543776.2011.539203 © 2011 Informa UK, Ltd. ISSN 1354-3776

Article highlights.

- A total of 24 patents and 125 articles covering laminin receptor precursor/laminin receptor (LRP/LR) and cancer, neurodegenerative diseases, viral diseases, bacterial and yeast infections for therapeutic approaches.
- Role of LRP/LR and laminin in metastatic cancer and apoptosis and possibilities for a targeted therapeutic intervention focusing on therapeutic LRP/LR antibodies.
- Molecular mechanism of prion disorders and the prion receptor LRP/LR as a target for therapeutic intervention using antibodies, siRNAs, heparan mimetics and receptor decoy mutants.
- Molecular biology of Alzheimer's disease and possibilities for a therapeutic intervention targeting key players such as laminin and amyloid β.
- Function of LRP/LR in viral diseases including dengue virus, Sindbis Virus and adeno-associated virus infections and possibilities for a therapeutic intervention targeting the receptor.
- Role of LRP/LR in bacterial and yeast infections presenting possibilities for alternative therapies.

This box summarizes key points contained in the article.

exhibiting a high affinity for laminin [4]. The 37 kDa LRP is thought to be a precursor of the 67 kDa LR [5,6]; however, the mechanism(s) regarding the formation of the 67 kDa LR isoform from the 37 kDa LRP precursor remains elusive [7]. The 37 kDa LRP is not only localized on the cell surface but also in the cytoplasm, perinuclear compartment and nucleus (for review: [3]). In the cytoplasm, the 37 kDa LRP (in the form of p40 ribosome-associated protein) is reported to function in the maturation of the 40S ribosomal subunit as well as protein synthesis. The 37 kDa LRP is reported to interact with prion proteins (PrPs) in the perinuclear compartment and to associate with histones in the nucleus (for review: [3]). The 37/67 kDa LRP/LR has also been reported to bind and mediate physiological responses induced by other ECM components such as elastin, cellular PrPs and carbohydrates (for review: [3]). Recent evidence has indicated that the 37/67 kDa LRP/LR is also involved in cell survival [8].

In addition to its roles as a receptor and mediator of ECM molecules, LRP/LR also plays a role in several pathological conditions. This receptor has been shown to serve as a receptor for infectious PrPs, several viruses (**Figure 1**) (for review: [3]) and respiratory tract pathogens [9]. Furthermore, the overexpression of LRP/LR, evident in several cancer types, has been demonstrated to enhance the invasiveness of cancer cells which ultimately leads to cancer metastasis [4,10]. Although LRP/LR is mainly known for its role in promoting cancer invasiveness, there is evidence that the 37/67 kDa LRP/LR may further be involved in apoptosis [11-13]. Thus, the 37/67 kDa LRP/LR could be a potential target for the development of anticancer therapeutics capable of impeding tumor invasiveness and inducing tumor cell apoptosis.

2. Patented biological approaches for therapeutic modification of LRP/LR in prion disorders

Prion disorders, also termed transmissible spongiform encephalopathies (TSEs), are an assembly of neurodegenerative diseases (for review: [3,14-19]) whose unique biology, prolonged incubation times and debilitating symptoms have created mounting public interest and served as catalysts to scientific research. These diseases manifest in both humans and animals. Animals may suffer from the following TSEs: scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in elk and deer as well as other forms of the disease in exotic zoo animals and ungulates [20] (for review: [15,21]). Human neurodegenerative disorders caused by prion aggregation include: Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome and Kuru (a historical prion disorder indigenous to the Fore tribe of New Guinea which was propagated through cannibalism (for review: [21])). The symptoms associated with the aforementioned disorders may involve: truncal or appendicular ataxia, myoclonous as well as tremor, chorea, seizures and cognitive difficulties.

It must be emphasized that prion disorders may be sporadic, familial (genetic) or infectious in nature (for review: [3]). The last is of the greatest concern in light of recent findings that infectious PrPs, in scrapie infected sheep and elk/deer suffering from CWD, colocalize with LRP/LR on human enterocytes [22] and the discovery that prions are secreted into the oral cavity of infected sheep [23,24]. These results suggest that prion disorders may be orally transmitted between individuals and contracted from the consumption of contaminated animal products which may exacerbate disease spread.

The pathogenic agent of prion disorders is composed solely of a particular type of protein (termed infectious PrP and given the designation (PrP^{Sc})) and in all probability lacks a nucleic acid component, as was proposed by Prusiner in the 1980s [25]. This infectious PrP is a pathogenic isoform of the normal host encoded glycoprotein PrP^{c} which is bound to the cell membrane via a glycosylphosphatidyl-inositol anchor [26]. While both isoforms of the PrP share an identical amino-acid sequence [27], they differ substantially with regard to conformation: the non-pathogenic PrP^{c} exhibits a greater α helix content than its infectious (PrP^{Sc}) counterpart. In addition to their structural disparities, the isoforms may be distinguished on the basis of susceptibility to proteinase K degradation. PrP^{c} is sensitive to this enzymatic digestion whilst PrP^{Sc} is resistant (for review: [28]).

The 37/67 kDa LRP/LR is fundamental for PrP^{c} and PrP^{Sc} internalization as was demonstrated in [29] in which the

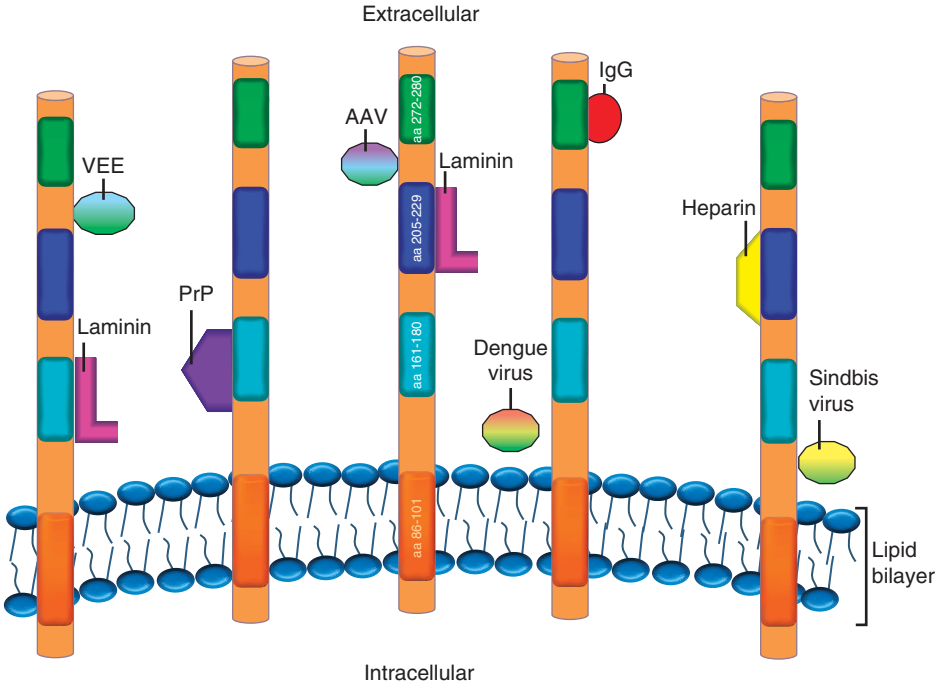


Figure 1. Schematic representation of the 37 kDa LRP. The 37 kDa LRP is 295 amino acids (aa) in length and consists of four functional domains with its C terminus exposed to the extracellular space. The transmembrane domain is located between amino acids 86 and 101, while a laminin/PrP-binding domain has been found to be positioned between amino acids 161 and 180. Further, a heparin/laminin-binding domain and a scFv/IgG1 antibody-binding domain have been shown to be located at amino-acid positions 205 – 229 and 272 – 280, respectively. Four viruses are known to bind to LRP/LR, namely VEE virus, AAV, Dengue virus and Sindbis virus. However, the binding domains of these aforementioned viruses to LRP/LR as of this time remain unclear and as such viral-LRP binding has been arbitrarily depicted within this diagram (for review: [3]).
AAV: Adeno-associated virus; LRP: Laminin receptor precursor; PrP: Prion protein; VEE: Venezuelan equine encephalitis.

enterocyte internalization of PrP^{BSE} was dependent on the presence of the aforementioned receptor. The endocytosis of both PrP isoforms is considered to occur through clathrin coated pits (**Figure 2**) [30] or caveolae [31] and the subsequent PrP^{Sc} accumulation ultimately leads to (neuronal) cell death and spongiosis, vacuolization and astrogliosis [32] evident in the brains of animals/humans suffering from the aforementioned diseases. It is noteworthy to add that LRP/LR exists in different isoforms [33], the most important of which are the 37 and 67 kDa isoforms referred to above and throughout the review.

We have demonstrated that the non-integrin 37/67 kDa receptor LRP/LR serves as a principal cell surface receptor for the host encoded PrP^{c} [34], as was originally proposed in [35], and may thereby function directly (as a mediator/intermediate between PrP^{c} and PrP^{Sc}) or indirectly (as a facilitator of the endocytic process) in the uptake of PrP^{Sc} and consequent disease establishment. The 37/67 kDa LRP/LR has been reported to have a fundamental role in the prion life cycle (for review: [3,15,36,37]). The receptor has a binding affinity for numerous biological molecules namely elastin [38], carbohydrates and of particular importance here, laminin [39] and heparan sulfate proteoglycans [7]. It is the laminin binding domain of the receptor that has a dual role as the PrP^{c} binding site.

Most therapeutics developed for either prophylaxis or treatment of prion disorders (for review: [3,15,36,40]) have been designed to either downregulate PrP^{c} expression, block the interaction between PrP^{c} and PrP^{Sc}, prevent PrP^{c} misfolding (polyene antibiotics, enhancement of chaperone levels and proteosomal activity [41,42]) or convert misfolded pathogenic PrP^{Sc} to its non-pathogenic form. As PrP^{c} is imperative for prion disease development, the silencing or knocking out of the PrP^{c} encoding gene (*Prn-p*) has been shown to prevent infection in mice [43] and in neuronal cells of cattle [44]. Furthermore, transgenic mice in which the gene has been knocked out ($PrP^{0/0}$) seemed to exhibit normal development, behavior, anatomy and reproductive potential [43]. However, the cellular PrP may have fundamental roles in signal transduction, normal sleep patterns and synaptic functions (for review: [14]). The inhibition thereof may result in reduced memory, learning difficulties and abnormalities of the hippocampus. Thus, as the physiological roles of PrP^{c} have not yet been adequately elucidated, its interruption may have multiple consequences. The 37/67 kDa LRP/LR may be an alternative therapeutic target. It is postulated that as this

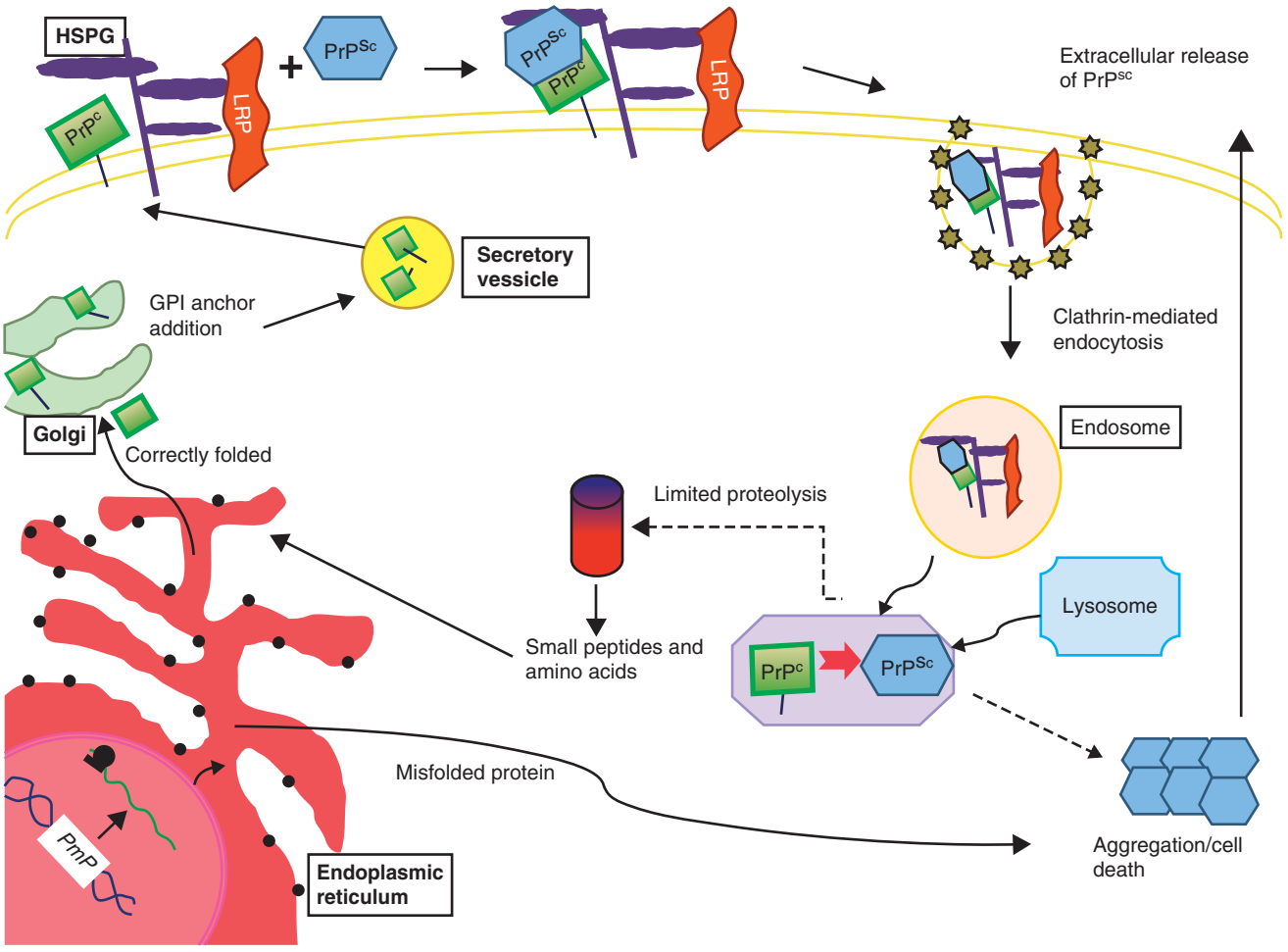


Figure 2. Model of infectious prion (PrP^{Sc}) internalization and replication through a PrP^{c}–LRP/LR–HSPG-dependent pathway. The normal cellular prion protein (PrP^{c}-green square) is encoded by *Prn-p* gene and its biosynthesis and post-translational modification (glycosylation) occur in the rER and Golgi apparatus, respectively. The proteins are then transported via secretory vesicles to the plasma membrane into which the proteins are anchored by glycosylphosphatidyl-inositol [26]. PrP^{c} binds to its specific receptor 37/67 kDa LRP-LR either directly or indirectly, through its octarepeat-binding domain, via HSPG co-receptors [7]. PrP^{Sc} binds specifically to PrP^{c} and this prion–LRP/LR–HSPG complex is internalized by clathrin-mediated endocytosis [30] or calveolae (not shown) into endosomes. Fusion of the heterodimeric complex-containing endosomes to lysosomes forms endolysosomes in which the PrP^{c} undergoes structural rearrangement and converts to the pathogenic PrP^{Sc} isoform which aggregates and ultimately leads to cell death. The sporadic forms of prion disorders may be the result of spontaneous conversion/misfolding of the prion protein PrP^{c} [14].
HSPG: Heparan sulfate proteoglycan; LRP/LR: Laminin receptor precursor/laminin receptor; rER: Rough endoplasmic reticulum.

receptor is directly/indirectly implicated in the PrP^{c}–PrP^{Sc} interaction, infectious agent internalization and propagation, therapies directed against the receptor may serve in treating prion disorders (for review: [3,15,18,19,36]).

2.1 LRP/LR mutant receptor decoys

The PrP^{c} and laminin binding domain, palindromic sequence LMWWML on LRP/LR, are located between amino acids 161 and 180 and this region [7,35] is located on the extracellular domain of this transmembrane protein. In lieu of this fact, Vana and Weiss developed an LRP mutant consisting solely of the extracellular domain, termed LRP 102 – 295::FLAG [45]. As this mutant thereby lacks the transmembrane domain it is secreted into the ECM as opposed to being inserted into the plasma membrane. However, as the laminin-PrP^{c} binding domain is still present, the mutant may bind to the infectious PrP^{Sc} isoforms in the ECM and thereby act as a decoy by preventing the interaction of PrP^{c}-PrP^{Sc} via the membrane-bound LRP/LR [45] (for review: [15]). In addition, a fragment or functional derivative (i.e., polyethylene glycol side chains to extend the biological activity of the soluble LRP/LR decoy in bodily fluids) of the receptor may

exert their effect via a similar mechanism. The administration of the decoy receptor into scrapie infected cells has proven successful in significantly reducing PrP^{Sc} accumulation [45] and scrapie-infected transgenic hemizygous mice, expressing the decoy mutant LRP102 – 295::FLAG displayed significantly increased incubation times/preclinical phases in comparison to the scrapie-infected wild-type mice [46] thus providing further evidence for the recommendation of this mutant as a therapeutic tool. The transgenic mice expressing the decoy mutant revealed no side effects.

2.2 RNAi–siRNAs as therapeutic agents

siRNAs (double-stranded RNA molecules) have been successfully used to inhibit the expression, by stimulating *Prn-p* gene silencing, of PrP^{c} as a means of reducing the quantity of PrP^{c} which may be converted to PrP^{Sc} and thereby hamper PrP^{Sc} accumulation [47]. Thus, siRNAs directed against the *Prn-p* mRNA may be exploited as both a preventative measure and therapy against prion diseases, as is proposed by a patent filed by Sakaguchi and Taira [48]. Viral vectors, particularly adenovirus, herpesvirus, retrovirus and lentivirus expression vectors, are the most preferable regards siRNA expression. Lentiviral packaged PrP^{c}-specific short hairpin RNAs have been shown to silence *Prnp* gene expression and thereby suppress PrP^{Sc} accumulation [49]. The use of siRNAs in this respect was documented in [50] and the use of antisense RNA or ribozymes to hinder the expression of LRP was originally described in a patent by Weiss [51]. Furthermore, Weiss and colleagues [52] used a lentiviral delivery system to introduce three different siRNAs directed against the LRP/LR into scrapie-infected neuronal cells ($N2aSc^{+}$), and demonstrated that this therapeutic approach is able to reduce LRP and PrP^{Sc} levels by 36 and 41%, respectively, and significantly prolong the preclinical phase. The treatment turned out not to induce any side effects in mice.

2.3 Polysulfated polyanions

2.3.1 *Heparan mimetics*

Heparan sulfate proteoglycans (HSPG) are molecules consisting of a protein core and glycosaminoglycan (GAG) side chains. These molecules function as co-receptors for the PrP–LRP interaction [7] as the octarepeat region of PrP acts an indirect binding domain for LRP via the HSPGs (**Figure 2**). Heparan sulfate mimetics (HMs) which have been developed through structural modification of HSPG sulfated GAGs (substitution of dextran with carboxymethyl, sulfate and hydrophobic groups) are able to interfere with the pathogenic PrP–LRP/LR interaction by out-competing the cellular HSPGs, impair prion endocytosis and thwart PrP^{Sc} synthesis [53]. Two such molecules HM2602 and HM5004 were effective in inhibiting infectious prion accumulation by inhibiting prion endocytosis and the structural conversion of PrP^{c} into PrP^{Sc} [53]. The former compound has been demonstrated to not only inhibit infectious prion accumulation but also increase the survival time of scrapie infected mice by ~ 14% *in vitro* [20]. In contrast, HM5004 failed to exert an effect *in vivo*. Such discrepancies may be attributed to the prion strain used. Moreover, it has been proposed by Safar *et al.* [54] that the exposure of PrP^{c} hydrophobic residues results in the structural rearrangements associated with the conversion of PrP^{c} to PrP^{Sc} and thus the higher the hydrophobicity of the HM (here HM2602) the stronger the interaction between the therapeutic and the PrP and thus the more effective the inhibition of PrP–LRP binding. Both heparan mimetics detailed above contain a potentially mutagenic benzylamide group and to avoid problems which may arise as a result hereof, HM derivatives such as CR36 which lack this functional group are being developed [55].

2.3.2 *Pentosane polysulfate (SP54)*

Pentosane polysulfates (PPS: poly-b-xylose-2,3-disulfonate) are polyglycoside molecules (for review: [15]) which have been reported to be effective in delaying the onslaught of prion disorders in rodents by prolonging incubation time [55] and inhibiting infectious PrP formation [56]. This therapeutic agent selectively binds to heparan binding sites on proteins and with regard to prion disorders outcompetes the normal cellular HSPGs for interaction with PrP and LRP/LR. In so doing, these compounds interfere with the interaction of PrP^{Sc} with the LRP/LR–HSPG cell–surface complex [53] and thus infectious prion internalization and life cycle. An alternative hypothesis is that SP54 alters the cellular location of the PrP^{c} precursor and this consequently serves as a deterrent to infectious prion internalization [53]. Furthermore, Microsens Biophage Ltd filed a patent proposing a set of certain conditions under which PPS binds to the pathological PrP^{Sc} but not to PrP^{c}, may thereby capture the infectious isoform and consequently reduce infection [57]. Results from *in vivo* studies have demonstrated that PPS was able to cure two mouse strains (VM and CBA) from two prion strains (22A and ME7) and reduce the binding of infectious prion by ~ 40% [58]. It is noteworthy to add that PPS prevents the *de novo* accumulation of PrP^{Sc} but does not destabilize pre-existing PrP^{Sc} [59] and may, therefore, be better suited as a prophylactic agent rather than as a therapeutic agent in prion disorders. A patent [60] covers PPS administered either alone or as a constituent of a composition comprising quinines, an agent having an affinity for lysosome or a reactive dye as a prophylactic tool.

2.4 Antibodies

Antibodies are gaining increasing favorability in the field of human therapeutics. Prion-strain specific antibodies may crossreact with both prion isoforms (mAb6H4) whilst others, such as mAb3F4 [61], are only able to bind specifically to PrP^{c} under native conditions. As a result thereof, such antibodies have had limited success with respect to inhibiting prion

infection and cell-to-cell propagation [61,62]. A monoclonal IgG antibody termed ICSM18, raised against PrP91 – 231, may react with both PrP^{c} and PrP^{Sc} and prevent prion infection and subsequent replication as was described in a patent filed by the Medical Research Council of London (Great Britain) [63].

Antibodies directed against infectious PrPs (typically prion strain specific) are highly sensitive tools which enable researchers to distinguish between the different isoforms of the PrP. Such antibodies include: 15B3 which specifically recognizes the infectious PrP in cattle by western blotting whilst not binding to the cellular counterpart in each of the aforementioned experimental animals [61] and 8G8, used in both western blotting and immunofluorescence microscopy, as it binds to infectious PrP^{BSE} but not PrP^{c} on human enterocytes [29]. A patent has been filed covering antibodies which bind specifically to the amino acids 106 – 126 of the aggregated protein and thereby allow researchers to distinguish between the two PrPs [64].

Anti-PrP specific antibodies were reported to cure scrapie infected cells from Scrapie, namely the Fab D18 [65] and the before mentioned monoclonal 6H4 [66]. Scrapie pathogenesis *in vivo* has been prevented by transgenic expression of an anti-PrP antibody in mice [67] and by passive immunotransfer of monoclonal anti-PrP antibodies termed ICSMs into mice [68].

Recently, peptide (2 – 100aa) combinations, of which one of the composite peptides is a PrP peptide or fragment thereof, have been constructed to mimic the tertiary structure of PrP^{Sc} and exploited as immunogens for the production of infectious PrP specific antibodies as is described by a patent filed by Copenhagen Biotech Assets Aps [69].

Furthermore, a patent was filed on the following finding: the gene encoding for the anti-prion antibody, which is introduced via viral vectors, may be transferred into stem cells and thereby serve as an active ingredient in the treatment of prion disorders, as a means of ensuring the continued production of the antibody *in vivo* and thus may have prophylactic potential and perhaps assist in the regaining of nerve function [70].

As LRP/LR has been proposed to fulfill an essential role in the pathogenesis of prion disorders, a patent has been filed covering antibodies directed against the receptor, or fragments thereof, for prion disease therapy as a means of disrupting the prion life cycle (**Figure 2**) and in general for the use in medicine [51]. Polyclonal antibodies (W3) which target the receptor were the first to be generated [35]. W3 on passive immunotransfer into PrP^{Sc} infected mice significantly reduced PrP^{Sc} accumulation and prolonged survival [71] and cured neuroblastoma cells from scrapie [50]. W3 turned out to reveal no side effects on passive immunization into mice [71]. However, W3 has a limited accessibility (amount) and their large size (150 kDa) hampers the ability of these immunoglobulins to traverse the BBB [72]. These factors thereby reduce their applicability as potential prion therapeutics. To circumvent these drawbacks, single chain antibodies (scFv), given the designations S18 and N3, were developed (by phage-display) [72] and described in a patent [73] in which the antibody-encoding cDNA is conveyed in an adeno-associated virus (AAV) expression vector. scFvs are composed of short segments of both the light and heavy chains of the full-length antibodies which are joined by a YOL linker and exhibit a molecular mass of ~ 30 kDa. These antibodies are thus more effective in penetrating the BBB and may thereby enter neural tissue in which prion-associated damage occurs. Zuber *et al.* [72] have demonstrated that scFvs are effective at reducing peripheral PrP^{Sc} propagation by ~ 40% when administered by passive immunotransfer but were unable to significantly prolong incubation and survival times. Similar results were observed when a recombinant AAV vector system was established for the *in vitro* delivery of the single chain antibodies (scFv-S18 and scFv-N3); a significant reduction (60 and 32%, respectively) of peripheral PrP^{Sc} was reported but incubation and survival times remained largely undiminished [74]. These results may be attributed to the lower stability of single chain antibodies in comparison to their full-length counterparts and their short half-life in blood (~ 12 h). As reported for W3 [71], the single chain antibodies directed against LRP/LR turned out also not to have any side effects in mice [72].

The mechanism by which the aforementioned antibodies interfere with PrP^{c}–LRP/LR binding remains ambiguous, although steric hindrance is likely to be crucial aspect of this disruption as the antibody and PrP^{c} do not bind the same domain thereby refuting the possibility of direct competition.

3. Patented therapeutic approaches targeting LRP/LR and laminin for the treatment of Alzheimer's disease

Affecting > 25 million people worldwide, Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder affecting the aging population. It has been proposed that this figure will increase to 81 million within the next 30 years [75]. The incidence of AD increases significantly with age, and the onslaught of symptoms begins at age 65 for most patients. Initial symptoms of AD include memory loss and mild cognitive impairment, followed by difficulties in speech and performing tasks. At this latter stage, behavioral and cognitive dysfunctions develop and are characterized by aggression, depression and paranoia. Furthermore, patients begin to experience difficulties with movement (ataxia) and exhibit extrapyramidal symptoms [76].

Brain sections from AD patients revealed amyloid plaques composed of the 4 kDa amyloid beta (Aβ) peptide. In order to further understand AD, the mechanism of Aβ generation was examined and illustrated that Aβ is generated from the proteolytic processing of the amyloid precursor protein

(APP). This transmembrane protein, 695 amino acid in length, is proteolytically cleaved by three enzymes, designated α-, β- and γ-secretase, thereby releasing various peptides (**Figure 3**). The non-amyloidogenic pathway involves cleavage of the APP within the Aβ sequence by α-secretase to release sAPPα. The remaining C-terminal fragment is subsequently cleaved by γ-secretase to release a 3 kDa peptide called p3, which is readily degraded. However, the amyloidogenic pathway results in the release of the Aβ peptide (**Figure 3**). During this pathological pathway, APP is cleaved by β-secretase at the start of the Aβ sequence to release sAPPβ and following γ-secretase cleavage of the remaining C-terminal fragment, Aβ is released (**Figure 3**) [77]. Owing to their hydrophobic nature, these Aβ peptides aggregate to form amyloid plaques and amyloid fibrils which are established on neuronal tissue and in the blood vessels of the brain, respectively. These protein aggregates are the causative agents leading to the neurodegeneration associated with AD. The afore-highlighted mechanism by which Aβ is generated and Aβ itself represent promising targets for AD therapy and as such are the focus of mounting research.

3.1 Therapeutic approaches for Alzheimer's disease targeting laminin

As has been previously stated, laminin is a 900 kDa ECM protein associated with cellular processes of growth, organization, differentiation and communication [78]. Studies have demonstrated that an interaction between laminin and Aβ promotes neurite outgrowth [79]. Further investigations have revealed that the A chain of the laminin protein exhibits an Aβ binding site and the association of laminin and Aβ proteins through this site inhibits fibrillogenisis: a process required for the formation of amyloid plaques [80,81]. Moreover, the addition of laminin results in the dissolution of preformed amyloid plaques. These findings have fuelled the investigation into laminin and laminin-derived proteins for the possible diagnosis and treatment of AD. The addition of Aβ binding laminin and specific laminin fragments in therapies has been patented [82] and these patents propose the production of pharmaceutical compositions of Aβ specific laminin-derived peptides as tools in AD diagnosis, through the peptide's ability to prevent fibril formation, as well as in AD therapy [83,84].

3.2 The roles of PrP^c and LRP/LR in the treatment of Alzheimer's disease

Infectious prions (PrP^{Sc}) are responsible for TSEs in a wide range of species. In humans, the infectious prion isoform leads to neurodegenerative diseases such as CJD amongst others. The cellular prion protein (PrP^c), however, has been suggested to a serve a neuroprotective role in the brain by protecting against various factors (such as oxidative and apoptotic stress) and promoting synapse generation, transmembrane signaling and ECM adhesion (for review: [85]). Due to its role in neuroprotection, the influence of PrP^c in AD was examined and the relationship between PrP^c and Aβ found to be inversely proportional such that PrP^c upregulation significantly reduces Aβ shedding whilst siRNA-mediated downregulation of the cellular prion protein results in an augmentation in Aβ levels [86]. This finding strongly suggests a protective role of PrP^c in AD by hampering Aβ shedding.

PrP^c not only reduces Aβ shedding but may also act as a receptor for the amyloid Aβ oligomers [87]. Aβ binding to PrP^c has been impeded by PrP-specific antibodies which also rescued synaptic plasticity in hippocampal slices from oligomeric Aβ, suggesting that PrP^c is a mediator of Aβ–oligomer-induced synaptic dysfunction [87]. In contrast to these findings, another report by Kessels *et al.* [88] indicates that Aβ-induced synaptic depression as well as loss of dendritic spines and blockage of long-term-synaptic potentiation occurs in hippocampal slices prepared from PrP^c-deficient animals [88]. Although this report confirms that oligomeric Aβ can interact with PrP^c, it intends that PrP^c is not the receptor responsible for synaptic perturbations caused by Aβ oligomers [88]. Lauren *et al.* hypothesized in their response to this article that the differences observed might be due differences in the Aβ oligomerization stati and/or different Aβ concentrations (discussion in [88]). A further report by Balducci *et al.* [89] confirms that Aβ oligomers interact with PrP^c but supports the finding of Kessels *et al.* [88], claiming that the Aβ oligomers are responsible for cognitive impairment in AD such as impairment of long-term memory and synaptic plasticity and not PrP^c.

The presence of amyloid plaques in patients suffering from CJD [90] supports the association between Aβ and prions. This may be attributed to the fact that a patient suffering from CJD would possess the infectious prion (PrP^{Sc}) and would thus have reduced concentrations of PrP^c to regulate Aβ shedding. Thus, it can be assumed that any therapy against PrP^{Sc} may further aid in prevention of AD by enhancing PrP^c availability for downregulation of Aβ shedding. Therefore, it may be valuable to examine whether therapies against prion disorders, such as those previously mentioned (section 1), may in addition function in preventing or delaying the onset of AD.

Gauczynski *et al.* reported that the 37/67 kDa LRP/LR acted as the receptor for both infectious prions (PrP^{Sc}) [53] and non-infectious PrP^c [34]. In light of the fact that PrP^c inhibits Aβ shedding [86], PrP^c acts as an interaction partner for Aβ oligomers [87-89] and may act as a receptor for Aβ oligomers [87], a finding which is controversially discussed [88,89], a possible contribution of the prion receptor LRP/LR in cell biology of Morbus Alzheimer cannot be excluded.

Therefore, it can be assumed/proposed that a possible relationship may exist between LRP/LR (the receptor for PrP^{Sc} and PrP^c) and Aβ, whose production is regulated by PrP^c.

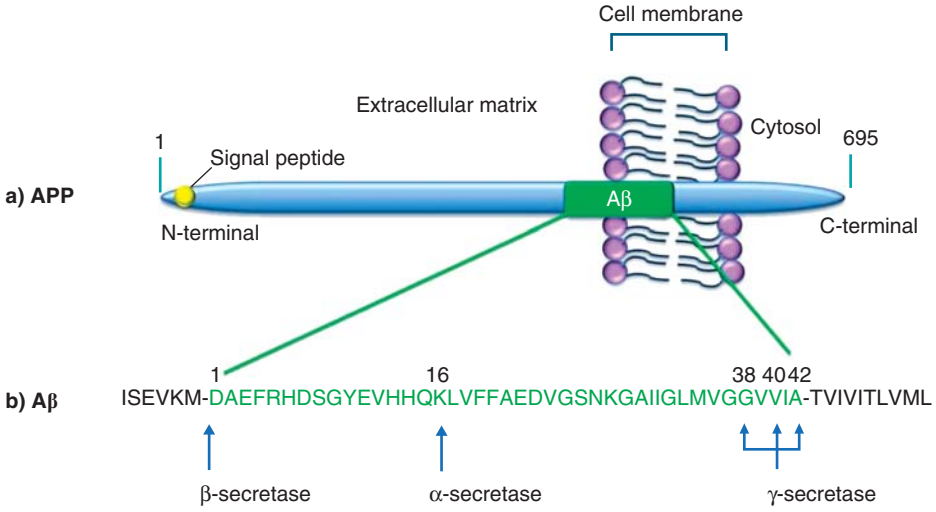


Figure 3. Schematic representation of the APP. (a) The location of APP, 695 amino acids in length, on the cell membrane is shown. (b) The Aβ sequence (one letter code) is shown in relation to APP. The cleavage sites for α-, β- and γ-secretase are shown within the APP protein, whereby it is evident that α-secretase cleavage precludes the production of Aβ peptide, while the β- and γ-secretase cleavage is necessary for shedding of the 4 kDa Aβ peptide.
Aβ: Amyloid β; APP: Amyloid precursor protein.

4. Patented biological approaches for therapeutic modification of LRP/LR in cancer

4.1 Current cancer therapies

In 2008, 12.7 million new cancer cases were reported worldwide [91]. Cancer remains the major cause of deaths globally with 7.6 million deaths attributed to the disease in 2008 [91]. Despite significant advances in cancer therapy over the last 2 decades. The relatively poor success of these therapies may be attributed to multiple factors including late diagnosis and the limitations of current therapeutic approaches. Currently, many patients suffering from the disease make use of a multi-disciplinary approach consisting of surgery, radiation therapy and chemotherapy in varying combinations. However, as the aforementioned strategies aim to kill cancer cells whilst exerting minimal adverse effects on normal cells, they are administered such that they do not exceed a specific limit without causing further harm to the patient (for review: [92]). Furthermore, if the tumor is metastatic in nature and thus multiple tissues are affected, the aforementioned treatment strategies are largely limited in their success and alternative approaches are required.

4.2 Targeted therapy

One alternative approach for cancer treatment represents targeted therapy which is a general term encompassing any form of medication or drug directed against a specific pathway (often via the blockage of essential molecules or receptors) suspected to play a significant role in cancer establishment and progression and thereby inhibits tumor growth or spread (for review: [93]). Immunotherapy is one class of targeted therapy and these drugs are usually a collection of monoclonal antibodies (mAbs) that bind specifically to antigens on the surface of the cells. mAbs are one of the most rapidly advancing forms of therapy and have been produced for the treatment of several cancer types. An example for such an antibody is the patented anti-CD20 antibody rituximab (Rituxan) produced by IDEC Pharmaceuticals Corp. [94] to form part of the management of relapsed low-grade non-Hodgkin's lymphoma (for review: [95]). Other novel approaches include gene therapy (for review: [96]), non-pathogenic bacteria and telomerase therapy [97].

4.3 37/67 kDa LRP/LR role in cancer

The 37/67 kDa LRP/LR has been reported to play a significant role in metastasis of various cancer types (for review: [3,36]). LRP/LR is a non-integrin LR that is involved in several tumorigenic processes, such as cell proliferation, cell viability, cell movement, cell adhesion and cell attachment [98]. One of the most significant molecular events involved in initiating metastasis involves the interaction between LRP/LR and laminin-1 which influences general laminin-mediated basement attachment [99] and, consequently, stimulates the local degradation of the basement membrane and movement of cells (**Figure 4**) [100]. Furthermore, a direct relationship between the overexpression of the 67 kDa form of the receptor and the increased degree of cancer cell invasiveness has been observed [99,101]. LR/laminin-induced metastasis has been observed in gastric cancer [102], colon carcinoma [103], colorectal carcinoma [104], cervical [105], breast [106], lung [107], ovarian [108], pancreatic [109] and prostate cancers [110]. A study using murine models

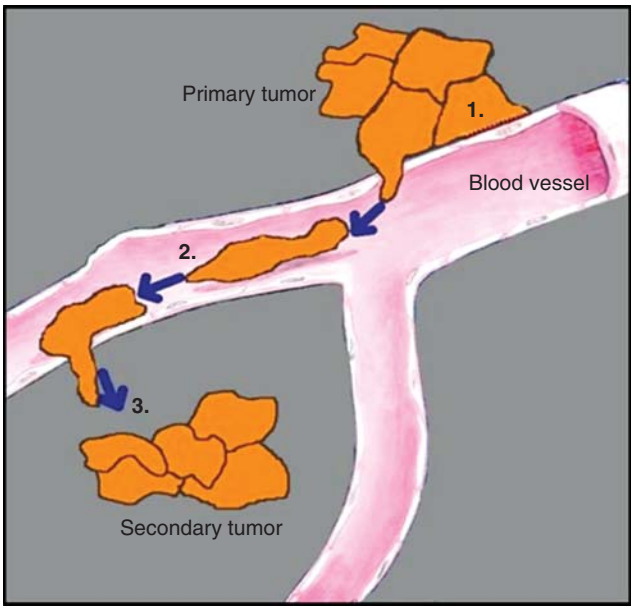


Figure 4. Illustration of the process of tumor metastasis. 1. Cells from original tumor attach to basement membrane of blood vessel via LRP/LR–laminin interaction. 2. The basal lamina is then digested by type IV collagenase allowing for tumor cell entry into the blood stream and migration. 3. Metastatic (secondary) tumor is then established by attachment of migrating cells to the blood vessel.
LRP/LR: Laminin receptor precursor/laminin receptor.

demonstrated that human fibrosarcoma (HT-1080) cells which were exposed to the anti-LRP antibody induced significantly reduced lung cancer metastasis compared to non-treated cells [111]. These findings suggest that the suppression of LRP expression hampers lung cancer cell proliferation and tumor formation [112]. Furthermore, application of the anti-LRP/LR antibody P4G resulted in a significant reduction of metastatic lung cancer in murine models [113].

A patent describing a soluble LRP, a mutein, fused protein, salt, functional derivative or fragment thereof that can bind to PrP^{Sc}, PrP^{c} or fragments thereof, an antibody or a fragment thereof that binds GST:LRP or fragments thereof, or an antisense RNA or ribozyme which reduces or inhibits the expression of LRP for use in medicine was granted for Germany and the UK [51]. Other patented therapies include the virus-based vectors that are able to target tumor cells particularly due to their attraction to high affinity LRs [114]. This therapy is, therefore, effective in the treatment of cancer as the tumor cells undergo necrosis following attachment to the LRs.

4.4 Anti-LRP/LR antibodies for the treatment of metastatic cancer

A significant reduction in the invasive potential of tumorigenic human fibrosarcoma cells (HT-1080) by the anti-LRP/LR specific antibodies scFv iS18, IgG1 iS18 as well as anti-LRP/LR tools such as siRNAs directed against LRP mRNA, and polysulfated glycanes such as heparan mimetics and pentosan polysulfate has been observed [4].

A patent describing antibodies directed against LRP for use in medicine has been granted for the UK and Germany [51]. A further patent was filed protecting specifically the single-chain antibody for the diagnosis and treatment of prion diseases and cancer [73]. Another patent was filed protecting these antibodies specifically for the treatment of several cancer types including B-cell chronic lymphocytic leukemia, non-Hodgkin's lymphoma, Hodgkin's lymphoma, lung cancer, colon carcinoma, mammary carcinoma, pancreatic carcinoma, prostate and metastasizing cancers [115].

A patented cancer vaccine containing epitopes of oncofetal antigens (OFAs) is able to target immature LRP [116]. Another filed patent includes a method for preparing an immunotherapeutic composition for the use in humans comprising: i) a plurality of OFA/LRP which stimulates T-helper lymphocytes in humans and ii) formulating two or more of the epitopes identified in i) with a carrier, thus forming the immunotherapeutic composition [117].

5LAC-23 represents a patented mAb which is effective in the diagnosis and treatment of cancerous diseases [118]. This particular antibody, or antigenic-binding fragments derived thereof, is able to bind to the LRP protein 37 kDa LRP. Specific antigenic moieties that can be targeted with 5LAC-23 are overexpressed in hepatocellular carcinoma cells and represent promising alternative antigens for the treatment of liver cancer [118]. 5LAC-23 treatment opportunities created by this discovery might also assist in cancer differentiation and diagnosis.

4.5 Improving antibody production and efficacy for the treatment of cancer

Despite the many discoveries and extensive ground-breaking research, mAbs are still unable to completely cure cancer; however, several innovative approaches could lead to drastic improvements in the efficacy of antibody-based therapies. The FDA has already approved six mAbs for the treatment of cancer [119] including: rituximab (non-Hodgkin's lymphoma), trastuzumab (breast cancer), bevacizumab (colorectal cancer), alemtuzumab (chronic lymphocytic leukemia), cetuximab (colorectal cancer) and panitumumab (colorectal cancer) [119]. Edrecolomab (Panorex), another mAb, which was approved in Germany in 1995 (for review: [95]), has been developed for the treatment of colorectal cancer. Furthermore, an immunoconjugate, gemtuzumab ozogamicin (Mylotarg) has also been approved and patented [120]. Metastatic cancer appears to be the main focus in the development of such antibodies as metastasis is a major contributing factor in the morbidity as well as mortality associated with cancer [99]. Although several methods by which antibodies may be produced exist, few are capable of producing mAbs as opposed to polyclonal antibodies. A polyclonal serum contains merely 5 – 10% of the required antibody (directed against the antigen used for immunization [71]). To address the problems associated with conventional antibody production methods, the hybridoma technique is used and involves the fusion of a short-living antibody producing cell with an immortal myeloma cell, resulting in the production of a single type of antibody [121].

Recent patient tumor response data gives an indication of the low success rate and further emphasizes the need to enhance the efficacy of the anti-cancer antibodies currently available. The previously mentioned anti-CD20 antibody rituxan, targeting non-Hodgkin's lymphoma, was shown to elicit a positive response in only 50% of the patients during a Phase II study (for review: [95]). Herceptin, a patented therapeutic antibody for the treatment of metastatic breast cancer, triggered only a 15% overall response rate during a Phase III clinical study (for review: [95]). Thus, these studies clearly indicate the potential of these therapies and with the application of novel strategies such as enhancing effector functions the efficacy of these antibodies could be improved.

4.6 Cancer and apoptosis

Cancer represents a disease characterized by uncontrolled cell growth as a result of unregulated cell proliferation or the lack of apoptosis or both (for review: [122]). Continuous cell growth is due to mutations in oncogenes (e.g., Ras) and tumor suppressor genes (e.g., p53 and Rb) that may thereby act by altering cell cycle checkpoint regulators and the DNA repair system (for review: [122]).

Apoptosis or programmed cell death is a physiological form of cell death that is well regulated in the cell (for review: [123,124]). This process is characterized by the disruption of the cytoskeletal elements of the cell as well as cell shrinkage, membrane blebbing, nuclear condensation and internucleosomal DNA fragmentation (for review: [124]). Apoptosis takes place in the cell via two major pathways, namely, the extrinsic and the intrinsic pathways (**Figure 5**). The blockage of the apoptotic process gives rise to numerous diseases, including cancer.

It has been demonstrated that alterations in the general apoptotic process may significantly affect tumor progression, and may thus be suggested that the inactivation of apoptosis may occur concurrently with tumor progression (for review: [122]) and may thereby be proposed that multiple cancer types induce such alterations as to allow for the evasion of apoptosis. Such an alteration may include mutations in p53 which could lead to loss of pro-apoptotic (e.g., Bax) gene regulation and consequently result in uncontrolled cell proliferation (for review: [122]). Moreover, LRP/LR has been implicated in the blockage of the apoptotic process [13]. The upregulation of LRP/LR has been reported in various cancerous cell lines [4,102-110]. SiRNA-mediated downregulation of LRP/LR stimulated the induction of apoptosis in Hep3B (transformed liver) cells as was detected by Annexin V/propidium iodide staining as well as by double staining with Annexin V and an antibody directed against the 37/67 kDa high affinity LR [13]. These results suggest that the 37/67 kDa LRP/LR receptor is fundamental in the maintenance of cell viability and could thus be a potential target for the treatment of cancers which display high expression of LRP/LR. A series of patents (section 4.3) have been filed encompassing antibodies for the treatment and vaccination of cancer [51,73,115,116,118].

The inhibition of carcinogenesis via the action of tea and tea polyphenols has been demonstrated in various animal models [125]. One such polyphenol of considerable interest in cancer treatment is (-)-epigallocatechin-3-gallate (EGCG). EGCG has been shown to bind to the 67 kDa LR in MCF-7 cells [126], and NMR spectroscopy was used to illustrate that EGCG binds to the BH3 pocket of anti-apoptotic function of Bcl-2 proteins [127]. However, the exact mechanism by which this compound exerts cancer preventative activity has not been completely elucidated.

Previous studies have established that the $\alpha_6\,\beta_4$ integrin is a receptor for the family of laminins [128-130]. RKO is a rectal carcinoma cell line that has been shown to lack expression of the β_4 integrin subunit of the $\alpha_6\beta_4$ LR. This cell line has also been shown to have a decreased interaction with laminin-1 when compared to similar cell lines that express $\alpha_6\beta_4$. Expression of full length β_4 cDNA and exogenous α_6 in the RKO cell line resulted in partial G1 arrest and the induction of apoptosis at a basal rate [131]. A correlation between apoptosis and LRP/LR has been suggested as the decreased LRP/LR expression resulted in the induction of apoptosis in HeLa (cervix carcinoma) cells [12].

Sindbis viral vectors are being researched as a mode of inducing apoptosis in cancerous cells. LRP/LR acts as one of the surface receptors in mammalian cells for the Sindbis virus and as LRP/LR is upregulated in cancer cells, Sindbis viruses are able to infect these cells at a higher rate. Sindbis virus

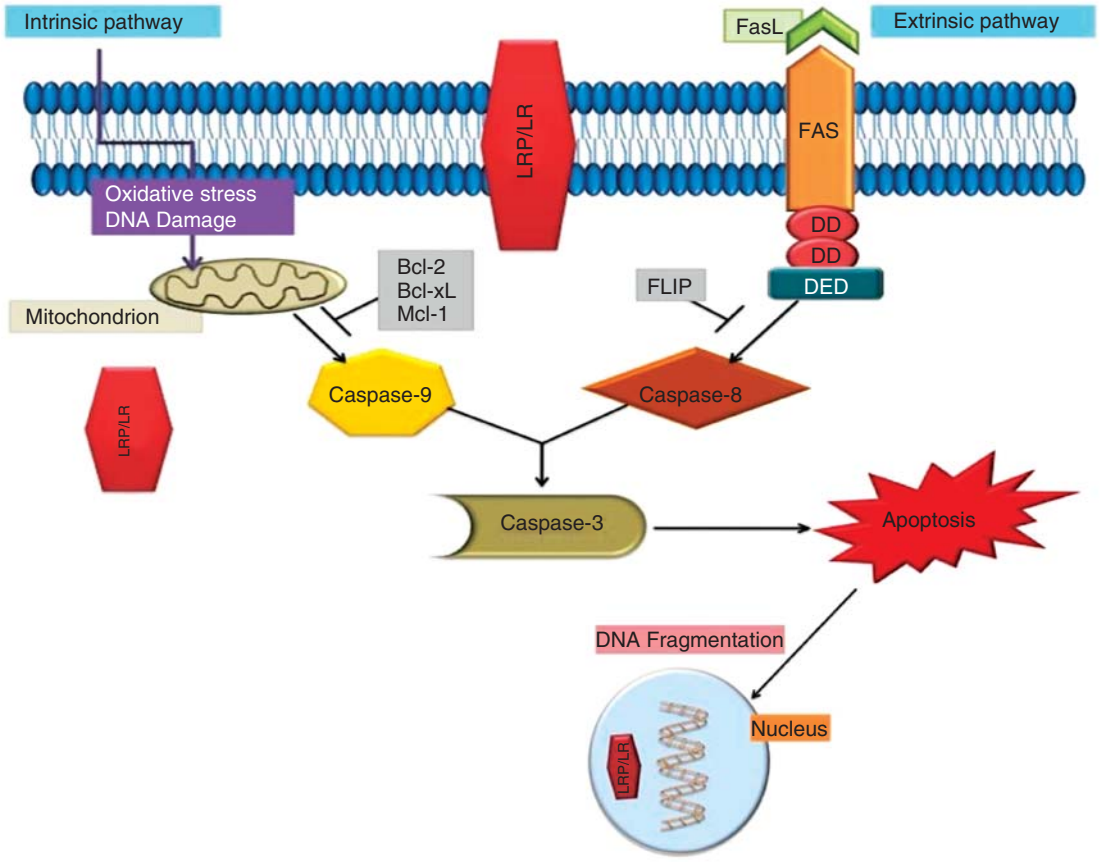


Figure 5. The two main apoptotic pathways in mammalian cells. The death receptor (extrinsic) apoptotic pathway is initiated by the binding of a ligand to a death receptor, e.g., FAS which results in the activation of caspase-8. The mitochondrial-dependent (intrinsic) apoptotic pathway is activated by intracellular signals such as oxidative stress or damaged DNA. These signals cause changes in the mitochondrial membrane, which in turn lead to the activation of caspase-9. The activation of caspase-8 and/or -9 leads to the activation of caspase-3 which initiates apoptosis by cleaving cytoskeletal proteins and activating enzymes which are involved in DNA fragmentation [123]. It is known that LRP/LR is present in the cell membrane, the cytosol and the nucleus [3] and is suggested that LRP/LR plays a role in the blockage of the apoptotic process [13]; however, the exact mechanism of this blockage is currently unknown.
LRP/LR: Laminin receptor precursor/laminin receptor.

infections have been shown to be highly apoptotic in mammalian cells. A patent has been filed for Sindbis viral vectors which exhibit apoptosis-inducing potential by binding to LRP/LR [132].

5. Patented biological approaches for the modulation of LRP/LR in viral diseases

The event that characterizes the initiation of viral infection involves the interaction between a virus and the host cell. The process of viral entry, which begins with viral–host attachment and eventually results in the entry of the viral genome within the cell, is dependent on the presence of specific receptors expressed on the surface of the host cell. It is the presence of these receptors that determines both host and tissue tropism (for review: [133]). HIV cell entry, for example, is initiated via the sequential interaction of the viral envelope glycoproteins with the CD4 receptor followed by one of the following chemokine receptors, CCR5 or CXCR4 [134]. Therapeutic targeting of this viral–host interaction continues to remain a promising strategy in the management of HIV/AIDS (for review: [135]). Numerous types of novel laminin polypeptides have been patented for the potential treatment of not only HIV infection, but a variety of other diseases including developmental and inflammatory disorders [136]. Therapeutic targeting of the HIV–host cell interaction when supplemented with other conservative techniques that target reverse transcriptase, for example [137-139], holds potential for the effective management of the infection (for review: [140,141]).

While CD4 and CCR5/CXCR4 were identified as binding partners of HIV, the high affinity LR was first identified by

Wang *et al.* [142] to act as a receptor for the Sindbis virus in mammalian cells. Within this study, hamster cells induced to overexpress the LR at the cellular surface were found to bind several-fold more Sindbis virus compared to those cells expressing lower levels of the receptor (**Figure 1**). Moreover, the 67 kDa LR has been described as a Sindbis virus receptor within mosquitoes [142]. Additionally, a laminin binding protein has been identified as a mediator of the Venezuelan equine encephalitis (VEE) virus attachment within mosquito cell culture (**Figure 1**) [143]. Two dimensional VOPBA has also revealed the interaction between the 37/67 kDa LRP/LR and Dengue virus serotypes 1, 2 and 3 [144]; while in a different study, the 37/67 kDa LRP/LR was shown to act as a receptor for AAV serotypes 2, 3, 8 and 9 (**Figure 1**) [145]. The identification of the 37/67 kDa LRP/LR as a receptor for the aforementioned mosquito-borne diseases has helped explain the unusually wide tropism of AAV, VEE virus, Dengue virus and Sindbis virus, and subsequently led to the development of gene therapy-based viral vectors targeting the LR [146].

Given the ability of LRP/LR to act as a receptor for the aforementioned viruses, the possible role that LRP/LR might play as a binding partner for other viruses and pathogens cannot be excluded. It is thus imperative that possible interactions of the 37/67 kDa LRP/LR with other disease-causing viruses be investigated.

6. Therapeutic approaches targeting LRP/LR for the treatment of other diseases

In addition to the multiple aforementioned roles which LRP/LR plays in the onset of various disorders and diseases, it has also been implicated in assisting certain bacteria cross the BBB [9]. Most bacteria can replicate in the bloodstream; however, only the three bacterial species (*Streptococcus pneumoniae, Neisseria meningitides* and *Haemophilius influenzae*) that are predominantly responsible for meningitis infections, can traverse the BBB [9]. This allows them to gain access to the cerebrospinal fluid where the immune defenses are initially restricted. However, in order to access the BBB, the pathogenic bacteria have to traverse the vascular epithelium. All three of these bacterial species utilize LRP/LR as a mediator for their entry into the BBB across the vascular epithelium [9]. *Streptococcus pneumoniae* encoded choline binding protein A (CbpA) binds to the BBB epithelium via the LRP/LR of the host via its R_2 domain which serves as a mediator for this attachment as described in a patent [147]. *Neisseria meningitides* and *H. influenzae* make use of similar strategies, except that they use PilQ or PorA and OmpP2, respectively, for the interaction with LRP/LR [9]. It is proposed that a common adhesion recognition site is present for these expressed proteins on the C terminus of LRP/LR [9].

A patent has been filed for a vaccine created to block these interactions by administering polypeptides mimicking the binding proteins expressed on the surface of these pathogenic bacterial cells. Furthermore, this vaccine consisted of antibodies directed against the polypeptides [148]. Another patented therapy focused on mimicking the R_2 domain to prevent CbpA binding to LRP/LR, thus blocking *S. pneumoniae* entry into the BBB [147]. It may also be assumed that strategies targeting and blocking LRP/LR would additionally impede the interactions with these binding proteins. Such a strategy could involve the use of antibodies directed against LRP/LR, as is observed in the treatment of prion disorders and cancer [36,115].

Candida albicans is an opportunistic pathogen able to parasitize various tissues of the human body. It has been demonstrated that the germ tubes of this pathogenic yeast facilitate attachment to host basement membranes by binding to laminin via a 68 kDa LR and this interaction thereby contributes to the establishment and progression of candidiasis [149]. Various approaches exist for blocking LRP/LR and it is possible that similar approaches may be undertaken to prevent *C. albicans* attachment by blocking the 68 kDa LR located on the yeast's germ tubes.

The LR is so widespread amongst living organisms that it is even present in the Archae Domain [150]. Its presence in archae bacteria such as the extreme halophile *Haloarcula marismortui* denotes an important conserved role for LRP/LR throughout evolution. However, most of the roles which LRP/LR assumes appear to be associated with disease; thus, it is valuable to keep examining the role of this receptor both in different species and diseases.

7. Conclusion

Heparan mimetics, siRNAs directed against LRP mRNA, a LRP decoy mutant and anti-LRP/LR specific antibodies have been proven to either downregulate or block the prion receptor LRP/LR. All tools either significantly increased the incubation time/preclinical phase (siRNAs [46], LRP decoy mutant [52]) or the survival (heparan mimetics such as HM 2602 [20] and anti-LRP specific antibody W3 [71]). PPS, proven to interfere with the prion-LRP/LR interaction on the cell surface [53], represents a drug for prion disorder treatment which has successfully cured Scrapie infected mice from prions [58].

Laminin was reported to dissolve amyloid plaques in AD [79], resulting in the production of laminin-derived peptides specific for the Aβ peptide for therapy and diagnosis of AD [83,84]. A possible role of LRP/LR in AD remains speculative.

LRP/LR plays a major role in metastatic cancer. Antibodies directed against LRP/LR, siRNAs downregulation of the receptor and polysulfated glycans significantly reduce the metastatic potential of fibrosarcoma cells [4]. LRP/LR hampers apoptosis [13] and, therefore, supports cancer progression by inhibiting programmed cell death. Thus, the receptor serves a fundamental role maintaining and stimulating cell viability.

Several viruses such as VEE virus, Sindbis virus, dengue virus and AAV use LRP/LR as initial attachment receptors.

The bacteria *S. pneumoniae*, *N. meningitis* and *H. influenzae* use LRP/LR as their mediator to pass the BBB to access the cerebrospinal fluid. *Candida albicans* germ tubes reveal that LRP/LR assist in attachment to host basement membranes.

Tools either blocking LRP/LR such as antibodies or tools which downregulate the LRP/LR level such as siRNAs might act as powerful tools for prophylaxis and therapy of metastatic cancer, neurodegenerative diseases as well as viral, bacterial and yeast infections.

8. Expert opinion

Prion diseases such as CJD which affect humans, BSE in cattle and CWD in elk and deer are exceptional disorders thought to be caused by misfolded protein aggregations. The dramatic reduction in BSE cases has lowered the risk of humans developing variant (v)CJD from the consumption of BSE-contaminated food, and consequently diminished the pressure for the development of effective prion disease therapeutics. No therapeutic for treatment of prion disorders is available, although a series of therapeutic approaches have been undertaken (for review: [15]). LRP/LR serving as a receptor for prions represents one of the novel targets for a therapeutic intervention in prion disorders. Although a significant prolongation of the incubation time/preclinical phase in Scrapie-infected mice has been achieved with siRNAs [52] and a LRP decoy mutant [46], we consider antibodies directed against the receptor as one of the most promising therapeutic LRP/LR tools for treatment of prion disorders. A series of patents cover LRP/LR specific antibodies, but only two cover their use in the treatment of prion disorders [51,73]. One of the most innovative therapeutic approaches involves the delivery of siRNAs directed against LRP mRNA by lentiviral vectors via microinjection [52]. A similar approach targeting PrP^c was reported earlier [49]. The low incidence rate for CJD (1/1 million inhabitants), concomitant with a negligible risk for humans achieving a vCJD infection by consumption of BSE-contaminated material may similarly result in reduced activities for the development of effective prion therapeutics or prophylaxis. However, as some CWD strains might indeed be transmissible to humans as was recently suggested [22], anti-LRP specific antibodies delivered by either passive immunotransfer or gene delivery systems such as AAV [74] might gain interest for further development.

AD represents a major problem for public health worldwide as the incidence rate is ~ 1/68 people. No therapeutic is as yet available and novel targets for AD treatment and prophylaxis are desired. One of the most promising targets is laminin due to its ability to resolve preformed amyloid plaques. A series of patents have been filed which encompass laminin and laminin-derived peptides for AD treatment.

LRP/LR acts as one of the key players in various metastatic cancer types by inducing cell adhesion and invasion though its interaction with the ECM component laminin-1 (for review: [3,36]). In addition, LRP/LR blocks apoptosis and thereby enhances cell viability [13]. LRP/LR represents an interesting molecule for targeted therapy in cancer for the reason that many different cancer types may be treated by knocking down or blocking LRP/LR. A series of therapeutic antibodies are on the market (for review: [3,95]); however, each antibody has only been approved for the treatment of select cancer types. Conversely, antibodies directed against LRP/LR do have the potential for the global treatment of many if not all metastatic cancer types. Patents have been filed encompassing epitopes of the OFA for cancer vaccination and a plurality of OFA/LRP as an immunotherapeutic composition for the use in humans [116,117]. A further patent has been filed encompassing the mAb 5LAC-23 for the diagnosis and treatment of cancer [118]. Moreover, a broader patent covers soluble LRP, ribozymes, antisense RNA and antibodies directed against LRP for the use in medicine [51]. Another set of patents has been filed covering LRP-specific antibodies for use in cancer therapy [73,115]. We consider antibodies directed against LRP/LR as the most important and siRNAs for LRP/LR downregulation as the most innovative tools for therapeutic intervention in various metastatic cancer types. Antibodies may be delivered by the oral route and encapsulated with ligands to target them to corresponding receptors expressed on the target cancer tissues. siRNAs and antibodies might be delivered by gene delivery systems such as lentiviral, adeno-associated or other viral vectors for targeted application to affected tissues or organs. We trust that anti-LRP specific antibodies possess huge potential to be further developed as therapeutic antibodies for treatment of many important cancer types worldwide. However, some years of development might be needed to attain approval for the use of LRP/LR specific antibodies as cancer therapeutics.

LRP/LR plays a further role as initial attachment receptors for viruses such as dengue virus, Sindbis virus, VEE virus and AAV. Dengue viruses infect ~ 50 million people worldwide a year and dengue viral infections are endemic in > 100 countries (http://www.who.int/en). As only supportive therapy is available for these viral infections, molecular tools targeting LRP/LR such as antibodies might, therefore, be promising candidates for a targeted therapeutic invention, especially for treatment of dengue virus infections. As Sindbis virus infections are highly apoptotic in mammalian cells, a patent encompassing Sindbis viral vectors with the potential to induce apoptosis in tumorigenic cells due to the binding and thereby blocking LRP/LR has been filed [132]. Sindbis virus vectors have in our opinion a high impact for cancer therapy.

Bacteria such as *S. pneumoniae*, the causative agent of meningitis, use LRP/LR to gain entry into cerebrospinal fluid via the BBB and the yeast *Saccharomyces cerevisiae* germ tubes express the receptor to facilitate attachment to basement membranes. *Saccharomyces cerevisiae* infections are very abundant in humans leading to candidiasis. Although we consider anti-LRP/LR tools, especially antibodies, as very suitable for a therapy of meningitis and *S. cerevisiae* fungal

infections, the high costs for this targeted antibody treatment may not be competitive with the more economical current conservative treatments such as the administration of antibiotics and fungicides for bacterial and fungal infections, respectively.

Regarding pharmaceutical developments, we estimate that antibodies directed against LRP/LR for the treatment of various metastatic cancer types might have the highest chances to receive approval from the authorities after successful animal trials and clinical studies and might reach the pharmaceutical market in a couple of years. The siRNA technology silencing LRP gene expression is promising; however, delivery systems such as recombinant lentiviral or adeno-associated viral vectors might struggle to receive approval even for clinical studies for the treatment of patients suffering from various metastatic cancer types. Antibodies directed against LRP have also the potential to reach the pharmaceutical market for the treatment of viral infections such as dengue or Sindbis virus infections. For both viral diseases only palliative therapies are available right now. Although LRP specific antibodies are promising alternative tools for the treatment of prion disorders, the low incident rates for CJD and related disorders worldwide might interfere with the approval of clinical studies on CJD patients.

Morbus Alzheimer has a high incidence rate worldwide and lacks any type of therapy. Laminin-derived peptides reported to dissolve amyloid plaques might have the greatest potential to reach the pharmaceutical market for the treatment of AD.

Acknowledgement

A Omar, K Jovanovic and B Da Costa Dias contributed equally to this work.

Declaration of interest

The authors are financially supported by the National Research Foundation (NRF), Republic of South Africa (RSA) and The University of the Witwatersrand. They declare no conflict of Interest.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Patarroyo M, Tryggvason K, Virtanen I. Laminin isoforms in tumor invasion, angiogenesis and metastasis. Semin Cancer Biol 2002;12(3):197-207
2. Givant-Horwitz V, Davidson B, Reich R. Laminin-induced signaling in tumor cells. Cancer Lett 2005;223(1):1-10
3. Mbazima V, Da Costa Dias B, Omar A, et al. Interactions between PrP(c) and other ligands with the 37-kDa/67-kDa laminin receptor. Front Biosci 2010;15:1150-63
4. Zuber C, Knackmuss S, Zemora G, et al. Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. J Mol Biol 2008;378(3):530-9
   - **Demonstrates that LRP/LR specific antibodies, siRNAs and polysulfated glycanes significantly reduce adhesion and invasion, key events in metastatic cancer.**
5. Landowski TH, Dratz EA, Starkey JR. Studies of the structure of the metastasis-associated 67 kDa laminin binding protein: fatty acid acylation and evidence supporting dimerization of the 32 kDa gene product to form the mature protein. Biochemistry 1995;34(35):11276-87
6. Buto S, Tagliabue E, Ardini E, et al. Formation of the 67-kDa laminin receptor by acylation of the precursor. J Cell Biochem 1998;69(3):244-51
7. Hundt C, Peyrin JM, Haik S, et al. Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. Embo J 2001;20(21):5876-86
8. Scheiman J, Tseng JC, Zheng Y, Meruelo D. Multiple functions of the 37/67-kd laminin receptor make it a suitable target for novel cancer gene therapy. Mol Ther 2010;18(1):63-74
   - **This report reveals that LRP/LR promotes cell cycle progression.**
9. Orihuela CJ, Mahdavi J, Thornton J, et al. Laminin receptor initiates bacterial contact with the blood brain barrier in experimental meningitis models. J Clin Invest 2009;119(6):1638-46
   - **Highlights a further role of LRP/LR in bacterial infections causing meningitis.**
10. Liu L, Sun L, Zhao P, et al. Hypoxia promotes metastasis in human gastric cancer by up-regulating the 67-kDa laminin receptor. Cancer Sci 2010;101(7):1653-60
11. Tachibana H, Fujimura Y, Yamada K. Tea polyphenol epigallocatechin-3-gallate associates with plasma membrane lipid rafts: lipid rafts mediate anti-allergic action of the catechin. Biofactors 2004;21(1-4):383-5
12. Kaneda Y, Kinoshita K, Sato M, et al. The induction of apoptosis in HeLa cells by the loss of LBP-p40. Cell Death Differ 1998;5(1):20-8
   - **The manuscript describes an inhibitory role of LRP/LR (LBP-40) in apoptosis.**
13. Susantad T, Smith DR. siRNA-mediated silencing of the 37/67-kDa high affinity laminin receptor in Hep3B cells induces apoptosis. Cell Mol Biol Lett 2008;13(3):452-64
   - **Confirms the inhibitory effect of LRP/LR on apoptosis in hepatitic cells through the use of siRNA technology.**
14. Linden R, Martins VR, Prado MA, et al. Physiology of the prion protein. Physiol Rev 2008;88(2):673-728
15. Ludewigs H, Zuber C, Vana K, et al. Therapeutic approaches for prion disorders. Expert Rev Anti Infect Ther 2007;5(4):613-30
16. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998;95(23):13363-83
17. Weissmann C. Molecular biology of prion diseases. Trends Cell Biol 1994;4(1):10-14
18. Zuber C, Ludewigs H, Weiss S. Therapeutic approaches targeting the prion receptor LRP/LR. Vet Microbiol 2007;123(4):387-93
19. Vana K, Zuber C, Nikles D, Weiss S. Novel aspects of prions, their receptor molecules, and innovative approaches for

TSE therapy. Cell Mol Neurobiol 2007;27(1):107-28

20. Adjou KT, Simoneau S, Sales N, et al. A novel generation of heparan sulfate mimetics for the treatment of prion diseases. J Gen Virol 2003;84(Pt 9):2595-603

21. Harris DA. Cellular biology of prion diseases. Clin Microbiol Rev 1999;12(3):429-44

22. Kolodziejczak D, Da Costa Dias B, Zuber C, et al. Prion Interaction with the 37-kDa/67-kDa Laminin Receptor on Enterocytes as a Cellular Model for Intestinal Uptake of Prions. J Mol Biol 2010;402:293-300

• **Highlights the use of an enterocyte model system to investigate the intestinal pathophysiology of alimentary prion infections. The study suggests that CWD may be orally transmissible to humans.**

23. Da Costa Dias B, Weiss SF. A kiss of a prion: new implications for oral transmissibility. J Infect Dis 2010;201(11):1615-16

24. Maddison BC, Rees HC, Baker CA, et al. Prions are secreted into the oral cavity in sheep with preclinical scrapie. J Infect Dis 2010;201(11):1672-6

• **The finding that prions are secreted into the oral cavity has implications for the oral transmissibility of prions.**

25. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982;216(4542):136-44

•• **The first report proposing that prions may cause transmissible spongiform encephalopathies.**

26. Stahl N, Borchelt DR, Hsiao K, Prusiner SB. Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell 1987;51(2):229-40

27. Stahl N, Baldwin MA, Teplow DB, et al. Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. Biochemistry 1993;32(8):1991-2002

28. Lasmezas CI, Weiss S. Molecular biology of prion disorders. In: Cary JW, Linz JE, Bhatnagar D, editors, Microbial foodborne diseases: mechanisms of pathogenesis and toxin synthesis. Technomics Publishing Co, Lancaster, USA; 2000. p. 495-537

29. Morel E, Andrieu T, Casagrande F, et al. Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. Am J Pathol 2005;167(4):1033-42

• **Manuscript suggesting a possible oral BSE transmission to humans via enterocytes dependent on LRP/LR.**

30. Shyng SL, Heuser JE, Harris DA. A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. J Cell Biol 1994;125(6):1239-50

31. Vey M, Pilkuhn S, Wille H, et al. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc Natl Acad Sci USA 1996;93(25):14945-9

32. Fraser H. The pathology of a natural and experimental scrapie. Front Biol 1976;44:267-305

33. Simoneau S, Haik S, Leucht C, et al. Different isoforms of the non-integrin laminin receptor are present in mouse brain and bind PrP. Biol Chem 2003;384(2):243-6

34. Gauczynski S, Peyrin JM, Haik S, et al. The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. EMBO J 2001;20(21):5863-75

•• **Highlighting LRP/LR as the receptor for the cellular prion protein.**

35. Rieger R, Edenhofer F, Lasmezas CI, Weiss S. The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. Nat Med 1997;3(12):1383-8

36. Vana K, Zuber C, Pflanz H, et al. LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. Infect Disord Drug Targets 2009;9(1):69-80

37. Rieger R, Lasmezas CI, Weiss S. Role of the 37 kDa laminin receptor precursor in the life cycle of prions. Transfus Clin Biol 1999;6(1):7-16

38. Hinek A, Wrenn DS, Mecham RP, Barondes SH. The elastin receptor: a galactoside-binding protein. Science 1988;239(4847):1539-41

39. Mecham RP. Receptors for laminin on mammalian cells. FASEB J 1991;5(11):2538-46

40. Trevitt CR, Collinge J. A systematic review of prion therapeutics in experimental models. Brain 2006;129(Pt 9):2241-65

• **A comprehensive review on prion therapeutics.**

41. Mange A, Nishida N, Milhavet O, et al. Amphotericin B inhibits the generation of the scrapie isoform of the prion protein in infected cultures. J Virol 2000;74(7):3135-40

42. Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. Nat Med 2004;10(Suppl):S10-17

43. Bueler H, Aguzzi A, Sailer A, et al. Mice devoid of PrP are resistant to scrapie. Cell 1993;73(7):1339-47

•• **Demonstration of the necessity of the cellular prion protein for prion infections.**

44. Richt JA, Kasinathan P, Hamir AN, et al. Production of cattle lacking prion protein. Nat Biotechnol 2007;25(1):132-8

45. Vana K, Weiss S. A trans-dominant negative 37kDa/67kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells. J Mol Biol 2006;358(1):57-66

46. Pflanz H, Vana K, Mitteregger G, et al. Scrapie-infected transgenic mice expressing a laminin receptor decoy mutant reveal a prolonged incubation time associated with low levels of PrPres. J Mol Biol 2009;388(4):721-9

47. Tilly G, Chapuis J, Vilette D, et al. Efficient and specific down-regulation of prion protein expression by RNAi. Biochem Biophys Res Commun 2003;305(3):548-51

48. Sakaguchi S, Taira K.; inventors, Nagasaki University, assignee. Method of inhibiting prion protein patent US2005/0053583A1; 2005

49. Pfeifer A, Eigenbrod S, Al-Khadra S, et al. Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. J Clin Invest 2006;116(12):3204-10

50. Leucht C, Simoneau S, Rey C, et al. The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. EMBO Rep 2003;4(3):290-5

51. University of the Witwatersrand, Weiss S, assignee. Pharmaceutical compositions comprising a soluble laminin receptor precursor or a

compound which blocks the interaction of the laminin receptor precursor and PrPSc or PrPc patent WO9853838A2; 1999

52. Pflanz H, Vana K, Mitteregger G, et al. Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. J Gen Virol 2009;90(Pt 1):269-74

53. Gauczynski S, Nikles D, El-Gogo S, et al. The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. J Infect Dis 2006;194(5):702-9

• **Primary demonstration that LRP/LR serves as a receptor for infectious prions.**

54. Safar J, Wille H, Itri V, et al. Eight prion strains have PrP(Sc) molecules with different conformations. Nat Med 1998;4(10):1157-65

55. Larramendy-Gozalo C, Barret A, Daudigeos E, et al. Comparison of CR36, a new heparan mimetic, and pentosan polysulfate in the treatment of prion diseases. J Gen Virol 2007;88(Pt 3):1062-7

56. Doh-ura K, Ishikawa K, Murakami-Kubo I, et al. Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. J Virol 2004;78(10):4999-5006

57. Microsens Biophage Limited, assignee. Binding of pathological forms of prion proteins patent US2005/0221404A1; 2005

58. Farquhar C, Dickinson A, Bruce M. Prophylactic potential of pentosan polysulphate in transmissible spongiform encephalopathies. The Lancet 1999;353(9147):117

•• **Describes pentosan polysulfate as a drug able to successfully cure two mouse strains from two scrapie strains.**

59. Caughey B, Raymond GJ. Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. J Virol 1993;67(2):643-50

60. Katsumi D, Murakami I.; inventors. Sangaku Renkei Kiko Kyushi KK, assignee. Composition for inhibiting production of pathological prion protein and method for inhibiting production of pathological prion protein patent JP2003040778A; 2003

61. Korth C, Stierli B, Streit P, et al. Prion (PrPSc)-specific epitope defined by a monoclonal antibody. Nature 1997;390(6655):74-7

•• **Description of the prion specific antibody 15B3.**

62. Feraudet C, Morel N, Simon S, et al. Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. J Biol Chem 2005;280(12):11247-58

63. Collinge J, Hawke S.; inventors, Medical Research Council, London, GB., assignee. Prion Inhibition patent US2006/0280745A1; 2006

64. University Court of the University of Edinburgh, University of Strathclyde Common Services Agency, Institute for Animal Health assignee. Novel antibodies against the prion protein and uses thereof patent Wo2007/144619A2; 2007

65. Peretz D, Williamson RA, Kaneko K, et al. Antibodies inhibit prion propagation and clear cell cultures of prion infectivity. Nature 2001;412(6848):739-43

66. Enari M, Flechsig E, Weissmann C. Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody. Proc Natl Acad Sci USA 2001;98(16):9295-9

67. Heppner FL, Musahl C, Arrighi I, et al. Prevention of scrapie pathogenesis by transgenic expression of anti-prion protein antibodies. Science 2001;294(5540):178-82

68. White AR, Enever P, Tayebi M, et al. Monoclonal antibodies inhibit prion replication and delay the development of prion disease. Nature 2003;422(6927):80-3

69. Heegard P, Jakobsen PH.; inventors, Copenhagen Biotech Assets Aps., assignee. Composite peptide compounds for the diagnosis and treatment of diseases caused by prion proteins patent WO2004/018511A2; 2004

70. Renomedix Inst., Inc., assignee. Remedy for prion disease and method of producing the same patent WO2005094846; 2005

71. Zuber C, Mitteregger G, Pace C, et al. Anti-LRP/LR antibody W3 hampers peripheral PrPSc propagation in scrapie infected mice. Prion 2007;1(3):207-12

72. Zuber C, Knackmuss S, Rey C, et al. Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases. Mol Immunol 2008;45(1):144-51

73. Affimed Therapeutics AG, Lmu Munich, assignee. Single-chain antibody acting against 37kDa/67kDa laminin receptor as tools for the diagnosis and therapy of prion diseases and cancer, production and use thereof patent DE10346627A1; WO2005035580A1; 2005

74. Zuber C, Mitteregger G, Schuhmann N, et al. Delivery of single-chain antibodies (scFvs) directed against the 37/67 kDa laminin receptor into mice via recombinant adeno-associated viral vectors for prion disease gene therapy. J Gen Virol 2008;89(Pt 8):2055-61

75. Ziegler-Graham K, Brookmeyer R, Johnson E, Arrighi HM. Worldwide variation in the doubling time of Alzheimer's disease incidence rates. Alzheimers Dement 2008;4(5):316-23

76. Burns A, Iliffe S. Alzheimer's disease. BMJ 2009;338:b158

77. Mills J, Reiner PB. Regulation of amyloid precursor protein cleavage. J Neurochem 1999;72(2):443-60

78. Beck K, Hunter I, Engel J. Structure and function of laminin: anatomy of a multidomain glycoprotein. FASEB J 1990;4(2):148-60

79. Koo EH, Park L, Selkoe DJ. Amyloid beta-protein as a substrate interacts with extracellular matrix to promote neurite outgrowth. Proc Natl Acad Sci USA 1993;90(10):4748-52

80. Castillo GM, Lukito W, Peskind E, et al. Laminin inhibition of beta-amyloid protein (Abeta) fibrillogenesis and identification of an Abeta binding site localized to the globular domain repeats on the laminin a chain. J Neurosci Res 2000;62(3):451-62

• **Highlighting the role of laminin as an inhibitor of Abeta fibrillogenesis. Laminin may be a natural substance able to hamper the development of Alzheimer's disease.**

81. Monji A, Tashiro K, Yoshida I, Tashiro N. Laminin inhibits Abeta42 fibril formation in vitro. Brain Res 1998;788(1-2):187-90

82. University of Washington, assignee. Therapeutic applications of

laminin-derived protein fragments patent US7314724B2; 2008

83. Proteotech, Inc., assignee. Small peptides for the treatment of Alzheimer's disease and other Beta-Amyloid protein fibrillogenisis disorders patent EP2172208A1; 2010

84. Proteotech, Inc., assignee. Peptides for the treatment of Alzheimer's Disease and other Beta-Amyloid protein fibrillogenisis disorders patent US2005/0153898A1; 2005

85. Westergard L, Christensen HM, Harris DA. The cellular prion protein (PrP(C)): its physiological function and role in disease. Biochim Biophys Acta 2007;1772(6):629-44

86. Parkin ET, Watt NT, Hussain I, et al. Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. Proc Natl Acad Sci USA 2007;104(26):11062-7

- **Description of a protective role of the cellular prion protein in the development of Alzheimer's disease.**

87. Lauren J, Gimbel DA, Nygaard HB, et al. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature 2009;457(7233):1128-32

- **A report on PrPc acting as a receptor for amyloid beta oligomers.**

88. Kessels HW, Nguyen LN, Nabavi S, Malinow R. The prion protein as a receptor for amyloid-beta. Nature 2010;466(7308):E3-4, discussion E4-5

- **A report arguing against PrPc acting as a receptor for amyloid beta oligomers.**

89. Balducci C, Beeg M, Stravalaci M, et al. Synthetic amyloid-beta oligomers impair long-term memory independently of cellular prion protein. Proc Natl Acad Sci USA 2010;107(5):2295-300

- **A further report also arguing that PrPc does not act as a receptor for amyloid beta oligomers.**

90. Kitamoto T, Tateishi J, Tashima T, et al. Amyloid plaques in Creutzfeldt-Jakob disease stain with prion protein antibodies. Ann Neurol 1986;20(2):204-8

91. Available from: http://globocan.iarc.fr [Cited]

92. Stegmeier F, Warmuth M, Sellers WR, Dorsch M. Targeted cancer therapies in the twenty-first century: lessons from imatinib. Clin Pharmacol Ther 2010;87(5):543-52

93. Sawyers C. Targeted cancer therapy. Nature 2004;432(7015):294-7

- **A comprehensive review on targeted cancer therapy.**

94. IDEC Pharmaceuticals Corporation, assignee. Variant IGG3 Rituxan and therapeutic use thereof patent US2002/0128448A1; 2002

95. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer 2001;1(2):118-29

- **A comprehensive review on antibody-based cancer therapy.**

96. Guinn BA, Mulherkar R. International progress in cancer gene therapy. Cancer Gene Ther 2008;15(12):765-75

97. Shay JW. Meeting report: the role of telomeres and telomerase in cancer. Cancer Res 2005;65(9):3513-17

98. Wewer UM, Taraboletti G, Sobel ME, et al. Role of laminin receptor in tumor cell migration. Cancer Res 1987;47(21):5691-8

- **Role of the non-integrin laminin receptor in tumor cell migration.**

99. Castronovo V, Taraboletti G, Sobel ME. Laminin receptor complementary DNA-deduced synthetic peptide inhibits cancer cell attachment to endothelium. Cancer Res 1991;51(20):5672-8

- **Describes the role of the non-integrin laminin receptor in cellular attachment.**

100. Montuori N, Selleri C, Risitano AM, et al. Expression of the 67-kDa laminin receptor in acute myeloid leukemia cells mediates adhesion to laminin and is frequently associated with monocytic differentiation. Clin Cancer Res 1999;5(6):1465-72

101. Rao CN, Castronovo V, Schmitt MC, et al. Evidence for a precursor of the high-affinity metastasis-associated murine laminin receptor. Biochemistry 1989;28(18):7476-86

- **Describes the role of the non-integrin laminin receptor in metastasis.**

102. de Manzoni G, Guglielmi A, Verlato G, et al. Prognostic significance of 67-kDa laminin receptor expression in advanced gastric cancer. Oncology 1998;55(5):456-60

103. Cioce V, Castronovo V, Shmookler BM, et al. Increased expression of the laminin receptor in human colon cancer. J Natl Cancer Inst 1991;83(1):29-36

104. Sanjuan X, Fernandez PL, Miquel R, et al. Overexpression of the 67-kD laminin receptor correlates with tumour progression in human colorectal carcinoma. J Pathol 1996;179(4):376-80

105. al-Saleh W, Delvenne P, van den Brule FA, et al. Expression of the 67 KD laminin receptor in human cervical preneoplastic and neoplastic squamous epithelial lesions: an immunohistochemical study. J Pathol 1997;181(3):287-93

106. Nadji M, Nassiri M, Fresno M, et al. Laminin receptor in lymph node negative breast carcinoma. Cancer 1999;85(2):432-6

107. Fontanini G, Vignati S, Chine S, et al. 67-Kilodalton laminin receptor expression correlates with worse prognostic indicators in non-small cell lung carcinomas. Clin Cancer Res 1997;3(2):227-31

108. van den Brule FA, Berchuck A, Bast RC, et al. Differential expression of the 67-kD laminin receptor and 31-kD human laminin-binding protein in human ovarian carcinomas. Eur J Cancer 1994;30A(8):1096-9

109. Pelosi G, Pasini F, Bresaola E, et al. High-affinity monomeric 67-kD laminin receptors and prognosis in pancreatic endocrine tumours. J Pathol 1997;183(1):62-9

110. Waltregny D, de Leval L, Menard S, et al. Independent prognostic value of the 67-kd laminin receptor in human prostate cancer. J Natl Cancer Inst 1997;89(16):1224-7

111. Gasparini G, Barbareschi M, Boracchi P, et al. 67-kDa laminin-receptor expression adds prognostic information to intra-tumoral microvessel density in node-negative breast cancer. Int J Cancer 1995;60(5):604-10

112. Menard S, Tagliabue E, Colnaghi MI. The 67 kDa laminin receptor as a prognostic factor in human cancer. Breast Cancer Res Treat 1998;52(1-3):137-45

113. Narumi K, Inoue A, Tanaka M, et al. Inhibition of experimental metastasis of human fibrosarcoma cells by anti-recombinant 37-kDa laminin

binding protein antibody. Jpn J Cancer Res 1999;90(4):425-31

• ***In vivo* study demonstrating a significant inhibition of metastatic lung cancer by the administration of a laminin receptor specific antibody.**

114. New York University, assignee. Tumor therapy with alphavirus-based and high affinity laminin receptor-targeted vectors patent WO02/076468A1; 2002

115. Affimed Therapeutics AG, Lmu Munich, assignee. Use of an antibody against the laminin receptor or laminin receptor precursor for the treatment or diagnosis of several cancer types patent WO2006/05954A2; EP1709972A1; 2006

116. South Alibama Medical Sciences, assignee. Cancer vaccines containing epitopes of ocofetal antigen patent US2006165709A1; 2006

117. South Alibama Medical Sciences US., assignee. Oncofetal specific monoclonal antibodies, methods of preparation and use patent WO86/03223; 1986

118. Mchale & Slavin PA, assignee. Laminin receptor 1 precursor protein (37LRP) epitope delineated by an hepatocellular carcinoma specific antibody patent US2005/0244899; 2005

119. Oldham RK, Dillman RO. Monoclonal antibodies in cancer therapy: 25 years of progress. J Clin Oncol 2008;26(11):1774-7

120. Kanisa Pharmaceuticals, assignee. Treatment of patients with cancer using a calicheamicin-antibody conjugate in combination with zosuquidar patent WO2007/008479A1; 2007

121. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity 1975. J Immunol 2005;174(5):2453-5

122. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57-70

•• **A comprehensive review on the hallmarks of cancer.**

123. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407(6805):770-6

•• **A comprehensive review on the biochemistry of apoptosis.**

124. Ferreira CG, Epping M, Kruyt FA, Giaccone G. Apoptosis: target of cancer therapy. Clin Cancer Res 2002;8(7):2024-34

125. Yang CS, Lambert JD, Hou Z, et al. Molecular targets for the cancer preventive activity of tea polyphenols. Mol Carcinog 2006;45(6):431-5

126. Tachibana H, Koga K, Fujimura Y, Yamada K. A receptor for green tea polyphenol EGCG. Nat Struct Mol Biol 2004;11(4):380-1

• **Identification of LRP/LR as a receptor for the green tea polyphenol EGCG, suggesting that one of the anti-tumorigenic mechanisms of ECGC may rely in blocking the receptor.**

127. Leone M, Zhai D, Sareth S, et al. Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins. Cancer Res 2003;63(23):8118-21

128. Lotz MM, Korzelius CA, Mercurio AM. Human colon carcinoma cells use multiple receptors to adhere to laminin: involvement of alpha 6 beta 4 and alpha 2 beta 1 integrins. Cell Regul 1990;1(3):249-57

129. Lee EC, Lotz MM, Steele GD Jr, Mercurio AM. The integrin alpha 6 beta 4 is a laminin receptor. J Cell Biol 1992;117(3):671-8

130. Tozeren A, Kleinman HK, Wu S, et al. Integrin alpha 6 beta 4 mediates dynamic interactions with laminin. J Cell Sci 1994;107(Pt 11):3153-63

131. Clarke AS, Lotz MM, Chao C, Mercurio AM. Activation of the p21 pathway of growth arrest and apoptosis by the beta 4 integrin cytoplasmic domain. J Biol Chem 1995;270(39):22673-6

132. Darby & Darby PC, assignee. Method for detecting cancer cells and monitoring cancer therapy patent US2005/0152838A1; 2005

133. Dimitrov DS. Virus entry: molecular mechanisms and biomedical applications. Nat Rev Microbiol 2004;2(2):109-22

• **A comprehensive review on virus entry mechanisms.**

134. Alkhatib G, Berger EA. HIV coreceptors: from discovery and designation to new paradigms and promise. Eur J Med Res 2007;12(9):375-84

135. Proudfoot AE, Power CA, Schwarz MK. Anti-chemokine small molecule drugs: a promising future? Expert Opin Investig Drugs 2010;19(3):345-55

136. Shanghai Biowindow Gene Development, Inc., assignee. A novel polypeptide – homo laminin protein B2 chain chain and polynucleotide encoding said polypeptide. patent WO01/90165A1; 2001

137. Weiss S, Konig B, Morikawa Y, Jones I. Recombinant HIV-1 nucleocapsid protein p15 produced as a fusion protein with glutathione S-transferase in Escherichia coli mediates dimerization and enhances reverse transcription of retroviral RNA. Gene 1992;121(2):203-12

138. Weiss S, Konig B, Muller HJ, et al. Synthetic human tRNA(UUULys3) and natural bovine tRNA(UUULys3) interact with HIV-1 reverse transcriptase and serve as specific primers for retroviral cDNA synthesis. Gene 1992;111(2):183-97

139. Weiss S, Hausl G, Famulok M, Konig B. The multimerization state of retroviral RNA is modulated by ammonium ions and affects HIV-1 full-length cDNA synthesis in vitro. Nucleic Acids Res 1993;21(21):4879-85

140. Coley W, Kehn-Hall K, Van Duyne R, Kashanchi F. Novel HIV-1 therapeutics through targeting altered host cell pathways. Expert Opin Biol Ther 2009;9(11):1369-82

• **A comprehensive review on novel HIV-1 therapeutics.**

141. Bhattacharya S, Osman H. Novel targets for anti-retroviral therapy. J Infect 2009;59(6):377-86

142. Wang KS, Kuhn RJ, Strauss EG, et al. High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. J Virol 1992;66(8):4992-5001

• **Describes LRP/LR as a receptor for Sindbis virus.**

143. Ludwig GV, Kondig JP, Smith JF. A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. J Virol 1996;70(8):5592-9

• **VEE virus is reported to utilize LRP/LR as a receptor.**

144. Tio PH, Jong WW, Cardosa MJ. Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3. Virol J 2005;2:25

145. Akache B, Grimm D, Pandey K, et al. The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. J Virol 2006;80(19):9831-6

- **The first report that AAV subtypes use LRP/LR as receptors.**

146. University of New York assignee. Tumour therapy with replication competent sindbis viral vectors patent WO2009105673(A2); 2009

147. St. Jude Children's Research Hospital, assignee. Synthetic Streptococcus pnuemoniae vaccine patent WO2008/039838; 2008

148. University of Nottingham, assignee. Laminin receptor binding proteins patent WO2009/144462A2; 2009

149. Bouchara JP, Tronchin G, Annaix V, et al. Laminin receptors on Candida albicans germ tubes. Infect Immun 1990;58(1):48-54

- **Presence of LRP/LR on Candida albicans (yeast) germ tubes.**

150. Ouzonis C, Kyrpides N, Sander C. Novel protein families in archaean genomes. Nucleic Acids Res 1995;23(4):565-70

- **Description of the presence of LRP/LR in Archae bacteria.**

Affiliation

Aadilah Omar[1], Katarina Jovanovic[1], Bianca Da Costa Dias[1], Danielle Gonsalves[1], Kiashanee Moodley[1], Robert Caveney[2], Vusi Mbazima[1] & Stefan FT Weiss[†1]
[†]Author for correspondence
[1]University of the Witwatersrand, School of Molecular and Cell Biology, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa (RSA)
Tel: +27 11 7176346; Fax: +27 11 7176351; E-mail: stefan.weiss@wits.ac.za
[2]University of the Witwatersrand, Wits Commercial Enterprise (Pty) Limited, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa (RSA)

Alimentary Prion Infections: Touchdown in the intestine

B. Da Costa Dias, K. Jovanovic, S.F.T. Weiss

Prion 5 (2011) 6-9

This brief review article discussed the modes of transmission of infectious prion proteins in prion disorders. This includes peroral and perenteral transmission as well proposed routes by which prion proteins are taken up by the gastrointestinal system. The presence of LRP/LR on the surface of enterocytes is thought to play a major role in the uptake and spread of infectious prion proteins. The transmission of infectious PrP is of general interest as this protein is known to readily cross the species barrier and cause Transmissible Spongiform Encephalopathies (TSEs). This is of particular importance as it poses a risk to human beings exposed to infected livestock, whereby transmission can occur and result in neurodegenerative diseases such as Creutzfeldt-Jakob Disease (CJD), Kuru and Fatal Familial Insomnia (FFI). The possibility of transmission in other neurodegenerative diseases such as Alzheimer's and Parkinson's diseases is also explored.

Prion 5:1, 6-9; January/February/March 2011; © 2011 Landes Bioscience

Alimentary prion infections

Touchdown in the intestine

Bianca Da Costa Dias, Katarina Jovanovic and Stefan F.T. Weiss*
School of Molecular and Cell Biology; University of the Witwatersrand; Johannesburg, Republic of South Africa

Neurodegenerative diseases are caused by proteinaceous aggregates, usually consisting of misfolded proteins which are often typified by a high proportion of β-sheets that accumulate in the central nervous system. These diseases, including Morbus Alzheimer, Parkinson disease and Transmissible Spongiform Encephalopathies (TSEs)—also termed prion disorders—afflict a substantial proportion of the human population and, as such, the etiology and pathogenesis of these diseases has been the focus of mounting research. Although many of these diseases arise from genetic mutations or are sporadic in nature, the possible horizontal transmissibility of neurodegenerative diseases poses a great threat to population health. In this article we discuss recent studies that suggest that the "non-transmissible" status bestowed upon Alzheimer and Parkinson diseases may need to be revised as these diseases have been successfully induced through tissue transplants. Furthermore, we highlight the importance of investigating the "natural" mechanism of prion transmission including peroral and perenteral transmission, proposed routes of gastrointestinal uptake and neuroinvasion of ingested infectious prion proteins. We examine the multitude of factors which may influence oral transmissibility and discuss the zoonotic threats that Chronic Wasting disease (CWD), Bovine Spongiform Encephalopathy (BSE) and Scrapie may pose resulting in vCJD or related disorders. In addition, we suggest that the 37 kDa/67 kDa laminin receptor on the cell surface of enterocytes, a major cell population in the intestine, may play an important role in the intestinal pathophysiology of alimentary prion infections.

Key words: prion, 37 kDa/67 kDa laminin receptor, CJD, BSE, CWD, scrapie, Alzheimer disease, Parkinson disease, intestine, enterocytes

Abbreviations: PrP^{c}, cellular prion protein; PrP^{Sc}, scrapie prion protein; TSE, transmissible spongiform encephalopathy; LRP/LR, non-integrin laminin receptor; M-cells, microfold cells; FDCs, follicular dendritic cells; CJD, Creutzfeldt-Jakob disease; BSE, Bovine Spongiform Encephalopathy; CWD, Chronic Wasting disease; ENS, enteric nervous system; CNS, central nervous system; Aβ, amyloid-beta

Submitted: 09/02/10

Accepted: 11/28/10

Previously published online: www.landesbioscience.com/journals/prion/article/14283

DOI: 10.4161/prion.5.1.14283

*Correspondence to: Stefan F.T. Weiss; Email: stefan.weiss@wits.ac.za

Many different mechanisms exist which underlie the etiology of the numerous neurodegenerative diseases affecting the human population. Amongst the most prominent are Morbus Alzheimer, prion disorders, Parkinson disease, Chorea Huntington, frontotemporal dementia and amylotrophic lateral sclerosis. The molecular mechanisms underlying these diseases vary; however, all neurodegenerative diseases share a common feature: they are caused by protein aggregation. The only neurodegenerative diseases proven to be transmissible are prion disorders. In contrast to frontotemporal dementia, recent evidence suggests that Alzheimer and Parkinson diseases may also be transmissible. Pre-symptomatic Alzheimer disease (APP23) mice exhibited an increase in the Alzheimer phenotype when brain homogenate of autopsied human Alzheimer disease patients and older, amyloid beta-(Aβ-) laden APP23 mice was injected into their hippocampi.[1] These findings suggest that the Aβ-abundant brain homogenate of Alzheimer disease patients may possess the ability to induce or supplement the overproduction of Aβ, possibly leading to the onset of Alzheimer disease.

The pathological feature associated with Parkinson disease is the formation of Lewy bodies in cell bodies and neuronal processes in the brain.[2] The main component of these protein aggregates is α-synuclein (reviewed in ref. 2). Autopsies of Parkinson disease patients revealed that Lewy bodies had formed on healthy

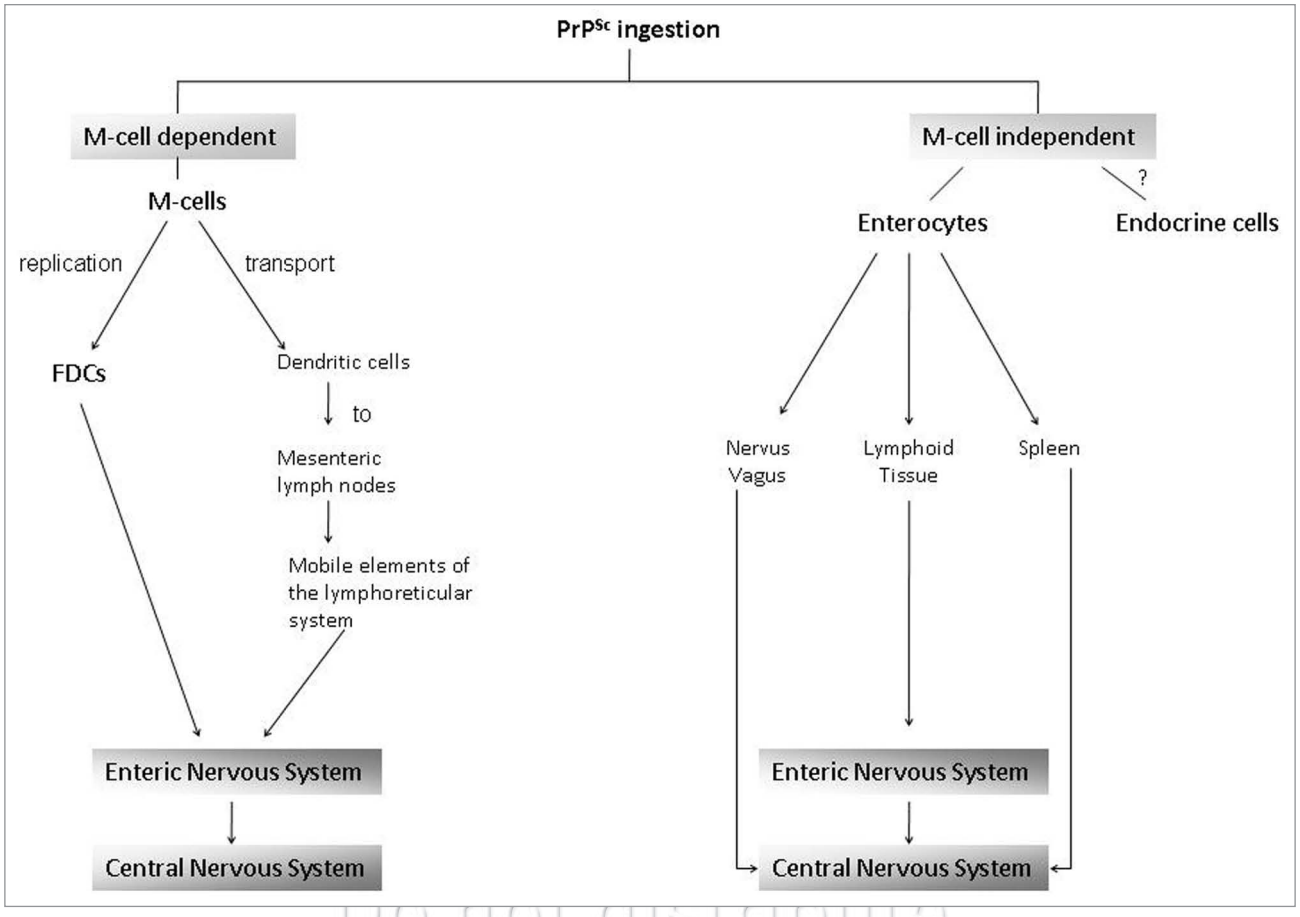


Figure 1. Proposed routes of gastrointestinal entry of ingested infectious prions (PrP^{Sc}) as well as possible pathways of amplification and transport to the central nervous system.

embryonic neurons that had been grafted onto the brain tissue of the patients several years before (prior to said examination).[3-5] It may thus be proposed that α-synuclein transmission is possible from diseased to healthy neurons, suggesting that Parkinson disease may be transmissible from a Parkinson disease patient to a healthy individual. These findings imply that Alzheimer and Parkinson diseases may be transmissible through tissue transplants and the use of contaminated surgical tools.[6]

Prion disorders, also termed Transmissible Spongiform Encephalopathies (TSEs), are fatal neurodegenerative diseases that affect the central nervous system (CNS) of multiple animal species. In lieu of the social, economic and political ramifications of such infections, as well as the possible intra- and interspecies transmissibility of such disorders, various routes of experimental transmission have been investigated including intracerebral, intraperitoneal, intraventricular, intraocular, intraspinal and subcutaneous injections (reviewed in ref. 7–9). However, such routes of transmission are not representative of the "natural" mechanism as the majority of prion disorders are contracted through ingestion of infectious prion (PrP^{Sc}) containing material. Thus, the peroral and perenteral prion transmission is of greatest consequence with respect to TSE disease establishment. Moreover, the presence of PrP^{Sc} in the buccal cavity of scrapie-infected sheep[10] (reviewed in ref. 11) and the possible horizontal transfer as a result hereof, as may be similarly proposed for animals suffering from other TSEs, may further contribute to the oral transmissibility of TSEs.

A number of model systems have been employed to study TSE transmissibility. Owing to ethical constraints, TSE transmissibility to humans via the oral route may not be directly investigated and as a result hereof, alternative model systems are needed. These may include the use of transgenic mice, cell lines which are permissive to infection[12] and experimental animals such as sheep, calves, goats, minks, ferrets and non-human primates (reviewed in ref. 9).

Intestinal entry of PrP^{Sc} has been proposed to occur via two pathways, the membranous (M) cell-dependent and M cell-independent pathways (**Fig. 1**).[13,14] The former involves endocytic M (microfold)-cells, which cover the intestinal lymphoid follicles (Peyer's patches)[14] and may take up prions and thereby facilitate the translocation of these proteins across the intestinal epithelium into the lymphoid tissues (reviewed in ref. 9) as has been demonstrated in a cellular model.[13] Following such uptake by the M cells, the prions may subsequently pass to the dendritic cells and follicular dendritic cells (FDCs) (**Fig. 1**), which allow for prion transport to the mesenteric lymph nodes and replication, respectively.[15] The prion

proteins may subsequently gain access to the enteric nervous system (ENS) and ultimately the central nervous system (CNS).[15]

However, prion intestinal translocation has been observed in the absence of M cells and has been demonstrated to be as a result of the action of polar, 37 kDa/67 kDa LRP/LR (non-integrin laminin receptor; reviewed in ref. 16–18) expressing enterocytes. Enterocytes are the major cell population of the intestinal epithelium and due to their ability to endocytose pathogens, nutrients and macromolecules,[19] it has been proposed that these cells may represent a major entry site for alimentary prions (**Fig. 1**).

Since enterocyte prion uptake has been demonstrated to be dependent on the presence of LRP/LR on the apical brush border of the cells,[14,20] the interaction between varying prion protein strains and the receptor[21-23] may be employed as a model system to study possible oral transmissibility of prion disorders across species as well as the intestinal pathophysiology of alimentary prion infections.[24] Moreover, the blockage of such interactions through the use of anti-LRP/LR specific antibodies has been reported to reduce PrP^{Sc} endocytosis[19] and thus these antibodies may serve as potential therapeutics to prevent infectious prion internalization and thereby prevent prion infections. It must be emphasized that the adhesion of prion proteins to cells is not solely dependent on the LRP/LR-PrP^{Sc} interactions;[24] however, this interaction is of importance with regards to internalization and subsequent pathogenesis.

We applied the aforementioned cell model to study the possible oral transmission of PrP^{BSE}, PrP^{CWD} and ovine PrP^{Sc} to cervids, cattle, swine and humans.[24] The direct transmission of the aforementioned animal prion disorders to humans as a result of dietary exposure and the possible establishment of zoonotic diseases is of great public concern. It must however be emphasized that the study investigated the co-localization of LRP/LR and various prion strains and not the actual internalization process.

PrP^{BSE} was shown to co-localize with LRP/LR on human enterocytes[24], thereby suggesting that PrP^{BSE} is transmissible to humans via the oral route which is widely accepted as the manner by which variant CJD originated. This suspicion was previously investigated using a macaque model, which was successfully perorally infected by BSE-contaminated material and subsequently lead to the development of a prion disorder that resembles vCJD.[25] These results, due to the evolutionary relatedness between macaques and humans, allowed researchers to confirm the oral transmissibility of PrP^{BSE} to humans. PrP^{BSE} may also potentially lead to prion disorder establishment in swine,[24] livestock of great economic and social importance.

The prion disorder affecting elk, mule deer and white-tailed deer is termed Chronic Wasting disease (CWD). Cases of the disease are most prevalent in the United States but are also evident in Canada and South Korea.[26,27] As the infectious prion isoform is reported to be present in the blood[28] and skeletal muscle,[29] hunting, consumption of wild venison and contact with other animal products derived from CWD-infected elk and deer may thereby pose a public health risk. Our studies demonstrate that PrP^{CWD} co-localizes with LRP/LR on human enterocytes[24] thereby suggesting a possible oral transmissibilty of this TSE to humans. This is, however, inconsistent with results obtained during intra-cerebral inoculation of the brains and spinal cords of transgenic mice overexpressing the human cellular prion protein (PrP^{c}),[26,27] which is essential for TSE disease establishment and progression. Further, discrepancies have also been reported with respect to non-human primates, as squirrel monkeys have been successfully intracerebrally inoculated with mule-deer prion homogenates,[30] while cynolmolgus macaques were resistant to infection.[31] CWD has been transmitted to ferrets, minks and goats[32] and as these animals may serve as domestic animals or livestock, secondary transmission from such animals to humans, through direct contact or ingestion of infected material, may be an additional risk factor that merits further scientific investigation.

Ovine PrP^{Sc} co-localization with LRP/LR on human and bovine enterocytes may be indicative of the infectious agents' ability to effect cross-species infections. The oral transmissibility of Scrapie has been confirmed in hamsters fed with sheep-scrapie-infected material.[33]

The discrepancies with regards to the transmissibility of certain infectious prion proteins when assessed by different model systems may be due to the experimental transmission route employed. Oral exposure often results in significantly prolonged incubation times when compared to intracerebral inoculation techniques and thus failure of transgenic mice and normal experimental animals to develop disease phenotypes after being fed TSE-contaminated material may not necessarily indicate that the infection process failed.[14] Apart from the route of infection, numerous other factors may influence transmission between species, including dose, PrP polymorphisms and genetic factors, the prion strain employed as well as the efficacy of prion transport to the CNS.[34] The degree of homology between the PrP^{c} protein in the animals serving as the infectious prion source and recipient has also been described as a feature limiting cross-species transmission.[34] The negative results, as referred to above, obtained upon prion-protein inoculation of animal models may have resulted due to the slow rate at which the infectious prion induces conformational conversion of the endogenous PrP^{c} in the animal cells and this in turn results in low levels of infectious prion replication and symptom development.[27]

Furthermore, even in the event that certain prion disorders are not directly transmissible to humans, most are transmissible to at least a single species of domestic animal or livestock. The infectious agents properties may be altered in the secondary host such that it becomes transmissible to humans (reviewed in ref. 35). Thus, interspecies transmission between animals may indirectly influence human health.

It is noteworthy to add that although the oral route of PrP^{Sc} transmission may result in prolonged incubation times, it may broaden the range of susceptible hosts. A common constituent of food is ferritin, a protein that is resistant to digestive enzyme hydrolysis and, due to its homology across species, it may serve as co-transporter of PrP^{Sc} and facilitate enterocyte internalization of the

infectious prion.[36] It may thus be proposed that prion internalization may occur via a ferritin-PrP^{Sc} complex even in the absence of co-localization between the infectious agent and LRP/LR such that many more cross-species infections (provided that the other infection factors are favorable) may be probable.[37] In addition, digestive enzymes in the gastrointestinal tract facilitate PrP^{Sc} binding to the intestinal epithelium and subsequent intestinal uptake[36] and thus depending on the individuals' digestive processes, the susceptibility to infection and the rate of disease development may vary accordingly. As a result hereof, though laboratory experiments in cell-culture and animal models may render a particular prion disorder non-infectious to humans, this may not be true for all individuals.

In lieu of the above statements, with particular reference to inconsistencies in reported results and the multiple factors influencing oral transmissibility of TSEs, further transmission studies are required to evaluate the zoonotic threat which CWD, BSE and Scrapie may pose through ingestion.

Acknowledgements

We thank the National Research Foundation (NRF), Republic of South Africa for financial support.

References

1. Meyer-Luehmann M, Coomaraswamy J, Bolmont T, Kaeser S, Schaefer C, Kilger E, et al. Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. Science 2006; 313:1781-4.
2. Olanow CW, Tatton WG. Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci 1999; 22:123-44.
3. Kordower JH, Brundin P. Lewy body pathology in long-term fetal nigral transplants: is Parkinson's disease transmitted from one neural system to another? Neuropsychopharmacology 2009; 34:254.
4. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. Nat Med 2008; 14:504-6.
5. Kordower JH, Chu Y, Hauser RA, Olanow CW, Freeman TB. Transplanted dopaminergic neurons develop PD pathologic changes: a second case report. Mov Disord 2008; 23:2303-6.
6. Brundin P, Melki R, Kopito R. Prion-like transmission of protein aggregates in neurodegenerative diseases. Nat Rev Mol Cell Biol 2010; 11:301-7.
7. Kimberlin RH, Cole S, Walker CA. Pathogenesis of scrapie is faster when infection is intraspinal instead of intracerebral. Microb Pathog 1987; 2:405-15.
8. Kimberlin RH, Walker CA. Pathogenesis of scrapie (strain 263K) in hamsters infected intracerebrally, intraperitoneally or intraocularly. J Gen Virol 1986; 67:255-63.
9. Weissmann C, Enari M, Klohn PC, Rossi D, Flechsig E. Transmission of prions. J Infect Dis 2002; 186:157-65.
10. Maddison BC, Rees HC, Baker CA, Taema M, Bellworthy SJ, Thorne L, et al. Prions are secreted into the oral cavity in sheep with preclinical scrapie. J Infect Dis 2010; 201:1672-6.
11. Da Costa Dias B, Weiss SF. A kiss of a prion: new implications for oral transmissibility. J Infect Dis 2010; 201:1615-6.
12. Neale MH, Mountjoy SJ, Edwards JC, Vilette D, Laude H, Windl O, et al. Infection of cell lines with experimental and natural ovine scrapie agents. J Virol 2010; 84:2444-52.
13. Ghosh S. Mechanism of intestinal entry of infectious prion protein in the pathogenesis of variant Creutzfeldt-Jakob disease. Adv Drug Deliv Rev 2004; 56:915-20.
14. Shmakov AN, Ghosh S. Prion proteins and the gut: une liaison dangereuse? Gut 2001; 48:443-7.
15. Davies GA, Bryant AR, Reynolds JD, Jirik FR, Sharkey KA. Prion diseases and the gastrointestinal tract. Can J Gastroenterol 2006; 20:18-24.
16. Mbazima V, Da Costa Dias B, Omar A, Jovanovic K, Weiss SFT. Interaction between PrP^c and other ligands with the 37 kDa/67 kDa laminin receptor. Front Biosci 2010; 15:1150-63.
17. Vana K, Zuber C, Pflanz H, Kolodziejczak D, Zemora G, Bergmann AK, et al. LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. Infect Disord Drug Targets 2009; 9:69-80.
18. Omar A, Jovanovic K, Da Costa Dias B, Gonsalves D, Moodley K, Caveney R, et al. Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. Expert Opin Ther Pat 2011; 21:35-53. DOI: 10.1517/13543776.2011.539203
19. Morel E, Andrieu T, Casagrande F, Gauczynski S, Weiss S, Grassi J, et al. Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. Am J Pathol 2005; 167:1033-42.
20. Shmakov AN, Bode J, Kilshaw PJ, Ghosh S. Diverse patterns of expression of the 67 kD laminin receptor in human small intestinal mucosa: potential binding sites for prion proteins? J Pathol 2000; 191:318-22.
21. Rieger R, Edenhofer F, Lasmezas CI, Weiss S. The human 37 kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. Nat Med 1997; 3:1383-8.
22. Gauczynski S, Nikles D, El-Gogo S, Papy-Garcia D, Rey C, Alban S, et al. The 37 kDa/67 kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. J Infect Dis 2006; 194:702-9.
23. Gauczynski S, Peyrin JM, Haik S, Leucht C, Hundt C, Rieger R, et al. The 37 kDa/67 kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. Embo J 2001; 20:5863-75.
24. Kolodziejczak D, Da Costa Dias B, Zuber C, Jovanovic K, Omar A, Beck J, et al. Prion interaction with the 37 kDa/67 kDa laminin receptor on enterocytes as a cellular model for intestinal uptake of prions. J Mol Biol 2010; 402:293-300.
25. Lasmezas CI, Deslys JP, Demaimay R, Adjou KT, Lamoury F, Dormont D, et al. BSE transmission to macaques. Nature 1996; 381:743-4.
26. Sandberg MK, Al-Doujaily H, Sigurdson CJ, Glatzel M, O'Malley C, Powell C, et al. Chronic wasting disease prions are not transmissible to transgenic mice overexpressing human prion protein. J Gen Virol 2010; 91:2651-7.
27. Tamguney G, Giles K, Bouzamondo-Bernstein E, Bosque PJ, Miller MW, Safar J, et al. Transmission of elk and deer prions to transgenic mice. J Virol 2006; 80:9104-14.
28. Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, Warren RJ, et al. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science 2006; 314:133-6.
29. Angers RC, Browning SR, Seward TS, Sigurdson CJ, Miller MW, Hoover EA, et al. Prions in skeletal muscles of deer with chronic wasting disease. Science 2006; 311:1117.
30. Marsh RF, Kincaid AE, Bessen RA, Bartz JC. Interspecies transmission of chronic wasting disease prions to squirrel monkeys (*Saimiri sciureus*). J Virol 2005; 79:13794-6.
31. Race B, Meade-White K, Race R, Chesebro B. Prion infectivity in fat of deer with chronic wasting disease. J Virol 2009; 83:9608-10.
32. Bartz JC, Marsh RF, McKenzie DI, Aiken JM. The host range of chronic wasting disease is altered on passage in ferrets. Virology 1998; 251:297-301.
33. McBride PA, Beekes M. Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie. Neurosci Lett 1999; 265:135-8.
34. Raymond GJ, Bossers A, Raymond LD, O'Rourke KI, McHolland LE, Bryant PK, 3rd, et al. Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. EMBO J 2000; 19:4425-30.
35. Belay ED, Maddox RA, Williams ES, Miller MW, Gambetti P, Schonberger LB. Chronic wasting disease and potential transmission to humans. Emerg Infect Dis 2004; 10:977-84.
36. Mishra RS, Basu S, Gu Y, Luo X, Zou WQ, Mishra R, et al. Protease-resistant human prion protein and ferritin are cotransported across Caco-2 epithelial cells: implications for species barrier in prion uptake from the intestine. J Neurosci 2004; 24:11280-90.
37. Sunkesula SR, Luo X, Das D, Singh A, Singh N. Iron content of ferritin modulates its uptake by intestinal epithelium: implications for co-transport of prions. Mol Brain 2010; 3:14.

Structural and mechanistic commonalities of amyloid-β and the prion protein

B. Da Costa Dias, K. Jovanovic, D. Gonsalves, S.F. Weiss

Prion 5 (2011) 126 – 137

This review article discussed the structural and functional similarities of the Aβ peptide and PrP^c and the similarities between the diseases these proteins cause (namely prion disorders and Alzheimer's disease). The several binding partners that these proteins have in common are also discussed. This leads to the proposal of a possible interaction between LRP/LR and Aβ due to the structural similarities between PrP^c and Aβ. Moreover, an interaction between LRP/LR and Aβ peptides is further corroborated by the fact that they share many of the same interaction partners (PrP^c, laminin and HSPGs).

Prion 5:3, 126-137; July/August/September 2011; © 2011 Landes Bioscience

Structural and mechanistic commonalities of amyloid-β and the prion protein

Bianca Da Costa Dias, Katarina Jovanovic, Danielle Gonsalves and Stefan F.T. Weiss*

School of Molecular and Cell Biology; University of the Witwatersrand; Johannesburg, Republic of South Africa (RSA)

Key words: Alzheimer disease, amyloid β, apoptosis, 37 kDa/67 kDa laminin receptor, prion proteins

Abbreviations: Aβ, amyloid beta/beta amyloid peptide; AD, Alzheimer disease; AICD, amyloid intracellular domain; APP, amyloid precursor protein; BACE, β-site APP cleaving enzyme; HSPGs, heparan sulphate proteoglycans; LRP/LR, non-integrin laminin receptor precursor; LTP, long-term potentiation; mGluR5, metabotrophic glutamate receptor; NMDA, N-methyl-D-aspartate receptor; $p75^{NTR}$, p75 neurotrophin receptor; PrP^{c}, cellular prion protein isoform; PrP^{Sc}, infectious prion protein isoform; RAGE, receptor for advanced glycosylation end products; TSE, transmissible spongiform encephalopathies

Amyloid beta (Aβ) is a major causative agent of Alzheimer disease (AD). This neurotoxic peptide is generated as a result of the cleavage of the Amyloid-Precursor-Protein (APP) by the action of β-secretase and γ-secretase. The neurotoxicity was previously thought to be the result of aggregation. However, recent studies suggest that the interaction of Aβ with numerous cell surface receptors such as N-methyl-D-aspartate (NMDA), receptor for advanced glycosylation end products (RAGE), P75 neurotrophin receptor ($P75^{NTR}$) as well as cell surface proteins such as the cellular prion protein (PrP^{c}) and heparan sulfate proteoglycans (HSPG) strongly enhances Aβ induced apoptosis and thereby contributes to neurotoxicity. This review focuses on the molecular mechanism resulting in Aβ-shedding as well as Aβ-induced apoptotic processes, genetic risk factors for familial AD and interactions of Aβ with cell surface receptors and proteins, with particular emphasis on the cellular prion protein. Furthermore, comparisons are drawn between AD and prion disorders and the role of laminin, an extracellular matrix protein, glycosaminoglycans and the 37 kDa/67 kDa laminin receptor (LRP/LR) have been highlighted with regards to both neurodegenerative diseases.

Alzheimer disease (AD), primarily defined by psychiatrist Alois Alzheimer in 1906, is a neurodegenerative disorder and currently exhibits a prevalence that "doubles approximately every five years from 0.5% at the common age of onset-65 years old."[1] This disease is the most common form of dementia afflicting the elderly and at present affects in excess of 37 million people globally[2] and it is predicted that 100 million people will be living with the disease by 2050.[3]

AD has received mounting scientific interest and has stimulated tireless research endeavours not only due to the complex mechanism by which it is caused; the multitude of contributing factors and contradictions which have arisen between hypotheses and acquired results, but also due to the rise in life expectancies[4] owing to the advent of modern medicine, which has socio-economic implications particularly in terms of strain placed upon national health systems.

*Correspondence to: Stefan F.T. Weiss; Email: stefan.weiss@wits.ac.za
Submitted: 06/10/11; Accepted: 07/24/11
DOI: 10.4161/pri.5.3.17025

Clinical Symptoms of Alzheimer Disease

AD related symptoms have been reported to occur in three stages. The initial symptoms include cognitive dysfunction, impaired short-term memory as well as language impairment and behavioural disturbances including paranoia, confusion, hallucinations and aggression.[4,5] Ultimately, AD patients exhibit motoric disturbances and a loss in co-ordination and encounter difficulties in performing simple daily tasks. Disease progression, although variable in the chronology of symptom development and severity, is proposed to occur over a decade.[3]

Neuropathology

Neuritic plaques are comprised primarily of amyloid β (Aβ) peptides, predominantly the 42 amino acid isoform designated $Aβ_{42}$ as it exhibits a greater propensity for aggregation[6] owing to an increased number of non-polar hydrophobic amino acid residues—isoleucine and alanine. These slow forming extracellular protein aggregates are localized around the synaptic terminals and axons of neurons in the limbic and associated cortices of the brain,[7] occurring first in the fronto cortex and progressing to the cortical regions.[8]

Neurofibrillary tangles are intracellular clusters of straight filaments of approximately 10 nm which appear helical when viewed under the electron microscope.[3] The filaments are composed of hyperphosporylated τ protein (a microtubule associated protein), which is also associated with the onset of frontotemporal dementia.[9] The abnormal phosphorylation of τ, which results in pathological consequences, may in turn be dependent on the presence of Aβ oligomers. It has been proposed that Aβ triggers p35 gene activation; the protein product is auto-catalytically cleaved to yield a p25 fragment which functions both as a transcription activator of the gene encoding for cyclin-dependent

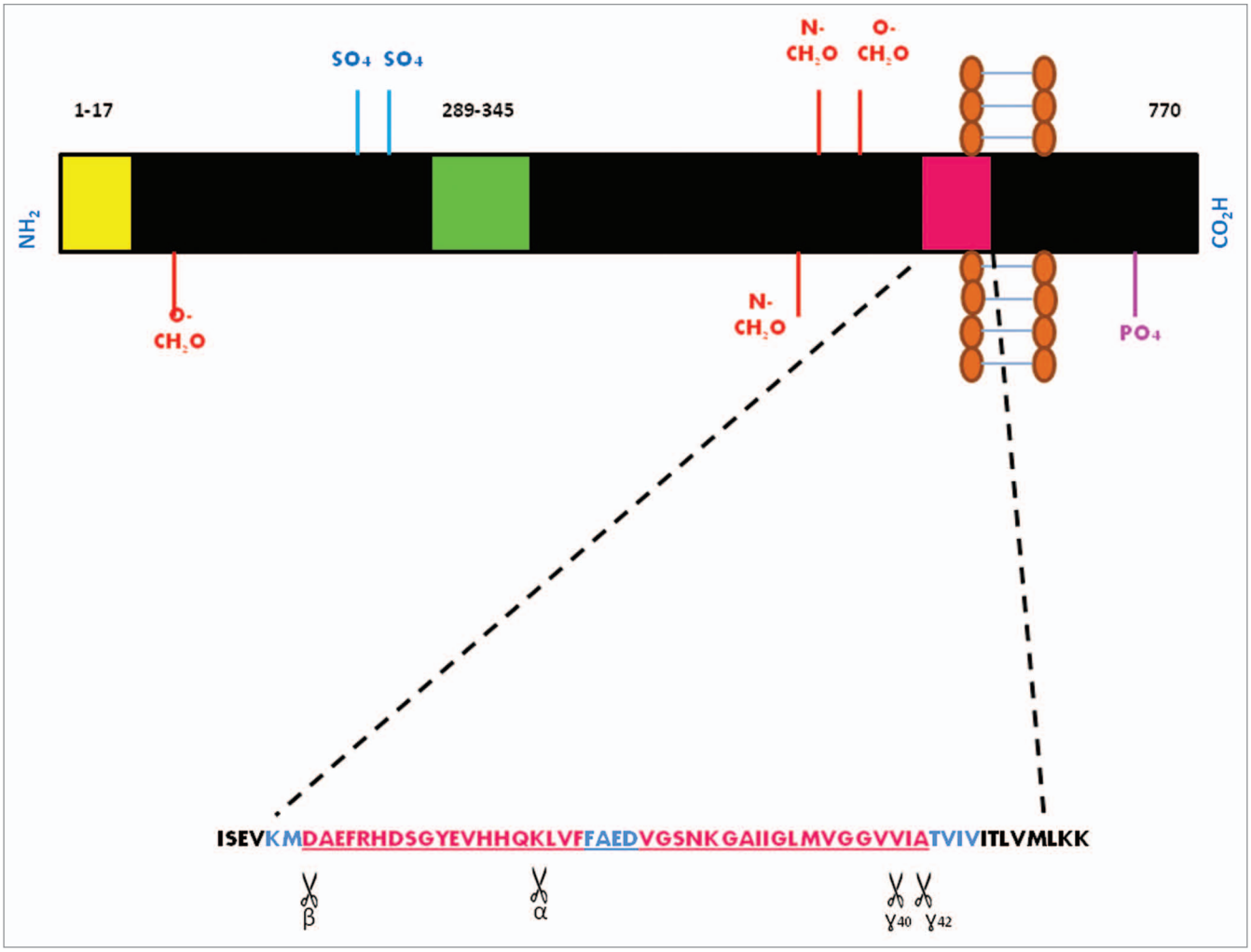


Figure 1. Schematic representation of the APP and the enzymatic cleavage sites located within the amyloid β sequence. (A) This transmembrane protein may be present in multiple isoforms APP695, APP751 and APP770, the latter is represented here. The regions of interest depicted in the diagram are: A signal peptide (yellow box) comprising 17 amino acid residues which ensures the protein is correctly transported to the cell surface; a 56 amino acid Kunitz-type serine protease inhibitor domain (KPI-green box) and the Aβ sequence.[4] In addition, the sites of post-translational modifications such as N- and O-linked sugars (NCH_2O and OCH_2O), phosphate (PO_4) and sulphate (SO_4) groups are shown. (B) The 40–42 amino acid Aβ sequence is highlighted above-the first 28 amino acid residues are polar and located on the extracellular domain of APP whilst the remaining residues are located within the 23 aa APP transmembrane domain and are non-polar. The enzymatic cleavage sites of β secretase, α secretase and γ secretase are depicted (Adapted from reference 112).

kinase 5 (Cdk5)—a τ phosphorylating kinase.[10,11] In addition, p25 forms a cytoplasmic complex with the cdk5 protein.

τ neurofibrillary tangles have been reported to occur prior to Aβ plaque deposition and the tangle load has been shown to correlate more closely to the severity of the dementia than the plaque level.[12-14] However, these tangles are not solely associated with AD and are instead characteristic pathological features of an array of neurodegenerative diseases and other disorders such as subacute sclerosing panencephalitis.[4] Aβ neuritic plaques, despite being present in healthy and AD diseased brains as well as patients suffering from dementia with Lewy bodies (DLB), are not the major pathological traits of any other disease.[4] Furthermore, mutations in the τ gene cause frontotemporal dementia as opposed to AD[15] whereas mutations which result in an overproduction of $A\beta_{42}$ (as shall be detailed below) cause familial AD. As a result hereof, it has been hypothesized that Aβ peptides are the central causative agents in AD.

The Molecular Basis of Alzheimer Disease

The amyloid precursor protein (APP) (**Fig. 1**) is a ubiquitous single transmembrane protein expressed in the cells of interest in neurodegenerative diseases namely neurons and astrocytes. The protein is encoded by an *APP* gene located on chromosome 21[16] and owing to alternative m-RNA splicing is present in three isoforms: APP695, APP751 and APP770,[17] the former being expressed in higher levels in neuronal tissues.[18] APP is post-translationally modified through the addition of N and O-linked sugar moieties. APP, largely through the action of sAPPα (a cleavage product of the non-amyloidogenic pathway), is vital for normal brain

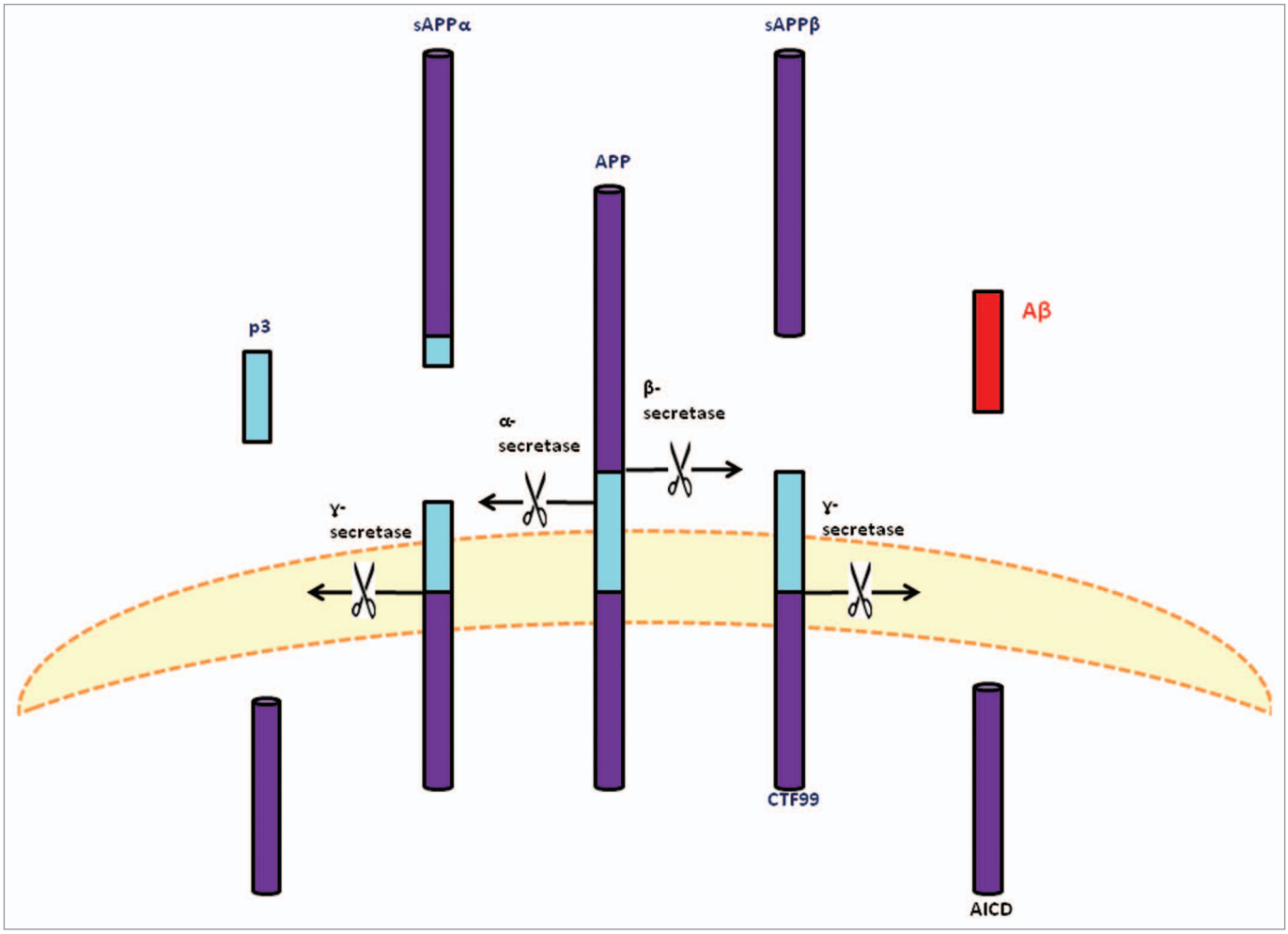


Figure 2. The proteolytic processing of the APP and its cleavage products. The amyloid precursor protein may be metabolized through two pathways. The first, depicted on the left, is termed the non-amyloidogenic pathways. This pathway involves the enzymatic cleavage by an α-secretase (presumably a member of the ADAM family) after residue 687, followed by γ-secretase mediated cleavage of the remaining carboxyl terminal fragment (CTF83), generating sAPPα, p3 and AICD (not shown), respectively. The amyloidogenic pathway (right) entails β-secretase mediated cleavage after residue 671, thereby releasing sAPPβ and the resultant CTF99 is cleaved by γ-secretase which results in Aβ shedding. The miscellaneous receptor, which may influence the process or serve as a receptor for either Aβ cleavage products, is hypothesized to be the 37 kDa/67 kDa Laminin Receptor Precursor/Laminin Receptor (LRP/LR) (adapted from reference 32).

development, long-term potentiation (LTP) and learning.[19] APP is rapidly metabolized within 45–60 min of expression via two pathways namely: a non-pathogenic non-amyloidogenic pathway and a Aβ synthesizing amyloidogenic pathway (**Fig. 2**). It must be emphasized that both pathways are present in normal healthy individuals and AD is caused either through the disproportionate favoring of the amyloidogenic cleavage or the retardation of the Aβ turnover rate.[17,20]

Amyloid Precursor Protein Processing

The non-amyloidogenic pathway is initiated by the α-secretase mediated cleavage of APP between residues APP687 and APP688,[21] which resides in the Aβ sequence thereby precluding Aβ formation and shedding. The released amino terminal ectodomain fragment is termed sAPPα (**Fig. 2**). The remaining 83aa carboxyl terminal fragment (CTF_{83}) is subsequently cleaved by γ-secretase thereby generating a soluble 3 kDa p3 fragment and a 57–59 aa amyloid intracellular domain (AICD) (**Fig. 2**) which exhibits transcriptional regulatory abilities as shall be discussed below (**Fig. 3**).

In contrast to the mechanism detailed above, the amyloidogenic pathway is initiated by β-secretase cleavage of APP (**Fig. 2**). This cleavage occurs 16 aa residues toward the amino terminal of the α-secretase cleavage site, between residues APP671 and APP672,[18] generating sAPPβ. The resultant CTF_{99} is also subjected to γ-secretase cleavage which produces a 4 kDa soluble Aβ monomer and AICD (**Fig. 2**). It is noteworthy to add that γ-secretase cleavage at positions APP712 and APP713 generate $Aβ_{40}$ while cleavage at APP714 results in $Aβ_{42}$ (**Fig. 1**).[22] Furthermore, as has been mentioned above, Aβ generation is a normal physiological process and Aβ levels in the cerebrospinal fluid and plasma of healthy individuals are in the 3–8 nM range[23] and 500 pM respectively.[24]

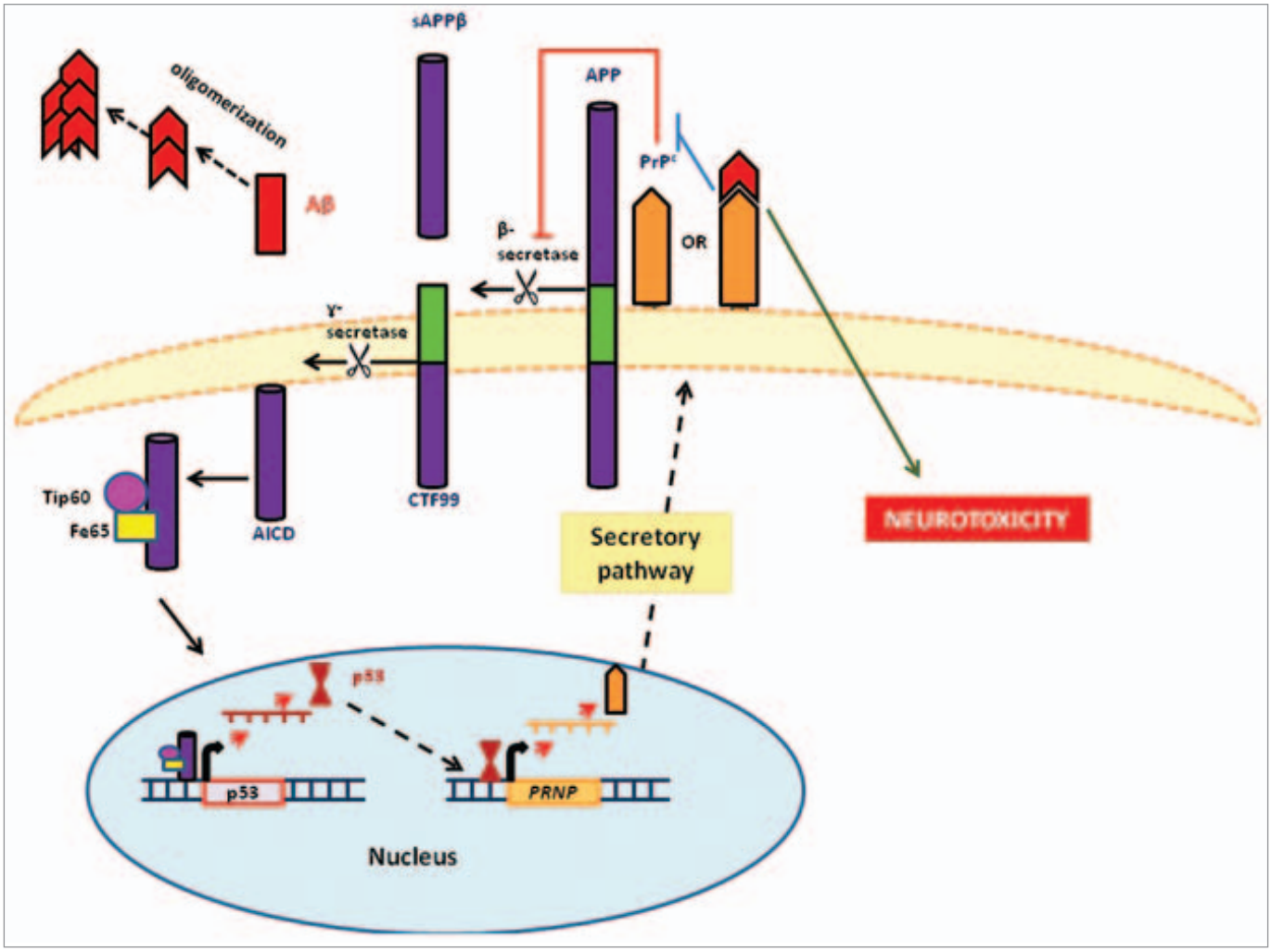


Figure 3. Feedback loop for the prion protein (PrP^c) mediated regulation of APP. The amyloid intracellular domain (AICD) amyloidogenic APP cleavage product, indirectly upregulates prion protein (PrP^c) expression through p53 gene activation. PrP^c consequently hampers β-secretase (BACE1) activity thereby reducing APP amyloidogenic processing and Aβ synthesis. In the presence of Aβ-oligomers, which preferentially bind to PrP^c, PrP^c is unable to inhibit β-secretase (BACE1) activity. This reduces the degree of regulatory control exerted on the amyloidogenic process resulting in increased levels of potentially toxic Aβ oligomers (adapted from reference 81).

Associated Enzymes

α-Secretase. The proteolytic abilities of this plasma membrane protein are attributable to the activities of multiple disintegrin and metalloproteinases (ADAMs) and ADAM 10, 17 and 19 are suggested to be of particular importance.[25-27]

β-Secretase. The predominant β-secretase in neural tissue is the β-site APP cleaving enzyme 1 (BACE1), although BACE2 isoforms are also present to a lesser extent.[28] The expression of these enzymes is upregulated in response to cellular stress in the form of oxidative stress, ischemia and the depletion of utilisable energy[29,30] and thus in AD patients as such stresses are augmented in diseased tissues. It must be noted that β-secretase is located in lipid raft domains of the plasma membrane and requires glycosaminoglycans to mediate effective cleavage,[1] characteristics analogous to those of the 37 kDa/67 kDa Laminin Receptor Precursor/Laminin Receptor (LRP/LR).[31,32]

γ-Secretase. γ-Secretase is a protein complex consisting of at least four proteins namely: presenilin 1 or 2 (aspartyl proteases which perform the catalytic function), nicastin (Nct), anterior pharynx defective-1 (Aph-1)/A and presenilin enhancer 2 (Pen 2).[33] Nct and Aph-1 are required for substrate recognition[34] which allow the enzyme to cleave in the order of 50 substrates.[35] The enzymatic components of this complex, the presenilins are transmembrane proteins with 6–9 transmembrane domains and both the amino and carboxy termini are cytoplasmic. Mutations in the *PSEN* genes encoding these proteins result in familial AD which is characterized by the early onset of typical symptoms, as shall be detailed below.

Familial Alzheimer Disease

AD may be inherited as an autosomal dominant disease, with the disease following Mendelian inheritance patterns in merely

5% of AD cases.[3] Familial AD is characterized by the onset of pathological symptoms before the age of 65[36] and is the result of mutations in three genetic loci (genes) namely: *APP* on chromosome 21 and the presenilin encoding genes (*PSEN1* and *PSEN2*) on chromosomes 14 and 1, respectively.[37]

APP gene. Twenty three missense mutations and two recessive mutations (a duplication and a trinucleotide deletion) have been reported in references 36 and 38 at various locations. The common consequence of all *APP* mutations is enhanced Aβ generation (be it $A\beta_{40}$, $A\beta_{42}$ or both)—the mechanism by which this is achieved, be it through the upregulation of APP expression, α-secretase inhibition or β-secretase stimulation, varies with each mutation.[39]

PSEN 1 gene. Within this gene 178 mutations, largely missense, have been identified and these are the most prevalent AD-related mutations in the general public.[36] The mutations are mainly localized near the transmembrane domains and hydrophilic loops.[40] β-secretase cleavage is affected by such mutations often resulting in an increased $A\beta_{42}/A\beta_{40}$ ratio either through enhanced synthesis of the more hydrophobic isoform[24] or a reduction in $A\beta_{40}$ generation.[41]

PSEN 2 gene. Mutations within this gene are less common as only 14 mutations have been reported to date.[36] However, the consequences of such mutations are indistinguishable from those described above with regards to *PSEN1* mutations.

Thus, all mutations leading to familial AD share a common modus operandi—each mutation serves to significantly augment the concentration of $A\beta_{42}$ isoform thereby providing additional support for the prevailing hypothesis that Aβ peptides are the causative agents of AD and consequently the central focus in the development of AD therapeutics.

Other Genetic Risk Factors

A single risk factor, the ε4 allele of the apolipoprotein E (APOE) gene on chromosome 19q is the only susceptibility factor which has consistently been reported to predispose people to late-onset AD.[42] However, recent genetic studies have revealed an array of potential risk genes at specific chromosomal regions, particularly those on chromosomes 6, 9, 10, 12 and 21.[43] Such novel risk genes include *CLU* which encodes for clusterin and *CR1* encoding for the complement C3b protein and *PICALM* encoding for the phosphatidylinositol binding clathrin assembly protein.[36] These genes are suggested to function in Aβ transport (escort) and endocytosis (*CLU*), Aβ clearance (*CR1*)[44] and APP recycling (*PICALM*). Moreover, the *PRNP* gene encoding for the cellular prion protein (PrP^c) and the possible Met129Val homozygosity thereby associated have also been described as potential risk factors.[45,46]

Focusing on the Causative Agent—the Aβ Peptide

Aβ peptides are amphiphilic 38–43 amino acid peptides[34] as the first 28 aa residues are polar and the remaining residues are non-polar in nature[8] (**Fig. 1**). As a result hereof, the peptides exhibit great differences in polarity at neutral pH and thus show a high propensity for aggregation.[47,48] The unfolded Aβ monomers may associate to form dimers, trimers and higher order oligomers which may ultimately form insoluble fibrils and plaques—a process termed nucleation dependent polymerization. This process in vitro is highly sensitive to experimental conditions and in vivo, such oligomers are predominantly dimeric as opposed to larger insoluble oligomers which are produced synthetically.[49]

Revisiting the Aβ Model

Neuritic plaques were initially regarded as the basis of AD. However, this is no longer the prevailing thought due to poor correlation between the degree of dementia and the anatomical plaque load.[50] The presence of said plaques in healthy individuals;[51] the onset of cognitive impairment in mice prior to plaque deposition[52] and the presence of intracellular Aβ oligomers suggesting that the extracellular deposits may not be the sole contributors to disease establishment. Current hypotheses suggest that enhanced levels of $A\beta_{42}$ or elevated $A\beta_{42}/A\beta_{40}$ peptide ratios increase the probability of developing Alzheimer disease (in line with the effects of the mutations stated above).[8] However, a remarkably larger body of research provides evidence for the latest hypothesis that neurotoxicity is mainly associated with Aβ oligomers, particularly dimers.[53]

Effects of Toxic Aβ Oligomers

It is suspected that Aβ oligomers are primarily deposited at small blood vessels and interactions between the peptides and receptors of the endothelial cells of the blood vessel wall activates inflammatory responses and cytokine release. These may ultimately lead to the destruction of the blood vessels, consequently reducing oxygen supply to neurons (rendering them ischemic) and finally resulting in neuronal death.[54] In the presence of Aβ oligomers neural associated-cells such as astrocytes and microglia would in addition activate the afore stated responses and the complement cascade.[54] Furthermore, Aβ-cell surface receptor interactions have been reported to cause oxidative damage by enhancing the synthesis of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide[54] which consequently contributes to protein degradation and lipid (such as myelin) oxidation, thereby slowing the rate of signal transmission[55] as well as the consolidation of new information, retrieval of memories and motor functions. Furthermore, these Aβ-receptor interactions induce the activation of apoptotic pathways and are thus an additional mechanism through which Aβ mediates its neurotoxicity. Moreover, the interaction of Aβ oligomers with cell-surface receptors such as metabotrophic glutamate receptors (mGluR5)[1,56] and N-methyl-D-aspartate receptors (NMDA),[1,57] disrupts ion homeostasis-particularly with respect to calcium ions (Ca^{2+}). These interactions allow increased Ca^{2+} to enter neurons, resulting in excitotoxicity, synaptic dysfunction and neuronal death.[58]

Table 1. Membrane associated proteins to which Aβ may bind and result in neurotoxic consequences[63]

Amyloid Precursor Protein (APP)
N-methyl-D-aspartate receptor (NMDA-R)
α7 Nicotininc Acetylcholine receptor (α7nAChR)
P75 Neurotrophin receptor ($P75^{NTR}$)
Integrins (particularly $\alpha_5\beta_1$)
Receptor for advanced glycosylation end products (RAGE)
Insulin receptor
Formyl peptide receptor-like-1 (FPRL1)
Scavenger receptors-classes A, B, BI
Heparan Sulfate Proteoglycans

Aβ Interactions

Aβ oligomers may be directly incorporated into the plasma membrane,[59-62] neuronal membranes and those of lysosomes, Golgi apparatus and endoplasmic reticulum.[63] In the former, Aβ is located in the lipid raft domains (the same cellular location as of 37 kDa/67 kDa LRP/LR) and disrupts membrane structure and function by forming Ca^{2+} channels which deregulates homeostasis.

Aβ interacts with a variety of proteins on the surfaces of neuronal and glial cells (**Table 1**). These include APP, NMDA receptors, integrins, α7 nicotininc acetylcholine receptors (α7nAchR), p75 neurotrophin receptors ($p75^{NTR}$), collagen-like Alzheimer amyloid plaque component precursor/collagen type XXV (CLAC-P/ColXXV), the receptor for advanced glycosylation end products (RAGE), serpin-enzyme complex receptor (SEC-R), insulin receptors, scavenger receptors on microglial cells and heparin sulphate proteoglycans (HSPGs).[63] The outcome of some of these Aβ-receptor/protein binding interactions are neuroprotective while the majority are toxic.[63] It is, however, noteworthy to add that the majority of these receptors—AAP, $p75^{NTR}$, CLAC-P/ColXXV, RAGE, SEC-R and integrins—are transmembrane receptors[63] as is the 37 kDa/67 kDa LRP/LR. Furthermore, τ has been proposed to be central in mediating Aβ toxicity.[64,65]

Laminin, a 850 kDa glycoprotein component of the basal membrane, functions in cell adhesion and basement membrane assembly. This cross-shaped glycoprotein is composed of three disulfide-bonded chains: α (of which there are five isoforms ranging from 200–400 kDa), β (a single 220 kDa isoform) and γ (a single isoform of 210 kDa).[66] The α-chain contains the Aβ binding site and the protein has also been reported to bind, via its IKAV neurite inducing site, to APP.[67] The Aβ-laminin interaction has been demonstrated to promote neurite outgrowth[68] and inhibit fibrillogenesis.[69]

The Prion Protein: A Central Factor in Alzheimer Disease

Prion proteins, encoded by the *PRNP* gene located on chromosome 20,[70] are cellular proteinaceous particles which have been implicated in pathogenesis, particularly with regards to the fatal neurodegenerative diseases termed transmissible spongiform encephalopathies (TSEs).[31,32,113] Two forms of the protein exist: the normal cellular prion protein (PrP^{c}) and the infectious form (PrP^{Sc}), the latter is considered the causative agent of TSEs.[71] PrP^{c} is composed of 250 amino acids containing an N-terminal domain and N-linked oligosaccharides, four proline and glycine-rich octarepeat regions and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor through which the protein is bound to the lipid raft domains of the plasma membrane. PrP^{c} constitutively cycles between the cell membrane and intra-cellular compartments via the endocytic pathway—a process mediated by the 37 kDa/67 kDa Laminin Receptor Precursor,[72] a high affinity receptor for PrP^{c}[73] and PrP^{Sc}.[72,74-76] The prion variants share a common amino acid sequence and differ primarily with regards to secondary structure (PrP^{Sc} exhibits greater β sheet proportion than PrP^{c}-consistent with its higher aggregation propensity) and proteinase K digestion (PrP^{Sc} resists degradation while PrP^{c} does not).[71,77]

PrP^{c} has a multitude of physiological functions[70] and the majority of these functions are dependent on the interaction of the prion protein with an array (>70)of proteins. These include ApoE, APP, HSPGs, RAGE, p75 NTR, amyloid β (proteins of importance with regards to Alzheimer disease as discussed above) and the 37 kDa/67 kDa laminin receptor precursor (LRP/LR).[73] The specific PrP^{c}-protein interactions and the functions thereof are beyond the scope of this paper but have been recently reviewed in reference 70. Owing to the potential role of PrP^{c} in Alzheimer disease and the central role of the PrP^{c} and PrP^{Sc}-LRP/LR interaction in TSEs[31] it may be proposed that a link between the two neurodegenerative diseases and more specifically the amyloid β and LRP/LR components may exist and it is plausible that PrP^{c} may facilitate possible AB-LRP/LR interactions.

Prion Protein's Influence on Alzheimer Disease

The cellular prion protein (PrP^{c}) has been identified as a high affinity receptor for Aβ oligomers[78] and residues PrP23–27 and PrP95–110 serve as Aβ binding sites.[79] The receptor specifically binds to Aβ oligomers as opposed to Aβ monomers or fibrils.[79] As the oligomers are considered to be the neurotoxic species, a PrP^{c}-Aβ interaction may influence synaptoxicity. However, due to contradictory results as to whether Aβ oligomers induce their toxic effects in a PrP^{c}-dependent or independent manner, the functional role of these binding interactions has not yet been firmly established.[1] Conversely PrP^{c} has been implicated in APP processing and has been reported to hamper β-secretase cleavage of APP thereby reducing Aβ generation (**Fig. 3**).[80] Furthermore, PrP^{c} has been shown to protect neurons against AD-promoting oxidative stress. Thus, PrP^{c} may serve a neuroprotective role in neuronal tissue.

Kellett and Hooper[81] have proposed a potential negative feedback loop which controls PrP^{c} expression and Aβ synthesis. PrP^{c} impedes the activity of β-secretase, thereby restraining amyloidogenic APP processing and reducing the concentrations of Aβ and AICD.[81] AICD is a transcription factor which regulates

the expression of p53-which in turn regulates *PRNP* expression[82]-therefore, PrP^c induced reduction of AICD ultimately results in downregulation of *PRNP* gene expression. It may be suggested that in AD patients, who exhibit increased levels of $A\beta_{42}$ oligomers, the binding of these oligomers to PrP^c may hamper the PrP^c regulation of β-secretase through steric inhibition or through enhanced PrP^c endocytosis and thereby prevent PrP^c-β-secretase interactions.[81]

Commonalities between Alzheimer Disease and Prion Disorders

The structural commonalities between prion proteins and Aβ include conserved histidine metal binding domains which result in the synthesis of ROS when the proteins bind to copper (Cu^{2+}) and zinc (Zn^{2+}) ions;[83,84] conserved tyrosines and methionine residues within the proteins' transmembrane regions and three GxxxG sequence repeats for transmembrane association.[85]

Comparable Processing and Post-Translational Modifications between APP and PrP^c

PrP^c may be released from the cell surface via two mechanisms-cleavage of the GPI anchor or cleavage by a member of the ADAM family (possibly ADAM10) between residues His111 and His112 which lie within the neurotoxic PrP106–PrP126 sequence (the region of PrP^c central in initiating the conformational change from PrP^c to PrP^{Sc} as well as the aggregation process).[86-90] This is analogous to the non-amyloidogenic APP processing pathway. However, cleavage may also occur upstream—at residue 88 (in the event of mutations in the *PRNP* gene) and thereby yield a neurotoxic product—PrP^{Sc}. This is not unlike the upstream positioning of the β-secretase cleavage site which yields potentially pathogenic Aβ peptides. In both TSEs and AD, non-fibril forming peptides, PrP106–126,[91] and Aβ oligomers are the neurotoxic agents initiating the disease. The resultant aggregates and plaques, although not the predominant agents, play a role in the pathogenic process.

In lieu of the above mentioned similarities in both the structure and neurotoxicity-inducing mechanisms of Aβ and PrP^c, it may be proposed that receptors fundamental in one process may be involved in the other. One such receptor, which is central to TSE disease establishment is the 37 kDa/67 kDa LRP/LR.

The 37 kDa/67 kDa Laminin Receptor Precursor/ Laminin Receptor

The 37 kDa/67 kDa LRP/LR is a multifunctional receptor (**Fig. 4**) located at the cell surface and is soluble in the cytoplasm and in the nucleus. At the cell surface the protein serves as a receptor for a variety of substrates including: ECM components such as elastin and laminin; viruses including Sindbis[92] Dengue,[93] Venezuelan equine encephalitis and Adeno-associated virus subtypes 2, 3, 8 and 9,[31,32] as well as cellular[73] and infectious prion proteins[72,74,75] (**Fig. 5**). Additionally, the receptor is present in the cytoplasm where it functions in the maturation of the 40S ribosomal subunit and protein synthesis[31,32] and in the nucleus in which it is associated with histones H2A, H2B and H4.[94]

LRP/LR is a transmembrane (TM) receptor (TMR) which is located at the cholesterol-rich lipid rafts and may employ HSPGs to mediate its binding interactions.[31,32] Aβ oligomers, as previously stated, have been shown to bind to an array of TM receptors and HSPGs or are alternatively inserted to lipid raft domains of the plasma membrane. Furthermore, the 37 kDa/67 kDa LRP/LR binds laminin (to which Aβ oligomers similarly bind), as well as PrP^c (a protein to which Aβ oligomers bind with high affinity). Thus, owing to the fact that Aβ and 37 kDa/67 kDa LRP/LR share a common cellular location and binding partners, as well as the likeness between the pathogenic agents and mechanisms in TSEs and AD, a relationship between Aβ and 37 kDa/67 kDa LRP/LR cannot be excluded. Moreover, a binding interaction between these proteins, be it direct or indirect, seems plausible and thereby warrants investigation.

The neurotoxicity of Aβ oligomers, especially with regards to the induction of oxidative stress and the deregulation of Ca^{2+} homeostasis may further be mediated by apoptosis.

Apoptosis

Apoptosis, primarily described by Kerr et al. in 1972, is a normal physiological process of particular importance during development, immune responses, tissue remodeling and the maintenance of normal cellular homeostasis.[95] Apoptotic events are regulated by multi-complex pathways which, although able to induce apoptosis independently of one another, are integrated and "cross talk" in the event that the apoptotic signal requires amplification to ensure the cell is committed to apoptosis.[96] These pathways are the extrinsic (death receptor) and intrinsic (mitochondrial) pathways (**Fig. 5**). An additional pathway, the granzyme/perforin pathway has also been proposed but shall not be discussed here.

The critical components of the said pathways/cascades are the caspases. These are highly conserved, hetero-tetrameric cysteine proteases which cleave aspartic residues and are synthesized as inactive zymogens (pro-caspases). Fourteen such caspases have been identified, seven of which are involved in apoptosis and the remaining (including caspases 1, 4 and 5) function in cytokine activation and inflammatory responses. Caspases may be subdivided into two groups-initiator caspases (caspases 2, 8, 9 and 10) which function at the beginning of the pathways and serve to cleave and activate the effector caspases (caspases 3, 6 and 7) which in turn cleave and activate cellular target protein and thereby induce apoptosis.

The extrinsic pathway (**Fig. 5**) is induced through signal protein-receptor interactions at the cell membrane, including FasL/FasR, tumor necrosis factor (TNF)α/TNFR1 (receptor), Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5.[96,97] The transmembrane receptors exhibit cytosolic death domains which facilitates the recruitment of adaptor proteins with the corresponding death domains as well as death effector domains. The latter domain in turn allows for the recruitment of multiple initiator procaspases (procaspase 8 and/or 10) and the resultant complex is termed the

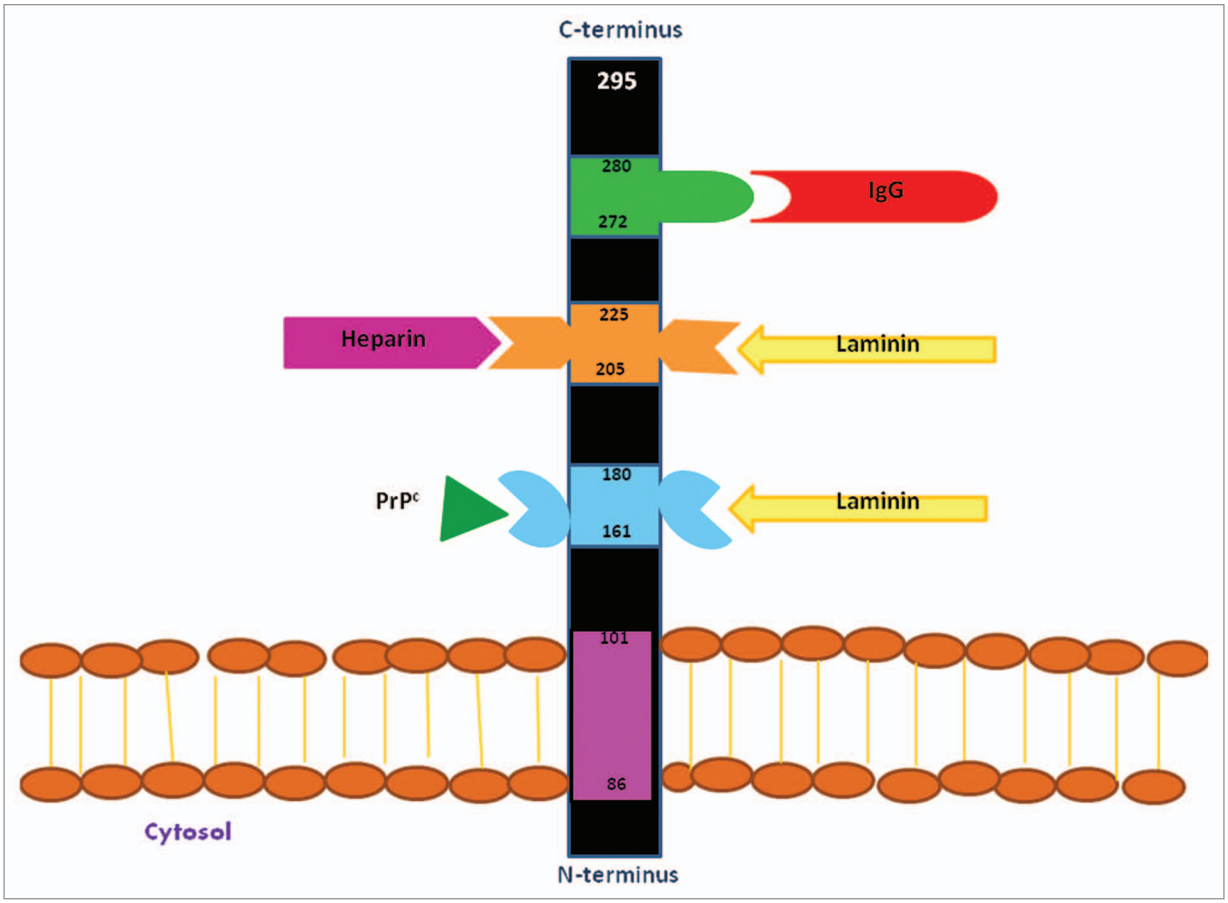


Figure 4. Schematic representation of the the functional domains of the 37 kDa/67 kDa Laminin Receptor Precursor/Laminin Receptor. This receptor, which is 295 amino acids in length, may be located at the cell surface, in the cytoplasm and the nucleus and displays different functional roles in each. A cell-surface associated form of the multi-functional protein is depicted here. The transmembrane domain of the receptor is located between amino acid residues LRP86-101. In this location the protein functions primarily as a receptor and encompasses four defined ligand-binding domains, including a prion protein binding domain (LRP161–180) and two laminin binding domains (LRP160–180 and LRP205–229), the latter functions as a heparin binding domain as well and an IgG-antibody binding domain (LRP272–280) (adapted from reference 32).

death inducing signaling complex (DISC). Within DISC, the high concentration of procaspases allows for the procaspases to auto-catalytically cleave and thereby activate each other and the activated enzymes subsequently activate effector caspases (usually caspases 3).[95,96]

Intracellular death signals and extracellular survival signals induce the intrinsic pathway in a receptor-independent manner. The intrinsic pathway centres around alterations in the permeability of the mitochondrial membrane and consequently the release of Ca^{2+} and cytochrome *c* (a component of the mitochondrial electron transport chain). This pathway is regulated by approximately 25 members of the Bcl-2 protein family. These proteins may either be anti-apoptotic (by sequestering Apaf-1 and inhibiting procaspase 9 activation) such as Bcl-2, Bcl-x, Bcl-X_L, Bcl-XS, Bcl-w and BAG or pro-apoptotic by promoting mitochondrial permeability through the formation of membrane channels such as Bax, Bak, Bid, Bad, Bim, Bik, Blk, Puma and Noxa.[95] Released cytochrome *c* binds to the cytosolic apoptotic protease activating factor-1 (Apaf-1) forming a heptamer termed the apoptosome, which subsequently recruits procaspase 9 which, upon cleavage-induced activation, activates caspases 3.

It is noteworthy to add that the MAP kinase, JNK and p53, activate Bim and Bax, Puma and Noxa respectively and are thus pro-apoptotic[98,99] p53 has also been reported to induce the expression of death receptors and thus promote apoptosis.[100,101]

The extrinsic pathway may induce the intrinsic pathway to amplify the apoptotic signal and does so through Bid. Activated caspase 8 cleaves Bid and the truncated form of this protein inhibits Bcl-2 (an anti-apoptotic protein) and thereby facilitates Apaf-1 induced procaspase 9 activation.[95]

Cellular Death

Activation of caspases 3, 6 and 7 ultimately leads to the morphological and biochemical alterations associated with apoptosis.[95] Caspase 3 activation results in the cleavage of the inhibitor of the caspases activated deoxyribonuclease (ICAD), thereby releasing the suppression of this enzyme and thus resulting in DNA fragmentation.[95] Furthermore, the effector caspases cleave

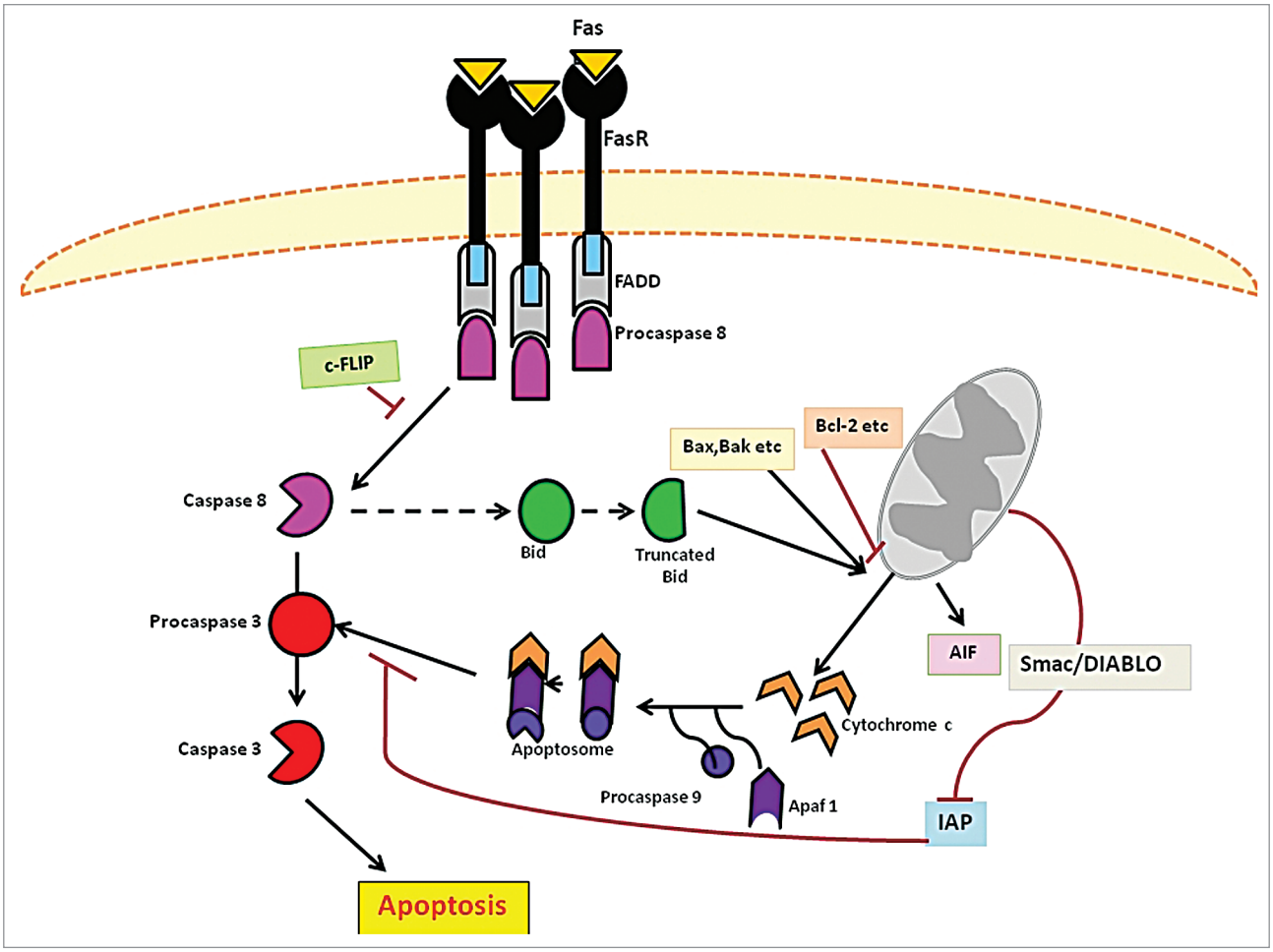


Figure 5. A schematic representation of the two classical apoptotic pathways in mammalian cells. The extrinsic is triggered at the cell surface by ligand (CD95L, FASL, TNFα) binding to death receptors (CD95, FASR, TNFR) and the ultimate formation of a death inducing signaling complex (DISC). Conversely the intrinsic pathway involves alterations in mitochondrion permeability as a result of intracellular signals such as DNA damage and oxidative stress. The activation of the aforementioned pathways leads to the cleavage and activation of initiator caspases 8 and caspases 9 respectively. These in turn activate the effector caspases 3 which facilitates DNA fragmentation and cytoskeletal protein degradation-leading to the morphological and physical features characteristic of apoptotic cells (adapted from reference 96).

cytoskeletal and associated proteins such as actin and gelsolin. The resultant disruption of the cytoskeleton consequently causes cellular shrinkage, cell membrane blebbing, disintegration of the cell into apoptotic bodies as well as defects in transport, division and signal transduction.[95]

Furthermore apoptosis may, in addition to caspase-mediated mechanisms, occur via processes independent of these cysteine proteases. One such route of programmed cell death (PCD) involves the nuclear translocation of the Apoptosis-inducing factor (AIF), a 57 kDa mitochondrial flavoprotein, upon stimulation by death signals. In the nuclear compartment AIF induces chromatin condensation and DNA fragmentation, the hallmarks of apoptosis, in a caspase independent manner.[102] An homologue of AIF, termed the AIF-homologous mitochondrion-associated inducer of death (AMID), similarly induces caspase-independent apoptosis.[103] In addition the mitochondrial release of endonuclease G has been reported to mediate caspase-independent DNA fragmentation.[104]

Evidence of Apoptosis in Alzheimer Disease

Alzheimer disease is characterized by enhanced oxidative stress and intracellular Ca^{2+} concentration-both of which may serve as stimuli to induce the intrinsic pathway.[95,105,106] Mutations in presenilins (particularly presenilin 2) may not only lead to familial Alzheimer disease but also render neurons more vulnerable to apoptosis. Further evidence for apoptosis in Alzheimer disease includes the detection of elevated levels of caspases 3, p53 and Bax in neurons of AD patients.[107] Aβ induces apoptosis through a number of mechanisms largely through the interaction of these peptides with cell surface receptors. Aβ activates microglial cells, through interaction with the scavenger receptors present on their surfaces (**Table 1**),[108] and this interaction results in the enhanced expression of TNFα (a ligand of the TNF death receptor) and thereby triggers the extrinsic pathway.[109] Aβ interactions with neuronal cell surface receptors ($p75^{NTR}$, FAS, TNFR1, RAGE, APP) (**Table 1**) may not only induce the production of reactive

oxygen species but may also, through signal transduction cascades, induce the expression of caspases and pro-apoptotic genes such as p53, p35 and tumor necrosis factors[110] as well as enhance mitochondrial permeability. It must be emphasised that these pro-apoptotic interactions need not necessarily involve death receptors as receptors lacking these domains (such as APP, RAGE, 37 kDa/67 kDa LRP/LR) may indirectly lead to apoptosis through such signaling pathways.

Apoptotic Potential of the Aβ-LRP/LR Interaction

The 37 kDa/67 kDa LRP/LR is a critical cell surface receptor with regards to the maintenance of cell viability and siRNA mediated downregulation of this receptor has been reported to induce apoptosis in transformed liver cells (Hep3B).[111] The significance of the receptor may be proposed to be a result of receptor interactions with cognate ligands and/or receptor mediated signaling. Thus the possibility that Aβ-LRP/LR interactions may inhibit these conventional interactions and thereby reduce the receptor's ability to promote cell viability may not be excluded. Furthermore, strong evidence suggests that Aβ-cell surface receptor interactions may be directly or indirectly apoptotic.[110] Therefore, owing to structural similarity and common cellular localization between the 37 kDa/67 kDa LRP/LR and receptors such as RAGE, $P75^{NTR}$ and others, if an Aβ-LRP/LR interaction is identified, further investigation of the possible apoptotic inducing potential of this Aβ-LRP/LR interaction is warranted as it may contribute to neurodegeneration and may be an alternative target for Alzheimer disease therapeutics.

Conclusion

Aβ induced neuronal apoptosis is mediated through the interactions of Aβ with the plasma membrane as well as cell surface proteins and receptors. The prion protein plays a vital role in Aβ aggregation and serves as a link between prion disorders and Alzheimer disease. The similarities between these neurodegenerative disorders offer alternatives for the development of therapeutic tools to target both diseases.

Disclosure of Potential Conflicts of Interest

Any opinion, findings and conclusions or recommendations express in this material are those of the author(s) and therefore the NRF do not accept any liability in regard thereto.

Financial Support

This work is based upon research supported by the National Research Foundation (NRF), the Republic of South Africa (RSA) and the Medical Research Council (MRC), the Republic of South Africa (RSA).

References

1. Forloni G, Balducci C. beta-amyloid oligomers and prion protein: Fatal attraction? Prion 2011; 5:10-5.
2. Mount C, Downton C. Alzheimer disease: progress or profit? Nat Med 2006; 12:780-4.
3. Palmer AM. Neuroprotective therapeutics for Alzheimer's disease: progress and prospects. Trends Pharmacol Sci 2011; 32:141-7.
4. Selkoe DJ. Alzheimer's disease: genes, proteins and therapy. Physiol Rev 2001; 81:741-66.
5. Burns A, Iliffe S. Alzheimer's disease. BMJ 2009; 338:158.
6. Jarrett JT, Berger EP, Lansbury PT Jr. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 1993; 32:4693-7.
7. Dickson DW. The pathogenesis of senile plaques. J Neuropathol Exp Neurol 1997; 56:321-39.
8. Pimplikar SW. Reassessing the amyloid cascade hypothesis of Alzheimer's disease. Int J Biochem Cell Biol 2009; 41:1261-8.
9. Iqbal K, Liu F, Gong CX, Grundke-Iqbal I. Tau in Alzheimer disease and related tauopathies. Curr Alzheimer Res 2010; 7:656-64.
10. Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 2000; 405:360-4.
11. Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. Nature 1999; 402:615-22.
12. Braak H, Braak E, Bohl J, Bratzke H. Evolution of Alzheimer's disease related cortical lesions. J Neural Transm Suppl 1998; 54:97-106.
13. Giannakopoulos P, Herrmann FR, Bussiere T, Bouras C, Kovari E, Perl DP, et al. Tangle and neuron numbers, but not amyloid load, predict cognitive status in Alzheimer's disease. Neurology 2003; 60:1495-500.
14. Josephs KA, Whitwell JL, Ahmed Z, Shiung MM, Weigand SD, Knopman DS, et al. Beta-amyloid burden is not associated with rates of brain atrophy. Ann Neurol 2008; 63:204-12.
15. Stanford PM, Halliday GM, Brooks WS, Kwok JB, Storey CE, Creasey H, et al. Progressive supranuclear palsy pathology caused by a novel silent mutation in exon 10 of the tau gene: expansion of the disease phenotype caused by tau gene mutations. Brain 2000; 123:880-93.
16. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 1991; 349:704-6.
17. Selkoe DJ. Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer's disease. Annu Rev Cell Biol 1994; 10:373-403.
18. Haass C, Hung AY, Selkoe DJ. Processing of beta-amyloid precursor protein in microglia and astrocytes favors an internal localization over constitutive secretion. J Neurosci 1991; 11:3783-93.
19. Ring S, Weyer SW, Kilian SB, Waldron E, Pietrzik CU, Filippov MA, et al. The secreted beta-amyloid precursor protein ectodomain APPs alpha is sufficient to rescue the anatomical, behavioral and electrophysiological abnormalities of APP-deficient mice. J Neurosci 2007; 27:7817-26.
20. Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, et al. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science 2010; 330:1774.
21. Anderson JP, Esch FS, Keim PS, Sambamurti K, Lieberburg I, Robakis NK. Exact cleavage site of Alzheimer amyloid precursor in neuronal PC-12 cells. Neurosci Lett 1991; 128:126-8.
22. Seubert P, Vigo-Pelfrey C, Esch F, Lee M, Dovey H, Davis D, et al. Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids. Nature 1992; 359:325-7.
23. Motter R, Vigo-Pelfrey C, Kholodenko D, Barbour R, Johnson-Wood K, Galasko D, et al. Reduction of beta-amyloid peptide42 in the cerebrospinal fluid of patients with Alzheimer's disease. Ann Neurol 1995; 38:643-8.
24. Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, et al. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med 1996; 2:864-70.
25. Fahrenholz F, Gilbert S, Kojro E, Lammich S, Postina R. Alpha-secretase activity of the disintegrin metalloprotease ADAM 10. Influences of domain structure. Ann NY Acad Sci 2000; 920:215-22.
26. Asai M, Hattori C, Szabo B, Sasagawa N, Maruyama K, Tanuma S, et al. Putative function of ADAM9, ADAM10 and ADAM17 as APP alpha-secretase. Biochem Biophys Res Commun 2003; 301:231-5.
27. Tanabe C, Hotoda N, Sasagawa N, Sehara-Fujisawa A, Maruyama K, Ishiura S. ADAM19 is tightly associated with constitutive Alzheimer's disease APP alpha-secretase in A172 cells. Biochem Biophys Res Commun 2007; 352:111-7.
28. Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, et al. BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional and synaptic functions. J Neurosci 2005; 25:11693-709.
29. O'Connor T, Sadleir KR, Maus E, Velliquette RA, Zhao J, Cole SL, et al. Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis. Neuron 2008; 60:988-1009.
30. Guglielmotto M, Aragno M, Autelli R, Giliberto L, Novo E, Colombatto S, et al. The upregulation of BACE1 mediated by hypoxia and ischemic injury: role of oxidative stress and HIF1alpha. J Neurochem 2009; 108:1045-56.

31. Omar A, Jovanovic K, Da Costa Dias B, Gonsalves D, Moodley K, Caveney R, et al. Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. Expert Opin Ther Pat 2011; 21:35-53.
32. Mbazima V, Da Costa Dias B, Omar A, Jovanovic K, Weiss SF. Interactions between PrP(c) and other ligands with the 37-kDa/67-kDa laminin receptor. Front Biosci 2010; 15:1150-63.
33. Wolfe MS. Inhibition and modulation of gamma-secretase for Alzheimer's disease. Neurotherapeutics 2008; 5:391-8.
34. Chow VW, Mattson MP, Wong PC, Gleichmann M. An overview of APP processing enzymes and products. Neuromolecular Med 2010; 12:1-12.
35. Wakabayashi T, De Strooper B. Presenilins: members of the gamma-secretase quartets, but part-time soloists too. Physiology 2008; 23:194-204.
36. Bettens K, Sleegers K, Van Broeckhoven C. Current status on Alzheimer disease molecular genetics: from past, to present, to future. Hum Mol Genet 2010; 19:4-11.
37. Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, et al. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 1995; 269:973-7.
38. Rovelet-Lecrux A, Hannequin D, Raux G, Le Meur N, Laquerriere A, Vital A, et al. APP locus duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy. Nat Genet 2006; 38:24-6.
39. Hardy J. Amyloid, the presenilins and Alzheimer's disease. Trends Neurosci 1997; 20:154-9.
40. Cruts M, Van Broeckhoven C. Presenilin mutations in Alzheimer's disease. Hum Mutat 1998; 11:183-90.
41. Kumar-Singh S, Theuns J, Van Broeck B, Pirici D, Vennekens K, Corsmit E, et al. Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. Hum Mutat 2006; 27:686-95.
42. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, et al. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci USA 1993; 90:1977-81.
43. Bertram L, Tanzi RE. Alzheimer's disease: one disorder, too many genes? Hum Mol Genet 2004; 13:135-41.
44. Gatz M, Reynolds CA, Fratiglioni L, Johansson B, Mortimer JA, Berg S, et al. Role of genes and environments for explaining Alzheimer disease. Arch Gen Psychiatry 2006; 63:168-74.
45. Dermaut B, Croes EA, Rademakers R, Van den Broeck M, Cruts M, Hofman A, et al. PRNP Val129 homozygosity increases risk for early-onset Alzheimer's disease. Ann Neurol 2003; 53:409-12.
46. Riemenschneider M, Klopp N, Xiang W, Wagenpfeil S, Vollmert C, Muller U, et al. Prion protein codon 129 polymorphism and risk of Alzheimer disease. Neurology 2004; 63:364-6.
47. Bitan G, Vollers SS, Teplow DB. Elucidation of primary structure elements controlling early amyloid beta-protein oligomerization. J Biol Chem 2003; 278:34882-9.
48. Teplow DB, Lazo ND, Bitan G, Bernstein S, Wyttenbach T, Bowers MT, et al. Elucidating amyloid beta-protein folding and assembly: A multidisciplinary approach. Acc Chem Res 2006; 39:635-45.
49. Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ. Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. J Physiol 2006; 572:477-92.
50. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 1991; 30: 572-80.
51. Nordberg A. Amyloid plaque imaging in vivo: current achievement and future prospects. Eur J Nucl Med Mol Imaging 2008; 35:46-50.
52. Lesne S, Kotilinek L, Ashe KH. Plaque-bearing mice with reduced levels of oligomeric amyloid-beta assemblies have intact memory function. Neuroscience 2008; 151:745-9.
53. Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, et al. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med 2008; 14:837-42.
54. Marchesi VT. Alzheimer's dementia begins as a disease of small blood vessels, damaged by oxidative-induced inflammation and dysregulated amyloid metabolism: implications for early detection and therapy. FASEB J 2011; 25:5-13.
55. Bartzokis G. Alzheimer's disease as homeostatic responses to age-related myelin breakdown. Neurobiol Aging 2011; 32:1341-71.
56. Renner M, Lacor PN, Velasco PT, Xu J, Contractor A, Klein WL, et al. Deleterious effects of amyloid beta oligomers acting as an extracellular scaffold for mGluR5. Neuron 2010; 66:739-54.
57. Bi X, Gall CM, Zhou J, Lynch G. Uptake and pathogenic effects of amyloid beta peptide 1–42 are enhanced by integrin antagonists and blocked by NMDA receptor antagonists. Neuroscience 2002; 112:827-40.
58. Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci 1992; 12:376-89.
59. Stefani M. Biochemical and biophysical features of both oligomer/fibril and cell membrane in amyloid cytotoxicity. FEBS J 2010; 277:4602-13.
60. Sepulveda FJ, Parodi J, Peoples RW, Opazo C, Aguayo LG. Synaptotoxicity of Alzheimer beta amyloid can be explained by its membrane perforating property. PLoS One 2010; 5:11820.
61. Qiu L, Lewis A, Como J, Vaughn MW, Huang J, Somerharju P, et al. Cholesterol modulates the interaction of beta-amyloid peptide with lipid bilayers. Biophys J 2009; 96:4299-307.
62. Sakurai T, Kaneko K, Okuno M, Wada K, Kashiyama T, Shimizu H, et al. Membrane microdomain switching: a regulatory mechanism of amyloid precursor protein processing. J Cell Biol 2008; 183:339-52.
63. Verdier Y, Penke B. Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. Curr Protein Pept Sci 2004; 5: 19-31.
64. Roberson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, et al. Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. Science 2007; 316:750-4.
65. Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, van Eersel J, et al. Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. Cell 2010; 142:387-97.
66. Beck K, Hunter I, Engel J. Structure and function of laminin: anatomy of a multidomain glycoprotein. FASEB J 1990; 4:148-60.
67. Kibbey MC, Jucker M, Weeks BS, Neve RL, Van Nostrand WE, Kleinman HK. beta-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin. Proc Natl Acad Sci USA 1993; 90:10150-3.
68. Koo EH, Park L, Selkoe DJ. Amyloid beta-protein as a substrate interacts with extracellular matrix to promote neurite outgrowth. Proc Natl Acad Sci USA 1993; 90:4748-52.
69. Castillo GM, Lukito W, Peskind E, Raskind M, Kirschner DA, Yee AG, et al. Laminin inhibition of beta-amyloid protein (Abeta) fibrillogenesis and identification of an Abeta binding site localized to the globular domain repeats on the laminin a chain. J Neurosci Res 2000; 62:451-62.
70. Mehrpour M, Codogno P. Prion protein: From physiology to cancer biology. Cancer Lett 2010; 290:1-23.
71. Prusiner SB. Prions. Proc Natl Acad Sci USA 1998; 95:13363-83.
72. Morel E, Andrieu T, Casagrande F, Gauczynski S, Weiss S, Grassi J, et al. Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. Am J Pathol 2005; 167:1033-42.
73. Gauczynski S, Peyrin JM, Haik S, Leucht C, Hundt C, Rieger R, et al. The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. EMBO J 2001; 20:5863-75.
74. Gauczynski S, Nikles D, El-Gogo S, Papy-Garcia D, Rey C, Alban S, et al. The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. J Infect Dis 2006; 194:702-9.
75. Kolodziejczak D, Da Costa Dias B, Zuber C, Jovanovic K, Omar A, Beck J, et al. Prion interaction with the 37-kDa/67-kDa laminin receptor on enterocytes as a cellular model for intestinal uptake of prions. J Mol Biol 2010; 402:293-300.
76. Da Costa Dias B, Jovanovic K, Weiss SF. Alimentary prion infections: Touchdown in the intestine. Prion 2011; 5:6-9.
77. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. Annu Rev Neurosci 2001; 24:519-50.
78. Lauren J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature 2009; 457:1128-32.
79. Chen S, Yadav SP, Surewicz WK. Interaction between human prion protein and amyloid-beta (Abeta) oligomers: role OF N-terminal residues. J Biol Chem 2010; 285:26377-83.
80. Parkin ET, Watt NT, Hussain I, Eckman EA, Eckman CB, Manson JC, et al. Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. Proc Natl Acad Sci USA 2007; 104:11062-7.
81. Kellett KA, Hooper NM. Prion protein and Alzheimer disease. Prion 2009; 3:190-4.
82. Vincent B, Sunyach C, Orzechowski HD, St. George-Hyslop P, Checler F. p53-Dependent transcriptional control of cellular prion by presenilins. J Neurosci 2009; 29:6752-60.
83. Bush AI. The metallobiology of Alzheimer's disease. Trends Neurosci 2003; 26:207-14.
84. Brown DR. Copper and prion disease. Brain Res Bull 2001; 55:165-73.
85. Barnham KJ, Cappai R, Beyreuther K, Masters CL, Hill AF. Delineating common molecular mechanisms in Alzheimer's and prion diseases. Trends Biochem Sci 2006; 31:465-72.
86. Vincent B, Paitel E, Saftig P, Frobert Y, Hartmann D, De Strooper B, et al. The disintegrins ADAM10 and TACE contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein. J Biol Chem 2001; 276:37743-6.
87. Cisse MA, Sunyach C, Lefranc-Jullien S, Postina R, Vincent B, Checler F. The disintegrin ADAM9 indirectly contributes to the physiological processing of cellular prion by modulating ADAM10 activity. J Biol Chem 2005; 280:40624-31.
88. Jobling MF, Stewart LR, White AR, McLean C, Friedhuber A, Maher F, et al. The hydrophobic core sequence modulates the neurotoxic and secondary structure properties of the prion peptide 106–126. J Neurochem 1999; 73:1557-65.
89. Tagliavini F, Prelli F, Verga L, Giaccone G, Sarma R, Gorevic P, et al. Synthetic peptides homologous to prion protein residues 106–147 form amyloid-like fibrils in vitro. Proc Natl Acad Sci USA 1993; 90: 9678-82.

90. Brown DR, Schmidt B, Kretzschmar HA. Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. Nature 1996; 380:345-7.
91. Salmona M, Malesani P, De Gioia L, Gorla S, Bruschi M, Molinari A, et al. Molecular determinants of the physicochemical properties of a critical prion protein region comprising residues 106–126. Biochem J 1999; 342:207-14.
92. Wang KS, Kuhn RJ, Strauss EG, Ou S, Strauss JH. High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. J Virol 1992; 66:4992-5001.
93. Thepparit C, Smith DR. Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. J Virol 2004; 78:12647-56.
94. Kinoshita K, Kaneda Y, Sato M, Saeki Y, Wataya-Kaneda M, Hoffmann A. LBP-p40 binds DNA tightly through associations with histones H2A, H2B and H4. Biochem Biophys Res Commun 1998; 253:277-82.
95. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007; 35:495-516.
96. Hengartner MO. The biochemistry of apoptosis. Nature 2000; 407:770-6.
97. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 2003; 10:26-35.
98. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995; 80:293-9.
99. Henry H, Thomas A, Shen Y, White E. Regulation of the mitochondrial checkpoint in p53-mediated apoptosis confers resistance to cell death. Oncogene 2002; 21:748-60.
100. Hussein MR, Haemel AK, Wood GS. Apoptosis and melanoma: molecular mechanisms. J Pathol 2003; 199:275-88.
101. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. Lancet Oncol 2003; 4:721-9.
102. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397:441-6.
103. Wu M, Xu LG, Li X, Zhai Z, Shu HB. AMID, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. J Biol Chem 2002; 277:25617-23.
104. van Loo G, Schotte P, van Gurp M, Demol H, Hoorelbeke B, Gevaert K, et al. Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. Cell Death Differ 2001; 8:1136-42.
105. Mattson MP, Tomaselli KJ, Rydel RE. Calcium-destabilizing and neurodegenerative effects of aggregated beta-amyloid peptide are attenuated by basic FGF. Brain Res 1993; 621:35-49.
106. Behl C, Davis JB, Lesley R, Schubert D. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 1994; 77:817-27.
107. Eckert A, Keil U, Marques CA, Bonert A, Frey C, Schussel K, et al. Mitochondrial dysfunction, apoptotic cell death and Alzheimer's disease. Biochem Pharmacol 2003; 66:1627-34.
108. El Khoury J, Hickman SE, Thomas CA, Cao L, Silverstein SC, Loike JD. Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. Nature 1996; 382:716-9.
109. Tan J, Town T, Paris D, Mori T, Suo Z, Crawford F, et al. Microglial activation resulting from CD40-CD40L interaction after beta-amyloid stimulation. Science 1999; 286:2352-5.
110. Small DH, Mok SS, Bornstein JC. Alzheimer's disease and Abeta toxicity: from top to bottom. Nat Rev Neurosci 2001; 2:595-8.
111. Susantad T, Smith DR. siRNA-mediated silencing of the 37/67-kDa high affinity laminin receptor in Hep3B cells induces apoptosis. Cell Mol Biol Lett 2008; 13:452-64.
112. Chen Q, Schubert D. Presenilin-interacting proteins. Expert Rev Mol Med 2002; 4:1-18.
113. Da Costa Dias B, Weiss SF. A kiss of a prion: new implications for oral transmissibility. J Infect Dis 2010; 201:1615-1616.

Global Alzheimer Research Summit: Basic and clinical research; Present and future Alzheimer research

D. Gonsalves*, K. Jovanovic*, B. Da Costa Dias and S.F.T Weiss

Prion 6 (2012) 1-4

* These authors contributed equally to the work

The Global Alzheimer Research Summit was held in September 2011 in Madrid, Spain. The conference was held over 2 days where many leaders in Alzheimer's disease research presented their findings. This meeting report highlights and summarizes the major points of discussion from each of the sessions namely Molecular Mechanisms, Genetics, Biomarkers, Diagnosis and Therapeutic Advances, Translational Research and Novel Aspects of Basic Research in AD. The importance of Biomarkers was repeatedly discussed during the course of the conference, as were the novel therapeutic strategies undergoing clinical trials (such as Bapineuzumab). Interesting avenues of research including effects of AD on mitochondria as well as the neuroprotective role of Reelin in AD were also presented.

Prion 6:1, 7-10; January/February/March 2012; © 2012 Landes Bioscience

Global Alzheimer Research Summit

Basic and clinical research
Present and future Alzheimer research

Danielle Gonsalves,† Katarina Jovanovic,† Bianca Da Costa Dias and Stefan F. T. Weiss*

School of Molecular and Cell Biology; University of the Witwatersrand; Johannesburg, Republic of South Africa (RSA)

†These authors contributed equally to this work.

Keywords: Alzheimer disease, amyloid beta, tau, β-secretase, γ-secretase, neurodegeneration, clinical trials, biomarkers, therapeutics, diagnosis

We report here on the proceedings of the Global Alzheimer Summit that took place September 22–23, 2011 in Madrid, Spain. As Alzheimer disease (AD) is the leading cause of neurodegeneration in elderly individuals and, as yet, has no effective therapeutic option, it continues to stimulate global research interests. At the conference, leaders in the field of AD research provided insights into current developments in various areas of research, namely molecular mechanisms, genetics, novel aspects of AD research and translational research. Emphasis was also placed on the importance of biomarkers in the diagnosis of AD and development of current therapeutic strategies.

Introduction

Alzheimer disease (AD) is the most prevalent form of dementia globally, affecting an excess of 36 million people, of which 7 million are found in Europe alone. Ten percent of individuals over 60 years of age and 50% of those over 85 years are afflicted with the disease.[1] To date, there is no cure for this disease and there are few effective palliative therapies available. Research is thus critical for the further understanding of the molecular basis of AD and the development of therapeutic interventions.

The Global Alzheimer Research Summit was held on the September 22–23, 2011 at the Palacio de Congresos de Madrid in Madrid, Spain. The conference was organized by the Queen Sofia Foundation and the Pasqual Maragall Foundation. The aim of this conference was to bring together leading experts in the field of AD and provide a comprehensive overview of the molecular mechanisms and genetics underlying this disease with the intent of providing a clear direction for future research into early diagnosis and therapeutic interventions.

The conference was divided into two sections: Health and Social Care Research (Learning to Live Better with Alzheimer Disease) and Basic and Clinical Research (Present and Future of Alzheimer Research), the latter of which will be the focus of this meeting report. There were five main areas of focus examined in this conference, namely molecular mechanisms, genetics, biomarkers, diagnosis and therapeutic advances, translational research and novel aspects of basic research in AD.

*Correspondence to: Stefan F.T. Weiss; Email: stefan.weiss@wits.ac.za
Submitted: 11/11/11; Revised: 11/21/11; Accepted: 11/23/11
http://dx.doi.org/10.4161/pri.6.1.18854

Molecular Mechanisms

The causative agent of AD is thought to be the 4 kDa neurotoxic amyloid β (Aβ) peptide. The proposed mechanism by which Aβ is generated involves the sequential proteolytic cleavage of the Amyloid Precursor Protein (APP) by β-secretase and the γ-secretase complex.[2-4] Sangram Sisodia (University of Chicago) opened the conference with an elucidating overview on the function of presenilin (PS), the catalytic subunit of the γ-secretase complex, in health and disease. He presented findings aimed at describing the γ-secretase complex and its function, as well as the importance of PS in autophagy. The differentiation and proliferation of neuronal progenitor cells was shown to be impaired in Familial AD (FAD)—linked PS1 variants suggesting a link between PS1 and neurogenesis.

Christian Haass (Ludwig-Maximilians University Munich) emphasized the unique role of the GxGD motif with regards to PS1 activity, drawing a parallel between this catalytic subunit and intramembrane cleaving aspartyl proteases. He further illustrated functional commonalities between the two in terms of their sequential substrate cleaving activities and the autoactivation which is a result thereof. γ-secretase modulation, as opposed to inhibition, was suggested as a means to prevent unwanted modifications of the essential Notch signaling pathway. First generation γ-secretase modulators (GSMs) have failed to prove effective in clinical trials, but second generation GSMs have been shown to reduce $A\beta_{42}$ production in cases exhibiting PS1 and PS2 mutations. Moreover, genome-wide RNA interference (RNAi) screening is being employed to identify endogenous GSMs.

Neurofibrillary tangles (NFTs) are one of the major pathological hallmarks of AD. The main constituent of these NFTs are paired helical filaments (PHF), which are assembled from the hyperphosphorylated microtubule associated protein, tau.[5] Virginia Lee (University of Pennsylvania) hypothesized that the

amount of normal tau available for microtubule stabilization would therefore be reduced and axonal transport compromised, leading to the neurodegeneration associated with AD. Lee focused her talk on targeting tau as a therapeutic tool for the treatment of AD, specifically focusing on Paclitaxel and Epothilone D. Animal trials employing Epothilone D have indicated its potential use in treating AD and other tauopathies.

Jesus Avila (Universidad de Autonoma de Madrid) discussed the role of glycogen synthase kinase 3 (GSK3), the enzyme responsible for the hyperphosphorylation of tau, in the impairment of neurogenesis and memory loss associated with AD. Avila suggested that the depletion of dentate gyrus (DG) stem cells within the hippocampus could be a cause for the inhibition of memory and learning associated with AD. The elevated Aβ levels observed in AD are thought to possibly promote the activation of GSK3. Adult neurogenesis was impaired in transgenic mice overexpressing GSK3 at the DG, and this correlated with a decrease in DG volume and hindered memory. Avila further illustrated that these memory impairments could be reversed so long as neuronal stem cells were present at the DG. It is therefore speculated that this could be a possible molecular mechanism underlying episodic memory loss as is noted in AD patients.

In the keynote lecture presented by the esteemed Dennis Selkoe (Harvard Medical School), the pathological effects of neuronal derived Aβ oligomers (as opposed to synthetic Aβ assemblies) were highlighted. Soluble Aβ oligomers, at nanomolar concentrations, were shown to not only inhibit long-term potentiation (LTP) but also induce long-term synaptic depression (LTD) while decreasing dendritic spine density in the hippocampus of normal rodent models. The administration of anti-Aβ antibodies directed against the N-terminus of Aβ prevented the aforementioned effects on LTP and LTD. Since the amyloid plaque cores themselves did not influence LTP or LTD, it was concluded that the soluble Aβ dimers and other low oligomeric number (low-n) Aβ species are the synaptotoxic species. Selkoe also discussed the ongoing phase III clinical trials of the anti-Aβ vaccine, Bapineuzumab.

Genetics

The apolipoprotein E (APOE) allele was the first major risk factor identified for the sporadic form of AD. However, despite our advances in the understanding of the genetics underlying this disease, over 50% of AD cases have no known genetic risk factors or components. Alison Goate (Washington University School of Medicine) and Sandra Barral (Columbia University) elaborated on their findings from several genome-wide association studies (GWAS) aimed at discovering novel risk factors by comparing AD cases to non-demented elderly controls. Nine novel risk factors (*CLU, PICALM, CR1, BIN1, MS4A4A cluster, ABCA7, CD2AP, CD33 and EPHA1*) were identified, which increase the risk for late onset Alzheimer disease (LOAD) by 10–15%. These novel risk factors are believed to play roles in a number of cellular pathways including lipid metabolism, the immune system and endocytosis. Barral further elaborated on the risk factors associated with LOAD, hypothesizing that multiple common variants underlie the cause of AD. The *APOE* ε4 allele is considered to be the risk factor most commonly associated with LOAD and increases risk in a dose-dependent manner, while the *APOE* ε2 allele reduces the risk for AD. Goate also described the use of cerebro-spinal fluid (CSF) biomarkers, i.e., CSF Aβ and tau, as endophenotypes for genes which may influence the expression levels of these CSF proteins. These analyses revealed that risk factors associated with AD correlate to CSF Aβ levels, while CSF tau levels corresponded to the rate of disease progression as opposed to risk.

In a plenary talk given by Kenneth Kosik (University of California), entitled "Stalking an Alzheimer's gene in the Colombian countryside," an interesting case was presented about a number of Colombian families residing in the state of Antioquia. These families, which include over 5,000 individuals, have been identified as carriers of one of the deadly mutations associated with early onset AD. This group of individuals presents an interesting opportunity for researchers and clinicians, through the use of genetic testing, to predict who will be afflicted with the disease and the possible age of onset. This scenario is ideal for Alzheimer "trialists" who believe that treatment of presymptomatic AD will prove most beneficial in delaying the age of onset—past the expected mean of 47 years of age, which is the case in this Colombian population.

Biomarkers, Diagnosis and Therapeutic Advances

Bruno Dubois (University of Paris) provided insight into new diagnostic criteria as proposed by the International Working Group for New Research Criteria for the Diagnosis of AD in 2007, moving away from the original criteria outlined by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA) in 1984. According to these new criteria, a clinician can make a clinical diagnosis of AD based on one major clinical criterion (e.g., episodic memory test with cued recall measures) and the presence of one or more biomarkers. This new classification system allows for the early diagnosis of AD at the prodromal stage. In terms of biomarkers, structural alterations, such as atrophy of the medial temporal lobe, can be assessed by Magnetic Resonance Imaging (MRI), while biological changes can be recorded by CSF analyses of tau and Aβ. Functional or molecular changes can be examined using neuro-imaging patterns or amyloid ligand retention on Positron Emission Tomography (PET) such as the Pittsburgh compound B (PiB-PET).

Dale Schenk (Elan Pharmaceuticals and Janssen Alzheimer Immunotherapy, LLC) and Jeffery Cummings (University of California) both highlighted the use of biomarkers, such as low CSF $A\beta_{42}$ and elevated CSF tau levels, as possible endpoints for clinical trials. The observed reduction in CSF $A\beta_{42}$ levels or an increase in PiB staining could be used as an indicator for dementia later in life, while a decrease in CSF tau levels correlates well with treatment efficacy. The use of such biomarkers in Phase II clinical trials can effectively increase the accuracy of reported therapeutic benefits using a smaller sample size, while reducing

the potential risk of entering into Phase III trials. As a representative of Elan Pharmaceuticals, South San Fransisco, Schenk also briefly spoke about Bapineuzumab and the ongoing Phase III trials.

Kaj Blennow (University of Gothenberg) further highlighted the use of biomarkers for monitoring the pathophysiological mechanisms central to AD. He reported on numerous studies that suggest that CSF biomarkers, including total tau reflecting neuronal degeneration, $A\beta_{42}$ reflecting plaque pathology and phosphorylated tau reflecting tau phosphorylation state and tangle pathology, have clinical use in the accurate diagnosis of prodromal AD. Blennow further elaborated on many of the same themes raised by Shenk and Cummings, suggesting the use of biomarkers in clinical trials to enhance patient selection for the trials and possibly increase the likelihood of identifying any significant clinical effects of the drugs in question. He further raised attention to the use of the shorter Aβ isoforms, namely $A\beta_{1-15}$ and $A\beta_{1-16}$ for the monitoring of γ-secretase inhibitor treatment, a biomarker found to be more sensitive compared with CSF $A\beta_{42}$ for monitoring this particular treatment option.

Mony de Leon (New York University School of Medicine) emphasized the combined use of both imaging and biomarkers in the pre-symptomatic diagnosis and identification of new mechanisms of AD. His team discovered two emergent AD related mechanisms: first, higher amyloid plaque load in the brain and reduced glucose metabolism was noted in pre-symptomatic patients whose mothers had a positive AD diagnosis and, second, higher plasma $A\beta_{40}$ levels were correlated with alterations in CO_2-linked hippocampal vasoreactivity. These findings suggest inherited maternal mutations in mitochondrial DNA could contribute to an increased AD risk and provide a vascular mechanism for the basis of Aβ-induced neurodegeneration.

Khalid Iqbal (New York State Institute for Basic Research in Developmental Disabilities) focused on the multifactoral nature of AD, suggesting that an inability to accurately identify various subgroups of AD and the assignment of these groups to specific clinical trials has hampered the development of effective AD therapies. It is his belief that the treatment of AD should not only target the inhibition of neurodegeneration but also stimulate neurogenesis and neuronal plasticity, and suggests that a careful balance between these two factors is necessary to reverse the cognitive damage inflicted by AD.

Bengt Winblad (Karolinska Institutet Alzheimer Disease Research Center) believes that we have achieved a partial degree of success in terms of the development of palliative therapies for the treatment of AD, but emphasizes a need for disease modifying drugs. He highlighted the ongoing clinical trials, many of which are based on the amyloid hypothesis of AD, but also acknowledged that a single therapy for AD is improbable. Winblad agreed with Iqbal in that multiple drugs targeting various pathways of AD will be needed to effectively manage this disease.

Although many clinical trials for BACE1 inhibitors have failed thus far, Martin Citron (Eli Lilly and Company) provided evidence for the fact that "BACE1 is druggable." The first orally available non-peptidic BACE1 (β-secretase) inhibitor, namely LY2811376, was recently generated using a fragment based chemistry strategy. LY2811376 was shown to significantly lower Aβ production in animal models, an effect which persists in humans. LY2811376 development was however terminated due to toxicology findings in preclinical studies. Citron's studies proved that BACE1 inhibition is a plausible therapeutic approach for the treatment of AD.

Translational Research

Oxidative and mitochondrial abnormalities were discussed in detail by George Perry (University of Texas at San Antonio). Impairments in mitochondrial function, fission and fusion events develop in the early stages of AD. A decrease in the expression levels as well as altered distribution of fission/fusion proteins DLP1, OPA1, Mfn1 and Mfn2C were reported in AD neurons, while a significant increase in Fis1 was noted. Moreover, APP and Aβ were found to be responsible for these changes in expression levels. The result thereof is a reduction in mitochondrial density, which consequently manifests as a decrease in spine numbers. Various structural changes are also evident in AD vulnerable neurons and include a reduction in the number and increase in the average size of mitochondria. However, it should be noted that the mitochondrial changes in AD are not solely from nor demonstrated to be the result of Aβ/APP alone as a similar effect of oxidative stress independent of Aβ is seen.

Lennart Mucke (Gladstone Institute of Neurological Diseases and University of California) discussed the key role of tau in Aβ induced changes in neuronal and cognitive function as well as intracellular transport. A reduction in tau effectively prevents neuronal dysfunction induced by Aβ oligomers in transgenic mouse models. Furthermore Aβ, tau and Fyn (a src family kinase) were found to function co-dependently. The receptor tyrosine kinase EphB2 is targeted for proteosomal degradation upon interacting with Aβ oligomers; this in turn affects NMDA-type glutamate receptors and ultimately results in synaptic deficits and memory and learning impairments.

Novel Aspects of Basic Research in AD

Eduardo Soriano (University of Barcelona) elucidated the intriguing link between Reelin (an extracellular matrix protein which functions in neuronal development) and AD. Reelin was shown to be an important protein in the brain controlling adult neurogenisis, glutamatergic neurotransmission and structural and functional properties of dendritic spines. This protein was found to regulate tau phosphorylation through GSK3 activity. Moreover, Reelin was shown not only to delay the accumulation of Aβ plaques but also to sequester the pathogenic oligomeric Aβ species within these plaques, preventing cognitive impairment. The Reelin-Aβ association disrupts the Reelin signaling cascade thus impairing the essential neuroprotective function of Reelin in the brain and leading to neurodegeneration.

The importance of side-chain oxidized oxysterol in AD pathogenesis was discussed by Angel Cedazo-Minguez (Karolinska Institutet Alzheimer Disease Research Center). It

was hypothesized that these oxysterol compounds were not by-products in cholesterol metabolism but rather play an important pathological role in the development of AD. Further he provided evidence linking hypercholesterolemia and hypertension to AD via disturbances in cholesterol metabolism and the overactivation of the brain rennin-angiotensin system ultimately resulting in disruptions in long-term potentiation (LTP).

Javier DeFelipe (Universidad Politecnica de Madrid) focused his talk on the dendritic spine alterations associated with AD. DeFelipe's group used powerful micro-anatomical tools to investigate the effects of tau aggregation on dendritic spines. It was found that non-aggregated pre-tangle tau proteins did not alter pyramidal neuron dendrites, as opposed to aggregated forms, which result in dendritic atrophy and spinal loss.

The Future of AD Research

Since Alois Alzheimer first described the disease in 1907,[6] our understanding of AD and its underlying molecular mechanisms has come a long way; however, many hurdles still remain. The continuing development of biomarkers and their use for not only the preclinical diagnosis of AD but also as endpoints for clinical trials will continue to change the face of AD research. It is now a reality that AD can be diagnosed in the prodromal stages. Numerous ongoing clinical trials offer hope to the millions of those afflicted by this devastating disease. The continued efforts of leading researchers in the field have offered a promising future for AD and its sufferers.

Disclosure of Potential Conflicts of Interest

Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and therefore the NRF do not accept any liability in regard thereto.

Financial Support

This work is based upon research supported by the National Research Foundation (NRF), Republic of South Africa and the University of the Witwatersrand Travel Award.

References

1. www.alzheimerinternacional2011.org/disease.html
2. Da Costa Dias B, Jovanovic K, Gonsalves D, Weiss SF. Structural and mechanistic commonalities of amyloid-beta and the prion protein. Prion 2011; 5:126-37; PMID:21862871; http://dx.doi.org/10.4161/pri.5.3.17025.
3. Mbazima V, Da Costa Dias B, Omar A, Jovanovic K, Weiss S. Interactions between PrPc and other ligands with the 37 kDa/67 kDa laminin receptor. Front Biosci 2010; 15:1150-63; PMID:20515747; http://dx.doi.org/10.2741/3667.
4. Omar A, Jovanovic K, Da Costa Dias B, Gonsalves D, Moodley K, Caveney R, et al. Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. Expert Opin Ther Pat 2011; 21:35-53; PMID:21110766; http://dx.doi.org/10.1517/13543776.2011.539203.
5. Ballatore C, Lee VM, Trojanowski JQ. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci 2007; 8:663-72; PMID:17684513; http://dx.doi.org/10.1038/nrn2194.
6. Möller HJ, Graeber MB. The case described by Alois Alzheimer in 1911. Historical and conceptual perspectives based on the clinical record and neurohistological sections. Eur Arch Psychiatry Clin Neurosci 1998; 248:111-22; PMID:9728729.

5. Research Articles

During the course of this PhD, the objectives were achieved and the results and findings thereof have been prepared for publication in 2 separate scientific journal articles.

The first of these articles, which has been published in Scientific Reports covered the findings obtained from objectives 1 – 6. The second article, which has been submitted to PLOS ONE for review, reports on the results acquired for objectives 7 – 9.

These articles are:

1. Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease
2. High resolution imaging study of interactions between the 37kDa/67kDa Laminin Receptor and APP, β-secretase and γ-secretase in Alzheimer's disease

Furthermore 2 research articles have been published; however, these did not reflect results obtained from my PhD. I was involved in the writing and editing of the manuscripts.

3. Anti-LRP/LR specific antibody IgG1-iS18 rescues cells from Aβ42 induced cytotoxicity
4. Prion interaction with the 37-kDa/67-kDa laminin receptor on enterocytes as a cellular model for intestinal uptake of prions

Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease

Katarina Jovanovic, Danielle Gonsalves, Bianca Da Costa Dias, Kiashanee Moodley, Uwe Reusch, Stefan Knackmuss, Clement Penny, Marco Weinberg, Melvyn Little & Stefan F.T. Weiss.

Scientific Reports 3, 2699; DOI:10.1038/srep02699 (2013)

This original research article was the first report on a role for LRP/LR in Alzheimer's disease. Here we found that blocking LRP/LR with the anti-LRP specific antibody IgG1-iS18 or knock down of LRP/LR using anti-LRP shRNA resulted in significant impedance in Aβ shedding. Furthermore anti-LRP/LR treatments resulted in a significant reduction in sAPPβ shedding, while cell surface expression levels of APP, β- and γ-secretase were unaltered. Immunofluorescence and confocal microscopy confirmed a high degree of co-localisation between LRP/LR and APP, β- and γ-secretase both intracellularly and on the cell surface. These findings suggested the possibility of an interaction between LRP/LR and APP, β- and/or γ-secretase. Due to the effects on sAPPβ shedding, the possibility of an interaction between LRP/LR and β-secretase was examined by means of a FLAG® co-immunoprecipitation assay, whereby it was found that a direct/indirect interaction existed between the 2 proteins. Taken together, these results not only implicate LRP/LR as a role player in AD and the amyloidogenic processing of APP, but also recommend IgG1-iS18 as a possible therapeutic for the treatment of AD (due to its ability to decrease Aβ shedding).

NB: All shRNA work in this manuscript was performed by Ms. Danielle Gonsalves as part of her MSc project. As such these results will not be discussed.

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
MOLECULAR NEUROSCIENCE
MECHANISMS OF DISEASE

Received
12 August 2013

Accepted
2 September 2013

Published
19 September 2013

Correspondence and requests for materials should be addressed to S.F.T.W. (stefan.weiss@wits.ac.za)

Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease

Katarina Jovanovic[1], Danielle Gonsalves[1], Bianca Da Costa Dias[1], Kiashanee Moodley[1], Uwe Reusch[2], Stefan Knackmuss[2], Clement Penny[3], Marc S. Weinberg[4], Melvyn Little[2] & Stefan F. T. Weiss[1]

[1]School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa, [2]Affimed Therapeutics AG, Technologiepark, Im Neuenheimer Feld 582, 69120 Heidelberg, Germany, [3]Department of Internal Medicine, University of the Witwatersrand, 7 York Rd, Johannesburg, 2193 Parktown, Republic of South Africa, [4]Antiviral Gene Therapy Research Unit (AGTRU), Department of Molecular Medicine & Haematology, School of Pathology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa.

Alzheimer's disease (AD) is the most prevalent form of dementia. The amyloid beta (Aβ) peptide is the predominant candidate aetiological agent and is generated through the sequential proteolytic cleavage of the Amyloid Precursor Protein (APP) by beta (β) and gamma (γ) secretases. Since the cellular prion protein (PrP^c) has been shown to regulate Aβ shedding, we investigated whether the cellular receptor for PrP^c, namely the 37 kDa/67 kDa Laminin Receptor (LRP/LR) played a role in Aβ shedding. Here we show that LRP/LR co-localises with the AD relevant proteins APP, β- and γ-secretase, respectively. Antibody blockage and shRNA knock-down of LRP/LR reduces Aβ shedding, due to impediment of β-secretase activity, rather than alteration of APP, β- and γ-secretase levels. These findings indicate that LRP/LR contributes to Aβ shedding and recommend anti-LRP/LR specific antibodies and shRNAs as novel therapeutic tools for AD treatment.

Alzheimer's disease (AD) is the most prevalent form of dementia afflicting in excess of 37 million people globally[1] and is associated with a multitude of genetic, environmental, epigenetic, dietary and lifestyle risk factors[2,3]. The neuropathological hallmarks of AD include intracellular neurofibrillary tangle formation (aggregates of hyper-phosphorylated microtubule associated protein, tau)[4] and extracellular Aβ plaque deposition[5]. The Aβ peptide and more specifically the 42 amino acid isoform ($A\beta_{42}$), is largely considered the primary disease causing agent in Alzheimer's disease (as Aβ accumulation is a pre-requisite for tau hyperphosporylation, the other AD-associated feature)[6,7]. Aβ is generated through the proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase (BACE1 - β site APP cleavage enzyme)[8] and γ-secretase (composed of 4 subunits of which the catalytic domain is composed of Presenilin (PS)[9]). The mechanisms underlying Aβ induction of neuronal loss (one of the key pathophysiological features of AD) are yet to be firmly established. However, it is proposed that Aβ may do so by eliciting alterations in signal transduction pathways through direct binding to cell surface receptors, such as N-Methyl-d-Aspartate (NMDA) receptors, insulin receptors or α-7 nicotinic receptors[10,11]. Alternatively, Aβ may alter signal transduction pathways indirectly via incorporation into lipid membranes of the plasma membrane and to a lesser extent cellular organelles[11,12]. This is thought to induce structural and functional alterations in lipid bound receptors and consequently results in aberrant signal transduction pathways[12].

In 2007, Parkin et al. demonstrated a link between cellular prion proteins (PrP^c) and the amyloidogenic processing of APP[13]. It was shown that PrP^c mediates a decrease in Aβ shedding by regulating β-secretase cleavage of APP. In addition, PrP^c was suggested to be a high affinity receptor for Aβ oligomers and vital in mediating the neurotoxic effects of Aβ[14]. PrP^c has also been reported to play an important role in synaptic and neuronal loss[15] as well as mediating toxic signalling induced by Aβ[16,17].

The extracellular matrix glycoprotein, laminin, similarly exhibits an Aβ binding site, namely the IKAV peptide sequence located on the alpha (α) chain of the tri-peptide[18]. However, the association between laminin and Aβ is reported to inhibit fibrillogenesis[18] and thereby thwart Aβ pathogenesis.

The 37 kDa/67 kDa laminin receptor (LRP/LR) (also known as LAMR, RPSA and p40) is a multifunctional protein located within the cholesterol-rich lipid raft domains of the plasma membrane, in the cytoplasm as well as

in the nucleus[19]. Associations between the receptor and a multitude of extracellular (laminin and elastin) and intracellular (cytoskeletal proteins, histones, heparan sulfate proteoglycans (HSPGs)) components have been described and are of physiological significance both in healthy and cancerous cells[20–24]. Moreover, it has been established that LRP/LR is a high affinity receptor for laminin and both the cellular and infectious prion protein isoforms (PrP^{c} and PrP^{Sc}, respectively)[25–28] and plays an important role in the binding, receptor mediated endocytosis and propagation of these proteins[29,30]. As LRP/LR and Aβ share the aforementioned mutual binding partners, we proposed that LRP/LR is implicated in AD pathogenesis. However, a relationship between these proteins has as yet not been investigated.

Table 1 | Pearson's Correlation Co-efficient for Co-localisation between LRP/LR and AD relevant proteins

AD relevant proteins	IF	Confocal
LRP/LR + APP	0.862	0.20
LRP/LR + β-secretase	0.915	0.53
LRP/LR + γ-secretase	0.938	0.57
LRP/LR + VLA6	0.583	-
LRP/LR + GFP	-	−0.51

The Pearson's Correlation co-efficient was employed to determine the degree of co-localisation between proteins of interest, where 1 indicates complete co-localisation and −1 is indicative of no co-localisation. The co-efficient was calculated for LRP/LR and AD relevant proteins APP, β- and γ-secretase respectively employing both Immunofluorescent Microscopy and Confocal Microscopy (employing fluorescently tagged proteins of interest).

Results

LRP/LR co-localises with APP, β- and γ-secretase on the cell surface. To assess whether LRP/LR and AD relevant proteins APP, β- and γ-secretase share a similar cell surface localisation, indirect immunofluorescence microscopy was employed. LRP/LR was shown to co-localise with APP (Fig. 1 and Fig. S1, a–d), β-secretase (Fig. 1 and Fig. S1, e–h), γ-secretase (Fig. 1 and Fig. S1, i–l) on the surface of non-permeabilised HEK293 (Fig. 1) and N2a cells (Fig. S1), as depicted by the yellow merged images. 2D-cytofluorograms (Fig. 1 and Fig. S1, d, h, l) reveal a yellow diagonal confirming co-localisation between the corresponding cell surface proteins. Pearson's Correlation co-efficient was employed to further confirm the observed results (Table 1). A Pearson's Correlation co-efficient of 1 is indicative of perfectly correlated proteins[31]. The obtained Pearson's correlation co-efficient between LRP/LR and the AD relevant proteins are all approximately within the 0.9 range (Table 1). An alternative laminin binding receptor, Very Late Antigen 6 (VLA6), employed as a negative control failed to co-localise with LRP/LR (Fig. 1 and Fig. S1, m–p, Table 1). The proximity of these proteins on the cell surface thereby suggests that an association/interaction between the receptor and AD relevant proteins is feasible.

LRP/LR co-localises with APP, β- and γ-secretase within the cell. To further investigate whether LRP/LR co-localises with APP, β- and

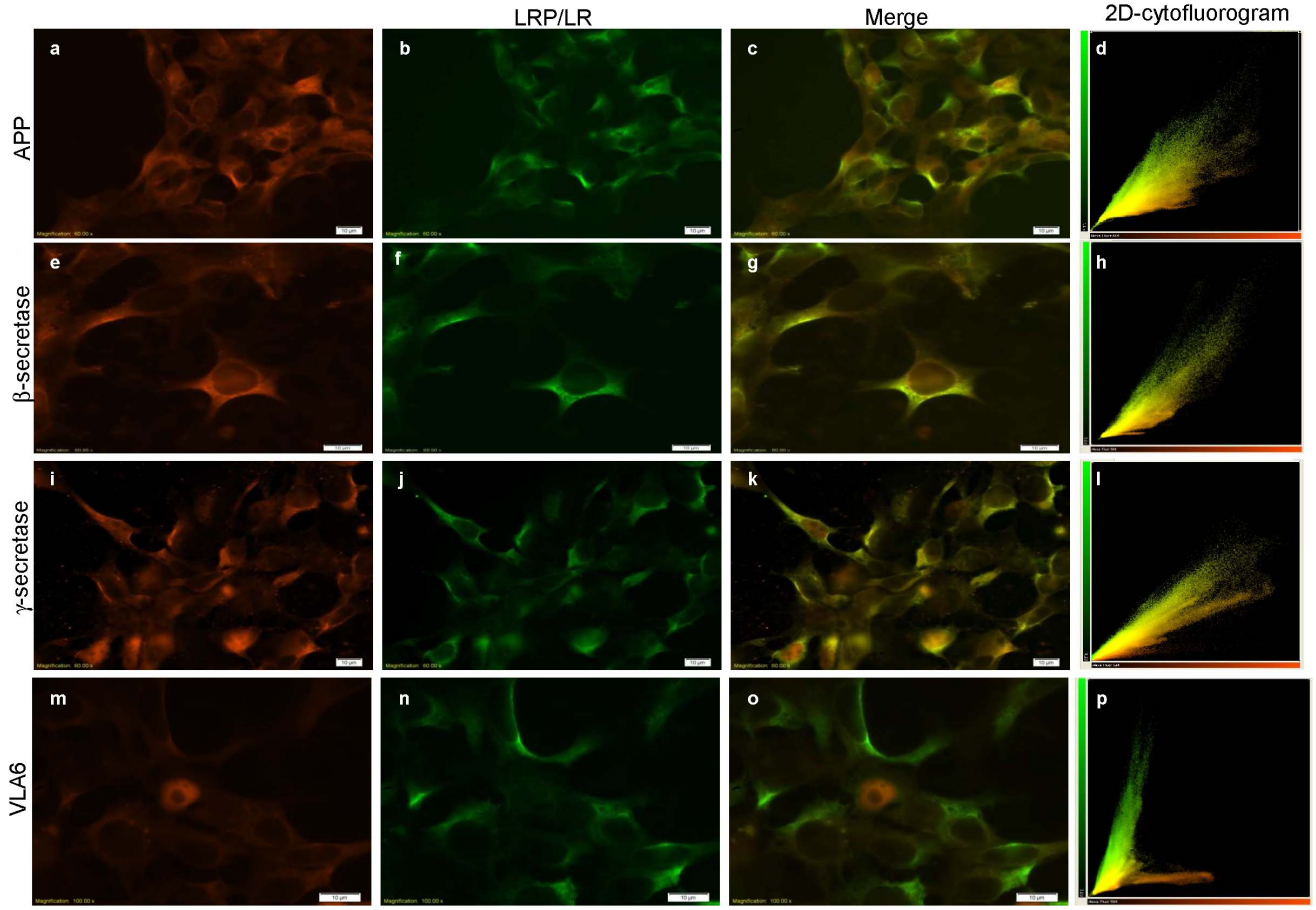


Figure 1 | Co-localisation of LRP/LR with the AD relevant proteins APP, β- and γ-secretase on the cell surace. Cell surface proteins on HEK293 cells were indirectly immunolabelled to allow for detection using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software. (a) APP, (e) β-secretase, (i) γ-secretase and (m) VLA6 were all indirectly labelled with Alexafluor 633, while an anti-human FITC conjugated antibody was used to label IgG1-iS18 bound to LRP/LR (b, f, j, n). The merged images between LRP/LR and relevant proteins are shown (c, g, k, o) and the corresponding 2D-cytofluorograms have been included to confirm the degree of co-localisation (d, h, l, p).

γ-secretase in subcellular locations other than the cell surface, HEK293 cells were transfected with plasmids encoding fluorescently tagged proteins and examined using confocal microscopy. APP-GFP, BACE1-GFP (β-secretase) and PS1-GFP (Presenilin 1 – the catalytic subunit of the γ-secretase) were respectively co-expressed with LRP-dsRed post transfection. The results obtained suggest that LRP/LR does not only co-localise with the AD relevant proteins on the cell surface but also intracellularly in the cytoplasm. From Fig. 2c, a greater degree of co-localisation is observed between APP (APP-GFP) and LRP/LR (LRP-dsRed) in the cytoplasm, and to a lesser extent in the nucleus of the HEK293 cells as shown by the intensity of the yellow stain. Fig. 2g highlights that the co-localisation between β-secretase (BACE1-GFP) and LRP/LR (LRP-dsRed) is confined to areas of the cytoplasm, but completely lacking in the nucleus. The co-localisation between γ-secretase (PS1-GFP) and LRP/LR (LRP-dsRed) reveals a similar relationship in the cytoplasm, however, there also appears to be a high degree of co-localisation on the cell membrane as emphasized by staining seen in Fig. 2k. The presence of any co-localisation between LRP/LR and γ-secretase is completely lacking in the nucleus of the cells. GFP was used as a negative control and shows very little co-localisation with LRP/LR as is evidenced by the weak yellow signal obtained in Fig. 2o. The 2D cytofluorograms (Fig. 2d, h, l) further confirm that co-localisation does occur between LRP/LR and the AD relevant proteins APP, β- and γ-secretase, as a distinct diagonal passing through quadrant 3 is seen. The 2D cytofluorogram between GFP and LRP/LR (Fig. 2P) shows a complete lack of a diagonal and does not pass through quadrant 3, therefore no co-localisation between LRP/LR and GFP is likely to exist other than by random chance (as is seen by the weak yellow signals in Fig. 2o). From Table 1, it is evident that a positive correlation exists between LRP/LR and APP, β- and γ-secretase, as the Pearson's co-efficient values are all above 0. The value seen for GFP and LRP (−0.51) is negative and indicates that there is a low level of correlation between the cellular localisation of these 2 proteins.

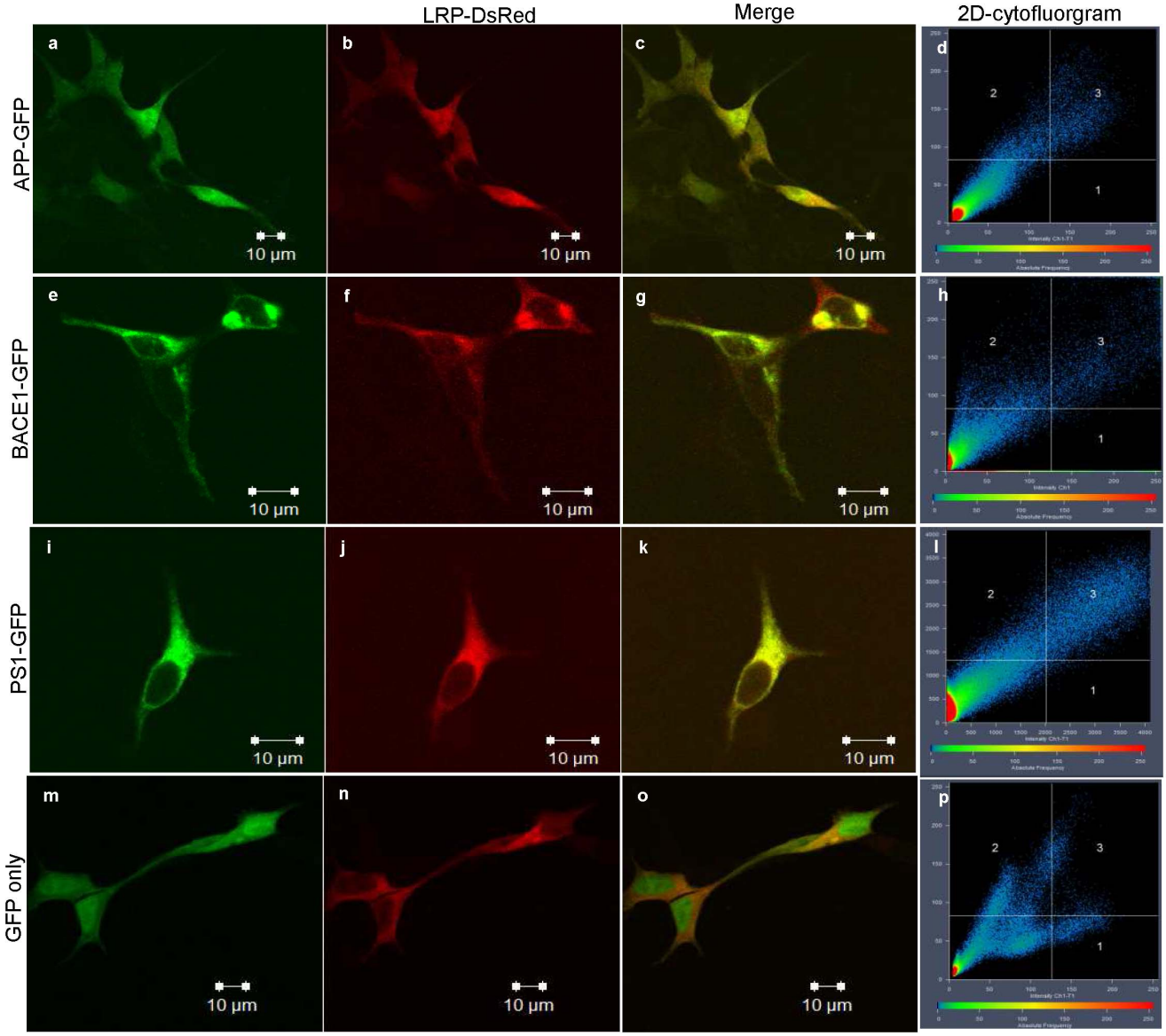


Figure 2 | Co-localisation of LRP-dsRed with APP-GFP, BACE1-GFP and PS1-GFP inside HEK293 cells. Fluorescently tagged (a) APP-GFP, (e) BACE1-GFP, (i) PS1-GFP and (m) GFP were co-expressed with LRP-dsRed (b, f, j, n) in HEK293 cells. Images were captured using the Zeiss LSM 780 confocal microscope and analysed using Zen 2010 software. The resulting merges between LRP-dsRed and APP-GFP (c), BACE1-GFP (g), PS1-GFP (k) and GFP (o) are shown and the corresponding 2D cytofluorograms for each merge have also been included (d, h, l, p) as a measure of the degree of co-localisation.

IgG1-iS18 and shRNA treatment targeting LRP/LR significantly reduces Aβ shedding. To investigate whether LRP/LR is involved in the amyloidogenic pathway and more specifically Aβ shedding into the extracellular space, cells were treated with the anti-LRP/LR specific antibody IgG1-iS18[32] and anti-cluster of differentiation (CD19) antibody IgG1-HD37[32] (negative control). Cellular incubation with IgG1-iS18 resulted in a significant reduction in Aβ concentration when compared to the no antibody control (Fig. 3a). Aβ levels were lowered by 47.6% in HEK293 cells (P = 0.0008) and 28.5% in SH-SY5Y cells (P = 0.0064) (Fig. 3a) after IgG1-iS18 treatment. To further assess the effects of IgG1-iS18 on Aβ shedding, a dose dependency assay was conducted using SH-SY5Y cells. Aβ shedding was significantly hampered by between 24% (for 25 μg/ml) and 43% (for 100 μg/ml) denoting that IgG1-iS18 impedes Aβ shedding in a dose-dependent fashion (Fig. 3b).

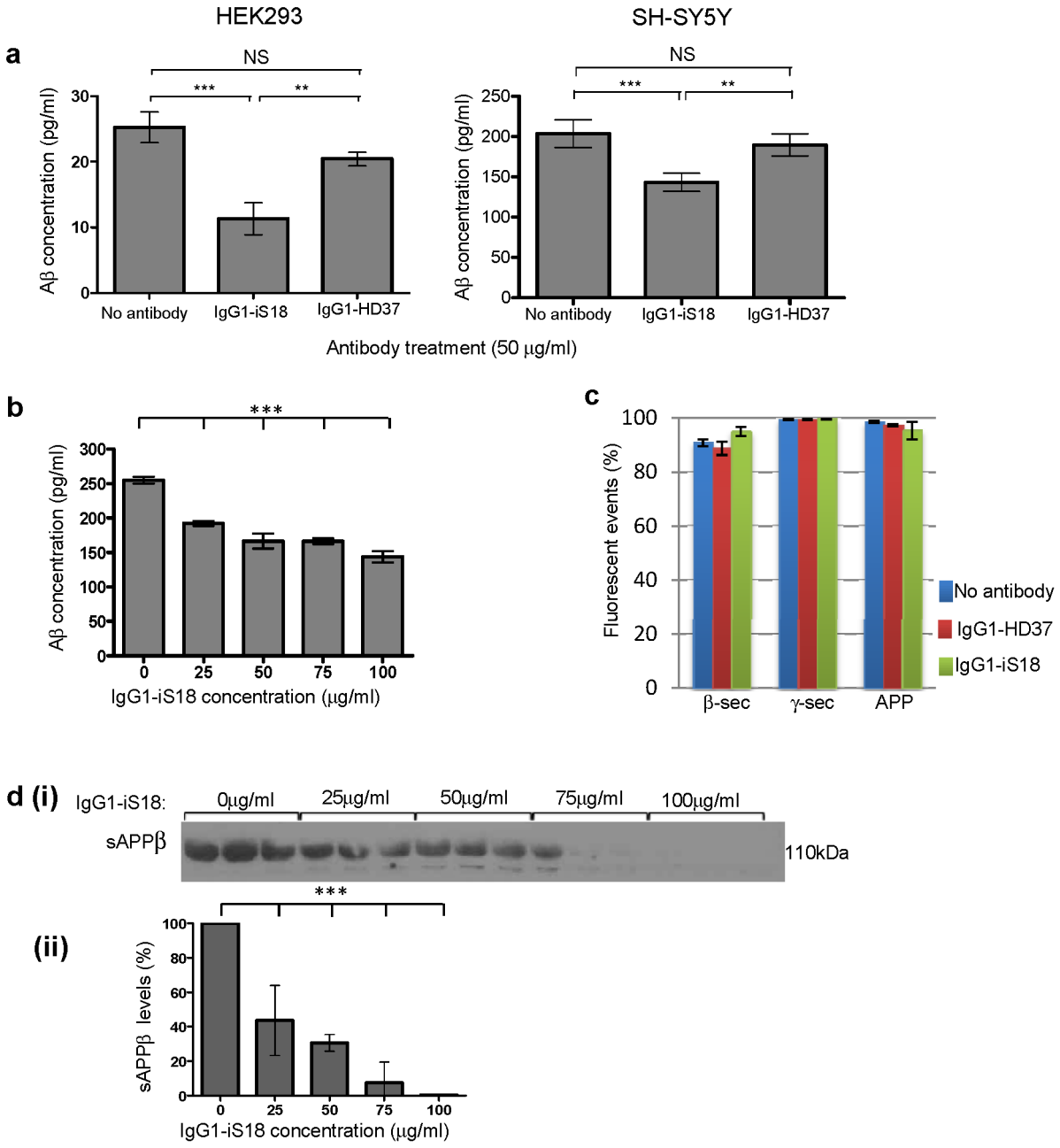


Figure 3 | Effects of IgG1-iS18. (a) Aβ levels in HEK293 and SH-SY5Y cells after treatment with IgG1-iS18 and IgG1-HD37 as detected by an Aβ ELISA after 18 hours of antibody incubation. Data shown (mean ± s.e.m) representative of three independent experiments (performed in triplicate) per cell line. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS not significant; Student's *t*-test. (b) Aβ concentrations after SH-SY5Y cells were treated with varying doses of IgG1-iS18 for 18 hours, as determined by an Aβ ELISA. Data shown (Mean ± s.d.) comparing Aβ levels of untreated cells (0 μg/ml) and IgG1-iS18 treated cells (25–100 μg/ml), ***$p < 0.0001$; n = 3; one way ANOVA. (c) Flow cytometric analysis of APP, β-secretase and γ-secretase levels on the surface of HEK293 cells post treatment with IgG1-iS18 (mean ± s.d., NS not significant, n = 3, Student's *t*-test). (d) (i) Western blot showing sAPPβ levels from cell culture medium after SH-SY5Y cells were treated with varying concentrations (0–100 μg/ml) of IgG1-iS18 for 18 hours. Western blot band intensities from three independent experiments were quantified using Quantity One 4.6 software. Gels have been cropped for clarity and conciseness purposes and have been run under the same experimental conditions. (d) (ii) Obtained band intensities were subsequently used to determine the percentage downregulation of sAPPβ. Data shown (mean ± s.d); ***$p < 0.0001$; One way ANOVA.

Owing to the ability of IgG1-iS18 to decrease Aβ shedding, it may be proposed that LRP/LR mediates this process. To further confirm this role in the amyloidogenic pathway, RNA interference technology, specifically short hairpin RNA (shRNA), was employed to downregulate LRP/LR expression. shRNA1 and shRNA7 resulted in a significant 42.85% and 16.42% decrease in LRP/LR expression levels, respectively, compared to the scrambled control (shRNAscr) (Fig. 4a). This downregulation correlated to a significant 16.88% and 11.95% decrease in Aβ shedding in HEK293 cells (for shRNA1 and shRNA7 respectively) (Fig. 4b). No significant difference was observed between mock-transfected and shRNAscr control transfected HEK293 cells (Fig. S3).

IgG1-iS18 and LRP/LR shRNA treatments do not alter cell surface expression of APP, β- and γ-secretase. To investigate whether LRP/LR influences the amyloidogenic pathway through altering cell surface protein expression levels of the aforementioned AD relevant proteins, flow cytometric analysis of the cell surface levels of APP, β-secretase and γ-secretase was performed post-antibody (IgG1-iS18 and IgG1-HD37) treatment in HEK293 cells (Fig. 3C and Fig. S4a). Both antibody treatment and downregulation of LRP/LR by shRNA1 and 7 (Fig. 4c, Fig. S4 b) did not significantly alter HEK293 cell surface expression levels of the APP, β- and γ-secretase in comparison to controls.

sAPPβ levels are affected by LRP blockade and downregulation. In an attempt to elucidate the mechanism whereby LRP/LR influences the amyloidogenic pathway, sAPPβ levels were assessed post-antibody (Fig. 3d(i)) and shRNA treatment (Fig. 4d (i)). Upon a dose dependent administration of IgG1-iS18 to SH-SY5Y cells, a

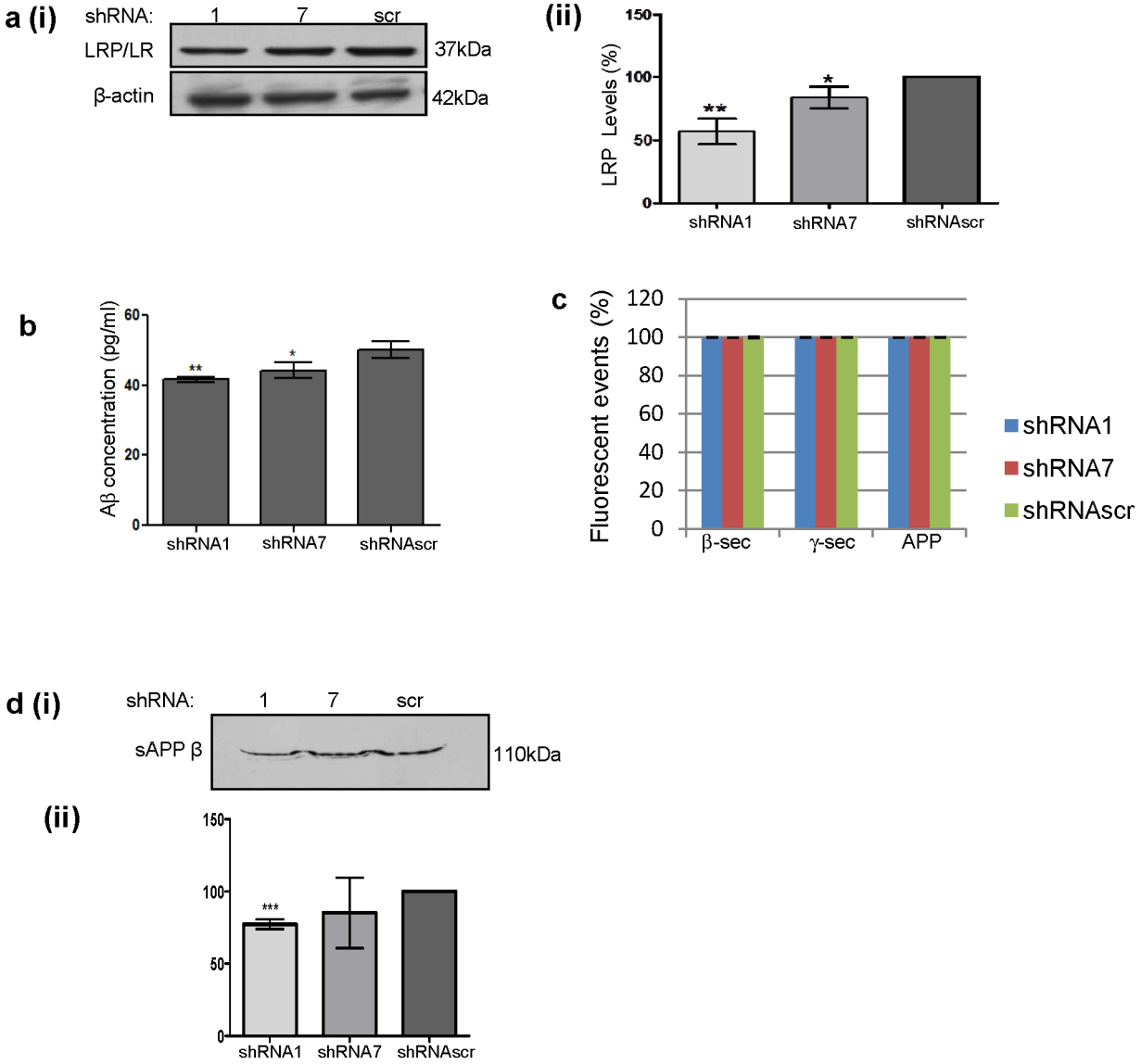


Figure 4 | Effects of LRP/LR downregulation by shRNA. (a) HEK293 cells were transfected with LRP/LR-specific shRNA1 and shRNA7 (as well as a scrambled control, shRNAscr). 72 hours post-transfection, cells were lysed and LRP/LR levels assessed by Western blotting. β-actin was used as a loading control. Western blot band intensities from three independent experiments were quantified using Quantity One 4.6 Software. (b) The Aβ concentration of the cell culture medium of shRNA-transfected HEK293 cells was analysed using an Aβ ELISA. Data shown (Mean ± s.d.) comparing Aβ levels of shRNA1 and shRNA7 to shRNAscr, *$p < 0.05$, **$p < 0.01$; $n = 3$; Student's *t*-test. (c) Flow cytometric analysis of APP, β-secretase and γ-secretase levels on the surface of shRNA-transfected HEK293 cells. Data shown (Mean ± s.d.); $n = 3$; Student's *t*-test. (d)(i) sAPPβ levels in shRNA-transfected HEK293 cells were analysed by Western blotting. Gels have been cropped for clarity and conciseness purposes and have been run under the same experimental conditions. (d)(ii) Band intensities were extracted as mentioned previously (Fig. 3 d) and used to calculate the percentage of downregulation of sAPPβ levels.

significant reduction in sAPPβ levels was observed across all antibody concentrations (Fig. 3d(ii)). Similar results were obtained for shRNA1 mediated LRP/LR downregulated HEK293 cells (Fig. 4d).

LRP/LR interacts with β-secretase. A FLAG® Immunoprecipitation assay was conducted using a FLAG-tagged variant of LRP (LRP::FLAG) which had the ability to bind to anti-FLAG M2 beads. Any protein interacting with LRP::FLAG would thus remain immobilised during subsequent washing steps and would be present in the eluate of the immunoprecipitation assay. A FLAG® Immunoprecipitation assay was thus performed to detect whether LRP/LR shows any interaction with β-secretase in order to further validate the co-localisation results observed between LRP/LR and β-secretase, as well as effects seen on sAPPβ shedding upon IgG1-iS18 incubation. From Fig. 5, it is evident that a distinct band is present in the eluate of the FLAG® Immunoprecipitation assay (Fig. 5, lane 4) corresponding in size to the band detected in the BACE1-GFP expressing cell lysate control lane (Fig. 5, lane 3). This suggests that an interaction does exist between LRP/LR and β-secretase, as only proteins that interact with LRP would be present in the eluate. CAT (chloramphenicol acetyl transferase) transfected cell lysates were used as a negative control. CAT was not present in the immunoprecipitation assay eluate, thus showing no interaction with LRP (Fig. S5).

Discussion

The co-localisation observed between LRP/LR and the relevant AD proteins (APP, β and γ-secretase) on both HEK293 and N2a cells, as assessed by immunofluorescence microscopy, indicates that a spatial overlap occurs between the fluorescently immunolabeled proteins (Fig. 1 and Fig. S1). Although the close cellular proximity of the proteins on the cell surface is not explicitly indicative of an interaction, it does imply that an association between these proteins and LRP/LR is possible.

Confocal microscopy was further utilized to examine whether LRP/LR co-localised with APP, β- and γ-secretase in sub-cellular locations other than the cell surface. From Fig. 2, it is evident that LRP/LR also shows a high degree of co-localisation with the AD relevant proteins within the cytoplasm. LRP/LR is known to be present in the cytoplasm[33] and lipid raft regions[34] of the cell membrane from where it was reported to co-localise with PrP^c in the early endosomes upon LRP/LR-dependent PrP^c endocytosis[29]. With regard to APP, it is proposed that this protein is internalized from the lipid raft region of the plasma membrane into early endosomes via either clathrin[35] or raft-mediated endocytosis[36]. β-secretase is also located in the lipid raft regions where it too is endocytosed into early endosomes[37] via the GTPase Arf6[38]. All 4 subunits of the γ-secretase have been found to be present in lipid raft regions of not only the plasma membrane[39], but of the Golgi and endosomal membranes[40] too. The presence of APP, β- and γ-secretase in the endosomes led to the finding that APP is preferentially cleaved by β-secretase at this site due to the lower pH within endosomes[8]. However, the three AD related proteins are also found in other subcellular locations, such as the Endopasmic reticulum, Golgi and *trans*-Golgi network; and recent studies have shown that APP cleavage can also occur at these sites[41,42]. This suggests a widespread distribution of APP and its cleavage proteins within various cellular structures and various internalization routes for each of the proteins involved. From our results, the intracellular distribution of APP, β- and γ-secretase is seen to be fairly widespread through the cytoplasm (Fig. 2a, e and i respectively) – due to the aforementioned cellular organelles in which they are found. From the literature it is evident that LRP/LR and the AD relevant proteins exist in similar cellular locations, and from the results obtained in Fig. 2 (b, f and j), LRP/LR is indeed found to be present in very similar intracellular regions (Fig. 2 c, g and k) as these proteins. This suggests that LRP/LR could be interacting with APP, β- and γ-secretase within the cellular structures in which they are found, or alternatively, could potentially be involved in the regulation of APP processing.

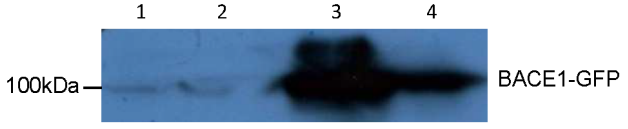


Figure 5 | LRP::FLAG Immunoprecipitation Assay Analysis. HEK293 cell lysates containing LRP::FLAG and either BACE1-GFP or CAT (Fig. S5) were subjected to Pull Down Assay analysis using Anti-FLAG M2 beads. After subsequent wash steps, eluted fractions were analysed using Immunoblotting. Lane 1 contained non-transfected (NT) cell lysate, lane 2 contained GFP transfected cell lysate, lane 3 contained BACE1-GFP transfected cell lysate and lane 4 contained the eluate from the FLAG® Immunoprecipitation assay between BACE1-GFP and LRP::FLAG. Gels have been cropped for clarity and conciseness purposes and have been run under the same experimental conditions.

LRP/LR blockage by IgG1-iS18 was seen to effectively impede Aβ shedding. This was further affirmed by shRNA mediated downregulation of the receptor. These results taken together suggest that LRP/LR plays a pivotal role in the amyloidogenic processing of APP.

Interestingly, LRP blockage did not result in modulation of cell surface levels of AD relevant proteins, thereby inferring that the influence of LRP/LR may rather be as a result of protein interactions.

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 LRP/LR impedes β-secretase activity. Similar results were seen when LRP/LR was downregulated, thus further corroborating the possibility of an interaction between these two proteins. These results implicate LRP/LR in the amyloidogenic process, specifically via promoting β-secretase activity. Since LRP/LR and β-secretase co-localize and sAPPβ shedding is significantly impeded by IgG1-iS18 and shRNA treatment, we proposed that an interaction between β-secretase and LRP/LR exists. This was verified upon performing a FLAG® Immunoprecipitation assay utilizing cell lysates of cells expressing LRP::FLAG and BACE1-GFP (Fig. 5). BACE1-GFP was detected in the eluate of the immunoprecipitation, and since only proteins binding to the LRP::FLAG protein would be present in this fraction, this strongly suggests that an interaction between LRP/LR and β-secretase does exist. However, as crude cell lysates expressing LRP::FLAG and BACE1-GFP were utilised for the assay, it is also possible that any interaction that exists between these two proteins may be an indirect one, mediated by another protein present in the cell lysate.

Efforts to develop effective therapies for AD have to take into consideration that targeting the secretases may cause off target effects, such as a disruption of BACE1 processing of CHL1 which is required for axonal guidance[43]. Here we imply that the role of β-secretase in the amyloidogenic pathway is augmented by LRP/LR, as the blockage of LRP/LR reduces the shedding of both sAPPβ and Aβ, and LRP/LR is seen to interact with β-secretase. The effects of hindering the interaction between β-secretase and LRP/LR would thus have to be examined in detail in future studies, to test for the effects it may have on the other physiological roles in which β-secretase is involved. Cell viability assays, however, have been performed using IgG1-iS18 on HEK293 cells, and no significant reduction in cell viability was observed[44], thus suggesting whatever interaction the antibody may be blocking between β-secretase and LRP/LR is not detrimental to the cells.

The result of the FLAG® Immunoprecipitation assay further confirmed the initial findings suggested by the co-localisation (Fig. 1) and confocal data (Fig. 2) that an interaction is likely to occur between LRP/LR and β-secretase both due to their proximity on

the cell surface and within the cell. Thus it further corroborates the possibility that interactions may also occur between LRP/LR and APP and/or γ-secretase.

In conclusion, we have identified a novel role for LRP/LR in AD and more specifically in enhancing β-secretase cleavage of APP. Blockage and downregulation of LRP/LR resulted in a significant reduction in Aβ levels suggesting that anti-LRP/LR specific antibodies and shRNAs could be used as possible alternative therapeutic tools for AD treatment.

Methods

Tissue culture. HEK293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin (P/S) solution. N2a cells were grown in Optimem with 10% FCS and 1% P/S. SH-SY5Y cells were cultured in 1 : 1 EMEM:F12 media supplemented with 15% FCS, 1% Non-Essential Amino Acids, 1% L-Glutamine and 1% P/S. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Immunofluorescence microscopy. HEK239 and N2a cells were seeded onto sterile microscope coverslips and incubated for 24 hours (37°C, 5% CO_2) allowing the cells to reach 50–70% confluency. The cells were fixed with 4% Paraformaldehyde (10 minutes, room temperature). The coverslips were rinsed 3 times in PBS and blocked in 0.5%PBS-BSA solution for 5–10 minutes. Following an additional wash in PBS, the coverslips were placed with the cells facing upwards on a microscope slide. 100 µl of primary antibody solution (diluted in 0.5%PBS-BSA) containing 1 : 150 IgG1-iS18 and either anti-APP (rabbit polyclonal IgG) (Abcam), anti-BACE (M-83) (rabbit polyclonal IgG) (Santa Cruz Biotechnology), anti-PEN-2 (FL-101) (rabbit polyclonal IgG) (Santa Cruz Biotechnology) or anti-very late antigen-6 (VLA6) CD49-f (rabbit monoclonal IgG) (Immunotech) was added to the cells. These slides were then incubated overnight at 4°C in moist containers. Cover slips were then rinsed 3 times in 0.5% PBS-BSA and placed on clean slides. The cells were treated with 100 µl of a secondary antibody solution containing goat anti-human FITC (Cell Lab) (1 : 350–1 : 400) (specific for IgG1-iS18) and Alexa Fluor® 633 goat anti-rabbit IgG (Invitrogen) (specific for the anti-APP, anti-BACE, anti PEN-2 or anti-VLA6 primary antibodies). After an hour's incubation in the dark, the coverslips were washed twice in 0.5% PBS-BSA and once in PBS and were subsequently mounted onto clean microscope slides using 75 µl Fluoromount (Sigma Aldrich). These slides were incubated at room temperature in the dark for 1–2 hours to allow the mounting medium to set.

Images were acquired at room temperature with 60× magnification using the Olympus IX71 Immunofluorescence Microscope and Olympus XM10 greyscale camera. analySIS Research Image Processing Software was used to capture the images and they were subsequently analysed (and 2D cytofluorograms were constructed) using CellSens Dimension Software.

Confocal microscopy. HEK 293 cells were transfected with plasmids encoding for LRP-dsRed and APP-GFP, BACE1-GFP or PS1-GFP respectively. 24 hours post transfection, coverslips were washed with PBS 3 times and then fixed with 4% Paraformaldehyde for 15 minutes. Coverslips were subsequently washed in PBS and mounted cell side down onto clean microscope slides using 75 µl Fluoromount (Sigma Aldrich). These slides were incubated at room temperature for 1–2 hours in the dark to allow the mounting medium to set.

Slides were viewed using the Zeiss LSM 780 confocal microscope and captured using the AxioCam MRm camera. Images were subsequently analysed using Zen 2010 imaging software.

Antibody treatment. HEK293 cells were grown to 50–70% confluency. The tissue culture medium was aspirated and replaced with media containing 50 µg/ml IgG1-iS18[32], 50 µg/ml IgG1-HD37[32], or no antibody. Each of the respective treatments were performed in triplicate. The cells were subsequently incubated at 37°C, 5%CO_2 for 18 hours.

LRP/LR target sequences and structure of shRNA1 and shRNA7. The complete shRNA expression cassettes were designed with the guide strand on the 3′arm, a poly T termination signal, and to include a full H1 RNA polymerase III promoter sequence. To prepare the shRNA cassettes, the H1 RNA Pol III promoter was used as a template in a nested PCR, whereby the sequences corresponding to the shRNAs were incorporated into two reverse primers (one for the primary PCR and one for the secondary PCR). The same forward primer, which is complementary to the start of the H1 promoter, was used in both. The PCR products coding for the shRNA expression constructs were sub-cloned into the pTZ57R/T vector (Fermentas). A scrambled shRNA (shRNAscr) that does not target any gene product was used as a negative control. See figure S2 for LRP/LR target sequence and shRNA 1 and shRNA7 structure.

Cell transfection. The construction of pCIneo-moLRP::FLAG[45] and pLRP-dsRed[46] have been described previously, while pEGFPN1-APP770[37] and pEGFPN1-BACE1[47] were generous gifts from Dr. Bradley T Hyman. pEGFP-PS1[48] was a kind gift from Dr. Oskana Berezovska. pEGFP-N1 (Clonetech) and pCDNA3/CAT (Invitrogen) were used as control plasmids. For confocal microscopy, HEK 293 cells were seeded onto coverslips in 6 well plates and incubated overnight. The following day, when cells were 30–50% confluent, calcium phosphate transfection was carried out using 86 µl of 1 × HBS, 5.1 µg of total plasmid DNA (i.e. 2.55 µg of each plasmid was used in co-transfections) and 5.1 µl of 2.5 M $CaCl_2$. Cells were incubated for 24 hours and then used for confocal microscopy. HEK293 cells were transfected with LRP/LR shRNA 1 and 7 according to the manufacturer's instructions, using *Trans*IT®-LT1 Transfection Reagent (Mirus). Transfected cells were incubated for 72 hours prior to analysis. For FLAG® Immunoprecipitation assays, HEK293 cells were seeded into 60 mm tissue culture plates and grown to 30–50% confluency overnight. Calcium phosphate transfection was carried out using 186 µl 1 × HBS, 11 µg of either pCIneo-moLRP::FLAG, pEGFPN1-BACE1 or pCDNA3/CAT and 11 µl 2.5 M $CaCl_2$. Cells were incubated for 72 hours prior to analysis.

Amyloid beta enzyme-linked immunosorbent assay (ELISA). The amyloid beta (Aβ) assay was performed using the Human Amyloid β (1 − x) Assay kit (Immuno-Biological Laboratories Co.,Ltd) – a solid phase ELISA. This assay was performed as per manufacturer's instructions using the tissue culture media after antibody and shRNA treatment (see above).

Flow cytometry. Cell lines were subjected to flow cytometric analysis following treatment with IgG1-iS18 or shRNA1 and 7. Cells were fixed using 4% paraformaldehyde (10 minutes,4°C), centrifuged at 1500 g (10 minutes, 4°C) and resuspended in 1 ml IsoFlow™ EPICS™ Sheath Fluid (Beckman Coulter). Half of the volume of each sample was treated with 30 ug/ml of either anti-APP (rabbit polyclonal IgG) (Abcam), anti-BACE (M-83) (rabbit polyclonal IgG) (Santa Cruz Biotechnology) or anti-PEN-2 (FL-101) (rabbit polyclonal IgG) (Santa Cruz Biotechnology) while remaining sample volume was incubated in IsoFlow™ EPICS™ Sheath Fluid (Beckman Coulter). Samples were incubated for 1 hour at room temperature and subsequently washed three times in IsoFlow™ EPICS™ Sheath Fluid (Beckman Coulter) (1700 g, 4°C). All samples were treated with 20 ug/ml of the corresponding goat anti-rabbit FITC coupled secondary antibody (Cell Lab) and incubated for 1 hour at room temperature. The samples were washed a further three times (as above) and the cell suspensions were transferred to flow cytometry tubes. The samples were analysed using Coulter EPICS® XL-MCL (for antibody treated samples) or the BD Accuri C6 (for shRNA treated samples) and 10 000–50 000 events recorded.

Western blotting. LRP/LR levels were determined by immunoblotting using IgG1-iS18 (1 : 10 000) and goat anti-human horseradish peroxidise (HRP) (1 : 10 000) (Cell Lab). sAPPβ levels were assessed by immunoblotting using sAPPβ-Wild type Rabbit IgG (1 : 1000) (Immuno-Biological Laboratories Co., Ltd) and goat anti-rabbit HRP (1 : 10 000) (Cell Lab). FLAG® Immunoprecipitation assay eluate samples were analysed using either anti-FLAG® (mouse monoclonal IgG) (1 : 5000) (Sigma-Aldrich), anti-BACE1 (rabbit polyclonal IgG fraction) (1 : 3000) (Abcam) or anti-Chloramphenicol Acetyl Transferase (rabbit IgG fraction) (1 : 5000)(Sigma-Aldrich). Anti-mouse HRP (1 : 5000) (Sigma-Aldrich) or anti-rabbit HRP (1 : 5000) (Sigma-Aldrich) were used as secondary antibodies. Immunodetection was carried out using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and X-ray film.

FLAG® immunoprecipitation assay. HEK293 cells were lysed 72 hours post-transfection with pCIneo-moLRP::FLAG, pEGFPN1-BACE1 or pCDNA3/CAT. Cell lysates expressing LRP::FLAG were then mixed with those containing either BACE1-GFP or CAT. These cell lysates were then subjected to a FLAG® Immunoprecipitation Kit (Sigma-Aldrich) analysis according to the manufacturer's instructions. Eluted samples were analysed via Immunoblotting.

1. Mount, C. & Downton, C. Alzheimer disease: progress or profit? *Nat Med* **12**, 780–784, http://dx.doi.org/nm0706-780 (2006).
2. Maloney, B. & Lahiri, D. K. The Alzheimer's amyloid beta-peptide (Abeta) binds a specific DNA Abeta-interacting domain (AbetaID) in the APP, BACE1, and APOE promoters in a sequence-specific manner: characterizing a new regulatory motif. *Gene* **488**, 1–12, http://dx.doi.org/S0378-1119(11)00262-9 (2011).
3. Holtzman, D. M., Herz, J. & Bu, G. Apolipoprotein e and apolipoprotein e receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb Perspect Med* **2**, a006312, http://dx.doi.org/10.1101/cshperspect.a006312 (2012).
4. Avila, J. Tau phosphorylation and aggregation in Alzheimer's disease pathology. *FEBS Lett* **580**, 2922–2927, http://dx.doi.org/S0014-5793(06)00270-5 (2006).
5. Gonsalves, D., Jovanovic, K., Da Costa Dias, B. & Weiss, S. F. Global Alzheimer Research Summit: Basic and clinical research: Present and future Alzheimer research. *Prion* **6**, http://dx.doi.org/18854 (2012).
6. Busciglio, J., Lorenzo, A., Yeh, J. & Yankner, B. A. beta-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. *Neuron* **14**, 879–888, http://dx.doi.org/0896-6273(95)90232-5 (1995).
7. Ardini, E. *et al*. Co-regulation and physical association of the 67-kDa monomeric laminin receptor and the alpha6beta4 integrin. *J Biol Chem* **272**, 2342–2345 (1997).
8. Vassar, R. *et al*. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735–741, http://dx.doi.org/7936 (1999).

9. Edbauer, D. *et al.* Reconstitution of gamma-secretase activity. *Nat Cell Biol* **5**, 486–488, http://dx.doi.org/10.1038/ncb960 (2003).
10. Verdier, Y. & Penke, B. Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. *Curr Protein Pept Sci* **5**, 19–31 (2004).
11. Da Costa Dias, B., Jovanovic, K., Gonsalves, D. & Weiss, S. F. Structural and mechanistic commonalities of amyloid-beta and the prion protein. *Prion* **5**, 126–137, http://dx.doi.org/17025 (2011).
12. Sepulveda, F. J., Parodi, J., Peoples, R. W., Opazo, C. & Aguayo, L. G. Synaptotoxicity of Alzheimer beta amyloid can be explained by its membrane perforating property. *PLoS One* **5**, e11820, http://dx.doi.org/10.1371/journal.pone.0011820 (2010).
13. Parkin, E. T. *et al.* Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U S A* **104**, 11062–11067, http://dx.doi.org/0609621104 (2007).
14. Lauren, J., Gimbel, D. A., Nygaard, H. B., Gilbert, J. W. & Strittmatter, S. M. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* **457**, 1128–1132, http://dx.doi.org/nature07761 (2009).
15. Kudo, W. *et al.* Cellular prion protein is essential for oligomeric amyloid-beta-induced neuronal cell death. *Hum Mol Genet* **21**, 1138–1144, http://dx.doi.org/ddr542 (2012).
16. Resenberger, U. K., Winklhofer, K. F. & Tatzelt, J. Cellular Prion Protein Mediates Toxic Signaling of Amyloid Beta. *Neurodegener Dis*, http://dx.doi.org/000332596 (2011).
17. Um, J. W. *et al.* Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat Neurosci* **15**, 1227–1235, http://dx.doi.org/nn.3178 (2012).
18. Castillo, G. M. *et al.* Laminin inhibition of beta-amyloid protein (Abeta) fibrillogenesis and identification of an Abeta binding site localized to the globular domain repeats on the laminin a chain. *J Neurosci Res* **62**, 451–462, http://dx.doi.org/10.1002/1097-4547(20001101)62:3451::AID-JNR15>3.0.CO;2-F (2000).
19. Mbazima, V., Da Costa Dias, B., Omar, A., Jovanovic, K. & Weiss, S. Interactions between PrPc and other ligands with the 37-kDa/67-kDa laminin receptor. *Frontiers in Bioscience* **15**, 1150–1163 (2010).
20. Moodley, K. & Weiss, S. F. Downregulation of the non-integrin laminin receptor reduces cellular viability by inducing apoptosis in lung and cervical cancer cells. *PLoS One* **8**, e57409, http://dx.doi.org/10.1371/journal.pone.0057409 PONE-D-12-36482 (2013).
21. Omar, A. *et al.* Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. *Expert Opin Ther Pat* **21**, 35–53, http://dx.doi.org/10.1517/13543776.2011.539203 (2011).
22. Khusal, R. *et al.* In vitro inhibition of angiogenesis by antibodies directed against the 37 kDa/67 kDa laminin receptor. *PLoS One* **8**, e58888, http://dx.doi.org/10.1371/journal.pone.0058888 (2013).
23. Khumalo, T. *et al.* Adhesion and Invasion of Breast and Oesophageal Cancer Cells Are Impeded by Anti-LRP/LR-Specific Antibody IgG1-iS18. *PLoS One* **8**, e66297, http://dx.doi.org/10.1371/journal.pone.0066297 PONE-D-13-07741 (2013).
24. Omar, A., Reusch, U., Knackmuss, S., Little, M. & Weiss, S. F. Anti-LRP/LR-specific antibody IgG1-iS18 significantly reduces adhesion and invasion of metastatic lung, cervix, colon and prostate cancer cells. *J Mol Biol* **419**, 102–109, http://dx.doi.org/S0022-2836(12)00207-0 (2012).
25. Gauczynski, S. *et al.* The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J* **20**, 5863–5875, http://dx.doi.org/10.1093/emboj/20.21.5863 (2001).
26. Gauczynski, S. *et al.* The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis* **194**, 702–709, http://dx.doi.org/JID36299 (2006).
27. Rieger, R., Edenhofer, F., Lasmezas, C. I. & Weiss, S. The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat Med* **3**, 1383–1388 (1997).
28. Hundt, C. *et al.* Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *EMBO J* **20**, 5876–5886, http://dx.doi.org/10.1093/emboj/20.21.5876 (2001).
29. Morel, E. *et al.* Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. *Am J Pathol* **167**, 1033–1042, http://dx.doi.org/167/4/1033 [pii] (2005).
30. Leucht, C. *et al.* The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. *EMBO Rep* **4**, 290–295, http://dx.doi.org/10.1038/sj.embor.embor768 (2003).
31. Dunn, K. W., Kamocka, M. M. & McDonald, J. H. A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol* **300**, C723–742, http://dx.doi.org/ajpcell.00462.2010 (2011).
32. Zuber, C. *et al.* Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *J Mol Biol* **378**, 530–539, http://dx.doi.org/S0022-2836(08)00164-2 (2008).
33. Romanov, V., Sobel, M. E., pinto da Silva, P., Menard, S. & Castronovo, V. Cell localization and redistribution of the 67 kD laminin receptor and alpha 6 beta 1 integrin subunits in response to laminin stimulation: an immunogold electron microscopy study. *Cell Adhes Commun* **2**, 201–209 (1994).
34. Patra, S. K., Rizzi, F., Silva, A., Rugina, D. O. & Bettuzzi, S. Molecular targets of (−)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts. *J Physiol Pharmacol* **59 Suppl 9**, 217–235 (2008).
35. Schneider, A. *et al.* Flotillin-dependent clustering of the amyloid precursor protein regulates its endocytosis and amyloidogenic processing in neurons. *J Neurosci* **28**, 2874–2882, http://dx.doi.org/28/11/2874 (2008).
36. Ehehalt, R., Keller, P., Haass, C., Thiele, C. & Simons, K. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. *J Cell Biol* **160**, 113–123, http://dx.doi.org/10.1083/jcb.200207113 (2003).
37. Kinoshita, A. *et al.* Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *J Cell Sci* **116**, 3339–3346, http://dx.doi.org/10.1242/jcs.00643 (2003).
38. Sannerud, R. *et al.* ADP ribosylation factor 6 (ARF6) controls amyloid precursor protein (APP) processing by mediating the endosomal sorting of BACE1. *Proc Natl Acad Sci U S A* **108**, E559–568, http://dx.doi.org/1100745108 (2011).
39. Chyung, J. H., Raper, D. M. & Selkoe, D. J. Gamma-secretase exists on the plasma membrane as an intact complex that accepts substrates and effects intramembrane cleavage. *J Biol Chem* **280**, 4383–4392, http://dx.doi.org/M409272200 (2005).
40. Vetrivel, K. S. *et al.* Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. *J Biol Chem* **279**, 44945–44954, http://dx.doi.org/10.1074/jbc.M407986200 (2004).
41. Griffiths, H. H. *et al.* Prion protein interacts with BACE1 protein and differentially regulates its activity toward wild type and Swedish mutant amyloid precursor protein. *J Biol Chem* **286**, 33489–33500, doi:M111.278556 (2011).
42. Pasternak, S. H., Callahan, J. W. & Mahuran, D. J. The role of the endosomal/lysosomal system in amyloid-beta production and the pathophysiology of Alzheimer's disease: reexamining the spatial paradox from a lysosomal perspective. *J Alzheimers Dis* **6**, 53–65 (2004).
43. Hitt, B. *et al.* beta-Site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1)-deficient mice exhibit a close homolog of L1 (CHL1) loss-of-function phenotype involving axon guidance defects. *J Biol Chem* **287**, 38408–38425, http://dx.doi.org/M112.415505 (2012).
44. Da Costa Dias, B. *et al.* Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from Ab42 induced cytotoxicity. *Sci. Rep.* **3**, 2702; http://dx.doi.org/10.1038/srep02702 (2013).
45. Vana, K. & Weiss, S. A trans-dominant negative 37 kDa/67 kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells. *J Mol Biol* **358**, 57–66, http://dx.doi.org/S0022-2836(06)00178-1 (2006).
46. Nikles, D. *et al.* Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins. *Biochim Biophys Acta* **1782**, 335–340, http://dx.doi.org/S0925-4439(08)00054-9 (2008).
47. von Arnim, C. A. *et al.* Demonstration of BACE (beta-secretase) phosphorylation and its interaction with GGA1 in cells by fluorescence-lifetime imaging microscopy. *J Cell Sci* **117**, 5437–5445, http://dx.doi.org/jcs.01422 (2004).
48. Uemura, K. *et al.* Allosteric modulation of PS1/gamma-secretase conformation correlates with amyloid beta(42/40) ratio. *PLoS One* **4**, e7893, http://dx.doi.org/10.1371/journal.pone.0007893 (2009).

Acknowledgements

This work is based upon research supported by the National Research Foundation (NRF), the Republic of South Africa (RSA). Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s), and therefore, the National Research Foundation does not accept any liability in this regard thereto. We thank Dr. Bradley T. Hyman (Harvard Medical School, Massachusetts, USA) for his generous gift of pEGFPN1-APP770 and pEGFPN1-BACE1. We also thank Dr. Oksana Berezovska (Massachusetts General Hospital, Massachusetts, USA) for his kind gift of pEGFP-PS1.

Author contributions

S.F.T.W. conceived and directed the project. K.J. and D.G. performed experiments. U.R., S.K., M.L. produced IgG1-iS18 and IgG1-HD37 antibodies. M.W. designed and assisted in production of shRNA; D.G. and K.M. produced the shRNA. C.P. assisted with both immunofluorescence and confocal micrsocopy. K.J., D.G., B.D.C.D. wrote manuscript and S.F.T.W. edited the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Jovanovic, K. *et al.* Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease. *Sci. Rep.* **3**, 2699; DOI:10.1038/srep02699 (2013).

Supplementary Information

*Correspondence and requests for materials should be referred to Stefan F.T. Weiss at Stefan.weiss@wits.ac.za

Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease

Katarina Jovanovic[1], Danielle Gonsalves[1], Bianca Da Costa Dias[1], Kiashanee Moodley[1], Uwe Reusch[2], Stefan Knackmuss[2], Clement Penny[3], Marc S. Weinberg[4], Melvyn Little[2] & Stefan F.T. Weiss[1]*

[1]School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa, [2]Affimed Therapeutics AG, Technologiepark, Im Neuenheimer Feld 582, 69120 Heidelberg, Germany, [3]Department of Internal Medicine, University of the Witwatersrand, 7 York Rd, Johannesburg, 2193 Parktown, South Africa, [4]Antiviral Gene Therapy Research Unit (AGTRU), Department of Molecular Medicine & Haematology, School of Pathology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa.

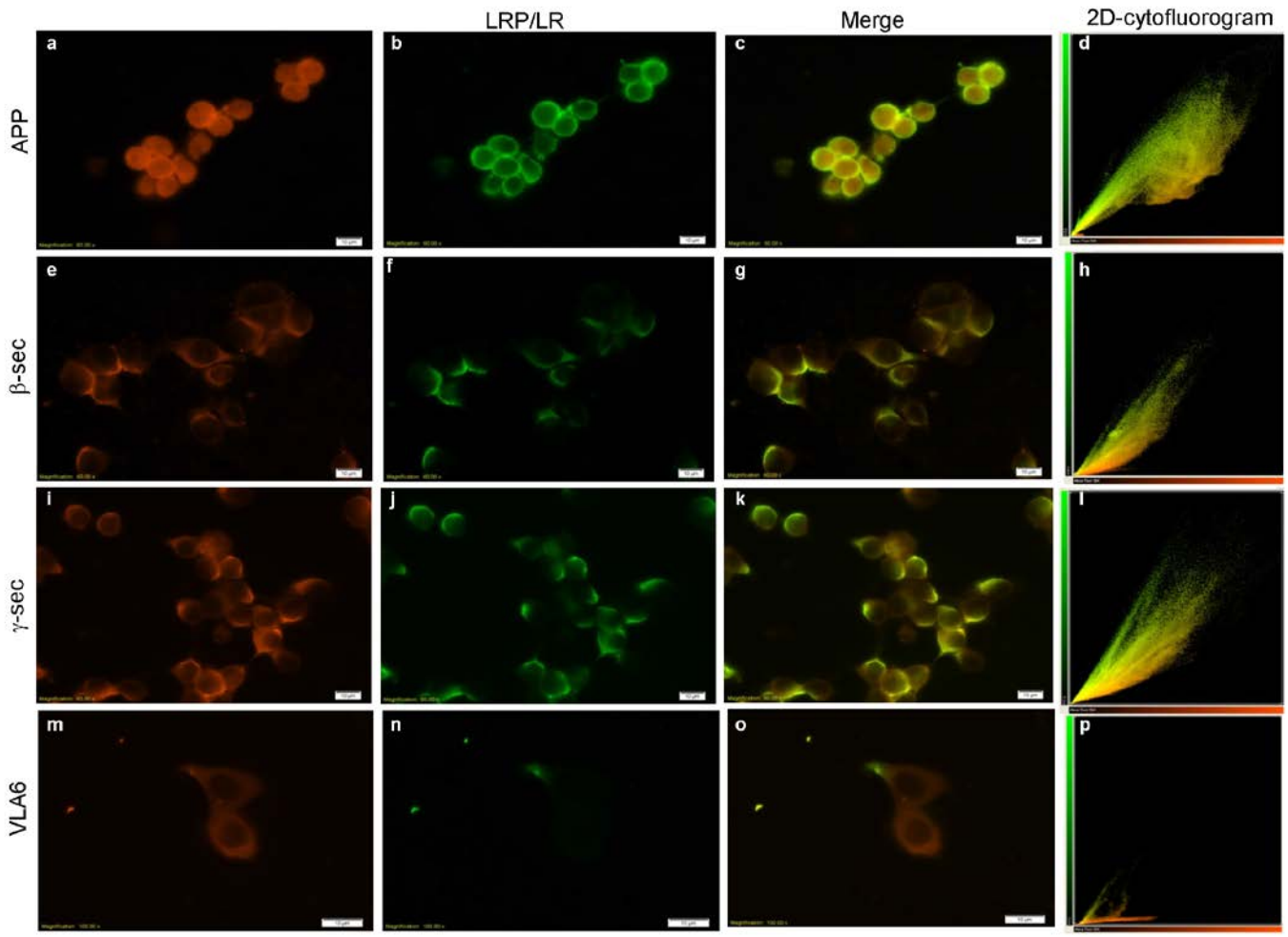


Fig S1| Co-localisation of LRP/LR with the AD relevant proteins APP, β- and γ-secretase. Cell surface receptors on N2a (mouse neuroblastoma) cells were indirectly immunolabelled to allow for detection using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software . (a), APP (detected by anti-APP (rabbit polyclonal IgG) (Abcam), (e), β-secretase (detected using anti-BACE (M-83) (rabbit polyclonal IgG) (Santa Cruz Biotechnology)), (i), γ-secretase (detected by anti-PEN-2 (FL-101) (rabbit polyclonal IgG) (Santa Cruz Biotechnology)) and (m), VLA6 (detected by anti-very late antigen-6 (VLA6) CD49-f (rabbit monoclonal IgG) (Immunotech) were all indirectly labelled with Alexaflour 633, while an anti-human FITC conjugated antibody (Cell Lab) was used to label LRP/LR (b, f, j, n). The merges between LRP/LR and relevant proteins are shown (c, g, k, o) and the corresponding 2D-cytofluorograms (acquired using CellSens Software) have been included to confirm the degree of co-localisation (d, h, l, p).

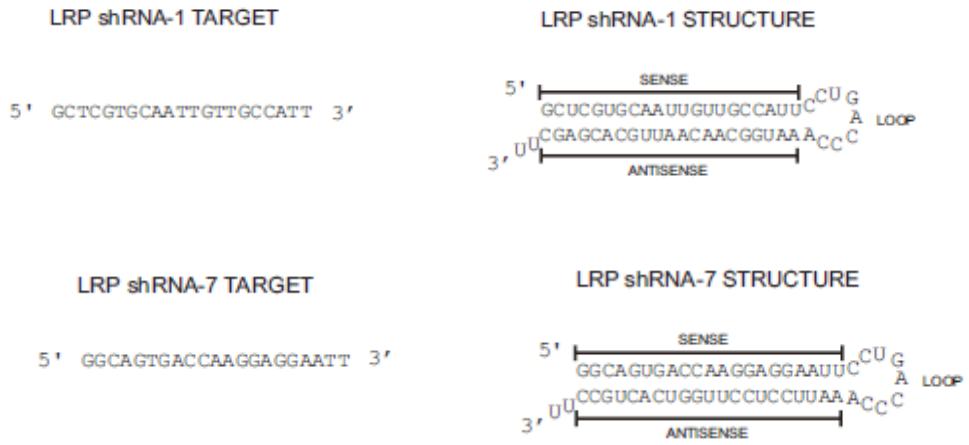


Figure S2| LRP/LR target sequences and structure of shRNA1 and shRNA7. shRNAs 1 and 7 were designed to target the LRP/LR sequences shown above. The hairpin structure of the shRNAs is also shown.

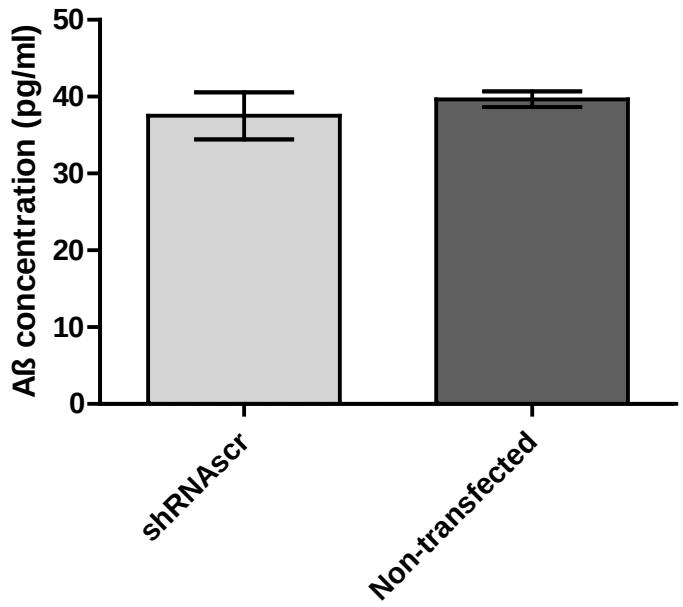
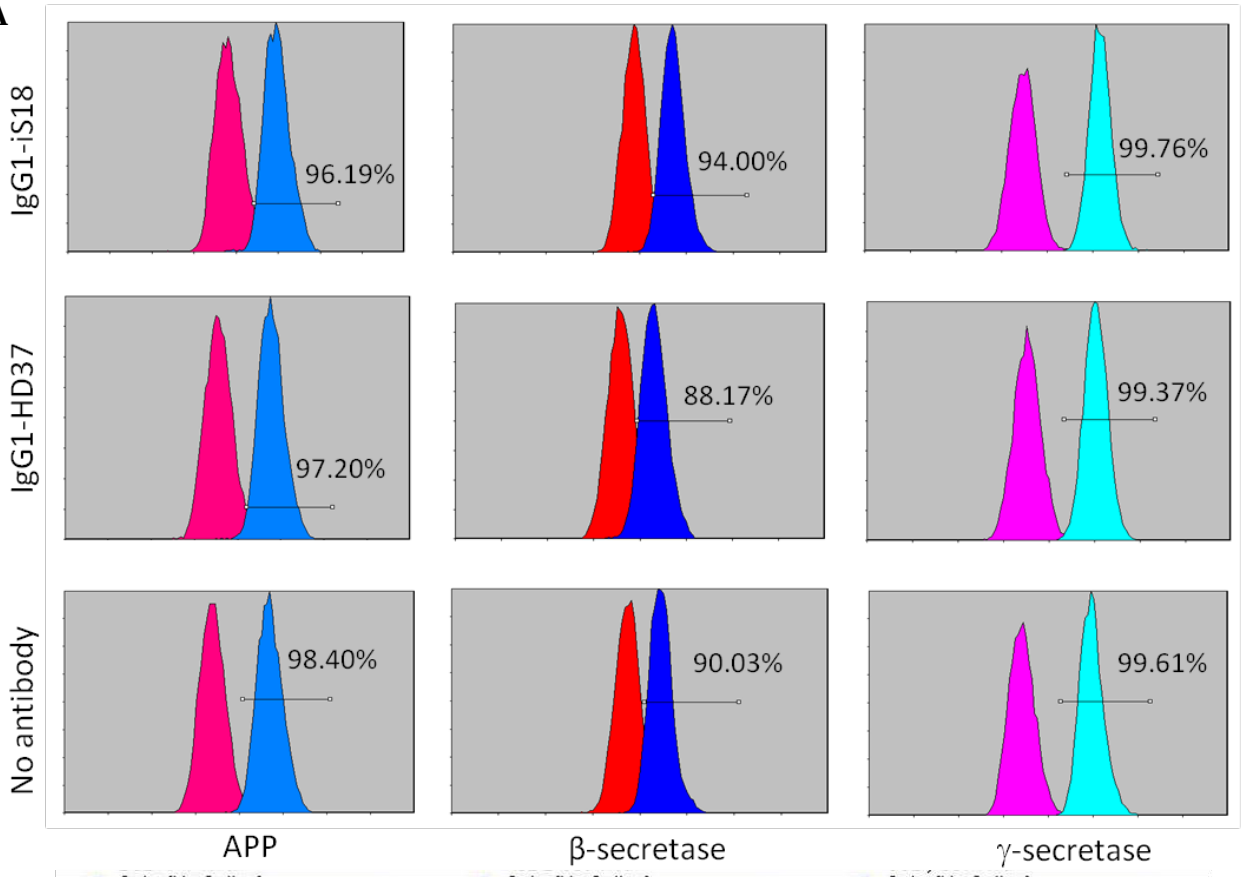


Figure S3| Aβ concentration of the cell culture medium of shRNAscr-transfected and mock-transfected HEK293 cells. HEK293 cells were either transfected with the scrambled control (shRNAscr) or mock-transfected with no plasmid. 72 hours post transfection, the Aβ concentration of the cell culture medium was analysed using an Aβ ELISA. Data shown (Mean±s.d); n=3; Student's *t*-test; $p>0.05$.

4A


IgG1-iS18
IgG1-HD37
No antibody
96.19%
94.00%
99.76%
97.20%
88.17%
99.37%
98.40%
90.03%
99.61%
APP
β-secretase
γ-secretase

B

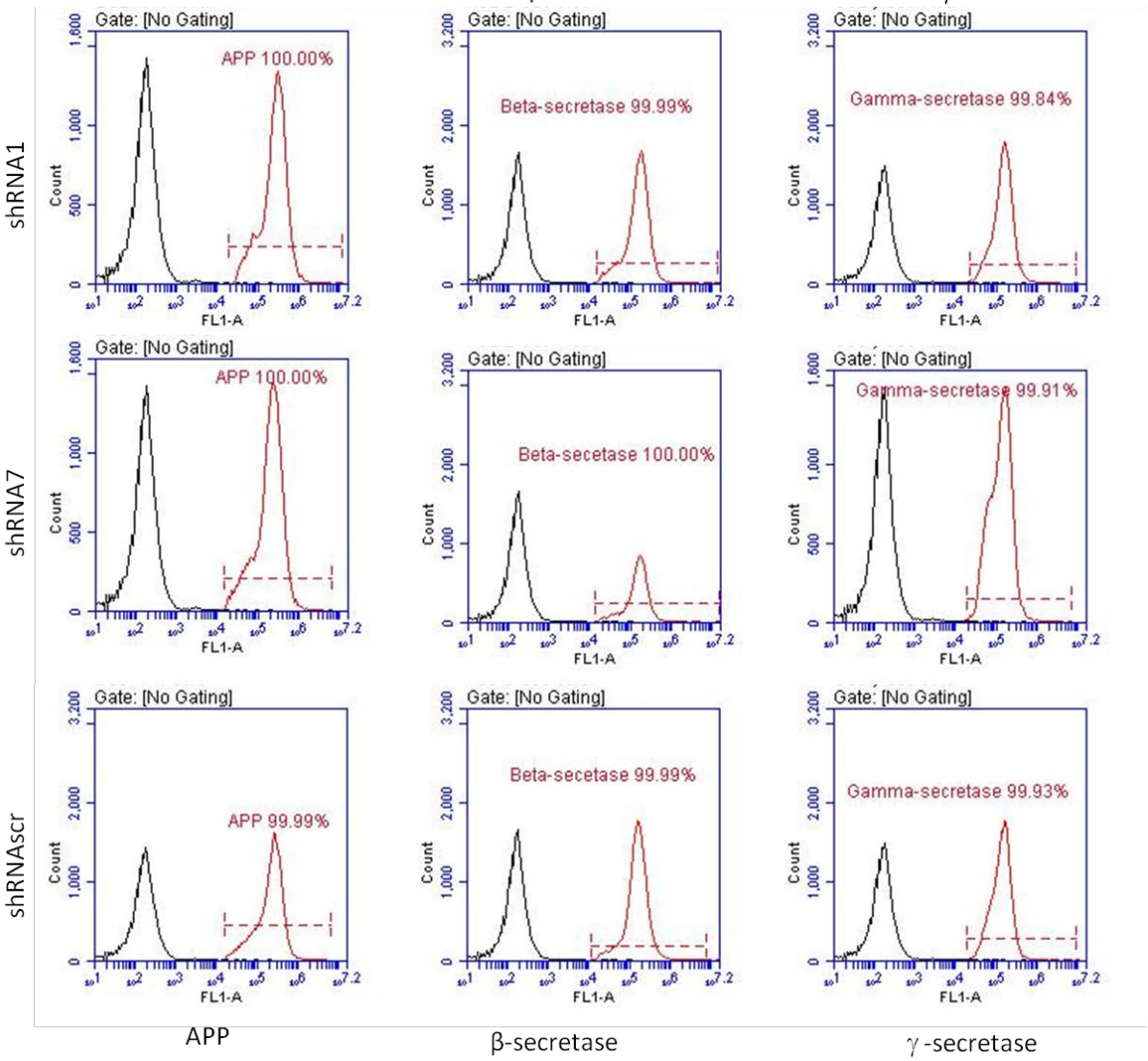

shRNA1
shRNA7
shRNAscr
Gate: [No Gating]
APP 100.00%
Beta-secretase 99.99%
Gamma-secretase 99.84%
APP 100.00%
Beta-secetase 100.00%
Gamma-secretase 99.91%
APP 99.99%
Beta-secetase 99.99%
Gamma-secretase 99.93%
Count
FL1-A
APP
β-secretase
γ-secretase

Figure S4| Flow cytometry histogram overlay plots for β-secretase, γ-secretase and APP after antibody or shRNA treatments. (A) HEK293 cells were incubated with either 50 μg/ml IgG1-iS18, IgG1-HD37 or no antibody for 18 hours after which APP, β- and γ-secretase cell surface levels were ascertained by flow cytometry (Coulter EPICS® XL-MCL). Images shown are averages of 3 independent experiments. (B) HEK293 cells were transfected with either shRNA1, shRNA7 or shRNAscr. 72 hours post transfection, the cell surface levels of APP, β- and γ-secretase were ascertained by flow cytometry (BD Accuri C6) using the methodology described above.

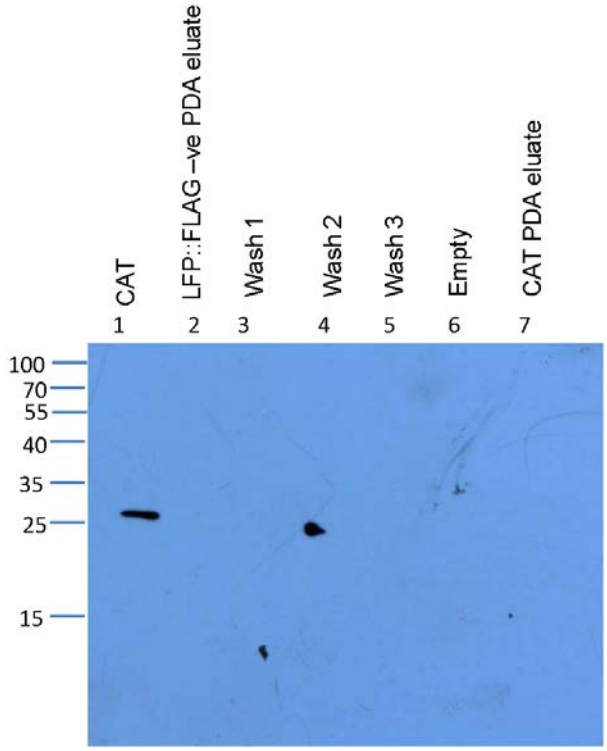


Figure S5| CAT fails to immunoprecipitate with LRP::FLAG. Cell lysates containing CAT (chloramphenicol acetyl transferase) were mixed with LRP::FLAG positive cell lysates and were subjected to a FLAG® Immunoprecipitation assay. CAT was only detected in the CAT cell lysate (positive control, lane 1) and possibly in residual amounts in the wash fractions of the assay (lane 4). It however is completely lacking in lane 7 which contains the eluate of the assay and hence all the proteins that would be interacting the LRP::FLAG protein.

High resolution imaging study of interactions between the 37kDa/67kDa Laminin Receptor and APP, β-secretase and γ-secretase in Alzheimer's disease

Katarina Jovanovic, Ben Loos, Clement Penny and Stefan F.T. Weiss

In Revision for PLOS ONE

As LRP/LR was seen to play a significant role in the shedding of Aβ, we sought to elucidate the possible molecular mechanisms by which LRP/LR exerted its influence. A study was thus undertaken to observe whether interactions existed between LRP/LR and the AD proteins APP, β- and γ-secretase. For this study, fluorescently tagged proteins of interest (namely APP-GFP, BACE1-GFP and PS1-GFP) were co-expressed with LRP-dsRed and numerous microscopic imaging techniques were used to analyse the samples. Firstly, maximum intensity projections obtained from z-stack images revealed a high degree of co-localisation between LRP-dsRed and APP-GFP, BACE1-GFP and PS1-GFP at the 200nm resolution. The co-localisation was limited to the cytoplasm and cell surface. However when super resolution structured illumination (SR-SIM) was performed due it is ability to resolve up to 80nm, co-localisation was only maintained between PS1-GFP and LRP-dsRed. The co-localisation between APP-GFP and LRP-dsRed was confined to only certain cytoplasmic regions and the cell surface, while all co-localisation was lost between LRP-dsRed and BACE1-GFP. Försters Resonance Energy Transfer was used to further verify whether LRP/LR interacted with the AD proteins as energy is only transferred when the two proteins in question are within 1-10nm of each other, a distance which is sufficiently small enough to allow for protein-protein interactions. Upon this analysis, an interaction was revealed between LRP/LR and PS1 of the γ-secretase, while no FRET was observed for LRP-dsRed with APP-GFP and BACE1-GFP. Taken together, these findings suggest that a direct interaction exists between LRP/LR and the γ-secretase, while the interaction between LRP/LR and β-secretase is most likely an indirect one, mediated by another molecule present in the cellular environment.

PLOS ONE

High resolution imaging study of interactions between the 37kDa/67kDa Laminin Receptor and APP, beta-secretase and gamma-secretase in Alzheimer's disease

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	High resolution imaging study of interactions between the 37kDa/67kDa Laminin Receptor and APP, beta-secretase and gamma-secretase in Alzheimer's disease
Short Title:	Interactions between LRP/LR and AD proteins
Corresponding Author:	Stefan F.T. Weiss, PhD University of the Witwatersrand Johannesburg, Gauteng SOUTH AFRICA
Keywords:	laminin receptor; LRP/LR; Alzheimer's Disease; BACE1; beta secretase; gamma secretase; PS1; APP; presenelin 1; prion; molecular mechanism; FRET; SR-SIM; confocal microscopy
Abstract:	Alzheimer's disease (AD) is the most prevalent form of dementia affecting the elderly. Neurodegeneration is caused by the amyloid beta (A-beta) peptide which is generated from the sequential proteolytic cleavage of the Amyloid Precursor Protein (APP) by the beta- and gamma- secretases. Previous reports revealed that the 37kDa/67kDa laminin receptor (LRP/LR) is involved in APP processing, however the exact mechanism by which this occurs remains largely unclear. This study sought to assess whether LRP/LR interacted with APP, beta- or gamma-secretase. Detailed confocal microscopy revealed that LRP/LR showed a strong co-localisation with APP, beta- and gamma-secretase at various sub-cellular locations. Superresolution Structured Illumination Microscopy (SR-SIM) showed that interactions were unlikely between LRP/LR and APP and beta-secretase, while there was strong co-localisation between LRP/LR and gamma-secretase at an 80nm resolution. FRET was further employed to assess the possibility of protein-protein interactions and only an interaction between LRP/LR and gamma-secretase was found. FLAG co-immunoprecipitation confirmed these findings as LRP/LR co-immunoprecipitated with gamma-secretase but failed to do so with APP. These findings indicate that LRP/LR exerts its influence on A-beta shedding via a direct interaction with the gamma-secretase and possibly an indirect interaction with the beta-secretase.
Order of Authors:	Katarina Jovanovic
	Ben Loos
	Clement Penny
	Stefan F.T. Weiss, PhD
Suggested Reviewers:	Juergen Goetz The University of Queensland j.goetz@uq.edu.au Alzheimer's Disease expert
	Jesus Avila Universidad Autonoma de Madrid javila@cbm.uam.es Alzheimer's Disease Expert
	David Holtzman Washington University holtzman@neuro.wustl.edu Alzheimer's Disease expert
	Gianluigi Forloni Mario Negri Institute for Pharmacological Research forloni@marionegri.it Neurodegenerative diseases specialist

High resolution imaging study of interactions between the 37kDa/67kDa Laminin Receptor and APP, β-secretase and γ-secretase in Alzheimer's disease

Katarina Jovanovic[1], Ben Loos[2], Clement Penny[3] and Stefan F.T Weiss[1]*

[1]School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa, [2]Department of Physiological Sciences, University of Stellenbosch, Stellenbosch, Republic of South Africa, [3]Department of Internal Medicine, University of the Witwatersrand, 7 York Rd, Johannesburg, 2193 Parktown, Republic of South Africa.

*Correspondence and requests for materials should be referred to Stefan F.T Weiss at Stefan.weiss@wits.ac.za

Alzheimer's disease (AD) is the most prevalent form of dementia affecting the elderly. Neurodegeneration is caused by the amyloid beta (Aβ) peptide which is generated from the sequential proteolytic cleavage of the Amyloid Precursor Protein (APP) by the β– and γ- secretases. Previous reports revealed that the 37kDa/67kDa laminin receptor (LRP/LR) is involved in APP processing, however the exact mechanism by which this occurs remains largely unclear. This study sought to assess whether LRP/LR interacted with APP, β- or γ-secretase. Detailed confocal microscopy revealed that LRP/LR showed a strong co-localisation with APP, β- and γ-secretase at various sub-cellular locations. Superresolution Structured Illumination Microscopy (SR-SIM) showed that interactions were unlikely between LRP/LR and APP and β-secretase, while there was strong co-localisation between LRP/LR and γ-secretase at an 80nm resolution. FRET was further employed to assess the possibility of protein-protein interactions and only an interaction between LRP/LR and γ-secretase was found. FLAG co-immunoprecipitation confirmed these findings as LRP/LR co-immunoprecipitated with γ-secretase but failed to do so with APP. These findings indicate that LRP/LR exerts its influence on Aβ shedding via a direct interaction with the γ-secretase and possibly an indirect interaction with the β-secretase.

Alzheimer's Disease (AD) is the most prevalent neurodegenerative disorder affecting the elderly population worldwide. There are an estimated 37 million people suffering from this disease[1] and due to the lack of any effective therapies, this number continues to rise and pose more of an economic and social burden[2]. Lack of understanding of the disease causing mechanisms have resulted in great difficulties in the development of effective therapeutic interventions and as yet, the only treatment strategies are merely palliative, despite numerous ongoing clinical trials[3].

The two hallmark features of AD are the formation of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein. Oligomeric Aβ is thought to be the candidate etiological agent for AD since it has been found to mediate neurotoxicity through interactions with many other proteins[4,5]. One such protein that has proved to be of significance in AD, is the cellular prion protein (PrP^c).

PrP^c is thought to have a neuroprotective role with regard to apoptosis and oxidative stress and also functions in cell signaling as well as synapse physiology[6], however, recent reports suggest an important role for PrP^c in mediating the toxicity caused by the Aβ peptide in AD. Lauren *et al*. showed that PrP^c acts as a high affinity receptor for Aβ peptides and thus mediates the impairment of synaptic plasticity[7]. Recently reports have further verified these findings by showing that PrP^c was required for the neurotoxicity caused by Aβ, through impairment of long term potentiation (LTP)[8] as well as by regulating the function of the N-methyl-D-aspartate receptor(NMDAR) – a function which is hindered due to the Aβ - PrP^c interaction and leads to excessive activity of the receptor thereby promoting neuronal damage[9]. PrP^c has also been implicated in neurotoxic signaling upon interaction with Aβ whereby Fyn kinase is activated and

leads to dendritic spine loss, lactate dehyrogenase activation and altered NMDAR expression of the plasma membrane of neurons [10,11].

The cellular receptor for both PrP^{c} [12] and its infectious isoform PrP^{Sc} [13] is the 37kDa/67KDa Laminin Receptor (LRP/LR). This multifunctional receptor has numerous physiological roles including cell adhesion, migration, survival and proliferation (for reviews see [14,15]). These roles are exploited by neoplastic cells whereby the receptor is involved in tumour metastasis[16,17,18], apoptosis[19] and angiogenesis[20].

Due to its role as the receptor for PrP^{c}, we examined whether LRP/LR may play some role in AD pathways. Blockage of LRP/LR with an anti-LRP/LR antibody (IgG1-iS18) or knock down of LRP/LR using anti-LRP shRNAs resulted in a significant reduction was seen both in Aβ levels[21] and in Aβ induced cytotoxicity[22]. As expression of APP, β- and γ-secretase were not affected upon antibody or shRNA treatment, an interaction between LRP/LR and one or more of the AD related proteins (APP, β- and γ-secretase) was deemed likely[21]. Since sAPPβ shedding was also impaired upon IgG1-iS18 and shRNA treatment, an interaction between LRP/LR and β-secretase was examined and co-immunoprecipitation revealed the existence of a (direct or indirect) interaction between the 2 proteins[21]. These findings revealed a novel role for LRP/LR in in AD. We thus aimed to further investigate whether LRP/LR interacts with the proteins which are central to AD, namely APP, β- and γ-secretase using high resolution imaging.

Materials and Methods

Cell Culture and transient transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 10% fetal calf serum and 1%

Penicillin/Streptomycin. Cells were incubated at 37°C with 5% CO_2. This cell line was obtained from American Tissue Culture Collection (ATCC). For transfections, cells were seeded onto sterile coverslips within the wells of a 6 well tissue culture dish (Corning) for all microscopy. For co-immunoprecipitation, cells were seeded into 60mm tissue culture dishes (Corning). Once the cells were 30 – 50% confluent, calcium phosphate transfection was performed as described previously[21]. The procedure for the generation of the plasmids encoding LRP-dsRed (pLRP-dsRed)[23] and LRP::FLAG (pCIneo-moLRP::FLAG)[24] has been described previously. Plasmids for APP-GFP (pEGFPN1-APP770) and BACE1-GFP (pEGFPN1-BACE1)[25] were a generous gift from Dr. Bradley T Hyman, while pEGFP-PS1 (coding for PS1-GFP)[26] was a kind gift from Dr. Oskana Berezovska. For confocal microscopy, pLRP-dsRed was co-transfected with pEGFPN1-APP770, pEGFPN1-BACE1 and pEGFP-PS1 respectively in a 1:1 ratio. pEGFP-N1 (Clonetech) and pDsRed-Express N1 (Clonetech) were used as controls. For co-immunoprecipitation, pCIneo-moLRP::FLAG, pEGFPN1-APP770 and pEGFP-PS1 were individually transfected in 60mm tissue culture dishes and incubated for 72 hours, after which the cells were lysed for further experiments.

Slide preparation

24 hours post transfection, coverslips with transfected cells (LRP-dsRed with APP-GFP, BACE1-GFP or PS1-GFP) were washed with PBS 3 times and then fixed with 4% Paraformaldehyde for 15 minutes. Coverslips were washed with PBS and mounted onto clean microscope slides using 75μl Fluoromount (Sigma Aldrich). Slides were incubated at room temperature for 1-2 hours in the dark to allow the mounting medium to set.

Confocal Microscopy

Slides were viewed using the Zeiss LSM 780 confocal microscope equipped with a GaAsp detector and images were acquired through z-stack acquisition, with an increment of ±0.4μm (depending on sample) between image frames. The AxioCam MRm camera was utilized to capture images. Cells expressing both LRP-dsRed and either APP-GFP, BACE1-GFP or PS1-GFP were selected and z-stack acquisition was performed. Images were displayed as maximum intensity projections and subsequently analysed for co-localisation using 2D cytofluorograms and fluorescence intensity line profiles obtained with the use of Zen 2010 imaging software.

SR-SIM Imaging

Slides were prepared as described and superresolution structured illumination (SR-SIM) was performed. Thin (0.1 μm) z-stacks of high-resolution image frames were collected in 5 rotations by utilizing an alpha Plan-Apochromat 100x/1.46 oil DIC M27 ELYRA objective, using an ELYRA S.1 (Carl Zeiss Microimaging) microscope equipped with a 488nm laser (100mW), 561nm laser (100mW) and Andor EM-CCD camera (iXon DU 885). Images were reconstructed using ZEN software (black edition, 2011, version 7.04.287) based on a structured illumination algorithm[27]. Analysis was performed on reconstructed superresolution images in ZEN.

FRET imaging

Cells were seeded onto coverslips, transfected as described above and Fluorescence Resonance Energy Transfer (FRET) acquisition and analysis was performed. Image frames were collected using confocal microscopy (LSM 780, Carl Zeiss) equipped with a LSM780 GaAsP detector, using a Plan-Apochromat 63x/1.4 Oil DIC M27 objective. Samples were excited with a 488nm and 561nm laser under utilization of a MBS 488/561 beam splitter. Emission was collected for

the donor-GFP channel at an emission window of 495nm-510nm, the FRET channel at an emission window of 586nm-600nm and the acceptor-dsRed channel at an emission window of 586nm-600nm, using the lamda setting. Appropriate controls for background, donor spectral bleedthrough (DSB) and Acceptor spectral bleedthrough (ASB) were prepared and GFP only control images as well as dsRed only control images were acquired. Sensitized emission FRET analysis was performed utilizing FRET_plus Version 3 for ZEN 2010. FRET (N-FRET, normalized, Xia) data were generated, correcting for emission/excitation crosstalk and normalizing for the concentration of donor and acceptor.

FLAG® Co-immunoprecipitation

Cells transfected with pCIneo-moLRP::FLAG, pEGFPN1-APP770 and pEGFP-PS1 were lysed 72 hours post-transfection. Cell lysates from cells expressing LRP::FLAG were mixed with those expressing either APP-GFP or PS1-GFP. Lysates were subjected to a FLAG® Co-immunoprecipitation assay (Sigma-Aldrich) according to the manufacturer's instructions and results were analysed via immunoblotting.

Immunoblotting

Co-immunoprecipitation assay eluates were assessed using immunoblotting. LRP::FLAG was detected using anti-FLAG® (mouse monoclonal IgG antibody) (1:5000) (Sigma-Aldrich) and Anti-mouse HRP antibody (1:5000) (Sigma-Aldrich). APP-GFP was detected using a rabbit monoclonal (Y188) antibody to Amyloid beta Precursor Protein (Abcam) (1:3000) and PS1-GFP was detected using rabbit monoclonal (EP2000Y) antibody against Presenillin 1 (Abcam) (1:3000). An anti-rabbit HRP antibody (1:5000) (Sigma-Aldrich) was employed for the detection of the rabbit primary antibodies. Immunodetection was

performed with SuperSignal West Pico Chemiluminescent Substrate and X-ray film (both Thermo Scientific).

Results and Discussion

Interactions between γ-secretase and LRP/LR

γ-secretase is composed of 4 sub-units namely Presenilin 1 or 2 (PS1 or PS2), nicastrin, anterior pharynx defective 1 and presenilin enhancer 2 [28]. The presenilin proteins form the catalytic domain of the γ-secretase complex as they contain two aspartyl residues, within transmembrane domains 6 and 7, which are responsible for the γ-secretase cleavage of APP [29]. The 4 subunit complex is found to function predominantly within cholesterol- and sphingolipid-rich lipid raft microdomains of membranes, most notably those of the plasma membrane [30], Trans Golgi Network as well as endosomes [31]. The cellular localizations of LRP/LR are limited to these same regions and the presence of this protein has also been noted in the Golgi apparatus [32]. The co-localisation observed from the maximum intensity projection between PS1-GFP and LRP-dsRed supports the notion that LRP/LR and γ-secretase appear to be located in similar cellular regions and compartments (Figure 1A). The 2D cytofluorogram accompanying the maximum intensity projection shows a clear diagonal passing through quadrant 3 indicating a high degree of co-localization between the signal in the green and red channels which results in the observed yellow pattern of co-localization. The line profile obtained from the maximum intensity projection of the z-stack analysis between PS1-GFP and LRP-dsRed (Figure S1) reveals that the spectra are well aligned and fluorescence intensity is strongest in the cytoplasm and plasma membrane. Due to the limited resolving power dictated by the wavelength utilized in laser scanning confocal microscopy, co-localization does not necessitate

functional protein – protein interaction. This can in part be overcome by an SR-SIM approach, which provides a resolution limit of approximately 80nm (compared to the 200nm resolution limit of laser scanning microscopy). Figure 1B reveals that even when SR-SIM is employed a high degree of co-localisation is still maintained between these two proteins, suggesting that these proteins are indeed in close proximity to one another. The expression of both PS1-GFP and LRP-dsRed is even more distinctly defined within the cytoplasm and on the plasma membrane when observed at this resolution. In order to further assess whether this is due to protein-protein interactions Förster Resonance Energy Transfer (FRET) was employed as in this technique, the donor and acceptor have to be within aproximity of 1-10nm to induce FRET. A well-defined FRET signal is observed in the normalized FRET scale panel (Figure 1C (i)), strongly suggesting protein-protein interaction, and not only co-localisation between γ-secretase and LRP/LR. This interaction appears to take place primarily in the cytoplasm and membrane region as revealed by the FRET signal distribution pattern, clearly observed in Figure 1C (ii) which shows a close up of the FRET signal obtained within an individual cell. LRP/LR and PS1 may be also interacting in the perinuclear region of the cell shown in Figure 1C (iii). Based on these results it is highly likely that a direct interaction does exist between LRP/LR and the PS1 subunit of the γ-secretase. In order to confirm this, FLAG® co-immunoprecipitation was performed using LRP::FLAG and PS1-GFP (Figure 1D). PS1 is clearly detected in the FLAG® co-immunoprecipitation assay eluate, denoting that PS1 has bound to LRP::FLAG (Figure 1D, lane 4). PS1 was detected within all of the control lanes, thus the protein visualized is most certainly cellular PS1 and not PS1-GFP. The band is seen at 36kDa which confirms that the proteins detected are most likely the homo-dimer of PS1 [33,34], as the expected band size for PS1 is 18kDa. This co-immunoprecipitation finding further substantiates the FRET data as it confirms the interaction between LRP/LR and PS1. These findings also suggest an explanation for the altered APP processing

observed upon the blockage and knockdown of LRP/LR [21], as these treatments may be interfering in or hampering the interaction between LRP/LR and γ-secretase.

Interactions between β-secretase and LRP/LR

β-secretase (also known as BACE1 - β-site APP cleaving enzyme) is a transmembrane aspartic protease which is predominantly localized in the Golgi and trans-Golgi network (TGN), as well as in endosomes where a slightly acidic pH provides the optimal environment for APP cleavage [35]. Initial reports suggested not only co-localisation between LRP/LR and β-secretase on the cell surface, but also that these two proteins also interacted, as shown by co-immunoprecipitation[21]. The findings presented in Figure 2 help to further understand the nature of the possible interactions between β-secretase and LRP/LR, where the maximum intensity projection (Figure 2A) constructed from Z-stack images (Figure S2) shows strong co-localisation between 2 proteins within the cytoplasm of the cell - most likely in the endosomes. This is further corroborated by the line profile in which the lines representing the intensity of BACE1-GFP and LRP-dsRed are not only aligned but also show the highest fluorescence intensities within the cytoplasm. However, when SR-SIM was employed, co-localisation was lost between LRP/LR and β-secretase, implying the proteins are situated approximately 100 – 200nm from each other. This diminishes the likelihood of a direct interaction between these two proteins, due to the spatial separation observed. The FRET findings further confirm this as no FRET occurred between the BACE1-GFP donor and the LRP-dsRed acceptor (Figure 2C). These findings imply that the interaction that was reported between LRP/LR and β-secretase [21] is most likely an indirect interaction mediated by another cellular protein, as cell lysates were employed for co-immunoprecipitation

studies. LRP/LR and β-secretase share several binding/interaction partners, the most notable one being PrP^c [36,37]. It is possible that since LRP/LR is the cellular receptor for PrP^c and plays an important role in regulating endocytosis[38] and propagation[39] of PrP^c, it may indirectly influence β-secretase activity. Another factor that may have led to the co-immunoprecipitation between LRP::FLAG and β-secretase is heparan sulphate proteoglycan (HSPG). Binding sites for HSPG have been located on LRP/LR and are implicated in regulating the interaction between LRP/LR and PrP^c [40]. Extensive studies have been performed on heparan sulphate, and analogues thereof, as therapeutic options for Alzheimer's Disease as this proteoglycan is seen to regulate β-secretase activity and significantly reduce the shedding of Aβ [41,42,43]. Thus, HSPG is another molecule which could be responsible for the indirect interaction suggested by the co-immunoprecipitation observed between LRP/LR and β-secretase [21] as it interacts with both of these proteins. PrP^c and HSPG could also be responsible for the decrease in sAPPβ (the cleavage product of β-secretase) levels upon anti-LRP/LR antibody and shRNA treatment[21] since they may be directly affected by these treatments resulting in an indirect effect on β-secretase activity which they are seen to regulate.

Interactions between APP and LRP/LR

Initially, an interaction between LRP/LR and APP seemed highly likely and would provide an explanation for the effects seen on Aβ shedding upon antibody and shRNA treatment of LRP/LR [21]. Initial findings also suggested that these two proteins co-localize when detected on non-permeabilised cells (indicating cell surface co-localisation) and within the cells (indicating intracellular co-localisation) [21]. Z-stacking was performed to further verify the sub-cellular localizations of these proposed interactions and to assess whether they were maintained throughout the cell (Figure S3). Maximum intensity projections were

constructed from the images obtained via Z-stacking and revealed a degree of overlap between the LRP-dsRed with APP-GFP signal as indicated by the resulting yellow colour when the images are merged (Figure 3A). The line profile constructed from the maximum intensity projection of the z-stack between APP-GFP and LRP-dsRed, shows the intensity of the red and green fluorescence along the line pasing through the cell of interest. These findings suggest a complete spatial overlap between the fluorescence observed in both the green and red channels. The observed intensity of both fluorescent channels is highest in the cytoplasm which further confirms the initial assumption that LRP/LR and APP are located in similar cellular locations. Findings in literature further support these data as it is known that APP is transported to the plasma membrane from where it is endocytosed from lipid raft regions into early endosomes[44,45]. LRP/LR is known to also be located in the lipid raft[46] as well as early endosomes[38]. Our SR-SIM data indicates a lower degree of co-localization between APP and LRP, which appears to be confined to the plasma membrane and certain cytoplasmic regions, presumably the endosomes in which both APP and LRP/LR are located (Figure 3B). This suggests the proteins are indeed in close proximity to one another, resulting in the distinct co-localization pattern. FRET was employed to further assess the viability of an interaction between LRP/LR and APP. This analysis revealed that no FRET occurred between APP-GFP and LRP-dsRed, as no signal was detected on the normalized FRET scale (Figure 3C). These findings suggest that no interaction takes place between APP and LRP/LR at this 1- 10nm scale. FLAG® Co-immunoprecipitation using a FLAG tagged LRP/LR variant (LRP::FLAG) further confirmed these results as no APP was detected in the eluate of the assay (Figure 3D, lane 6). The results obtained from figure 3 collectively suggest that although LRP/LR and APP are found in similar cellular locations in which they co-localize, the proximity between them is still present when SR-SIM is employed (i.e. 80nm), but lost at the nanometer scale required for FRET to

take place thus suggesting it is highly unlikely for any interaction to occur between these two proteins.

Interactions between GFP and LRP/LR

GFP was used a negative control for these studies as it failed to co-localize with LRP/LR. This is evidenced by the maximum intensity projection (Figure 4A) obtained from z-stack analysis (Figure S4). The 2D cytofluorogram reveals a complete absence of a diagonal passing through quadrant 3 confirming the lack of co-localisation between GFP and LRP-dsRed. The line profile obtained shows two distinct patterns of fluorescence with no alignment of the red and green channels which further confirms the low degree of co-localization between these proteins. These findings also validate the co-localization seen between LRP-dsRed and APP, β- and γ-secretase as it indicates that the observed co-localization is as a result of the proximity of LRP/LR to the AD proteins and not just the GFP labels

.

Conclusion

In light of this study, we have found that LRP/LR is seen to co-localise with APP, β- and γ-secretase on the cell surface as well as intracellularly within the cytoplasm. This co-localisation is limited to 200nm when observed for BACE1 and LRP/LR, 80nm between APP and LRP/LR and less than 10nm for LRP/LR and PS1. These results reveal a novel interaction between LRP/LR and the PS1 catalytic subunit of the γ-secretase complex (as confirmed by co-immunoprecipitation) and suggest that the previously observed interaction between LRP/LR and BACE1 is likely an indirect interaction only. These findings further highlight the role of LRP/LR in Alzheimer's Disease.

Acknowledgements

This work is based upon research supported by the National Research Foundation (NRF), the Republic of South Africa (RSA). Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s), and therefore, the National Research Foundation does not accept any liability in this regard thereto.

We would like to acknowledge Dr. Bradley T Hyman (Harvard Medical School, Massachusetts, USA) for his generous gift of pEGFPN1-APP770 and pEGFPN1-BACE1. We also acknowledge Dr. Oksana Berezovska (Massachusetts General Hospital, Massachusetts) for the kind gift of pEGFP-PS1.

REFERENCES

1. Mount C, Downton C (2006) Alzheimer disease: progress or profit? Nat Med 12: 780-784.

2. Wimo A, Jonsson L, Bond J, Prince M, Winblad B (2013) The worldwide economic impact of dementia 2010. Alzheimers Dement 9: 1-11 e13.

3. Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M (2010) Alzheimer's disease: clinical trials and drug development. Lancet Neurol 9: 702-716.

4. Kudo W, Lee HP, Zou WQ, Wang X, Perry G, et al. (2012) Cellular prion protein is essential for oligomeric amyloid-beta-induced neuronal cell death. Hum Mol Genet 21: 1138-1144.

5. Verdier Y, Penke B (2004) Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. Curr Protein Pept Sci 5: 19-31.

6. Westergard L, Christensen HM, Harris DA (2007) The cellular prion protein (PrP(C)): its physiological function and role in disease. Biochim Biophys Acta 1772: 629-644.

7. Lauren J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature 457: 1128-1132.

8. Barry AE, Klyubin I, Mc Donald JM, Mably AJ, Farrell MA, et al. (2011) Alzheimer's disease brain-derived amyloid-beta-mediated inhibition of LTP in vivo is prevented by immunotargeting cellular prion protein. J Neurosci 31: 7259-7263.

9. You H, Tsutsui S, Hameed S, Kannanayakal TJ, Chen L, et al. (2012) Abeta neurotoxicity depends on interactions between copper ions, prion protein, and N-methyl-D-aspartate receptors. Proc Natl Acad Sci U S A 109: 1737-1742.

10. Um JW, Strittmatter SM (2013) Amyloid-beta induced signaling by cellular prion protein and Fyn kinase in Alzheimer disease. Prion 7: 37-41.

11. Um JW, Nygaard HB, Heiss JK, Kostylev MA, Stagi M, et al. (2012) Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. Nat Neurosci 15: 1227-1235.

12. Gauczynski S, Peyrin JM, Haik S, Leucht C, Hundt C, et al. (2001) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. EMBO J 20: 5863-5875.

13. Gauczynski S, Nikles D, El-Gogo S, Papy-Garcia D, Rey C, et al. (2006) The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. J Infect Dis 194: 702-709.

14. Mbazima V, Da Costa Dias B, Omar A, Jovanovic K, Weiss S (2010) Interactions between PrPc and other ligands with the 37-kDa/67-kDa laminin receptor. Frontiers in Bioscience 15: 1150-1163.

15. Omar A, Jovanovic K, Da Costa Dias B, Gonsalves D, Moodley K, et al. (2011) Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. Expert Opin Ther Pat 21: 35-53.

16. Zuber C, Knackmuss S, Zemora G, Reusch U, Vlasova E, et al. (2008) Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. J Mol Biol 378: 530-539.

17. Omar A, Reusch U, Knackmuss S, Little M, Weiss SF (2012) Anti-LRP/LR-specific antibody IgG1-iS18 significantly reduces adhesion and invasion of metastatic lung, cervix, colon and prostate cancer cells. J Mol Biol 419: 102-109.

18. Khumalo T, Reusch U, Knackmuss S, Little M, Veale RB, et al. (2013) Adhesion and Invasion of Breast and Oesophageal Cancer Cells Are Impeded by Anti-LRP/LR-Specific Antibody IgG1-iS18. PLoS One 8: e66297.

19. Moodley K, Weiss SF (2013) Downregulation of the non-integrin laminin receptor reduces cellular viability by inducing apoptosis in lung and cervical cancer cells. PLoS One 8: e57409.

20. Khusal R, Da Costa Dias B, Moodley K, Penny C, Reusch U, et al. (2013) In vitro inhibition of angiogenesis by antibodies directed against the 37kDa/67kDa laminin receptor. PLoS One 8: e58888.

21. Jovanovic K, Gonsalves D, Da Costa Dias B, Moodley K, Reusch U, et al. (2013) Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease. Sci Rep 3: 2699.

22. Da Costa Dias B, Jovanovic K, Gonsalves D, Reusch U, Knackmuss S, et al. (2013) Anti-LRP/LR specific antibody IgG1-iS18 rescues cells from Aβ42 induced cytotoxicity. Scientific Reports 3: 2702.

23. Nikles D, Vana K, Gauczynski S, Knetsch H, Ludewigs H, et al. (2008) Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins. Biochim Biophys Acta 1782: 335-340.

24. Vana K, Weiss S (2006) A trans-dominant negative 37kDa/67kDa laminin receptor mutant impairs PrP(Sc) propagation in scrapie-infected neuronal cells. J Mol Biol 358: 57-66.

25. von Arnim CA, Tangredi MM, Peltan ID, Lee BM, Irizarry MC, et al. (2004) Demonstration of BACE (beta-secretase) phosphorylation and its interaction with GGA1 in cells by fluorescence-lifetime imaging microscopy. J Cell Sci 117: 5437-5445.

26. Uemura K, Lill CM, Li X, Peters JA, Ivanov A, et al. (2009) Allosteric modulation of PS1/gamma-secretase conformation correlates with amyloid beta(42/40) ratio. PLoS One 4: e7893.

27. Heintzmann R, Cremer CG. Laterall modulated excitation microscopy: improvement of resolution by using a diffraction grating. In: Bigio IJ, Schneckenburger H, Slavik J, Svanberg K, Viallet PM, editors; 1999; Stockholm, Sweden. SPIE digital Library. pp. 185-196.

28. Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, et al. (2003) Reconstitution of gamma-secretase activity. Nat Cell Biol 5: 486-488.

29. Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, et al. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 398: 513-517.

30. Chyung JH, Raper DM, Selkoe DJ (2005) Gamma-secretase exists on the plasma membrane as an intact complex that accepts substrates and effects intramembrane cleavage. J Biol Chem 280: 4383-4392.

31. Vetrivel KS, Cheng H, Lin W, Sakurai T, Li T, et al. (2004) Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J Biol Chem 279: 44945-44954.

32. Shmakov AN, Bode J, Kilshaw PJ, Ghosh S (2000) Diverse patterns of expression of the 67-kD laminin receptor in human small intestinal mucosa: potential binding sites for prion proteins? J Pathol 191: 318-322.

33. Schroeter EH, Ilagan MX, Brunkan AL, Hecimovic S, Li YM, et al. (2003) A presenilin dimer at the core of the gamma-secretase enzyme: insights from parallel analysis of Notch 1 and APP proteolysis. Proc Natl Acad Sci U S A 100: 13075-13080.

34. Herl L, Lleo A, Thomas AV, Nyborg AC, Jansen K, et al. (2006) Detection of presenilin-1 homodimer formation in intact cells using fluorescent lifetime imaging microscopy. Biochem Biophys Res Commun 340: 668-674.

35. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286: 735-741.

36. Parkin ET, Watt NT, Hussain I, Eckman EA, Eckman CB, et al. (2007) Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. Proc Natl Acad Sci U S A 104: 11062-11067.

37. Griffiths HH, Whitehouse IJ, Baybutt H, Brown D, Kellett KA, et al. (2011) Prion protein interacts with BACE1 protein and differentially regulates its activity toward wild type and Swedish mutant amyloid precursor protein. J Biol Chem 286: 33489-33500.

38. Morel E, Andrieu T, Casagrande F, Gauczynski S, Weiss S, et al. (2005) Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. Am J Pathol 167: 1033-1042.

39. Leucht C, Simoneau S, Rey C, Vana K, Rieger R, et al. (2003) The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. EMBO Rep 4: 290-295.

40. Hundt C, Peyrin JM, Haik S, Gauczynski S, Leucht C, et al. (2001) Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. EMBO J 20: 5876-5886.

41. Scholefield Z, Yates EA, Wayne G, Amour A, McDowell W, et al. (2003) Heparan sulfate regulates amyloid precursor protein processing by BACE1, the Alzheimer's beta-secretase. J Cell Biol 163: 97-107.

42. Patey SJ, Yates EA, Turnbull JE (2005) Novel heparan sulphate analogues: inhibition of beta-secretase cleavage of amyloid precursor protein. Biochem Soc Trans 33: 1116-1118.

43. Beckman M, Holsinger RM, Small DH (2006) Heparin activates beta-secretase (BACE1) of Alzheimer's disease and increases autocatalysis of the enzyme. Biochemistry 45: 6703-6714.

44. Schneider A, Rajendran L, Honsho M, Gralle M, Donnert G, et al. (2008) Flotillin-dependent clustering of the amyloid precursor protein regulates its endocytosis and amyloidogenic processing in neurons. J Neurosci 28: 2874-2882.

45. Ehehalt R, Keller P, Haass C, Thiele C, Simons K (2003) Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol 160: 113-123.

46. Patra SK, Rizzi F, Silva A, Rugina DO, Bettuzzi S (2008) Molecular targets of (-)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts. J Physiol Pharmacol 59 Suppl 9: 217-235.

Figure Legends

Figure1. LRP/LR interacts with PS1 of the γ-secretase complex. (A) Co-localization between PS1-GFP and LRP-dsRed is shown in the maximum intensity profile for PS1-GFP and LRP-dsRed. The 2D cytofluorogram confirms co-localization as a diagonal is observed passing through quadrant 3. Line profile reveals aligned spectra suggestive of high degree of co-localisation. (B) SR-SIM reveals that PS1-GFP and LRPdsRed show a high degree of co-localisation. A diagonal passes through quadrant 3 of the 2D cytofluorogram suggesting that there is still a high degree of co-localisation between the 2 proteins. Scale bar: 5μm. (C)(i) FRET analysis with PS1-GFP donor and LRP-dsRed acceptor. Distinct FRET signal is detected on the normalized FRET scale. Scale bar: 20μm. (ii) Enlarged view of single cell from (i) showing that energy is transferred in the cytoplasmic and plasma membrane regions of the cell. (iii) Enlarged veiw from cell in (i) showing FRET signal occurs in perinuclear membrane. (D) FLAG® co-immunoprecipitation assay was performed utilizing lysates of cells transfected with LRP::FLAG and PS1-GFP. Immunoblot performed using rabbit monoclonal antibody (EP2000Y) for Presenillin 1. Lane (1) Non transfected cell lysates, (2) cell lysates expressing GFP alone, (3) cell lysates expressing PS1-GFP, (4) FLAG® co-immunoprecipitation eluate of assay performed using cell lysates expressing LRP::FLAG and PS1-GFP. Detected bands are 36kDa indicating PS1 dimers and not PS1-GFP.

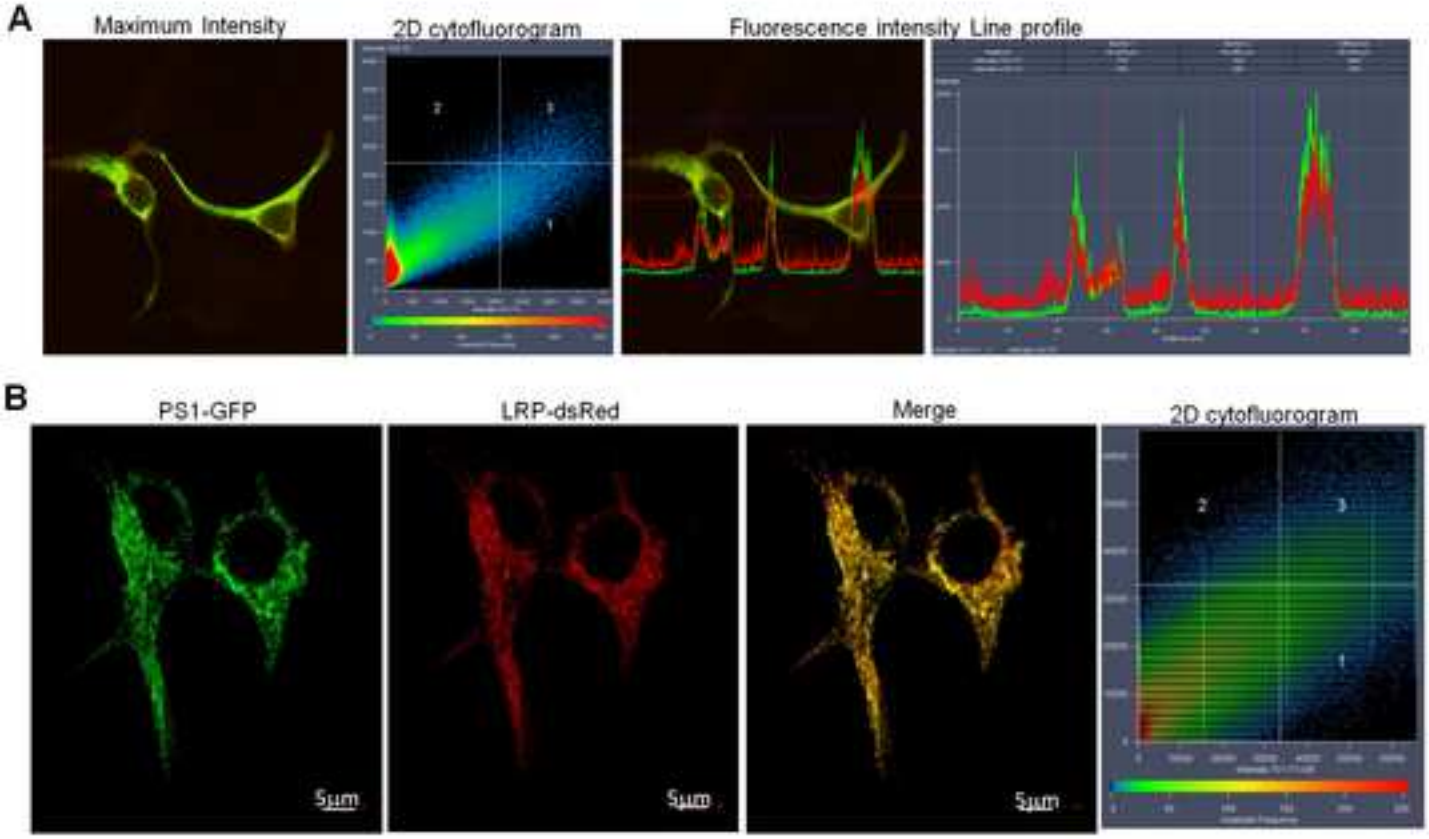
Figure 2. β-secretase fails to co-localize and interact with LRP/LR at high resolutions (A) Maximum intensity profile obtained from z-stack analysis reveals strong cytoplasmic co-localisation between BACE1-GFP and LRP-dsRed. Diagonal in 2D cytofluorogram indicates high degree of co-localization, as is confirmed by the line profile. (B) SR-SIM analysis of HEK293 cell expressing BACE1-GFP and

LRP-dsRed. No co-localisation occurs between these proteins. The 2D cytofluorogram confirms the absence of co-localisation as there is no signal detected in quadrant 3. Scale bar: 5μm (C) FRET did not occur between the donor BACE1-GFP and the acceptor LRP-dsRed as no signal was detected on the normalized FRET scale. Scale bar: 20μm.

Figure 3. Co-localization occurs between APP and LRP/LR without the presence of an interaction. (A) Maximum intensity profile obtained from Z-stack analysis revealing co-localisation between APP-GFP and LRP-dsRed in the cytoplasm of HEK293 cells. 2D cytofluorogram reveals the co-distribution of green (APP-GFP) and red (LRP-dsRed) pixels. A diagonal is indicative of a high degree of co-localization. Line profile displaying the fluorescence intensities of the 2 colour channels along a line of interest and reveals co-localisation occuring specifically within the cytoplasmic regions. (B) SR-SIM analysis reveals that APP-GFP and LRP-dsRed co-localise to a lesser extent. Co-localisation is highest at the cell surface and in limited cytoplasmic locations. Scale bar: 10μm. (C) FRET was performed using APP-GFP as donor and LRP-dsRed as acceptor. No FRET signal was observed on the normalized FRET scale indicating that no energy transfer occurred from BACE1-GFP to LRPdsRed. Scale bar: 20μm. (D) FLAG® co-immunoprecipitation analysis using lysates of cells expressing LRP::FLAG and APPwt. Anti-APP antibody (Y188) was utilised for detection. Lane (1) cell lysates containing with LRP::FLAG, (2) cell lysate with overexpressed APPwt (APP positive control), (3) FLAG® co-immunoprecipitation assay eluate of assay performed using APPwt overexpressing cell lysates but no LRP::FLAG (i.e. Anti-FLAG M2 bead binding control), (4) BAP fusion protein (positive control for FLAG® co-immunoprecipitation assay), (5) empty lane, (6) FLAG® co-immunoprecipitation assay eluate of sample with LRP::FLAG and APPwt, (7 – 9) Wash fractions 1 - 3 of APPwt + LRP::FLAG FLAG® co-immunoprecipitation assay post overnight binding step.

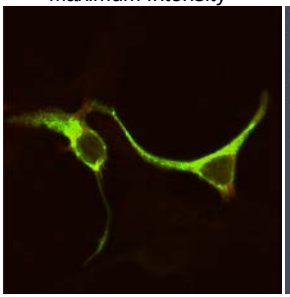
Figure 4. LRP/LR fails to co-localize with GFP. (A) Maximum intensity projection obtained from z-stack analysis of GFP and LRP-dsRed shows poor co-localisation between the 2 proteins. This is further confirmed by the lack of a clear diagonal passing through quadrant 3 and the line profile which shows no alignment between the 2 colour channels

Figure 1 A and B
Click here to download high resolution image

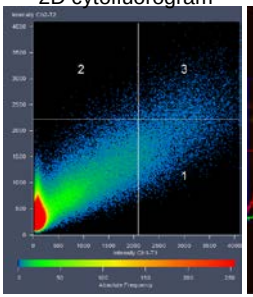


A

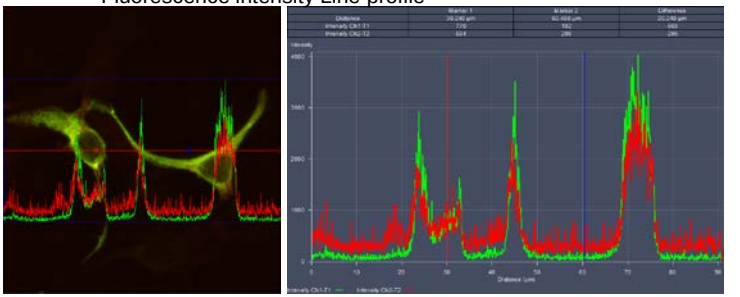
Maximum Intensity



2D cytofluorogram

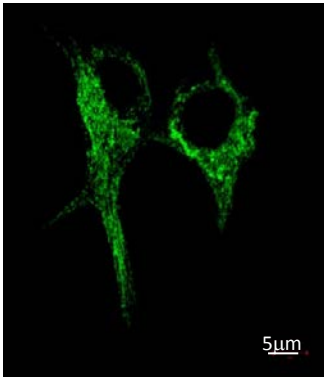


Fluorescence intensity Line profile



B

PS1-GFP



LRP-dsRed

Merge

2D cytofluorogram

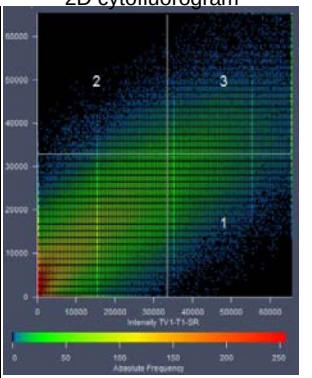
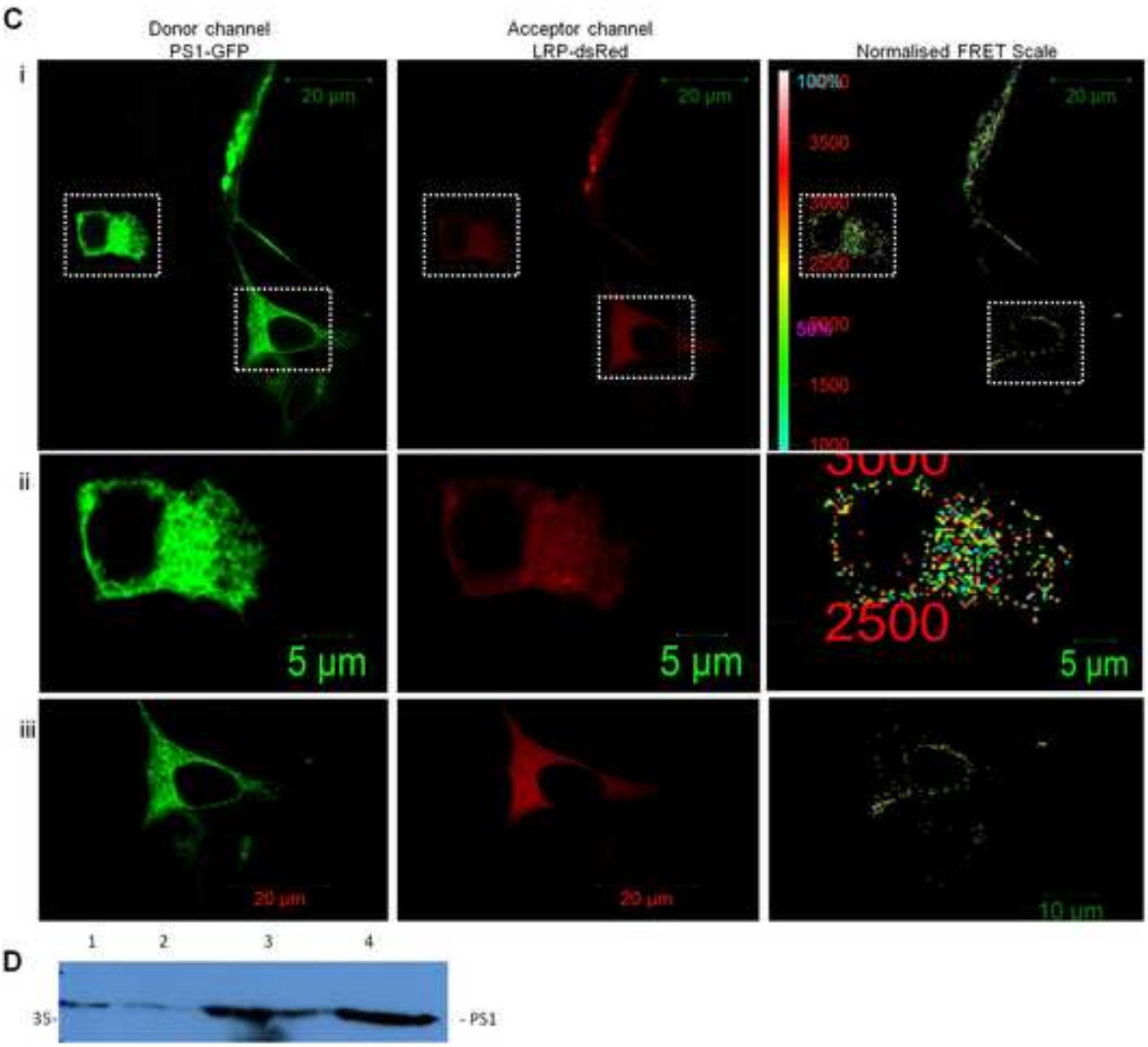


Figure 1 C and D
Click here to download high resolution image



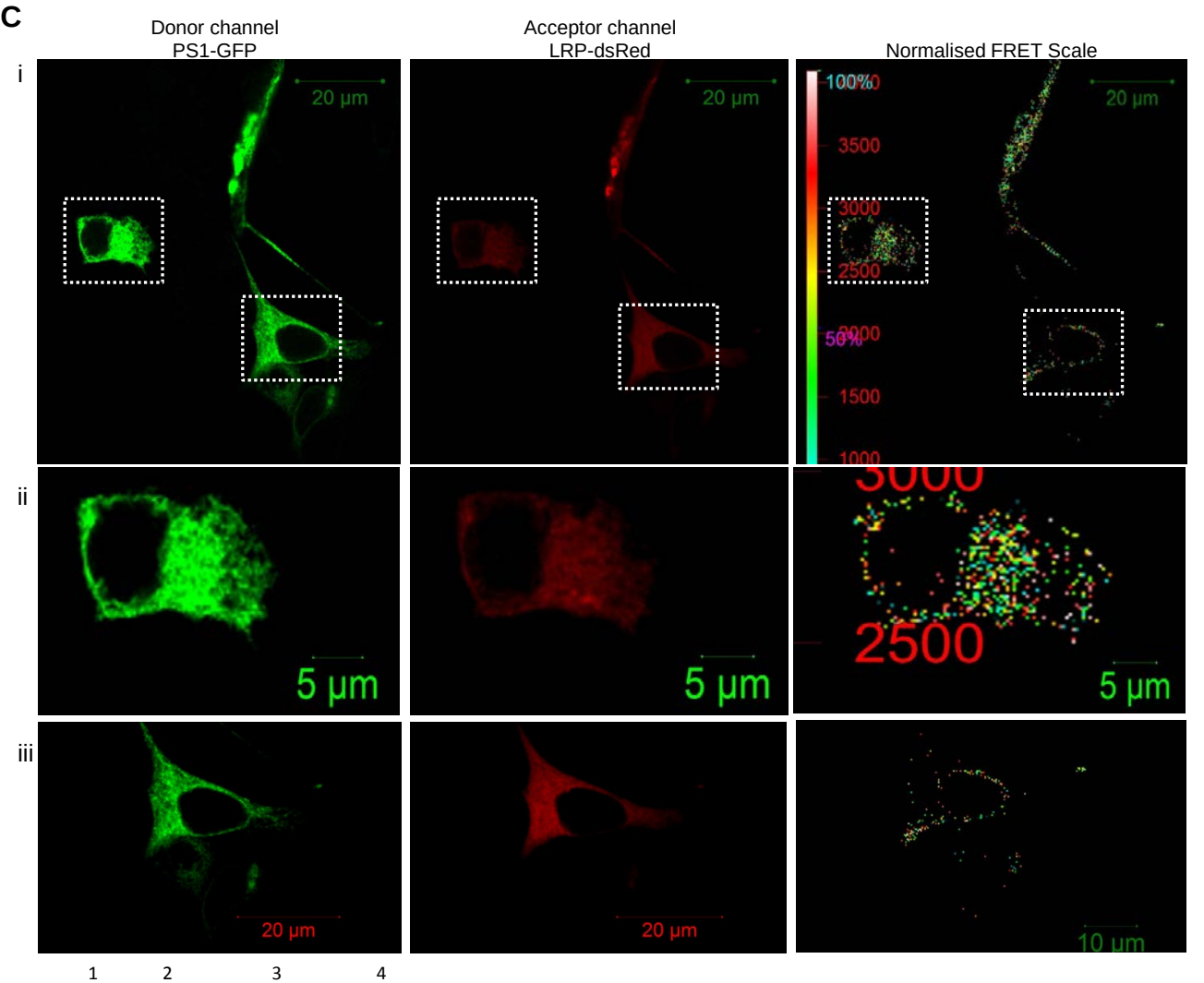
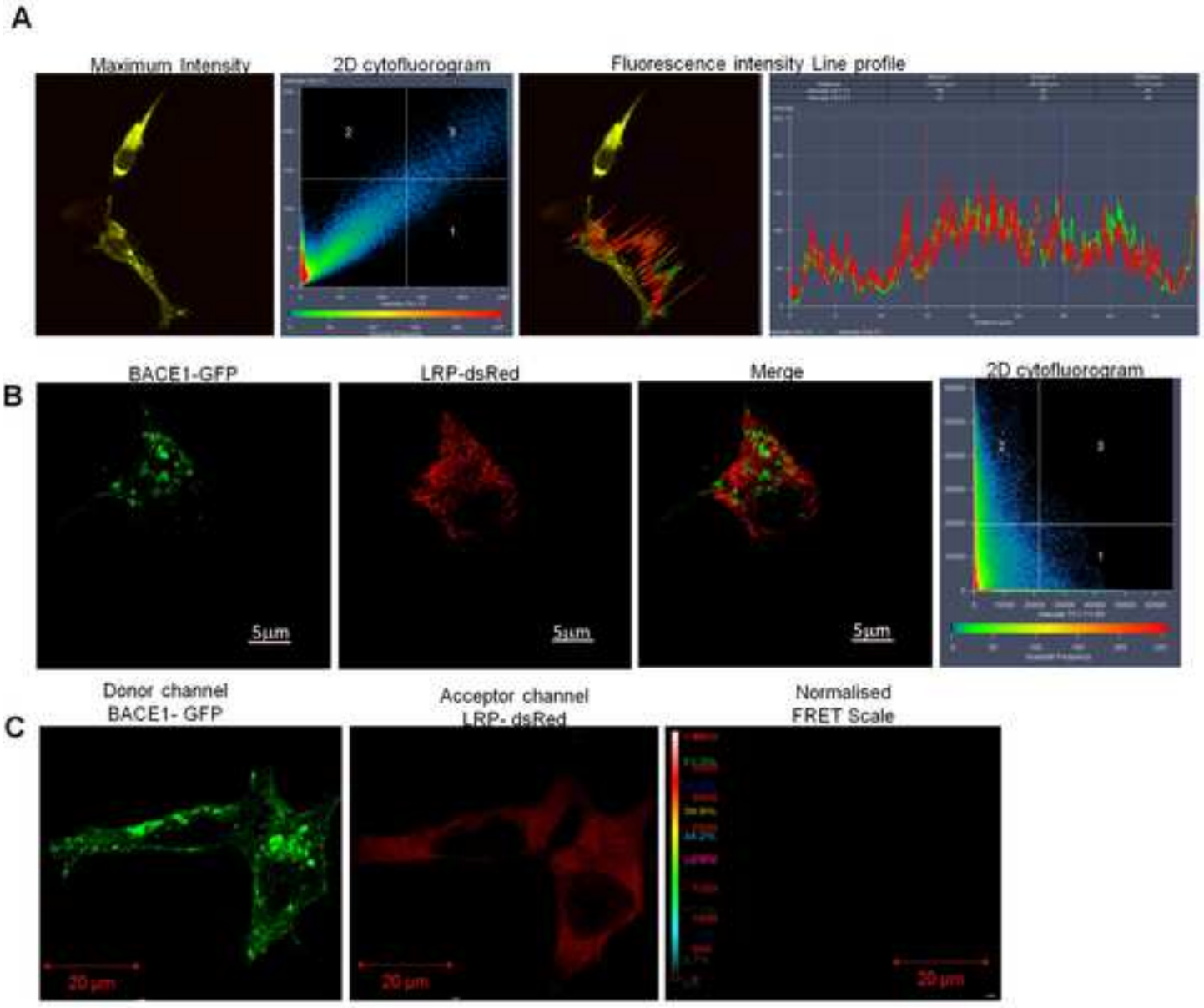
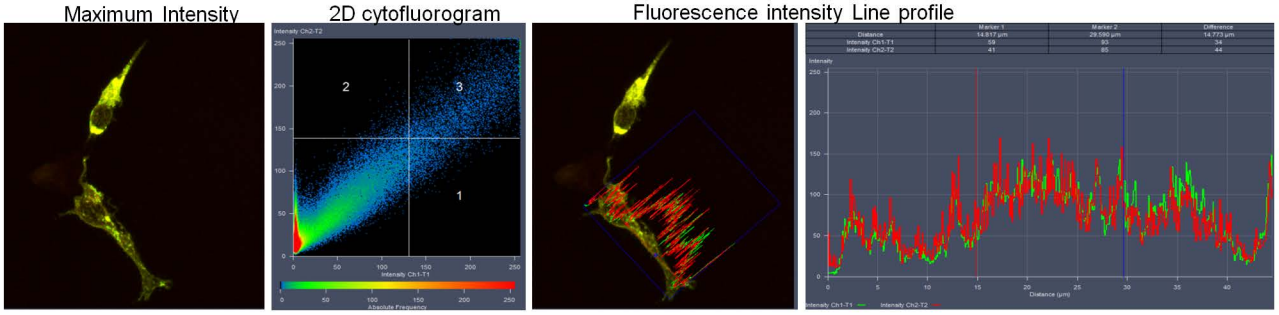

C
Donor channel
PS1-GFP
Acceptor channel
LRP-dsRed
Normalised FRET Scale
i
ii
iii
20 µm
5 µm
100%
3500
3000
2500
2000
1500
1000
50%
2500
1 2 3 4
D
40-
35-

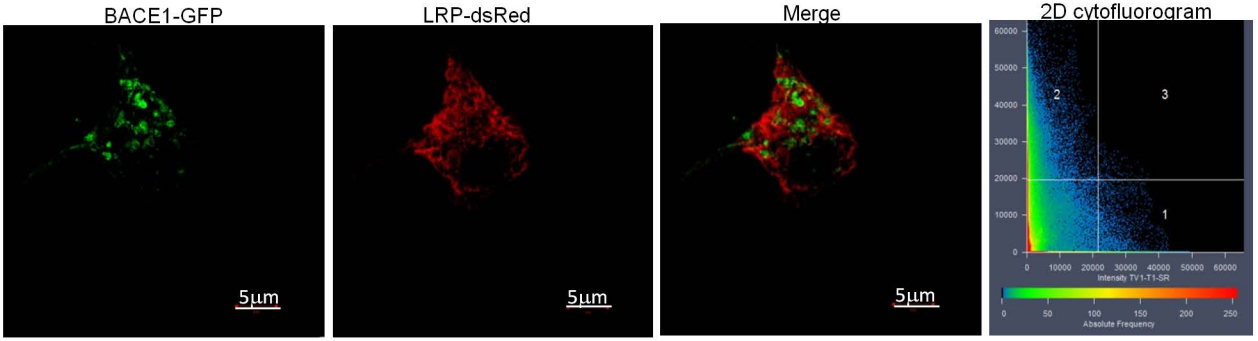
Figure 2
Click here to download high resolution image



A


Maximum Intensity
2D cytofluorogram
Fluorescence intensity Line profile
2
3
1
Intensity Ch1-T1
Absolute Frequency
Distance (µm)

B


BACE1-GFP
LRP-dsRed
Merge
2D cytofluorogram
5µm
5µm
5µm
2
3
1
Intensity TV1-T1-SR
Absolute Frequency

C

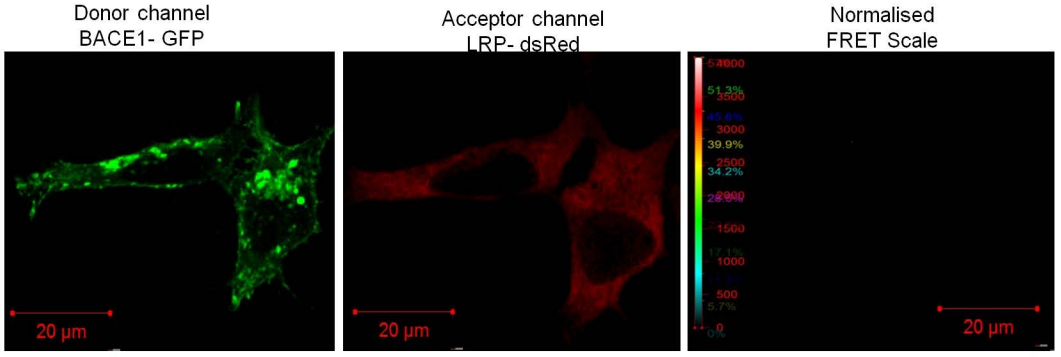
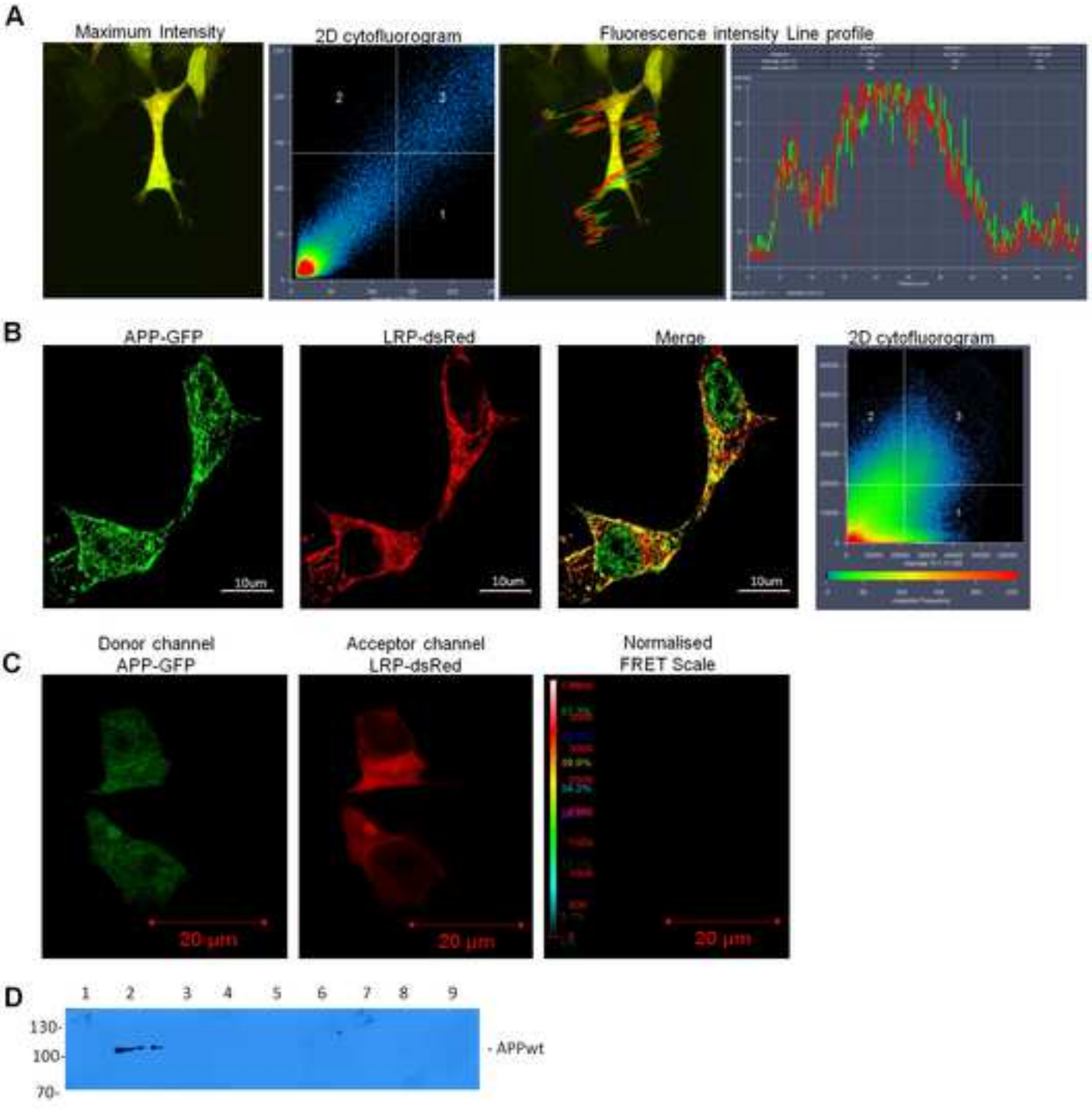

Donor channel
BACE1- GFP
Acceptor channel
LRP- dsRed
Normalised
FRET Scale
20 µm
20 µm
20 µm

Figure 3
Click here to download high resolution image



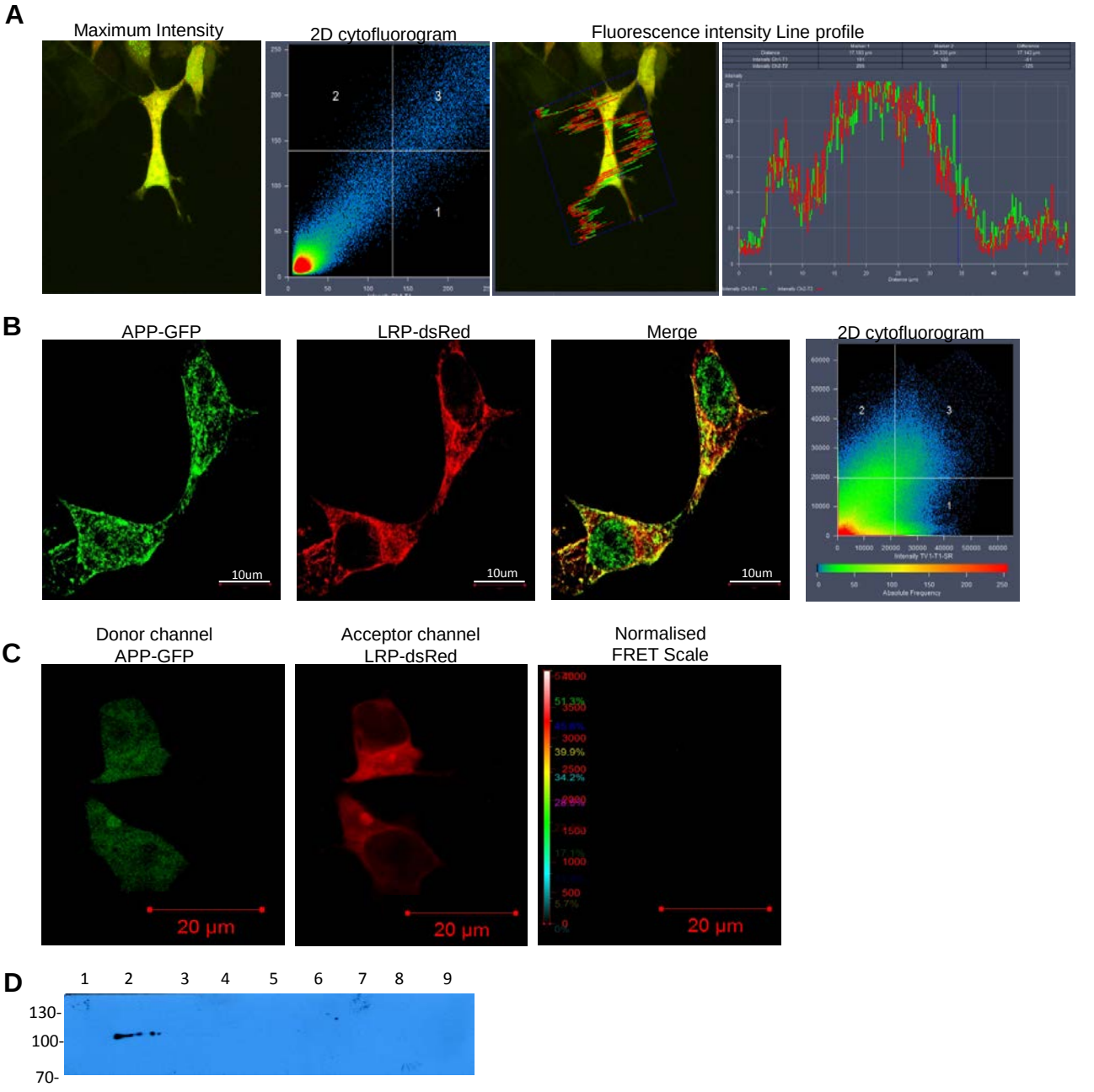
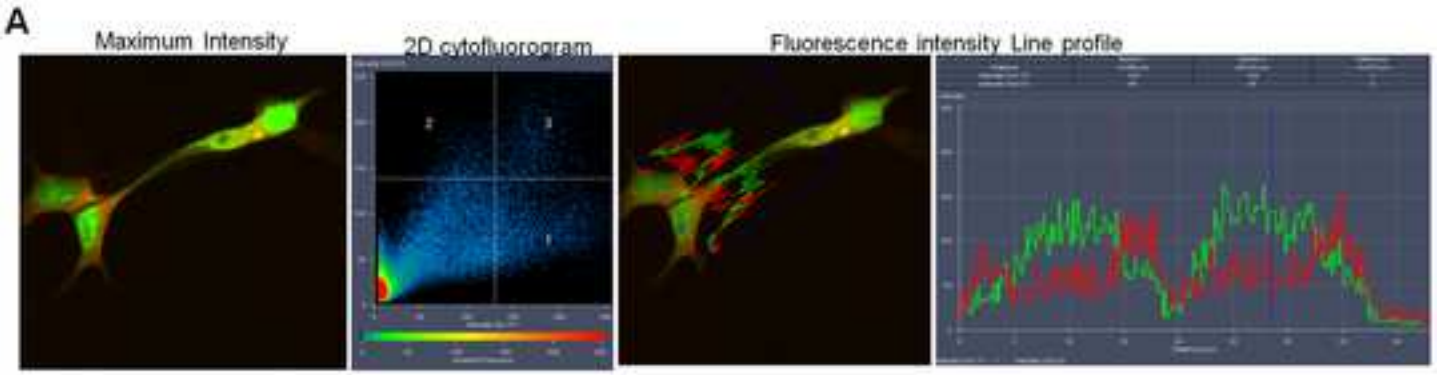
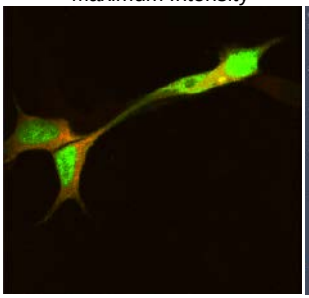

A
Maximum Intensity
2D cytofluorogram
Fluorescence intensity Line profile
2
3
1
B
APP-GFP
LRP-dsRed
Merge
2D cytofluorogram
10um
10um
10um
2
3
1
C
Donor channel
APP-GFP
Acceptor channel
LRP-dsRed
Normalised
FRET Scale
20 µm
20 µm
20 µm
51.3%
39.9%
34.2%
D
1 2 3 4 5 6 7 8 9
130-
100-
70-

Figure 4
Click here to download high resolution image

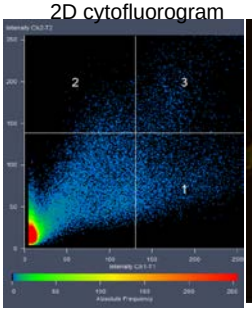


A

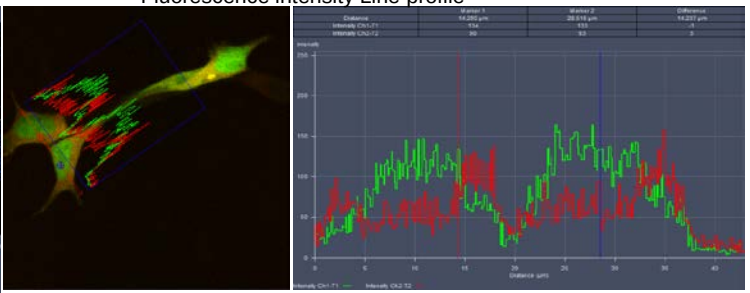
Maximum Intensity



2D cytofluorogram



Fluorescence intensity Line profile



Supporting Information

Click here to download Supporting Information: Supplementary information.docx

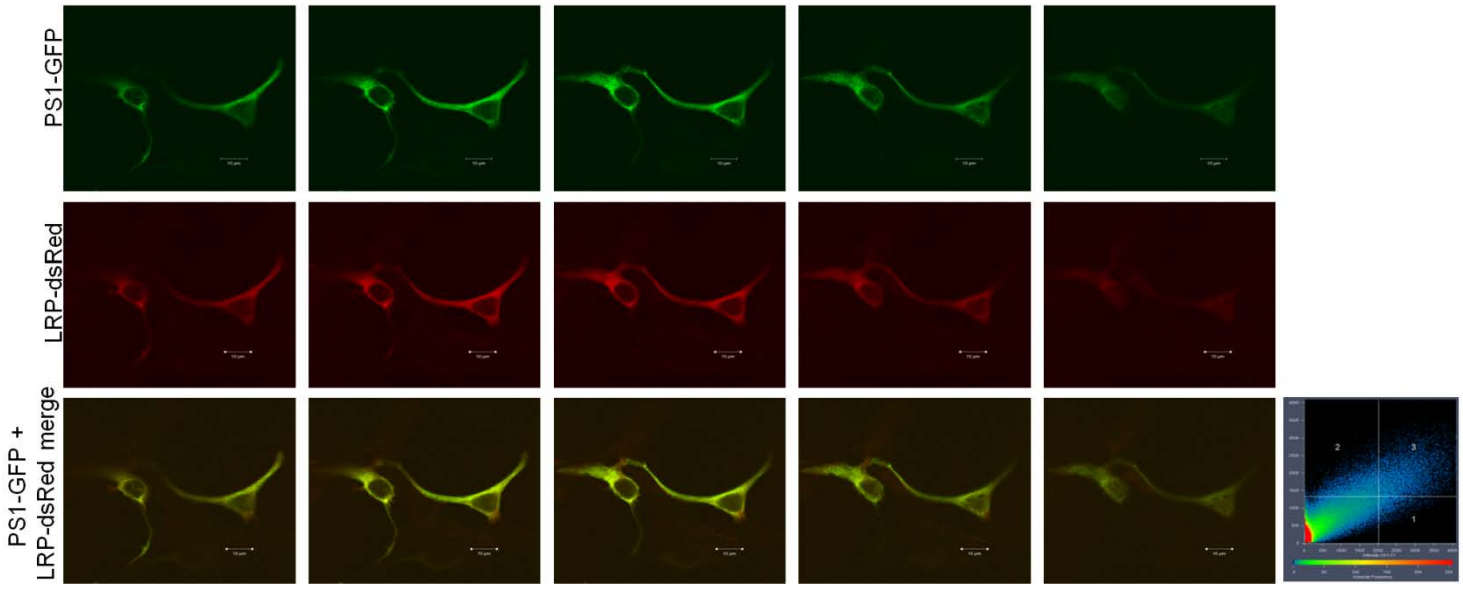


Figure S1. LRP-dsRed and PS1-GFP co-localise in the cytoplasmic and cell membrane surface of HEK293 cells. Z-stack analysis of images captured using HEK293 cells expressing PS1-GFP (top row), LRP-dsRed (middle row) and merge of both proteins (bottom row). The merge and 2D cytofluorogram both reveal a high degree of co-localisation between LRP-dsRed and PS1-GFP particularly within the cytoplasmic and plasma membrane region of the cells. A clear diagonal passes through quadrant 3 of the 2D cytofluorogram confirms the co-localization. Sections: 1.1μm; scale bars: 10μm.

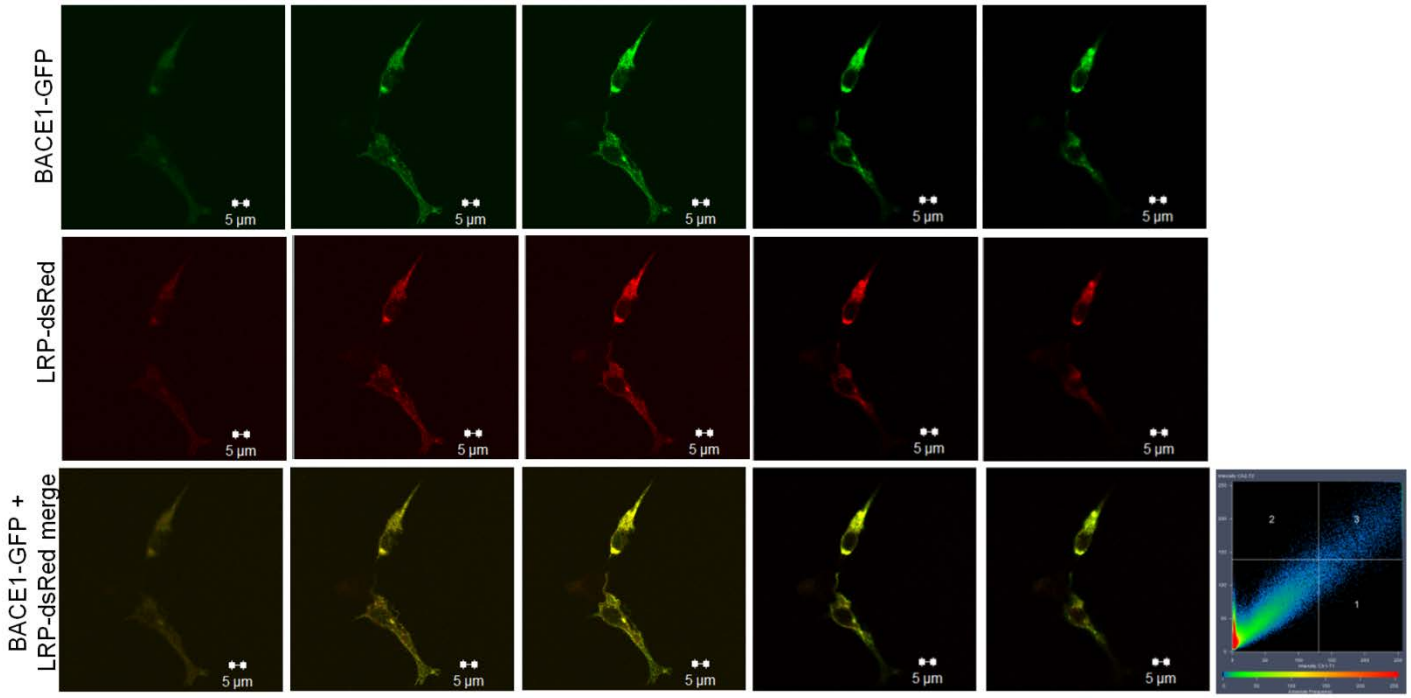


Figure S2. LRP-dsRed and BACE1-GFP co-localise in the cytoplasm of HEK293 cells. z-stack analysis revealing signal distribution of BACE1-GFP (top row), LRP-dsRed (centre row) and the merge of both proteins (bottom row). Distinct co-localisation exists between LRP-dsRed and BACE1-GFP within cytoplasmic regions of HEK293 cells. 2D cytofluorogram reveals a weak diagonal through quadrant 3 suggesting that co-localisation does occur but to a lesser degree. Sections: 0.39µm; scale bars: 5µm.

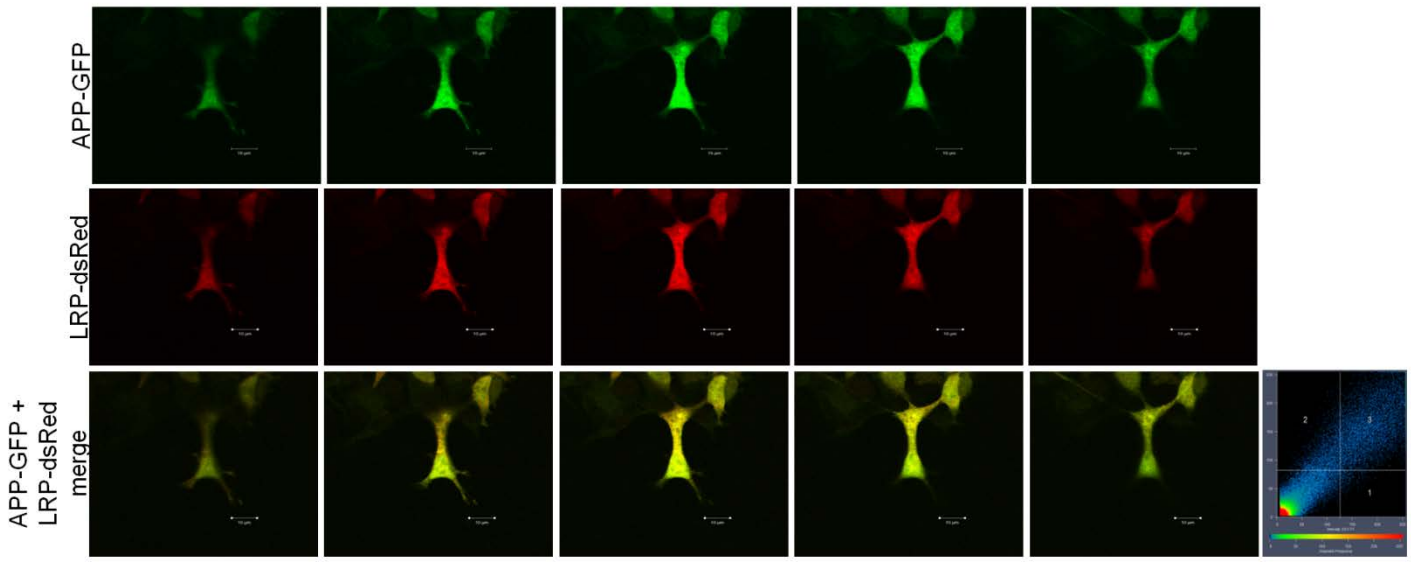


Figure S3. LRP-dsRed and APP-GFP co-localise in the cytoplasm of HEK293 cells. Z-stack image analysis representing subcellular localisations of APP-GFP (top row) and LRP-dsRed (middle row). Merged image (bottom row) shows APP-GFP and LRP-dsRed co-localising in the cytoplasmic regions. 2D-cytofluorogram confirms this result as is indicated by a diagonal passing through quadrant 3. Sections: 1μm; scale bar: 10μm.

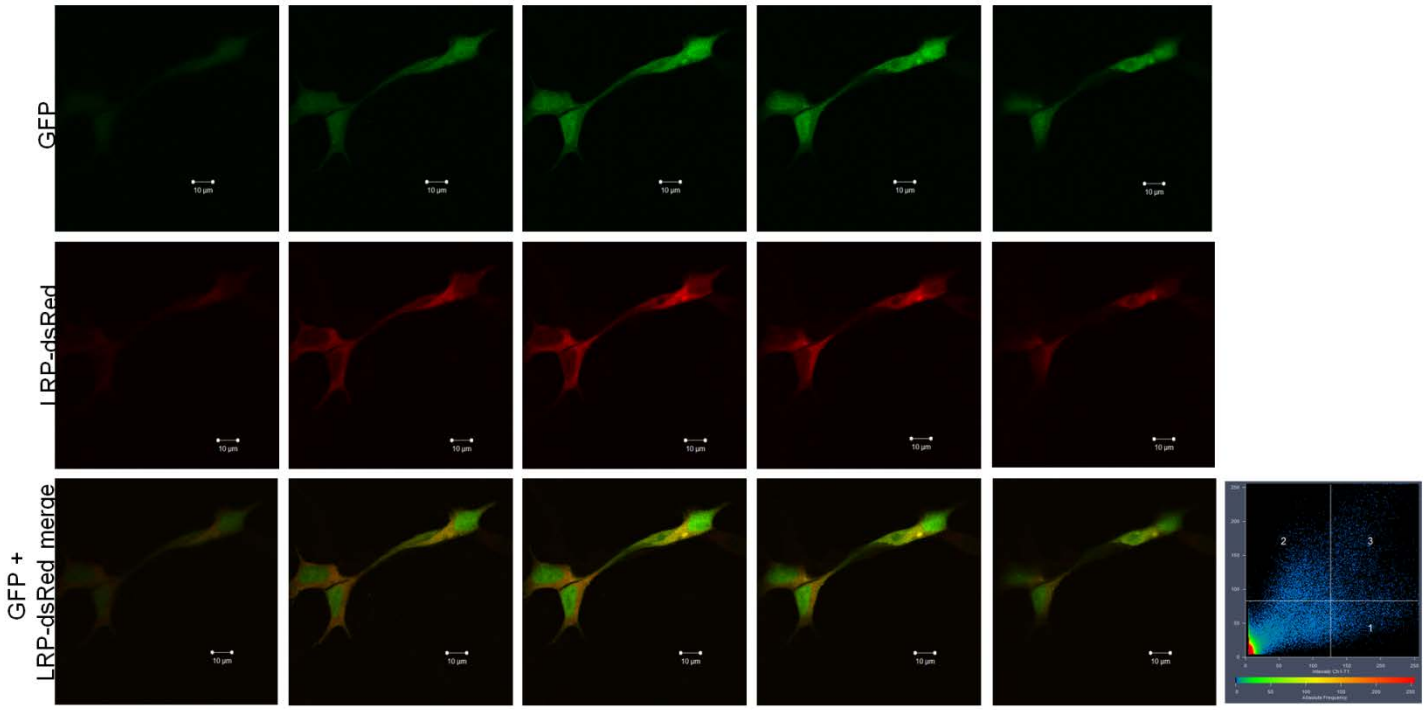


Figure S4. LRP-dsRed and GFP fails to co-localise. HEK293 cells were transfected with either pEGFPN1, pLRP-dsRed or both plasmids. Z-stack analysis was then performed to analyse whether LRP-dsRed co-localized with only GFP. Images show that the 2 proteins fail to co-localise as there is a weak yellow signal in the merge images. The 2D-cytofluorogram shows weak co-localisation as most signals occur in quadrants 1 and 2 (representing the green and red fluorescent channels). Very little signal is detected in quadrant 3 (the quadrant representing the yellow from the overlap of green and red fluorescence). Sections: 1µm; scale bars: 10µm.

Anti-LRP/LR specific antibody IgG1-iS18 rescues cells from Aβ42 induced cytotoxicity

Bianca Da Costa Dias, Katarina Jovanovic, Danielle Gonsalves, Uwe Reusch, Stefan Knackmuss, Clement Penny, Melvyn Little & Stefan Weiss

Scientific Reports 3, 2702; DOI:10.1038/srep02702 (2013).

A study was undertaken to examine whether an interaction existed between LRP/LR and the Aβ peptide, and furthermore to understand the nature of this interaction. FLAG® co-immunoprecipitation revealed that an interaction does exist between Aβ and LRP/LR. Furthermore, the nature of this interaction was shown to be cytotoxic as exogenous addition of Aβ resulted in a reduction in cell viability. Cells were rescued from cytotoxicity upon addition of IgG1-iS18 suggesting that LRP/LR plays a role in Aβ induced cytotoxicity. This was confirmed by shRNA knockdown of LRP/LR where the same cell rescuing effects were seen. These findings show that LRP/LR not only interacts with Aβ, but also suggest that this interaction may be of a cytotoxic nature. The results presented herein further recommend anti-LRP/LR antibodies and shRNA as therapeutics for AD.

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
MOLECULAR NEUROSCIENCE
MECHANISMS OF DISEASE

Received
22 July 2013

Accepted
3 September 2013

Published
19 September 2013

Correspondence and requests for materials should be addressed to S.F.T.W. (stefan.weiss@wits.ac.za)

Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity

Bianca Da Costa Dias[1], Katarina Jovanovic[1], Danielle Gonsalves[1], Kiashanee Moodley[1], Uwe Reusch[2], Stefan Knackmuss[2], Clement Penny[3], Marc S. Weinberg[4], Melvyn Little[2] & Stefan F. T. Weiss[1]

[1]School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa, [2]Affimed Therapeutics AG, Technologiepark, Im Neuenheimer Feld 582, 69120 Heidelberg, Germany, [3]Department of Internal Medicine, University of the Witwatersrand, 7 York Rd, Johannesburg, 2193 Parktown, Republic of South Africa, [4]Antiviral Gene Therapy Research Unit (AGTRU), Department of Molecular Medicine & Haematology, School of Pathology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa.

Alzheimer's disease (AD) is characterized by neurofibrillary tangles, senile plaques and neuronal loss. Amyloid beta (Aβ) is proposed to elicit neuronal loss through cell surface receptors. As Aβ shares common binding partners with the 37 kDa/67 kDa laminin receptor (LRP/LR), we investigated whether these proteins interact and the pathological significance of this association. An LRP/LR-$A\beta_{42}$ interaction was assessed by immunofluorescence microscopy and pull down assays. The cell biological effects were investigated by 3-(4,5-Dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide and Bromodeoxyuridine assays. LRP/LR and $A\beta_{42}$ co-localised on the cell surface and formed immobilized complexes suggesting an interaction. Antibody blockade by IgG1-iS18 and shRNA mediated down regulation of LRP/LR significantly enhanced cell viability and proliferation in cells co-treated with $A\beta_{42}$ when compared to cells incubated with $A\beta_{42}$ only. Results suggest that LRP/LR is implicated in $A\beta_{42}$ mediated cytotoxicity and that anti-LRP/LR specific antibodies and shRNAs may serve as potential therapeutic tools for AD.

Neurodegenerative diseases represent the fourth major cause of global mortality after ischaemic heart disease, cerebrovascular disease and trachea, bronchus and lung cancers. Alzheimer's Disease (AD) is the predominant progressive dementing neurodegenerative disorder afflicting the elderly[1] and is characterized by "positive" and "negative" lesions including amyloid beta plaques, neurofibrillary tangles and neuronal, neuropil and synaptic loss respectively[2,3]. Many of the neuronal perturbations in AD are attributable to and probably induced by the amyloid beta (Aβ) peptide[2]. The Aβ fragment is derived from the transmembrane region of the Amyloid Precursor Protein (APP). Although Aβ is a normal physiological peptide, elevated concentrations of the peptide, which consequently results in the onslaught of AD, are generated either through the misappropriate favouring of the amyloidogenic processing of APP or a decline in Aβ clearance or degradation[4]. The amyloid plaques are predominantly composed of the $A\beta_{42}$ isoform which has a higher aggregation propensity[5] and neural toxicity[6] than the 40 amino acid isoform ($A\beta_{40}$) which predominates in non-diseased brains. However, the prevailing sentiment is that the plaques themselves are not the pathological agents but rather contribute to neural dysfunction through the distortion of neuronal morphology (within a 50 μm radius[7,8]) and by hampering neurotransmission[9]. Rather, it is the soluble Aβ oligomers which are deemed neurotoxic.

The proposed mechanisms whereby Aβ has been reported to impair neuronal function are numerous. A common thread in Aβ induced cytotoxicity and neuronal dysfunction is the requirement for an interaction between the neurotoxic peptide and cellular components, of greatest importance are the lipid membranes and cellular receptors[10].

Owing to the hydrophobic nature of the peptide, Aβ may readily associate with and be subsequently incorporated into plasma[11,12], nucleosomal and lysosomal membranes. This may result in membrane structure distortion and the formation of ion-permissible (of particular concern is Ca^{2+}) channels, the resultant ion influx may induce cytotoxicity[13,14].

Several of the factors thought to contribute to AD, namely oxidative stress, protein degradation, lipid oxidation and slowed signal transmission may be attributed to Aβ interaction with cell surface receptors[15–17]. These include, but are not limited to, N-methyl-D-aspartate receptors (NMDAR), integrins (particularly $\alpha_5\beta_1$), insulin receptors, α-7 nicotinic acetylcholine receptors (α7nAChR), the receptor for advanced glycation end products (RAGE), Ephrin-type B2 receptor (EphB2) and the cellular prion protein (PrP^c)[1,10]. Aβ may thwart NMAR activation and the resultant induction of long term potentiation (LTP) by desensitizing the receptor to synaptic glutamate[10,18] or by prompting receptor internalization[10]. This in turn results in aberrant signaling cascades and ultimately results in synaptic dysfunction and neuronal death.

Although the association between Aβ and PrP^c has been one of mounting interest over the past decade, its biological influence remains to be definitively characterized. It has been suggested that PrP^c plays a role in mediating the devastating effects of Aβ oligomers particularly neuronal and synaptic toxicity and LTP impedance[19] as well as stimulating pro-apoptotic signal transduction cascades[20]. On the contrary a neuroprotective role for PrP^c has been proposed as the protein was reported to hinder β-secretase cleavage of APP[21].

A receptor of noted physiological importance which binds to PrP^c and is implicated in PrP^c internalization is the 37 kDa/67 kDa laminin receptor (LRP/LR)[22]. This multifunctional protein is located in multiple cellular compartments namely the nucleus, cytosol and within the lipid raft domains of the plasma membrane[23,24]. LRP/LR exhibits binding affinities for a multitude of cellular components including: extracellular matrix (ECM) molecules, laminin-1 being of greatest physiological relevance with regard to cellular adhesion, survival and migration as well as cytoskeletal, ribosomal and histone proteins and PrP^c [23,24]. LRP/LR is also of pathological importance as the receptor has been shown to be central in prion protein uptake, propagation and progression of prion disorders[25–27]. Furthermore, LRP/LR plays a central role in metastatic cancer and antibodies targeting the receptor have been reported to significantly impede adhesion and invasion of numerous cancer types, namely fibrosarcoma[28], lung, cervical, colon, prostate[29], breast and oesophageal cancer[30] as well as inhibit *in vitro* angiogenesis[31].

As Aβ toxicity has been posited to be mediated through its association with the lipid raft region of the plasma membrane and its interactions with plasma membrane anchored proteins, and LRP/LR shares mutual binding partners with Aβ (laminin[32] and PrP^c), we aimed to examine whether LRP/LR and Aβ interact on the cell surface and to investigate whether LRP/LR plays a central role in Aβ induced cytotoxicity.

Results

LRP/LR co-localises with Aβ on the cell surface. Indirect immunofluorescence is regularly employed to provide a preliminarily indication of potential interactions at the cell surface[33,34]. Here too this methodology was employed to investigate whether endogenous LRP/LR and Aβ are located in close proximity on the cell surface, which would thereby indicate that an association between these proteins is conceivable. Co-localization of LRP/LR and Aβ was observed on the surface of non-permeabilized HEK293 and N2a cells (Fig. 1c and Fig. 1o, respectively). 2D cytofluorograms represent both green and red fluorescence and the resultant yellow diagonal (Fig. 1d and Fig. 1p) reveals that the fluorescence from both proteins is jointly distributed. These images, in addition to the highly positive Pearson's correlation coefficient (Table 1), verify that LRP/LR and Aβ co-localize on the cell surface. LRP/LR did not co-localize with the Very Late Antigen 6 (VLA6), a laminin binding integrin, (Fig. 1g and Fig. 1s). This was indicated by the 2D-cytofluorogram (Fig. 1h and Fig. 1t) as well as the very low Pearson's correlation coefficient (Table 1).VLA6 thereby served as the negative control[25]. Therefore, owing to the cell surface proximity of LRP/LR and Aβ, an association between these proteins is feasible.

Interaction of Aβ with LRP/LR. Although co-localisation studies between LRP/LR and Aβ proved the proximity of the proteins on the cell surface, this finding merely indicates that an interaction between these proteins is feasible. Therefore a pull down assay was performed to investigate definitively whether a stable interaction exists. Recombinantly expressed LRP::FLAG was immobilized on the anti-FLAG® M2 agarose beads, as highlighted by the red arrow (Fig. 2a and 2e) as it is present in the eluted sample. The identity of the band was further authenticated by immunoblotting (Fig. 2b). Co-incubation of anti-FLAG® M2 beads with LRP::FLAG containing cell lysate to which 100 ng/ml of synthetic $A\beta_{42}$ was applied resulted in the immobilization of both proteins. The presence of $A\beta_{42}$ in the eluted sample (Fig. 2a) was confirmed by equivalent polypeptide position in Fig. 2a lane 6 containing pure, synthetic $A\beta_{42}$ (2 μg). The presence of both proteins in eluted samples (Fig. 2a - lane 5) implies that an association exists. The relevant controls are shown in Fig. 2c–f.

IgG1-iS18 rescues cells from Aβ mediated cytotoxicity. A MTT cell viability assay was employed to assess the cytotoxicity of synthetic amyloid beta ($A\beta_{42}$) at various concentrations on HEK293FT, N2a and SHSY5Y cells (Fig. 3 a–c). Exogenous application of 200 nM and 500 nM $A\beta_{42}$ significantly reduced cell viability in HEK293 cells (Fig. 3a). Co-incubation of cells with 50 μg/ml anti-LRP/LR specific antibody IgG1-iS18 and 500 nM $A\beta_{42}$ significantly enhanced cell viability (Fig. 3a). Similar results, albeit at different $A\beta_{42}$ concentrations were observed for SH-SY5Y (Fig. 3b) and N2A (Fig. 3c) cells. The decrease in cell viability observed in N2a cells (Fig. 3c) was shown to be as a result of hampered cellular proliferation (Fig. 3d). Protocatechuic acid (PCA) an apoptosis inducing agent was employed, at a concentration of 8 mM, as the positive control. Antibody, IgG1-iS18, treatment alone in the absence of Aβ does not significantly enhance cellular viability in all the model cell lines employed (Fig. S1), thereby negating the possibility that IgG1-iS18 non-specifically enhances cellular viability.

To confirm that LRP/LR plays a role in Aβ toxicity, and that the IgG1-iS18 effects observed are not owing to the possible lack of antibody specificity, RNA interference technology and more specifically short hairpin RNAs (shRNAs) were employed to down regulate LRP/LR. When compared to the shRNAscr control, shRNA1.1 transfection resulted in a 20.52% reduction in LRP/LR expression, whilst shRNA7.6 transfection produced a significant 67.46% reduction in LRP/LR expression levels (Fig. 4a and 4b). LRP/LR down regulation (mediated by the aforementioned shRNAs), in the presence of varying concentrations of exogenously administered $A\beta_{42}$, resulted in a significant enhancement in cell viability (Fig. 4c) and cellular proliferation (Fig. 4d). These results are analogous to those obtained employing IgG1-iS18. No significant difference amongst untreated, mock transfected and shRNAscr transfected cells HEK293 cells was observed with regards to both cellular viability (Fig. S2a) and proliferation (Fig. S2b).

Discussion

LRP/LR and Aβ were demonstrated to share close cell surface proximity by indirect immunofluorescence microscopy (Fig. 1c and Fig. 1o) and these results were considered as a primary indication of a potential interaction between these proteins on the cell surface. However, supplementary systems are commonly required to verify the interaction proposed by immunofluorescence data.

In an attempt to confirm the proposed interaction between LRP/LR and the neurotoxic $A\beta_{42}$ peptide, as revealed by co-localization results, pull down assays were performed. The presence of both proteins in the eluted sample suggests that an association between

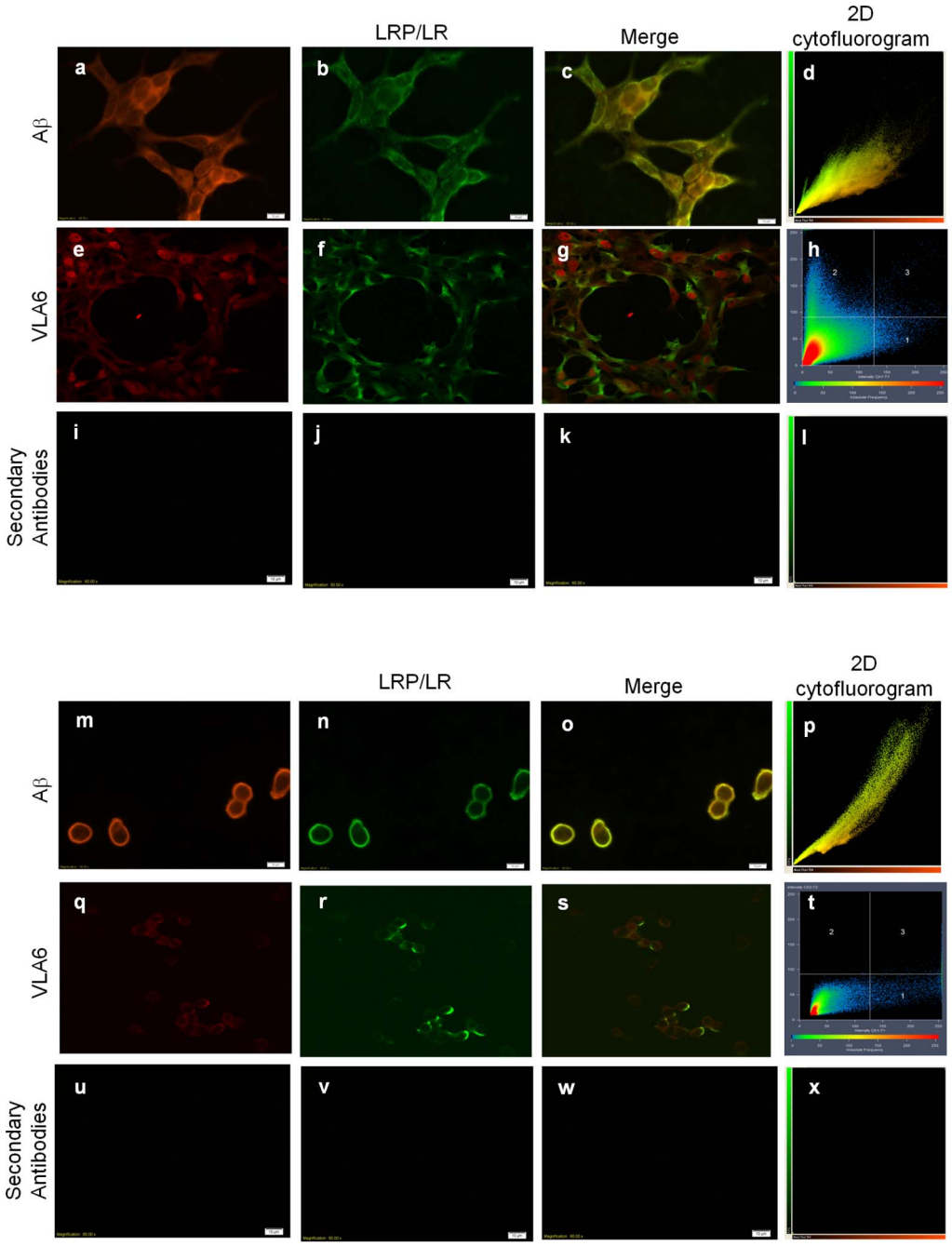


Figure 1 | Cell surface co-localisation between LRP/LR with Aβ. (a) Endogenous cell surface LRP/LR and Aβ on HEK293 (upper panel) and N2a (lower panel) cells were indirectly immunolabelled. Aβ was indirectly detected using anti-β-amyloid (22–35) (Sigma) and anti-rabbit Alexafluor 633 antibodies (Fig. 1a, m). LRP/LR was detected employing anti-IgG1-iS18 (human) and anti-human-FITC (Cell lab) antibodies (Fig. 1b, f and Fig. 1n, r). Merged images (Fig. 1c, o) and 2D-cytofluorograms (Fig. 1d, p) (acquired using CellSens Software) verified the co-localization. The negative control, Very Late Antigen 6 (VLA6) was detected employing anti-VLA6 and anti-rabbit Alexaflour 633 antibodies (Fig. 1e, q). The merged images (Fig. 1g, s) and 2D-cytofluorograms (Fig. 1h, t) demonstrated that VLA6 and LRP/LR do not co-localize on the cell surface. Secondary antibody controls are shown in Fig. 1i-l and Fig. 1u-x. Fluorescence was detected and resultant images acquired using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software. Scale bars are 10 μm.

Table 1 | Pearson's Correlation Co-efficient for LRP/LR, Aβ and VLA6 cell surface co-localization

	HEK293	N2a
LRP/LR + Aβ	0.926	0.969
LRP/LR + VLA6	0.30	0.12

LRP and $A\beta_{42}$ exists. However, the exclusivity of this interaction could not be verified owing to the presence of contaminant bands present within the eluted sample lane (Fig. 2a, lane 5). These polypeptides may represent numerous LRP ligands, possibly including laminin, PrP^{c}, actin, tubulin[35], heparin sulphate proteoglycans as well as ribosomal and histone components. The control shall be briefly discussed. Anti-FLAG® M2 agarose beads were subjected to incubation in the presence of lysis buffer (Fig. 2c) as well as cell-lysates

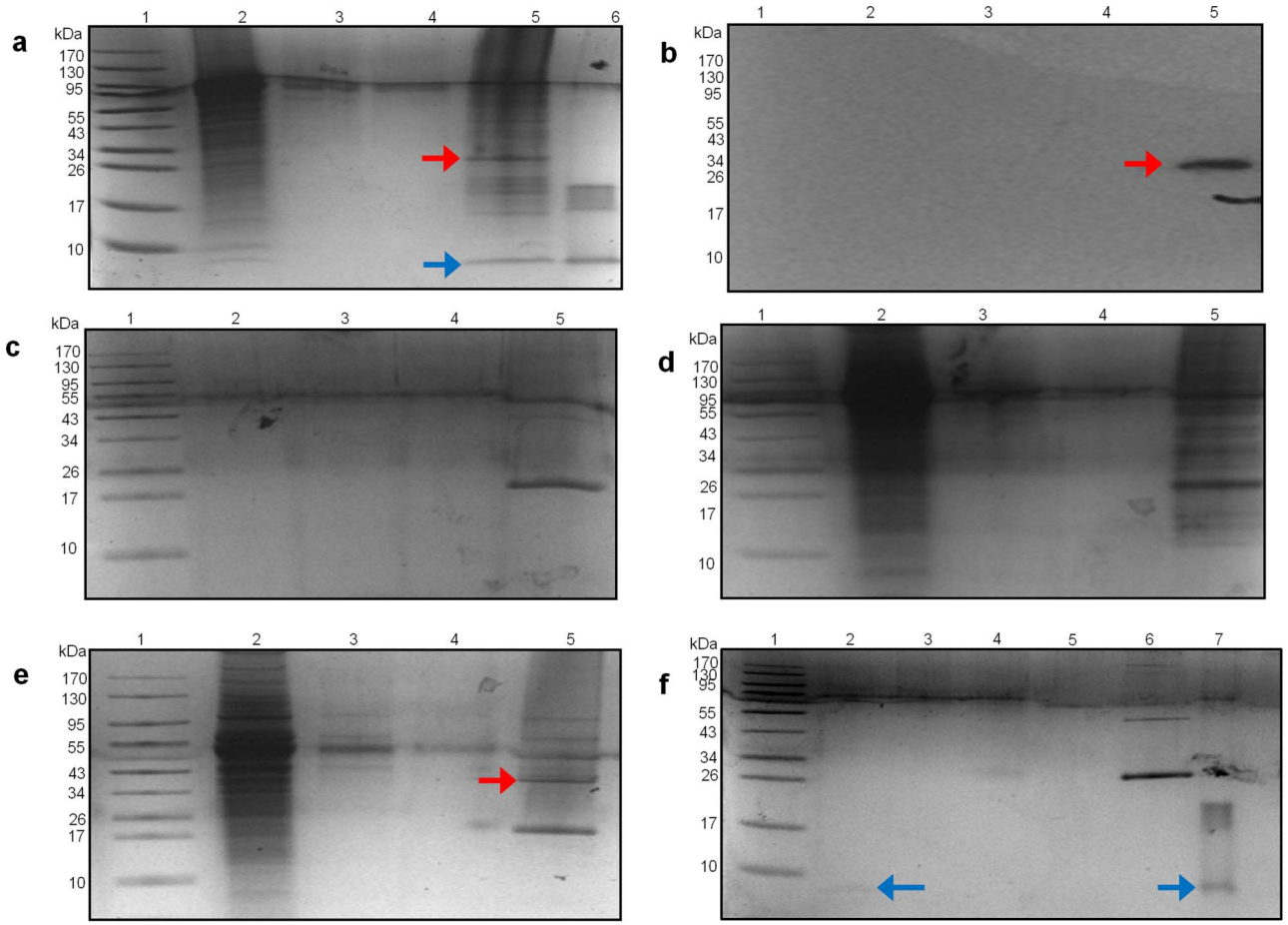


Figure 2 | LRP/LR as a potential Aβ-interacting protein. Pull down assays were employed using FLAG® Immunoprecipitation kit (Sigma Aldrich), to investigate the proteins detectable in unbound samples (lane 2), wash steps (lanes 3 and 4) and eluted samples (Fig. 2a–e: lane 5 and Fig. 2f: lane 6) and 2 μg of synthetic $A\beta_{42}$ (positive control) (Fig. 2a: lane 6 and Fig. 2f: lane 7). (a) Cell lysates containing recombinantly expressed LRP/LR::FLAG were co-incubated with exogenous Aβ. (b) Immunoblot employed to validate the position of LRP::FLAG (~38 kDa). Figures represent anti-FLAG® M2 beads incubated with (c) lysis buffer, (d) non-transfected HE293 cell lysates, (e) HEK293 cell lysates of cells transfected with pCIneo::FLAG as well as (f) pure synthetic $A\beta_{42}$ in the absence of cell lysate. Samples were resolved on 16% Tris-tricine SDS PAGE gels and stained with Coomassie Brilliant Blue. Blue and red arrows are indicative of $A\beta_{42}$ and LRP::FLAG respectively.

lacking recombinant LRP::FLAG expression (Fig. 2d). Furthermore, cell lysates in which LRP::FLAG was recombinantly expressed were analysed and column immobilization was confirmed (Fig. 2E). In addition, this control served to demonstrate the number of cellular components which were able to bind to LRP::FLAG (Fig. 2e). Fig. 2f, served to assess whether the "sticky" nature of $A\beta_{42}$ allowed it to bind to the affinity column in the absence of the tagged protein. Upon analysis of 10 μg of synthetic $A\beta_{42}$, the peptide was present in the unbound soluble fraction (Fig. 2f, lane 2) thereby illustrating that the presence of $A\beta_{42}$ in the eluted sample of the Fig. 2a, lane 5, was owing to an immobilizing interaction with LRP.

As an interaction between LRP/LR and $A\beta_{42}$ has been proposed an investigation into the influence of such an interaction on AD pathogenesis, specifically cellular survival, was justifiable. Significant reductions in cellular viability across all three cell lines were observed at varying concentrations of exogenously administered synthetic $A\beta_{42}$ (Fig. 3a–c). More notably, upon co-incubation of cells with the $A\beta_{42}$ peptide and anti-LRP/LR specific antibody IgG1-iS18, a significant enhancement in cell viability was observed (Fig. 3a–c). These results were further confirmed by shRNA mediated down regulation of LRP/LR (Fig. 4c), thereby demonstrating that the cell rescuing abilities of IgG1-iS18 are not owing to a lack of antibody specificity. Thus, it may be suggested that LRP/LR may be implicated in Aβ mediated cytotoxicity and the association between these proteins may be pathological in nature. It is plausible that this association may be pathological in nature as PrP^c has been reported to be important in mediating the synapotoxic effects of $A\beta$[19] and the neuroprotective role of PrP^c may be inhibited upon its binding to Aβ. Thus both PrP^c and its cell surface receptor LRP/LR[25] may be implicated in mediating this pathological role.

Furthermore, to assess whether the impediment of cellular proliferation contributed to reduced cell viability (Fig. 3a–c), the proliferative potential of N2a cells incubated with varying $A\beta_{42}$ concentrations was evaluated. Cellular proliferation was similarly hampered in the presence of $A\beta_{42}$ and IgG1-iS18 (Fig. 3d) rescued cells from this effect. This result was further corroborated by enhancement in cellular proliferation observed when Aβ was administered to cells in which LRP/LR was down regulated by shRNAs (Fig. 4d). Therefore, it may be proposed that the LRP/LR-$A\beta_{42}$ interaction may possibly result in aberrant proliferative cell signaling pathways. Under physiological conditions, LRP/LR promotes cellular survival, reported through the activation of the Mitogen activated protein (MAP) kinase signal transduction pathway[36]. It is plausible that an interaction between LRP/LR and $A\beta_{42}$ may foil the receptor mediated initiation of proliferative pathways.

In conclusion, it has been demonstrated that an LRP/LR-$A\beta_{42}$ interaction occurred on the cell surface and antibody blockade of LRP/LR by IgG1-iS18 or shRNA mediated down regulation of LRP/

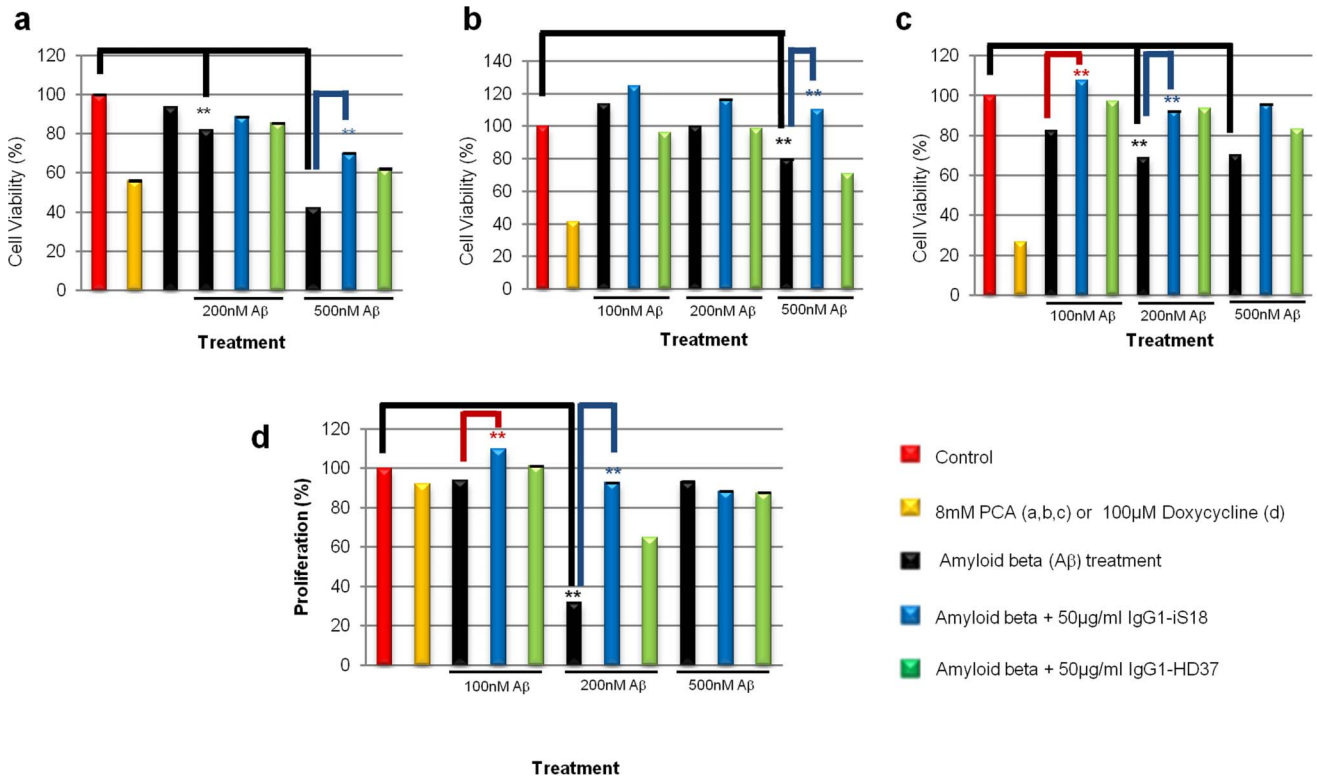


Figure 3 | Cell rescuing effects of anti-LRP/LR antibody IgG1-iS18. (a) Cellular viability of HEK293 cells, as determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) assay, post exogenous treatment with synthetic $A\beta_{42}$ and upon co-incubation with anti-LRP/LR IgG1-iS18 or IgG1-HD37 (negative control). The cell viability was assessed 48 h post treatment and the no antibody control was set to 100%. SH-SY5Y (b) and N2a cells (c) were exposed to similar treatments. (d) Cellular proliferation of N2a cells as determined by colorimetric 5-bromo-2′-deoxyuridine (BrdU) non-isotopic immunoassay (Calbiochem®), allowing 4 h for BrdU incorporation into cultured cells. Error bars represent sd. **$p < 0.01$; Student's *t*-test.

LR rescued cells from $A\beta_{42}$ induced cytotoxicity and impedance of proliferation. These results suggest that LRP/LR may contribute to $A\beta_{42}$ mediated pathogenesis in AD and that anti-LRP/LR specific antibodies and shRNAs directed against the receptor mRNA may show promise in the quest for effective AD disease-modulating therapeutics.

Methods

Immunofluorescence Microscopy. HEK239FT and N2a cells were seeded onto microscope coverslips and incubated until a confluency of 50–70% was attained. The cells were subsequently fixed with 4% Paraformaldehyde (10 minutes, room temperature), rinsed thrice with 1xPBS and blocked in 0.5%PBS-BSA (5–10 minutes). Post blocking, coverslips were additionally washed in PBS and placed such that the cell-free side came into contact with the microscope slide. 100 μl of primary antibody solution (diluted in 0.05%PBS-BSA) containing 1 : 150 IgG1-iS18 (human), 1 : 150 anti-VLA6 or 1 : 100 anti-β-amyloid (22–35) (rabbit (Sigma) was administered to the cells. Post an overnight incubation at 4°C in moist containers, coverslips were again washed thrice in 0.5% PBS-BSA and placed on clean slides. A 100 μl volume of a secondary antibody solution containing1 : 300 goat anti-human FITC (Cell Lab) and 1 : 300 goat anti-rabbit IgG conjugated to Alexa Fluor® 633 (Invitrogen) were administered to cells and incubated for an hour in the dark. Post incubation, coverslips were washed twice in 0.5% PBS-BSA and once in PBS and mounted onto clean microscope slides using 50 μl Fluoromount (Sigma Aldrich). The Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software were employed to detect fluorescence and acquire images, respectively. Images were analysed and 2D cytofluorograms were constructed using Cell Sens Software.

Pull down assay. HEK293 cells were transfected via calcium phosphate methodology with a pCIneo-LRP::FLAG plasmid for 72 hours at 37°C, 5% CO_2. Pull down experimental samples were composed of 200 μl of HEK293 whole cell lysates in which LRP::FLAG was recombinantly expressed and 10–20 μl of synthetic $A\beta_{42}$ (Sigma-Aldrich) was exogenously administered. Assays were performed using FLAG® Immunopercipitation Kit (Sigma-Aldrich) according to manufacturer's instructions. Samples were subsequently electrophorectically analysed and gels were stained with Coomassie Blue. LRP::FLAG was detected via immunoblotting using murine anti-FLAG antibody (1 : 4000) (Sigma-Aldrich) and goat anti-mouse HRP (1 : 10 000) (Beckman Coulter).

3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. HEK293, N2a and SH-SY5Y cells were seeded in a 96 well plate as to attain 50–70% confluency within 24 hours and incubated in a 5% CO_2 humidified atmosphere at 37°C. Post incubation, synthetic neurotoxic Amyloid beta (Aβ) peptide (Sigma Aldrich) was administered to the cells in varying concentrations (100 nM, 200 nM and 500 nM respectively) to determine the affect thereof on cell viability. In addition, untreated controls (cells incubated in DMEM) as well as positive controls (cells incubated with 8 mM protocatechuic acid(PCA)-an apoptosis inducing agent) were included. Furthermore, cells were additionally co-incubated with Aβ (at the concentrations listed above) as well as either 50 μg/ml IgG1-iS18 antibody or 50 μg/ml IgG1-HD37 antibody (Affimed Therapeutics). Treated cells were incubated (37°C, 5% $C0_2$) for 48 hours, following which 20 μl of 1 mg/ml MTT was added to each well and the cells subsequently incubated (37°C, 5%CO_2) for 2 hours. After incubation, culture media was aspirated and 180 μl of DMSO added to each well to lyse the cells and dissolve the formazan crystals formed within the cells. The absorbance was recorded at 570 nm using an ELISA microtiter plate reader and the percentage survival of the cells, relative to the non-treated controls, calculated. Three separate experiments were performed, each in triplicate.

Bromodeoxyuridine (BrdU) proliferation assay. HEK293, N2a and SH-SY5Y cells were seeded in a 96 well plate as to attain 50–70% confluency within 24 hours and incubated in a 5% CO_2 humidified atmosphere at 37°C. Post incubation, synthetic neurotoxic Amyloid beta (Aβ) peptide (Sigma Aldrich) was administered to the cells in varying concentrations (100 nM, 200 nM and 500 nM respectively) in the presence or absence of IgG1-iS18 or IgG1-HD37. Proliferative potential of treated cells was assessed as per manufacturer's instructions for BrdU Proliferation Assay Kit (Calbiochem®). Three separate experiments were performed, each in triplicate.

Production of shRNA directed against LRP/LR mRNA. shRNAs were designed to be expressed from the H1 RNA Pol III Promoter. shRNA1.1 was designed to be homologous to murine sequences reported in previous studies and shRNA7.6 was designed using The RNAi Consortium. The expression cassettes comprised of a full H1 RNA Pol III promoter sequence, a poly T termination signal and the guide strand on the 3′ arm. The shRNA expression cassettes were generated using nested PCR in

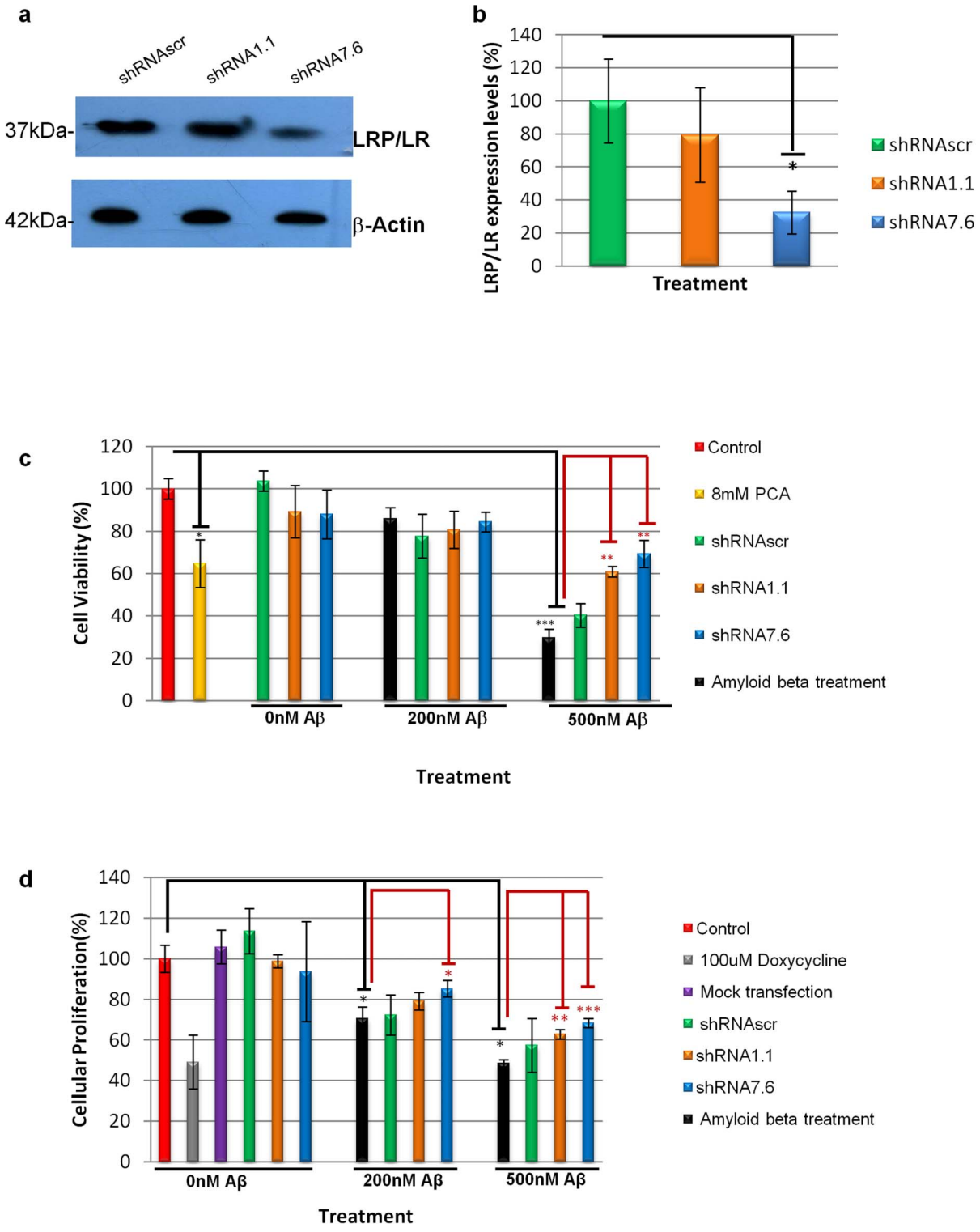


Figure 4 | shRNA-mediated downregulation of LRP/LR and the effects thereof. (a) HEK293FT cells were transfected with shRNAscr, shRNA1.1 and shRNA7.6 using the *Trans*IT®-LT1 Transfection reagent. 72 h post transfection total LRP/LR levels were assessed by Western blotting. β-actin was employed as a loading control. Gels have been cropped for clarity and conciseness purposes and have been run under the same experimental conditions. (b) Bar graph depicting percentage LRP/LR down regulation was generated by quantifying the Western blot band intensities of three independent experiments employing Quantity One 4.6 Software. To assess the role of LRP/LR in Aβ, toxicity 24 h post transfection, varying concentrations of synthetic Aβ was exogenously administered to cells. 72 h post transfection (48 h post Aβ incubation) cellular viability was assessed by MTT assay (c) and cellular proliferation was assessed by BrdU assay (d) Error bars represent sd. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ Student's *t*-test.

which the H1 RNA Pol III promoter served as the template. The forward primer was complementary to that of the H1 RNA Pol III promoter and the shRNA sequences were incorporated into the reverse primers. The resultant PCR products, which coded for the shRNA expression constructs were subsequently cloned into the pTZ57R/T vector (Fermentas).An shRNA that does not target any gene, herein termed scrambled shRNA (shRNAscr), served as the negative control. The LRP/LR target sequence as well as the structure of shRNA1.1 and shRNA7.6 are described in Jovanovic et al., (2013)[37].

Cellular transfection with shRNA directed against LRP/LR mRNA. The *Trans*IT®–LT1 Transfection reagent (Mirus) was employed to transfect LRP/LR shRNA 1 and 7 into HEK293 cells as per the manufacturer's instructions.

Western blotting. Post 72 h transfection of HEK293 cells with shRNAs, cells were lysed and total LRP/LR levels were determined by Western blotting employing IgG1-iS18 (1 : 10 000) and goat anti-human horseradish peroxidase (HRP) (1 : 10 000) (Cell Lab) antibodies, respectively. Western blot band intensities were quantified using Quantity One 4.6 Software.

Assessing cell viability and proliferation post cellular transfection with shRNA. Synthetic $A\beta_{42}$, at varying concentrations, was exogenously administered to transfected cells, 24 h post transfection. Thereafter cells were incubated in the presence of Aβ for an additional 48 h prior to analysis by MTT and BrdU assay respectively.

Statistical evaluation. Student's *t*-tests were used to analyse the data and obtain *p* values. All statistical evaluations were performed using GraphPad Prism (version 5.03) software.

1. Verdier, Y. & Penke, B. Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. *Curr Protein Pept Sci* **5**, 19–31 (2004).
2. Serrano-Pozo, A., Frosch, M. P., Masliah, E. & Hyman, B. T. Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med* **1**, a006189, doi:10.1101/cshperspect.a006189 a006189 [pii] (2011).
3. Gonsalves, D., Jovanovic, K., Da Costa Dias, B. & Weiss, S. F. Global Alzheimer Research Summit: basic and clinical research: present and future Alzheimer research. *Prion* **6**, 7–10, doi:18854 [pii] 10.4161/pri.6.1.18854 (2012).
4. Kakiya, N. *et al.* Cell surface expression of the major amyloid-beta peptide (Abeta)-degrading enzyme, neprilysin, depends on phosphorylation by mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) and dephosphorylation by protein phosphatase 1a. *J Biol Chem* **287**, 29362–29372, doi:M112.340372 [pii] 10.1074/jbc.M112.340372 (2012).
5. Jarrett, J. T., Berger, E. P. & Lansbury, P. T., Jr. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**, 4693–4697 (1993).
6. Saido, T. C. Alzheimer's disease as proteolytic disorders: anabolism and catabolism of beta-amyloid. *Neurobiol Aging* **19**, S69–75 (1998).
7. Spires-Jones, T. L. *et al.* Impaired spine stability underlies plaque-related spine loss in an Alzheimer's disease mouse model. *Am J Pathol* **171**, 1304–1311, doi:S0002-9440(10)62393-0 [pii] 10.2353/ajpath.2007.070055 (2007).
8. Koffie, R. M. *et al.* Oligomeric amyloid beta associates with postsynaptic densities and correlates with excitatory synapse loss near senile plaques. *Proc Natl Acad Sci U S A* **106**, 4012–4017, doi:0811698106 [pii] 10.1073/pnas.0811698106 (2009).
9. Hyman, B. T. *et al.* Quantitative analysis of senile plaques in Alzheimer disease: observation of log-normal size distribution and molecular epidemiology of differences associated with apolipoprotein E genotype and trisomy 21 (Down syndrome). *Proc Natl Acad Sci U S A* **92**, 3586–3590 (1995).
10. Mucke, L. & Selkoe, D. J. Neurotoxicity of Amyloid beta-Protein: Synaptic and Network Dysfunction. *Cold Spring Harb Perspect Med* **2**, a006338, doi:10.1101/cshperspect.a006338 a006338 [pii] (2012).
11. Stefani, M. Biochemical and biophysical features of both oligomer/fibril and cell membrane in amyloid cytotoxicity. *FEBS J* **277**, 4602–4613, doi:10.1111/j.1742-4658.2010.07889.x (2010).
12. Sepulveda, F. J., Parodi, J., Peoples, R. W., Opazo, C. & Aguayo, L. G. Synaptotoxicity of Alzheimer beta amyloid can be explained by its membrane perforating property. *PLoS One* **5**, e11820, doi:10.1371/journal.pone.0011820 (2010).
13. Demuro, A. *et al.* Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. *J Biol Chem* **280**, 17294–17300, doi:M500997200 [pii] 10.1074/jbc.M500997200 (2005).
14. Lin, H., Bhatia, R. & Lal, R. Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology. *FASEB J* **15**, 2433–2444, doi:10.1096/fj.01-0377com 15/13/2433 [pii] (2001).
15. Marchesi, V. T. Alzheimer's dementia begins as a disease of small blood vessels, damaged by oxidative-induced inflammation and dysregulated amyloid metabolism: implications for early detection and therapy. *FASEB J* **25**, 5–13, doi:25/1/5 [pii] 10.1096/fj.11-0102ufm (2011).
16. Bartzokis, G. Alzheimer's disease as homeostatic responses to age-related myelin breakdown. *Neurobiol Aging* **32**, 1341–1371, doi:S0197-4580(09)00271-1 [pii] 10.1016/j.neurobiolaging.2009.08.007 (2011).
17. Da Costa Dias, B., Jovanovic, K., Gonsalves, D. & Weiss, S. F. Structural and mechanistic commonalities of amyloid-beta and the prion protein. *Prion* **5**, 126–137, doi:17025 [pii] 10.4161/pri.5.3.17025 (2011).
18. Li, S. *et al.* Soluble oligomers of amyloid Beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. *Neuron* **62**, 788–801, doi:S0896-6273(09)00387-0 [pii] 10.1016/j.neuron.2009.05.012 (2009).
19. Kudo, W. *et al.* Cellular prion protein is essential for oligomeric amyloid-beta-induced neuronal cell death. *Hum Mol Genet* **21**, 1138–1144, doi:ddr542 [pii] 10.1093/hmg/ddr542 (2012).
20. Resenberger, U. K. *et al.* The cellular prion protein mediates neurotoxic signalling of beta-sheet-rich conformers independent of prion replication. *EMBO J* **30**, 2057–2070, doi:emboj201186 [pii] 10.1038/emboj.2011.86 (2011).
21. Parkin, E. T. *et al.* Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U S A* **104**, 11062–11067, doi:0609621104 [pii] 10.1073/pnas.0609621104 (2007).
22. Leucht, C. *et al.* The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. *EMBO Rep* **4**, 290295–, doi:10.1038/sj.embor.embor768 embor768 [pii] (2003).
23. Mbazima, V., Da Costa Dias, B., Omar, A., Jovanovic, K. & Weiss, S. F. Interactions between PrP(c) and other ligands with the 37-kDa/67-kDa laminin receptor. *Front Biosci* **15**, 1150–1163, doi:3667 [pii] (2010).
24. Omar, A. *et al.* Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. *Expert Opin Ther Pat* **21**, 35–53, doi:10.1517/13543776.2011.539203 (2011).
25. Gauczynski, S. *et al.* The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J* **20**, 5863–5875, doi:10.1093/emboj/20.21.5863 (2001).
26. Hundt, C. *et al.* Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *EMBO J* **20**, 5876–5886, doi:10.1093/emboj/20.21.5876 (2001).
27. Gauczynski, S. *et al.* The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis* **194**, 702–709, doi:JID36299 [pii] 10.1086/505914 (2006).
28. Zuber, C. *et al.* Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *J Mol Biol* **378**, 530–539, doi:S0022-2836(08)00164-2 [pii] 10.1016/j.jmb.2008.02.004 (2008).
29. Omar, A., Reusch, U., Knackmuss, S., Little, M. & Weiss, S. F. Anti-LRP/LR-specific antibody IgG1-iS18 significantly reduces adhesion and invasion of metastatic lung, cervix, colon and prostate cancer cells. *J Mol Biol* **419**, 102–109, doi:S0022-2836(12)00207-0 [pii] 10.1016/j.jmb.2012.02.035 (2012).
30. Khumalo, T. *et al.* Adhesion and Invasion of Breast and Oesophageal Cancer Cells Are Impeded by Anti-LRP/LR-Specific Antibody IgG1-iS18. *PLoS One* **8**, e66297, doi:10.1371/journal.pone.0066297 PONE-D-13-07741 [pii] (2013).
31. Khusal, R. *et al.* In vitro inhibition of angiogenesis by antibodies directed against the 37 kDa/67 kDa laminin receptor. *PLoS One* **8**, e58888, doi:10.1371/journal.pone.0058888 PONE-D-12-36507 [pii] (2013).
32. Kibbey, M. C. *et al.* beta-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin. *Proc Natl Acad Sci U S A* **90**, 10150–10153 (1993).
33. Lacor, P. N. *et al.* Synaptic targeting by Alzheimer's-related amyloid beta oligomers. *J Neurosci* **24**, 10191–10200, doi:24/45/10191 [pii] 10.1523/JNEUROSCI.3432-04.2004 (2004).
34. Manczak, M., Calkins, M. J. & Reddy, P. H. Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: implications for neuronal damage. *Hum Mol Genet* **20**, 2495–2509, doi:ddr139 [pii] 10.1093/hmg/ddr139 (2011).
35. Venticinque, L., Jamieson, K. V. & Meruelo, D. Interactions between laminin receptor and the cytoskeleton during translation and cell motility. *PLoS One* **6**, e15895, doi:10.1371/journal.pone.0015895 (2011).
36. Givant-Horwitz, V., Davidson, B. & Reich, R. Laminin-induced signaling in tumor cells: the role of the M(r) 67,000 laminin receptor. *Cancer Res* **64**, 3572–3579, doi:10.1158/0008-5472.CAN-03-3424 64/10/3572 [pii] (2004).
37. Jovanovic, J. *et al.* Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease. *Sci. Rep.* **3**, 2699; doi:10.1038/srep02699 (2013).

Acknowledgments

This work is based upon research supported by the National Research Foundation (NRF), the Republic of South Africa (RSA). Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s), and therefore, the National Research Foundation does not accept any liability in this regard thereto.

Author contributions

Conceived and designed the experiments: S.F.T.W. Design of shRNA: M.W. Performed experiments: B.D.C.D., D.G. and K.M. Assisted with the immunofluorescence microscopy: C.P. Antibody (IgG1-HD37) production: U.R., S.K. and M.L. Analysed data: B.D.C.D. Wrote the manuscript: B.D.C.D., K.J. and D.G. Edited the manuscript: B.D.C.D. and S.F.T.W.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Da Costa Dias, B. *et al.* Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity. *Sci. Rep.* **3**, 2702; DOI:10.1038/srep02702 (2013).

Prion interaction with the 37-kDa/67-kDa laminin receptor on enterocytes as a cellular model for intestinal uptake of prions

D. Kolodziejczak*, B. Da Costa Dias*, C. Zuber*, K. Jovanovic, A. Omar, J. Beck, K. Vana, V. Mbazima, J. Richt, B. Brenig and S.F. Weiss

Journal of Molecular Biology 402 (2010) 293-300

*These authors contributed equally to this work

This article reports on the use of enterocytes as a model system for the intestinal uptake of infectious prion proteins. PrP^{Sc} (infectious ovine prions for the disease called Scrapie), PrP^{BSE} (infectious prions causing Bovine Spongiform Encephalopathies) and PrP^{CWD} (responsible for Chronic Wasting Disease in ungulates) were all applied to enterocyte cell lines derived from human, bovine, ovine, porcine and cervid hosts. This paper showed that LRP/LR aids in the intestinal uptake of cross-species prion proteins after oral ingestion, resulting in zoonotic spread of prion disorders.

doi:10.1016/j.jmb.2010.06.055 J. Mol. Biol. (2010) 402, 293–300

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Prion Interaction with the 37-kDa/67-kDa Laminin Receptor on Enterocytes as a Cellular Model for Intestinal Uptake of Prions

Dominika Kolodziejczak[1]†, Bianca Da Costa Dias[2]†, Chantal Zuber[1]†, Katarina Jovanovic[2], Aadilah Omar[2], Julia Beck[3], Karen Vana[1], Vusi Mbazima[2], Juergen Richt[4], Bertram Brenig[3] and Stefan F. T. Weiss[2]*

[1]*Laboratorium für Molekulare Biologie, Genzentrum, Institut für Biochemie der LMU, München, Feodor-Lynen-Strasse 25, D-81377 München, Germany*

[2]*School of Molecular and Cell Biology, University of the Witwatersrand, Wits 2050, Johannesburg, Republic of South Africa*

[3]*Tierärztliches Institut der Universität Göttingen, Burckhardtweg 2, D-37077 Göttingen, Germany*

[4]*Diagnostic Medicine/ Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA*

Received 16 March 2010; received in revised form 10 June 2010; accepted 25 June 2010
Available online 13 July 2010

Edited by J. Karn

Enterocytes, a major cell population of the intestinal epithelium, represent one possible barrier to the entry of prions after oral exposure. We established a cell culture system employing enterocytes from different species to study alimentary prion interaction with the 37-kDa/67-kDa laminin receptor LRP/LR. Human, bovine, porcine, ovine, and cervid enterocytes were cocultured with brain homogenates from cervid, sheep, and cattle suffering from chronic wasting disease (CWD), scrapie, and bovine spongiform encephalopathy (BSE), respectively. PrP^{CWD}, ovine PrP^{Sc} and PrP^{BSE} all colocalized with LRP/LR on human enterocytes. PrP^{CWD} failed to colocalize with LRP/LR on bovine, porcine, and ovine enterocytes. Ovine PrP^{Sc} colocalized with the receptor on bovine enterocytes, but failed to colocalize with LRP/LR on cervid and porcine enterocytes. PrP^{BSE} failed to colocalize with the receptor on cervid and ovine enterocytes. These data suggest possible oral transmissibility of CWD and sheep scrapie to humans and may confirm the oral transmissibility of BSE to humans, resulting in zoonotic variant Creutzfeldt–Jakob disease. CWD might not be transmissible to cattle, pigs, and sheep. Sheep scrapie might have caused BSE, but may not cause transmissible spongiform encephalopathy in cervids and pigs. BSE may not be transmissible to cervids. Our data recommend the enterocyte model system for further investigations of the intestinal pathophysiology of alimentary prion infections.

Keywords: CWD; prion; laminin receptor; scrapie; variant Creutzfeldt–Jakob disease

*Corresponding author. E-mail address: stefan.weiss@wits.ac.za.

† D.K., B.D.C.D., and C.Z. contributed equally to this work.

Abbreviations used: CWD, chronic wasting disease; BSE, bovine spongiform encephalopathy; TSE, transmissible spongiform encephalopathy; PrP, prion protein; FACS, fluorescence-activated cell scanning; 2D, two-dimensional; PBS, phosphate-buffered saline; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; P/S, penicillin/ streptomycin; NEAA, nonessential amino acids.

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders affecting both humans and animals. In contrast to other "protein misfolding diseases" such as morbus Alzheimer's disease or Huntington's disease, prion diseases may be infectious. According to the protein-only hypothesis,[1,2] scrapie prion PrP^{Sc}, an abnormal form of cellular prion PrP^{c}, is thought to be the major infectious constituent of these diseases that accumulates in the central nervous system, resulting in neuronal loss and spongiform degeneration in the brain.

TSEs can be transmitted within a single animal species (intraspecies) or among different species (interspecies). The oral transmission of prion disease has been widely observed; for example, bovine spongiform encephalopathy (BSE) has been suggested to originate from the consumption, by cattle, of sheep-scrapie-contaminated food.[3] In addition, it has been widely accepted that ingestion of BSE-infected meat resulted in the development of human variant Creutzfeldt–Jakob disease,[4] showing the transmissibility of BSE prions to humans. Extensive experimental studies have been conducted on the transmission of prions to other animal species,[5–7] and the results demonstrated altered incubation times and survival or an inability to transmit the disease.[8–11] This phenomenon has been termed the "species barrier." In addition to the significance of the variety of prion strains and differences in the prion protein (PrP) structure, the route of infection is also an important factor that must be taken into consideration. This has been illustrated by the fact that pigs are intracerebrally and intraperitoneally infectable with the BSE agent, whereas oral infection and disease transmission have failed.[9] Currently, BSE and variant Creutzfeldt–Jakob disease represent orally acquired TSEs, but little is known about the mechanisms underlying the infection process. After ingestion of prion-contaminated food, the TSE-causing agent has to cross the intestinal epithelial cell barrier, where both M-cells (microfold cells)[12] and enterocytes are proposed to mediate prion uptake and transport.[13,14] Enterocytes constitute the major cell population in the intestine[15] expressing PrP^{c} on their surface.[16] A protease-resistant PrP is transcytosed across human enterocytes independently of endogenous PrP^{c} expression,[17] suggesting an alternative receptor-mediated prion uptake mechanism. Furthermore, the 37-kDa/67-kDa laminin receptor LRP/LR is expressed on the apical brush border of enterocytes[18] and has been shown to be responsible for the binding of both PrP^{c}[19] and infectious prions.[20] Moreover, recent studies on human enterocytes (Caco-2/TC7) revealed LRP/LR-dependent binding and internalization of bovine prions (PrP^{BSE}) that have been impeded by preincubation with the anti-LRP/LR-specific antibody W3.[14] W3 has been reported to be a therapeutic tool in a murine scrapie model,[21] and single-chain antibodies directed against the LRP/LR interfered with scrapie propagation[22,23] and may potentially serve as therapeutic antibodies (for review, see Refs. 24–27).

The spread of chronic wasting disease (CWD), a prion disease affecting free-range cervids, represents a severe risk in some regions of North America and parts of Canada due to hunting of this game and consumption of infected meat. Although transmission experiments with transgenic mice propose a species barrier to CWD prions in humans,[28] oral transmission of CWD to humans via contaminated food cannot be excluded.

In the present study, we investigated the intestinal pathophysiology of alimentary prion infections by determining the prion uptake capacity of enterocytes.

Results

Cell surface LRP/LR levels on human and animal enterocytes

In view of the fact that LRP/LR has been identified as a receptor for PrP^{c}[19] and PrP^{Sc},[20] respectively, we examined whether the cell surface level of the receptor potentially correlates with the PrP uptake capacity of enterocytes. Cell surface LRP/LR levels of five different enterocyte species have been determined by fluorescence-activated cell scanning (FACS) analysis (Fig. 1). A high LRP/LR level has been observed on cervid (66.34%; Fig. 1a), human (72.01%; Fig. 1b), and bovine (40.78%; Fig. 1d) enterocytes, respectively, in comparison to relatively low LRP/LR levels on porcine (16.05%; Fig. 1c) and ovine (3.69%; Fig. 1e) enterocytes (Table 1).

PrP^{CWD} colocalizes with LRP/LR on human enterocytes

To investigate whether CWD prions might bind via LRP/LR, we cultured human enterocytes (Caco-2/TC7) in the absence or in the presence of CWD-infected brain homogenates and stained them for LRP/LR (polyclonal antibody W3) and PrP^{CWD} (anti-PrP antibody 8G8), respectively. Immunofluorescence microscopy, followed by two-dimensional (2D) cytofluorogram analysis (Fig. 2, histograms, bottom), revealed that CWD brain-derived PrP colocalizes with LRP/LR on the cell surface of Caco-2/TC7 cells (Fig. 2e–g). PrP^{CWD} colocalized with LRP/LR on cervid enterocytes (Fig. 2a–c). In contrast, no colocalization of LRP/LR and PrP^{CWD}

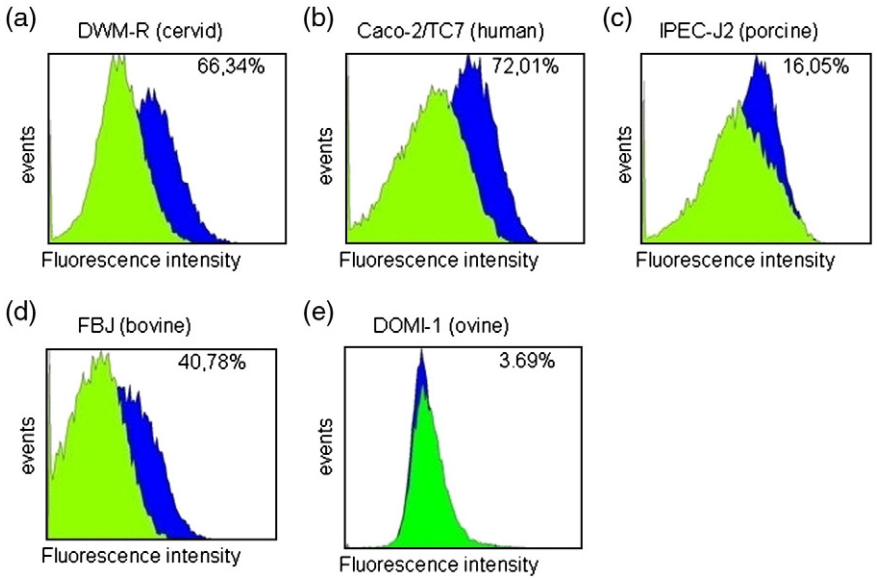


Fig. 1. Detection of cell surface LRP/LR levels on human and animal enterocytes by FACS analysis. (a) Cervid (DWM-R), (b) human (Caco-2/TC7), (c) porcine (IPEC-J2), (d) bovine (FBJ), and (e) ovine (DOMI-1) enterocytes were analyzed for LRP/LR surface level. LRP/LR levels were detected using the anti-LRP/LR antibody scFv S18 (blue curve). Anti-C9 scFv antibody was employed as negative control (green curve). Twenty thousand cells were counted per experiment. One of five representative graphs is shown for each cell line.

(Fig. 2i–k) on porcine enterocytes was observed. Similarly, both bovine and ovine enterocytes failed to display surface colocalization of PrP^{CWD} with LRP/LR (Fig. 2m–o and q–s). In order to exclude the possibility that the PrP fluorescence signal resulted from any unspecific antibody recognition, we stained cells with the 8G8 antibody in the absence of brain homogenates (Fig. 2d, h, l, p, t; Table 1).

Both ovine PrP^{Sc} and PrP^{BSE} colocalize with LRP/LR on human and bovine enterocytes

Human (Caco-2/TC7), cervid (DWM-R), bovine (FBJ), porcine (IPEC-J2), and ovine (DOMI-1) enterocytes were cultured in the presence of brain homogenates from ovine-scrapie-infected animals (ovine PrP^{Sc}). Staining of LRP/LR (by anti-LRP/LR W3) and ovine PrP^{Sc} (by 8G8), respectively, revealed colocalization of PrP^{Sc} with LRP/LR on the cell surface of Caco-2/TC7 cells (Fig. 3e–g, 2D cytofluorogram analysis, bottom). Furthermore, ovine PrP^{Sc} colocalizes with LRP/LR on bovine enterocytes (Fig. 3m–o). In contrast, ovine PrP^{Sc} (Fig. 3a–c) did not colocalize with LRP/LR on cervid enterocytes. According to 2D cytofluorography, sheep PrP^{Sc} failed to colocalize with LRP/LR on porcine enterocytes (Fig. 3i–k). Moreover, intraspecies binding analysis revealed that ovine PrP^{Sc} demonstrates merely an "in-part" colocalization with LRP/LR on ovine enterocytes (DOMI-1) (Fig. 3q–s; Table 1).

Human, cervid, bovine, ovine, and porcine enterocytes, respectively, were incubated with BSE-infected brain homogenate (PrP^{BSE}) and stained with antibodies W3 and 8G8, respectively, for LRP/LR and PrP^{BSE}. Immunofluorescence studies, followed by 2D cytofluorogram analysis, revealed colocalization of LRP/LR and PrP^{BSE} on Caco-2/TC7 cells (Fig. 4e–g). PrP^{BSE} colocalized with the laminin receptor on the cell surface of bovine enterocytes (Fig. 4m–o). In contrast, according to 2D cytofluorogram analysis, no colocalization of PrP^{BSE} and LRP/LR on cervid and ovine enterocytes (Fig. 4a–c and q–s, respectively) was detected. PrP^{BSE}, however, colocalized in part with

Table 1. Colocalization of infectious PrPs of different species with LRP/LR on the cell surface of human and animal enterocytes

	Cervid (DWM-R)	Human (Caco-2/TC7)	Porcine (IPEC-J2)	Bovine (FBJ)	Ovine (DOMI-1)
PrP^{CWD}	+	+	−	−	−
Ovine PrP^{Sc}	−	+	−	+	In part
PrP^{BSE}	−	+	In part	+	−
Cell surface LRP/LR levels (%)	66.34	72.01	16.05	40.78	3.69

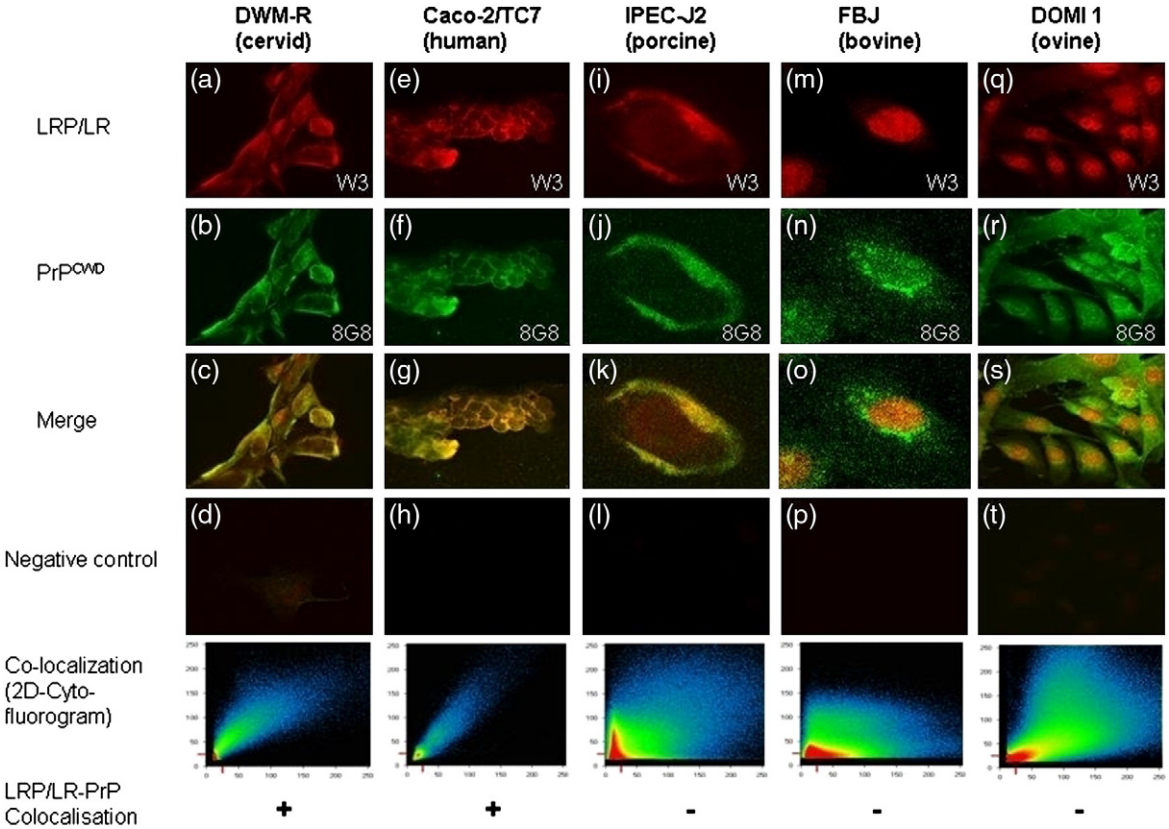


Fig. 2. Binding and colocalization of PrP^{CWD} with LRP/LR on human and cervid enterocytes. The 37-kDa/67-kDa LRP/LR was detected with the anti-LRP/LR antibody W3 on (a) cervid (DWM-R), (e) human (Caco-2/TC7), (i) porcine (IPEC-J2), (m) bovine (FBJ), and (q) ovine (DOMI-1) enterocytes. PrP^{CWD} was detected with the anti-PrP antibody 8G8 on (b) cervid, (f) human, (j) porcine, (n) bovine, and (r) ovine enterocytes. Merging of both stainings is shown on (c) cervid, (g) human, (k) porcine, (o) bovine, and (s) ovine enterocytes, respectively. Two-dimensional cytofluorograms showing the joint distribution of green and red and the area of colocalization representing the intersection line are plotted for each double-stained image (2D cytofluorogram, lower panels). For control (d, h, l, p, t), PrP staining was carried out by incubating cells with 8G8 in the absence of brain homogenates. All cells were incubated with a total concentration of 50 μg of protein from corresponding homogenates.

LRP/LR on porcine enterocytes (Fig. 4i– k; Table 1). We want to highlight that PrP^{BSE} and ovine PrP^{Sc} colocalize with LRP/LR on human enterocytes to a similar extent (compare Figs. 3 and 4).

Discussion

We established a cell culture model, including enterocytes from different species with heterogeneous morphology, to study the intestinal pathophysiology of alimentary prion infections by determining the prion uptake capacity of enterocytes. The laminin receptor plays an important role in prion propagation and presents a target for therapeutic approaches (for review, see Refs. 24–27 and 29). Brain homogenates originating from animals suffering from prion diseases were applied to human and animal enterocytes, respectively, and LRP/LR-dependent PrP binding was analyzed by immunofluorescence microscopy. Enterocytes represent the model of choice, as this cell type has been demonstrated to bind and internalize BSE prions via the 37-kDa/67-kDa LRP/LR.[14]

Colocalization of BSE prions with LRP/LR on Caco-2/TC7 cells confirmed that endocytosis of bovine prions by Caco-2/TC7 cells is LRP/LR dependent,[14] providing evidence for the important role of enterocytes, in conjunction with LRP/LR, in the oral transmission of BSE to humans. The lack of colocalization of BSE prions with LRP/LR on cervid enterocytes possibly suggests that BSE might not be transmissible to cervids. We could not detect a colocalization of BSE prions with LRP/LR on ovine enterocytes, which might suggest that BSE may not be orally transmitted to sheep. However, some reports indicate an oral transmission of BSE to sheep,[30-32] which might be dependent on the genotype of the recipient.[33] LRP/LR only exhibited an "in-part" colocalization with PrP^{BSE} on porcine

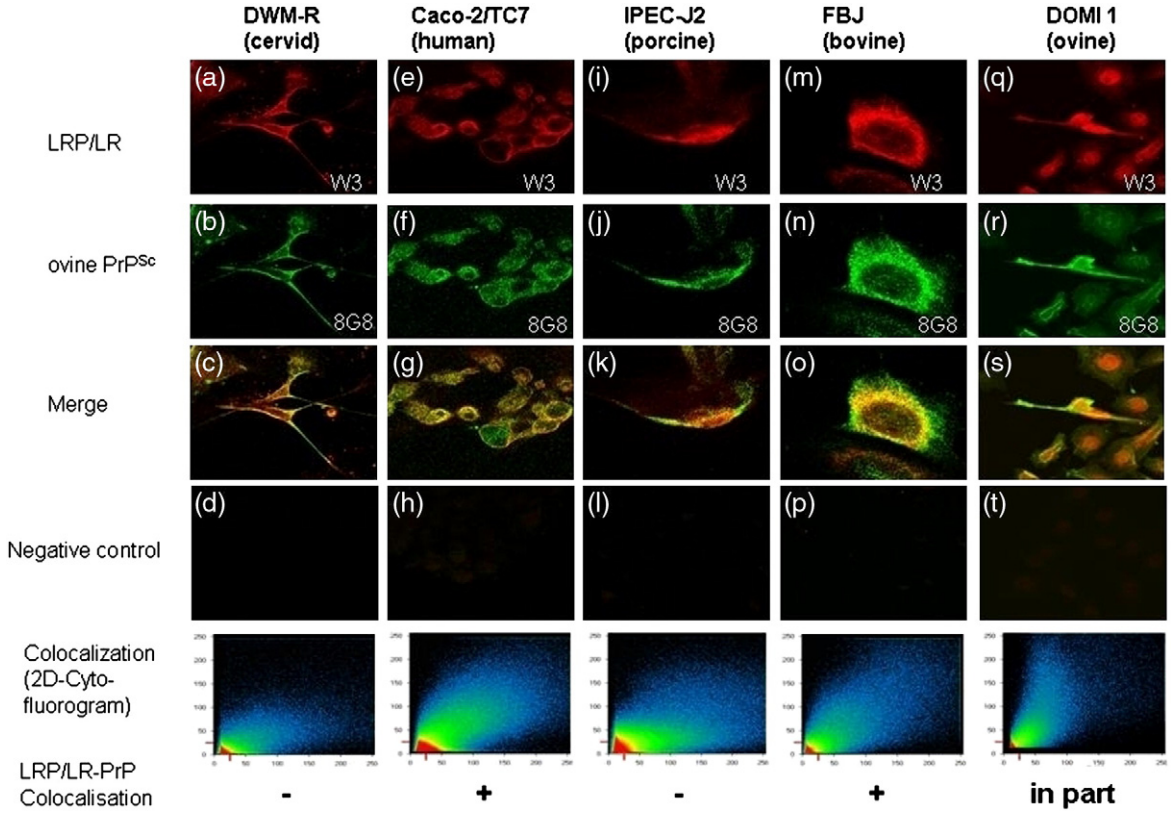


Fig. 3. Binding and colocalization of ovine PrP^{Sc} with LRP/LR on human and bovine enterocytes. The 37-kDa/67-kDa LRP/LR was detected on (a) cervid (DWM-R), (e) human (Caco-2/TC7), (i) porcine (IPEC-J2), (m) bovine (FBJ), and (q) ovine (DOMI-1) enterocytes by the anti-LRP/LR antibody W3. Ovine PrP^{Sc} was detected by the anti-PrP antibody 8G8 on (b) cervid, (f) human, (j) porcine, (n) bovine, and (r) ovine enterocytes. Merging of corresponding micrographs is shown for (c) cervid, (g) human, (k) porcine, (o) bovine, and (s) ovine enterocytes. Two-dimensional cytofluorograms showing joint distribution and colocalization are plotted (lower panels). All cells were incubated with a total concentration of 50 μg of protein. For control (d, h, l, p, t), cells were stained with 8G8 in the absence of infected brain homogenates.

enterocytes, thereby providing a potential explanation for the failure to induce porcine infection with BSE by the oral route.[9]

The fact that both PrP^{CWD} and ovine PrP^{Sc} colocalize with LRP/LR on human enterocytes possibly suggests that PrP^{CWD} and ovine PrP^{Sc} may be orally transmitted to humans, potentially giving rise to new zoonotic diseases. In contrast, PrP^{CWD} failed to colocalize with LRP/LR on bovine, ovine, and porcine enterocytes, suggesting that PrP^{CWD} may not be transmissible to sheep, cattle, or pigs via the oral route.

Sheep scrapie prions colocalize with LRP/LR on bovine enterocytes, supporting the sheep origin hypothesis stating that BSE originated from the oral consumption, by vegetarian sheep, of meat and bone meal from cattle suffering from BSE.[3] The failure of colocalization between ovine PrP^{Sc} and LRP/LR on cervid and porcine enterocytes may suggest that sheep scrapie might not be orally transmissible to elk, deer, and pigs. It has been demonstrated very recently that prions are secreted into the oral cavity of scrapie-infected sheep.[34] This finding suggests a possible oral intraspecies/interspecies transmissibility of prions (for comment, see Da Costa Dias and Weiss[35]). In light of our findings (Table 1), we cannot exclude an oral transmission of ovine PrP^{Sc} to humans and cattle; transmission to the former may possibly result in another zoonotic disease, whereas transmission to the latter confirms the sheep origin hypothesis for the development of BSE.[3] It must be emphasized, however, that our model system investigates colocalization of the prion receptor LRP/LR with different prion species, which does not necessarily allow for conclusions on the oral transmissibility of prion disorders but rather allows for inferences with regard to the intestinal pathophysiology of prion diseases.

Observed differences in the colocalization of prions to LRP/LR on the surface of species-specific enterocytes might be due to the individual strain of prion employed.

FACS analyses revealed that 72.01% of human enterocytes, 66.34% of cervid enterocytes, 40.78% of

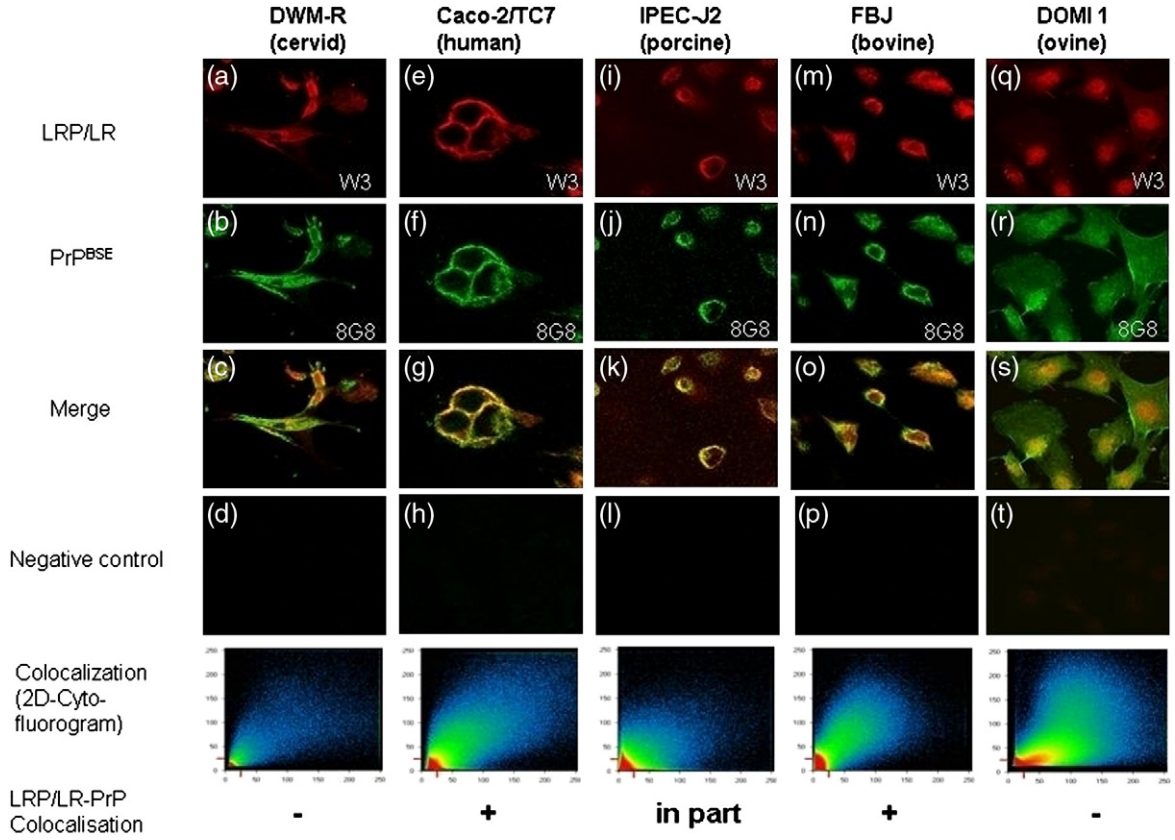


Fig. 4. Binding and colocalization of BSE prions with LRP/LR on human and bovine enterocytes. (a–d) Cervid (DWM-R), (e–h) human (Caco-2/TC7), (i–l) porcine (IPEC-J2), (m–p) bovine (FBJ), and (q–t) ovine (DOMI-1) enterocytes were incubated with BSE-infected brain homogenate (total protein concentration, 50 μg). LRP/LR was detected with the anti-LRP/LR-specific antibody W3 (a, e, i, m, q), and BSE PrPs were detected with the antibody 8G8 (b, f, j, n, r). Merging of corresponding micrographs is shown for (c) cervid, (g) human, (k) porcine, (o) bovine, and (s) ovine enterocytes. Two-dimensional cytofluorograms displaying the area of colocalization of the laminin receptor and BSE prions on the cell surface are plotted. For control (d, h, l, p, t), cells were stained with 8G8 in the absence of infected brain homogenates.

bovine enterocytes, 16.05% of porcine enterocytes, and 3.69% of ovine enterocytes, respectively, express LRP/LR on the cell surface (Fig. 1; Table 1). Since no convincing colocalization of PrP^{CWD}, ovine PrP^{Sc}, and PrP^{BSE} with LRP/LR has been observed on porcine and ovine enterocytes (revealing low LRP/LR levels of 16.05% and 3.69%, respectively), we speculate that a minimum level of LRP/LR on enterocytes might be required for an efficient prion infection by the oral route.

We cannot exclude the possibility that prions might use alternative infection pathways, besides the LRP/LR-mediated PrP internalization process, to enter enterocytes such as M-cells,[36] which might also mediate prion uptake, further leading to prion propagation in gut-associated lymphoid tissues.[37] As recently demonstrated, BSE prions bind to and become internalized by human enterocytes in an LRP/LR-dependent manner.[14] As shown in this work, CWD and sheep scrapie prions also bind to human enterocytes via LRP/LR. The importance of the laminin receptor as a target for therapeutic approaches[22,23,38,39] has already been demonstrated. From our colocalization studies and the finding that the anti-LRP/LR antibody W3 prevented the binding of BSE prions to enterocytes,[14] we conclude that the laminin receptor LRP/LR may play a vital role in the oral transmission of prion diseases.

Our data recommend the enterocyte model system for further studies investigating the intestinal pathophysiology of other alimentary prion infections.

Materials and Methods

Isolation of primary ovine enterocytes

A female Leine sheep (>18 months) was euthanized by intravenous injection of T61® and Narcoren®. A segment of the duodenum distal to the pylorus was taken and rinsed with Krebs–Ringer buffer to remove food particles.

Duodenal segments were digested in phosphate-buffered saline (PBS) containing 0.8 U/ml collagenase, 0.8 U/ml dispase, and 5 mM $CaCl_2$ for 30 min at 37 °C. The mucosa was scrapped off carefully from the underlying musculature and digested additionally for 10 min at 37 °C with 0.8 U/ml dispase and 5 mM $CaCl_2$. Mucosal enterocytes were separated from other cells by sequential filtration through a 1000-μm sieve and a 300-μm sieve. The filtrate was centrifuged at 200***g*** for 5 min and washed in PBS with 100 U/ml penicillin/100 μg/ml streptomycin. The pellet was resuspended in Medium 199 with Earle's salts, 2 mM L-glutamine, 25 mM Hepes, 15% fetal calf serum (FCS), and 50 μg/ml gentamicin (Gibco Invitrogen). Cell yield and viability were determined by 0.4% trypan blue. About 4×10^5 to 6×10^5 cells/cm^2 were seeded on 6-cm Petri dishes in Medium 199.

Tissue culture of enterocytes

Caco-2/TC7 cells (human enterocytes; provided by M. Rousset) were grown in Dulbecco's modified Eagle's medium (DMEM), 4500 mg/l D-glucose, 2 mM Glutamax, 20% FCS, 1% penicillin/streptomycin (P/S), and 1% nonessential amino acids (NEAA; Gibco Invitrogen). FBJ cells (bovine enterocytes; provided by R. Riebe) were maintained in DMEM/minimum essential medium with Hank's salts (1:1), 1000 mg/l D-glucose, 2 mM Glutamax, 10% FCS, 1% P/S, 1% NEAA (Gibco Invitrogen), and 500 mg/l $NaHCO_3$. DWM-R cells (cervid enterocytes; provided by R. Riebe) were cultured in Iscove's modified Dulbecco's medium/F12 Nutrient Mix (1:1), 2 mM Glutamax, and 10% FCS (Gibco Invitrogen). IPEC-J2 cells (porcine enterocytes) were cultured in DMEM/F12 Nutrient Mix (1:1), 1000 mg/l D-glucose, 2 mM Glutamax, 5% FCS, 1% P/S, 1% NEAA, 0.1% insulin–transferrin–selenium, and 5 ng/ml epidermal growth factor (Gibco Invitrogen). After 2 days of growth in Medium 199, adult enterocytes were cultured in Iscove's modified Dulbecco's medium/F12 Nutrient Mix (1:1), 2 mM Glutamax, and 10% FCS (Gibco Invitrogen). All cell types were grown at 37 °C with 5% CO_2.

Brain homogenate preparation

Brains samples from infected cattle (BSE), white-tailed deer (CWD), and sheep (scrapie) were homogenized to 20% (wt/vol) in PBS at 4 °C.

Fluorescence-activated cell scanning

Cultured cells were detached with 1 mM PBS/ethylenediaminetetraacetic acid, centrifuged at 1200 rpm at 4 °C, and fixed in 4% paraformaldehyde (4 °C). The cell surface LRP/LR levels of each cell type were stained by the single-chain anti-LRP/LR antibody scFv S18.[22] scFv C9, an antibody against hepatitis B surface protein, was used as negative control. Primary antibodies were diluted 1:50 in FACS buffer (0.01% sodium azide, 20 mM ethylenediaminetetraacetic acid, and 2% FCS in 1× PBS) and incubated for 1 h at 4 °C. After three washing steps, the cells were incubated with the secondary antibody c-*myc* FITC (1:50; Santa Cruz Biotechnology) and resuspended in FACS buffer for analysis.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. The primary antibody (8G8) and the secondary antibodies [Alexa Fluor® 488 goat anti-mouse IgG (H+L) and Alexa Fluor® 633 goat anti-rabbit IgG (H+L); Molecular Probes and Invitrogen] were diluted in PBS/0.3% Triton X-100. Cells were incubated overnight at 4 °C with primary antibodies (1:150), followed by incubation with secondary antibodies (1:300). Immunofluorescence backgrounds were estimated by the signal obtained in cells incubated with noninfectious brain homogenates or by fluorescence of infected cells when secondary antibodies were applied alone. Examination was performed by confocal fluorescence microscopy (Zeiss LSM 510).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (grant WE 2664/2-1), the National Research Foundation, Republic of South Africa and the NIH grant NIAID-NIH PO1 AI 77774-01, USA.

References

1. Griffith, J. S. (1967). Self-replication and scrapie. *Nature*, **215**, 1043–1044.
2. Prusiner, S. B. (1989). Scrapie prions. *Annu. Rev. Microbiol.* **43**, 345–374.
3. Wilesmith, J. W., Wells, G. A., Cranwell, M. P. & Ryan, J. B. (1988). Bovine spongiform encephalopathy: epidemiological studies. *Vet. Rec.* **123**, 638–644.
4. Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drummond, D., Suttie, A. *et al.* (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, **389**, 498–501.
5. Bruce, M., Chree, A., McConnell, I., Foster, J., Pearson, G. & Fraser, H. (1994). Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos. Trans. R. Soc. London Ser. B*, **343**, 405–411.
6. Chesebro, B. (2003). Introduction to the transmissible spongiform encephalopathies or prion diseases. *Br. Med. Bull.* **66**, 1–20.
7. Prusiner, S. B. (1994). Molecular biology and genetics of prion diseases. *Philos. Trans. R. Soc. London Ser. B*, **343**, 447–463.
8. Pattison, I. H. (1965). Scrapie in the welsh mountain breed of sheep and its experimental transmission to goats. *Vet. Rec.* **77**, 1388–1390.
9. Wells, G. A., Hawkins, S. A., Austin, A. R., Ryder, S. J., Done, S. H., Green, R. B. *et al.* (2003). Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. *J. Gen. Virol.* **84**, 1021–1031.
10. Collinge, J., Palmer, M. S., Sidle, K. C., Hill, A. F., Gowland, I., Meads, J. *et al.* (1995). Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature*, **378**, 779–783.

11. Scott, M. R., Peretz, D., Nguyen, H. O., Dearmond, S. J. & Prusiner, S. B. (2005). Transmission barriers for bovine, ovine, and human prions in transgenic mice. *J. Virol.* **79**, 5259–5271.
12. Heppner, F. L., Christ, A. D., Klein, M. A., Prinz, M., Fried, M., Kraehenbuhl, J. P. & Aguzzi, A. (2001). Transepithelial prion transport by M cells. *Nat. Med.* **7**, 976–977.
13. Okamoto, M., Furuoka, H., Horiuchi, M., Noguchi, T., Hagiwara, K., Muramatsu, Y. *et al.* (2003). Experimental transmission of abnormal prion protein (PrP^{sc}) in the small intestinal epithelial cells of neonatal mice. *Vet. Pathol.* **40**, 723–727.
14. Morel, E., Andrieu, T., Casagrande, F., Gauczynski, S., Weiss, S., Grassi, J. *et al.* (2005). Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. *Am. J. Pathol.* **167**, 1033–1042.
15. Booth, C. & Potten, C. S. (2000). Gut instincts: thoughts on intestinal epithelial stem cells. *J. Clin. Invest.* **105**, 1493–1499.
16. Morel, E., Fouquet, S., Chateau, D., Yvernault, L., Frobert, Y., Pincon-Raymond, M. *et al.* (2004). The cellular prion protein PrP^{c} is expressed in human enterocytes in cell–cell junctional domains. *J. Biol. Chem.* **279**, 1499–1505.
17. Mishra, R. S., Basu, S., Gu, Y., Luo, X., Zou, W. Q., Mishra, R. *et al.* (2004). Protease-resistant human prion protein and ferritin are cotransported across Caco-2 epithelial cells: implications for species barrier in prion uptake from the intestine. *J. Neurosci.* **24**, 11280–11290.
18. Shmakov, A. N., Bode, J., Kilshaw, P. J. & Ghosh, S. (2000). Diverse patterns of expression of the 67-kD laminin receptor in human small intestinal mucosa: potential binding sites for prion proteins? *J. Pathol.* **191**, 318–322.
19. Gauczynski, S., Peyrin, J. M., Haik, S., Leucht, C., Hundt, C., Rieger, R. *et al.* (2001). The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J.* **20**, 5863–5875.
20. Gauczynski, S., Nikles, D., El-Gogo, S., Papy-Garcia, D., Rey, C., Alban, S. *et al.* (2006). The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J. Infect. Dis.* **194**, 702–709.
21. Zuber, C., Mitteregger, G., Pace, C., Kretzschmar, H. A., Zerr, I. & Weiss, S. (2007). Anti-LRP/LR antibody W3 hampers peripheral PrP^{Sc} propagation in scrapie infected mice. *Prion*, **1**, 207–212.
22. Zuber, C., Knackmuss, S., Rey, C., Reusch, U., Rottgen, P., Frohlich, T. *et al.* (2008). Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases. *Mol. Immunol.* **45**, 144–151.
23. Zuber, C., Mitteregger, G., Schuhmann, N., Rey, C., Knackmuss, S., Rupprecht, W. *et al.* (2008). Delivery of single-chain antibodies (scFvs) directed against the 37/67 kDa laminin receptor into mice via recombinant adeno-associated viral vectors for prion disease gene therapy. *J. Gen. Virol.* **89**, 2055–2061.
24. Ludewigs, H., Zuber, C., Vana, K., Nikles, D., Zerr, I. & Weiss, S. (2007). Therapeutic approaches for prion disorders. *Expert Rev. Anti-Infect. Ther.* **5**, 613–630.
25. Zuber, C., Ludewigs, H. & Weiss, S. (2007). Therapeutic approaches targeting the prion receptor LRP/LR. *Vet. Microbiol.* **123**, 387–393.
26. Vana, K., Zuber, C., Nikles, D. & Weiss, S. (2007). Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy. *Cell. Mol. Neurobiol.* **27**, 107–128.
27. Vana, K., Zuber, C., Pflanz, H., Kolodziejczak, D., Zemora, G., Bergmann, A. K. & Weiss, S. (2009). LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. *Infect. Disord. Drug Targets*, **9**, 69–80.
28. Kong, Q., Huang, S., Zou, W., Vanegas, D., Wang, M., Wu, D. *et al.* (2005). Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models. *J. Neurosci.* **25**, 7944–7949.
29. Mbazima, V., Da Costa Dias, B., Omar, A., Jovanovic, K. & Weiss, S. (2010). Interactions between PrP^{c} and other ligands with the 37 kDa/67 kDa laminin receptor. *Front. Biosci.* **15**, 1150–1163.
30. Foster, J. D., Hope, J. & Fraser, H. (1993). Transmission of bovine spongiform encephalopathy to sheep and goats. *Vet. Rec.* **133**, 339–341.
31. Foster, J. D., Parnham, D., Chong, A., Goldmann, W. & Hunter, N. (2001). Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats. *Vet. Rec.* **148**, 165–171.
32. Foster, J. D., Parnham, D. W., Hunter, N. & Bruce, M. (2001). Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. *J. Gen. Virol.* **82**, 2319–2326.
33. Bellworthy, S. J., Dexter, G., Stack, M., Chaplin, M., Hawkins, S. A., Simmons, M. M. *et al.* (2008). Oral transmission of BSE to VRQ/VRQ sheep in an experimental flock. *Vet. Rec.* **162**, 130–131.
34. Maddison, B. C., Rees, H. C., Baker, C. A., Taema, M., Bellworthy, S. J., Thorne, L. *et al.* (2010). Prions are secreted into the oral cavity in sheep with preclinical scrapie. *J. Infect. Dis.* **201**, 1672–1676.
35. Da Costa Dias, B. & Weiss, S. F. (2010). A kiss of a prion: new implications for oral transmissibility. *J. Infect. Dis.* **201**, 1615–1616.
36. Ghosh, S. (2002). Intestinal entry of prions. *Z. Gastroenterol.* **40**, 37–39.
37. Glaysher, B. R. & Mabbott, N. A. (2007). Role of the GALT in scrapie agent neuroinvasion from the intestine. *J. Immunol.* **178**, 3757–3766.
38. Pflanz, H., Vana, K., Mitteregger, G., Renner-Muller, I., Pace, C., Kuchenhoff, H. *et al.* (2009). Scrapie-infected transgenic mice expressing a laminin receptor decoy mutant reveal a prolonged incubation time associated with low levels of PrP^{res}. *J. Mol. Biol.* **388**, 721–729.
39. Pflanz, H., Vana, K., Mitteregger, G., Pace, C., Messow, D., Sedlaczek, C. *et al.* (2009). Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. *J. Gen. Virol.* **90**, 269–274.

6. Discussion

Since the first report on the Aβ peptide was published in 1984 [32], the molecular mechanisms underlying the aetiology of Alzheimer's Disease have become the focal point of countless research efforts globally. The mounting body of work has elucidated much on not only the genetic and environmental factors underlying this devastating disease, but also on the numerous mechanisms by which neurodegeneration is elicited and the molecular components thereof.

The symptomatic progressive memory loss and cognitive dysfunction of AD result from the neurotoxicity brought about by a complex interplay of proteins within the neuronal tissue. Processes leading to the onset of AD are thought to begin many years before the first symptoms are observed. This is due to the accumulation of neurotoxic events and loss of synapses as well as neuronal injury. All of these features are attributed to the accrual and aggregation of the 4 kDa amyloid beta peptide and the subsequent cellular changes which result in the hyperphosphorylation of the tau protein, leading to the formation of neurofibrillary tangles.

Aβ is produced through the proteolytic processing of APP by the β- and γ-secretases at various cellular locations; after which Aβ is shed or transported into the extracellular space whereby it aggregates to form dimers, oligomers (the most neurotoxic form of Aβ), fibrils and eventually plaques which are deposited onto the neuronal tissue. The processes leading to the production of Aβ are thus of great importance as the understanding of these molecular mechanisms could give insights into how they can be targeted for future therapeutic strategies. Thus an ever-growing number of proteins have been revealed to somehow affect either the production or mediation of neurotoxicity caused by the Aβ peptide. One such protein of great importance to AD pathology is the cellular prion protein (PrP^c). A direct interaction exists between this protein and the β-secretase. PrP^c has been shown to regulate APP processing through this interaction[122,123]. Recent findings also suggest that PrP^c is required for Aβ induced neurotoxicity as well as synaptic and neuronal injury. Thus, PrP^c is of importance in the processes central to AD pathology.

For this reason, an investigation was undertaken to further examine whether the cellular receptor for PrP^c, namely the 37kDa/67kDa laminin receptor (LRP/LR), might also be implicated in AD etiopathenogenesis. LRP/LR is the cellular receptor for not only cellular prion proteins [93], but infectious variants (PrP^{Sc}) too[141], thus many therapeutic strategies have been developed targeting LRP/LR for the treatment of prion disorders. The current antibody of choice for targeting LRP/LR is IgG1-iS18 which has been shown to impede adhesion and invasion in numerous neoplastic cell lines [143,144,145].

The following discussion is based on the work published in the 2 research articles composed of the results obtained from this PhD. In order to investigate the possibility of a role for LRP/LR in AD, the anti-LRP/LR antibody IgG1-iS18 was used to effectively block the receptor and thus limit its functionality by hindering interactions that it may form with its ligands and interaction partners. On this premise, HEK293 (human embryonic kidney) and SH-SY5Y (human neuroblastoma) cells were treated with 50μg/ml of IgG1-iS18, IgG1-HD37 (control antibody directed against CD19) [143] or no antibody for 18 hours. Aβ shedding was then quantified by conducting an Aβ specific solid phase ELISA for the determination of Aβ concentration within the cell culture medium. Any disparity between the Aβ levels detected in the samples treated with IgG1-iS18 compared to the controls could thus be attributed to the impedance of LRP/LR activity upon IgG1-iS18 addition. A significant reduction of (on average) 14pg/ml (±47%) in Aβ concentration was observed in HEK293 cells treated with IgG1-iS18 compared to the controls. A similar effect was observed in the SH-SY5Y cells whereby a reduction of approximately 60pg/ml (± 29%) was noted. Furthermore, upon applying increasing concentrations of IgG1-iS18, a dose-dependent reduction in Aβ levels was observed correlating to increasing IgG1-iS18 concentration. These findings showed that blockage of LRP/LR with IgG1-iS18 significantly obstructed the amyloidogenic processing of APP so as to result in a significantly lowered amount of Aβ being produced. These findings thus suggest a putative role for LRP/LR in AD.

An attempt was made to further understand the mode of action by which LRP/LR exerted its effects on the amyloidogenic pathway. Firstly, cell surface expression levels of APP, β- and –γ-secretase on HEK293 cells were determined post IgG1-iS18 and IgG1-HD37 treatment. As no significant differences were detected in protein expression when compared to the no antibody control samples, it was concluded that the mode of action of LRP/LR in AD was not

that of gene-expression modulation of the proteins necessary for Aβ production. Furthermore, sAPPβ levels were detected after SH-SHY5Y cells had been treated with varying concentrations of IgG1-iS18. The reason for this is that sAPPβ is the product of the initial cleavage in the amyloidogenic pathway, performed by the β-secretase. Thus sAPPβ can be used as an indicator of β-secretase activity. Immunoblot analysis revealed that sAPPβ levels were reduced in a dose dependent manner corresponding to the concentration of IgG1-iS18 added. This shows that blockage of LRP/LR results in a significant inhibition of β-secretase activity, which would suggest that LRP/LR either interacts with β-secretase (directly or indirectly) or has the ability to influence its functionality by some other mechanism.

Immunofluorescence and confocal microscopy was employed to verify the possible of interactions between LRP/LR and the AD proteins APP, β- and γ-secretase and FLAG® co-immunoprecipitation assays were used to verify the validity of these possible interactions. A high degree of co-localisation was revealed between APP and LRP/LR when immunofluorescence microscopy was performed on non-permeabilised HEK293 cells. This co-localisation was maintained when confocal microscopy was performed on HEK293 cells co-expressing APP-GFP and LRP-dsRed. Z-stack analysis (confocal microscopy which scans though of the sample along the z-axis, take numerous sections through the cells) allows for the study of intracellular co-localisation within various cellular locations. This showed that the co-localisation between LRP-dsRed and APP-GFP was not only limited to the cell surface but occurred also to a great extent within cytoplasmic regions. Analysis tools such as 2D-cytofluorograms and line profiles were used to validate the degree of co-localisation. Super resolution structured illumination microscopy (SR-SIM) is an advanced form of confocal microscopy allowing for a higher resolution (80nm) between two points (confocal laser scanning microscopy has a 200nm resolution limit). This technique was used to give further insight into the proximity between the proteins of interest. At this resolution, the degree of co-localisation between APP-GFP and LRP-dsRed was greatly diminished in comparison to the co-localisation observed with confocal microscopy. Co-localisation was maintained in certain cellular regions, most notably on the cell surface, and within certain cytoplasmic regions. This is expected as LRP/LR and APP are both known to be found in lipid raft microdomains on the plasma membrane [149,150]. Furthermore, APP is internalised into endosomes via either clathrin or raft-mediated endocytosis [63], while LRP/LR has also been detected in endosomes after the internalisation of PrP^c [136]. APP processing has also been

studied in the trans-golgi-network (TGN) and golgi apparatus. This suggests that the sites for the co-localisation that remains between APP and LRP/LR are likely to be the lipid raft regions of the plasma membrane and within the endosomes. This is in line with the finding that LRP/LR plays a role in the amyloidogenic processing of APP, as it co-localises with APP in the subcellular locations where it is known that APP proteolytic processing occurs.

The co-localisation between LRP/LR and β-secretase showed similar results when immunofluorescence microscopy was employed as a high degree of cell surface staining was observed. Furthermore confocal microscopy and z-stack analysis confirmed that co-localisation was also highly prevalent within the cytoplasmic regions of the cells. However, when SR-SIM analysis was performed between BACE1-GFP and LRP-dsRed, co-localisation was completely lacking. This suggests that the proximity between these 2 proteins is greater than 80nm – a distance not likely to warrant any direct protein interactions; however, it does not exclude the possibility of an indirect interaction mediated by another molecule. β-secretase is located in lipid raft regions of the cell membrane whereby it is internalised into early endosomes via Arf6 [106]. β-secretase cleavage of various APP isoforms has been observed in the TGN, golgi apparatus and in the secretory pathway[123,151]. Thus the co-localisation observed between β-secretase and LRP-dsRed, which appears to be strongest within cytoplasmic regions, could be as a result of the two proteins co-localising within either one or more of the abovementioned cellular organelles.

Studies into the co-localisation between γ-secretase and LRP/LR focused on the PS1 subunit of the γ-secretase complex as it is the catalytic subunit responsible for the proteolytic processing of APP and release of the Aβ peptide. All four sub-units of the γ-secretase are required for full activity of the complex, and the correct assembly of this complex is a highly regulated process [87]. Many quality controls are in place, often leading to recycling of the subunits in different subcellular locations until they are correctly assembled into functional γ-secretase complexes [152]. This accounts for the 'spatial paradox' which has been described for the disparity between the co-localisation of PS1 and its substrate APP [153,154]. The majority of PS1 is localised in the endoplasmic reticulum (ER), intermediate compartment, and *cis*-Golgi (the early biosynthetic compartments); however it is found to be active in post-Golgi locations, including lipid raft regions of the plasma membrane and endosomal membrane [89,155,156,157,158]. Co-localisation between LRP/LR was initially found on the

cell surface of HEK293 cells by immunofluorescence microscopy and later confirmed using confocal microscopy which revealed a high degree of intracellular co-localisation between PS1-GFP and LRP-dsRed. Co-localisation was still maintained when SR-SIM was employed, revealing strong localisation within cytoplasmic regions of the cell and the plasma membrane. The cytoplasmic co-localisation between these two proteins was seen to a greater extent than what had been observed for the samples expressing LRP-dsRed with either APP-GFP or BACE1-GFP. This suggested that the likelihood of an interaction between LRP/LR and the PS1 sub-unit of the γ-secretase was greater than with the remaining AD proteins, APP and β-secretase.

While co-localisation studies provide a good indication for whether interactions between two proteins are plausible, they do not necessarily imply that an interaction does exist as it merely reveals the proximity between the proteins within their cellular environment. Thus in order to overcome this, two methodologies which confirm protein-protein interactions were employed. The first was Förster Resonance Energy Transfer (FRET), whereby if two fluorescent proteins are within a proximity of 1 – 10nm, energy can be transferred from the donor fluorescent protein to the acceptor. This causes the acceptor to emit light without being directly stimulated [159]. APP-GFP, BACE1-GFP and PS1-GFP were used as donor proteins due to their GFP tags, while the dsRed tag of LRP-dsRed was the acceptor. No energy transfer was detected between APP-GFP and LRP-dsRed suggesting that these 2 proteins are not within 10nm of each other. This finding greatly diminished the likelihood of an interaction between APP and LRP/LR, and upon testing for co-immunoprecipitation between LRP::FLAG and APPwt, no interaction was found between these proteins showing that APP failed to co-immunoprecipitate with LRP/LR.

No FRET was observed between BACE1-GFP and LRP-dsRed, denoting that these proteins were found at a distance greater than 10nm from each other. This is in line with the SR-SIM findings, since no co-localisation was detected at 80nm, it is unlikely that FRET would be detected due to the distance between the proteins. However, co-immunoprecipitation analysis, revealed that BACE1-GFP does co-immunoprecipitate with LRP::FLAG. This finding suggests that an interaction does take place between β-secretase and LRP/LR, a finding which may appear to be contradictory to the FRET data. It is easily explained upon analysis of the methodology used. A FLAG® co-immunoprecipitation assay utilises whole cell

lysates, thus any interactions that are detected may be indirect interactions facilitated by the presence of another protein/molecule in the cell lysates. Upon examination of the literature, there are a few options that could apply. PrP^c has been reported to interact directly with the prodomian of Golgi-localised β-secretase, and furthermore affects its activity and localisation [122,123]. β-secretase has also been found to interact with heparin sulphate proteoglycans (HSPGs), which also regulate β-secretase activity and reduce the shedding of Aβ thus making them important molecules for therapeutic research [160,161,162]. Interaction domains have been located on LRP/LR for not only PrP, but also for HSPGs, which are involved in LRP/LR dependent internalisation of PrP [134]. Thus, it is likely that either HSPG or PrP^c could be responsible for the indirect interaction observed between LRP/LR and β-secretase. This would also provide an explanation for the effects observed on both Aβ and sAPPβ shedding upon IgG1-iS18 blockage of LRP/LR, as it is possible that this could have disrupted in the internalisation of HSPG and PrP^c or their interactions with β-secretase.

A transfer of energy was observed between PS1-GFP and LRP-dsRed upon FRET analysis. This finding not only suggests that an interaction exists between LRP/LR and PS1 of the γ-secretase, but also reveal the cellular regions where energy FRET occurred (i.e. the location of the interaction). Signals were clearly observed on the plasma membrane of the cells, and in cytoplasmic regions. The FRET signals also revealed novel co-localisation in the perinuclear region of the cell. LRP/LR is known to occur in this subcellular location [163] and as mentioned, the majority of PS1 is localised to the early biosynthetic compartments which are known to exist in close proximity to the nucleus, i.e. perinuclear regions. Since FRET was detected between PS1-GFP and LRP-dsRed, these proteins must be within 1 – 10nm of each other. This proximity strongly suggests that a direct protein-protein interaction does occur between PS1 and LRP/LR. Co-immunoprecipitation was confirmed between LRP::FLAG and PS1, suggesting that an interaction between LRP/LR and the PS1 subunit of the γ-secretase does exist, and due to the proximity of the two proteins within the cellular milieu, it is highly likely that this interaction is a direct one. The blockage of LRP/LR with IgG1-iS18 may have impeded the shedding of Aβ by disrupting the interaction between LRP/LR and PS1. The mode of action by which IgG1-iS18 results in reduced Aβ shedding could also be a combination of hindering the interactions seen between LRP/LR and both β- and γ-secretase.

While this project has elucidated a novel role for LRP/LR in Alzheimer's disease, the exact underlying mechanism remains to be elucidated. It is evident that IgG1-iS18 clearly impeded the production of Aβ, suggesting that LRP/LR is involved in the amyloidogenic processing of APP. Examination of the binding sites on LRP/LR reveals that IgGs bind between aa 272 – 280 of LRP/LR. Due to the large size of IgG antibodies (±150 kDa) and the high affinity binding between LRP/LR and IgG1-iS18, the binding of PrP^c and HSPGs to LRP/LR could be prevented due to steric hindrance. Alternatively, the binding of IgG1-iS18 may induce allosteric hindrance of the interactions between PrP^c and HSPGs and their receptor LRP/LR due to conformational changes induced by the binding of the antibody. This would effectively inhibit the interactions between LRP/LR and its ligands, possibly by prohibiting the functionality of LRP/LR with regard to the role it plays in the internalisation and binding of its interaction partners. Internalisation of PrP^c could be encumbered in either of these ways, effectively preventing the interaction between β-secretase and PrP^c. The same could be applied to HSPG as the binding site on LRP/LR is between aa205 – 229, which is closer to the antibody binding site. Disrupting the LRP/LR – HSPG interaction could possibly indirectly alter the β-secretase – HSPG interaction, resulting in altered APP cleavage. If the integrity of PrP^c and HSPGs interactions with β-secretase were affected upon LRP/LR incubation with IgG1-iS18, it would present an explanation for the reductions seen in sAPPβ levels. Since Aβ is released from the C99 β-CTF by γ-secretase cleavage, it is most likely a (steric or allosteric) disruption in the direct interaction between LRP/LR and the γ-secretase that resulted in the reduced Aβ levels upon IgG1-iS18 incubation.

Elucidation of the exact mechanisms underlying all these observations could be of great benefit to AD research. The potential to reduce Aβ shedding via LRP/LR blockade presents a novel potential therapeutic option for the treatment of this devastating disease. Many of prominent clinical trials for AD, such as the antibody bapineuzumab (directed against the free N-terminus of Aβ)[164] or the γ-secretase inhibitors (R)-flurbiprofen[165] and semagacestat[166] have all proven unsuccessful in the past due to harmful off-target affects. Currently an antibody directed against the mid-regions of Aβ, solanezumab, is showing benefits in decreasing the rate of cognitive decline in mild AD patients, with minimal side effects [167], however no improvements are seen in moderate cases of AD. To date, clinical trials have highlighted many issues that need to be addressed for future therapeutic strategies, and have also shown the importance of antibodies in disease treatment. IgG1-iS18 should

thus be examined further in terms of its therapeutic potential. Firstly, animal trials would need to be conducted to examine whether the decline in Aβ levels upon IgG1-iS18 treatment correlates to improvements in cognitive ability, while having minimal off target effects. Furthermore, if animal trials prove to be successful, clinical trials will have to be performed to validate the efficacy of this treatment option in human patients.

Thus in conclusion, LRP/LR is involved in Aβ shedding as blockage of this cellular receptor with the humanised IgG1-iS18 antibody resulted in a reduced production of not only Aβ, but of the cleavage product of β-secretase, sAPPβ, as well. Interactions have been established between LRP/LR and the secretases responsible for the proteolytic processing of APP. An indirect interaction was found between LRP/LR and the β-secretase, while the interaction between γ-secretase and LRP/LR was confirmed to be a direct one. These findings taken together present LRP/LR as a novel target for AD treatment strategies, and furthermore highlight the possibility of using the anti-LRP/LR specific antibody IgG1-iS18 as a potential therapeutic for the treatment of Alzheimer's disease.

7. References

1. Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297: 353-356.
2. Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81: 741-766.
3. Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, et al. (2002) Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. Cell 110: 55-67.
4. Mbazima V, Da Costa Dias B, Omar A, Jovanovic K, Weiss S (2010) Interactions between PrPc and other ligands with the 37-kDa/67-kDa laminin receptor. Frontiers in Bioscience 15: 1150-1163.
5. Omar A, Jovanovic K, Da Costa Dias B, Gonsalves D, Moodley K, et al. (2011) Patented biological approaches for the therapeutic modulation of the 37 kDa/67 kDa laminin receptor. Expert Opin Ther Pat 21: 35-53.
6. Selkoe DJ (2011) Alzheimer's disease. Cold Spring Harb Perspect Biol 3.
7. Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, et al. (2005) Global prevalence of dementia: a Delphi consensus study. Lancet 366: 2112-2117.
8. Ziegler-Graham K, Brookmeyer R, Johnson E, Arrighi HM (2008) Worldwide variation in the doubling time of Alzheimer's disease incidence rates. Alzheimers Dement 4: 316-323.
9. Qiu C, De Ronchi D, Fratiglioni L (2007) The epidemiology of the dementias: an update. Curr Opin Psychiatry 20: 380-385.
10. Waldemar G, Dubois B, Emre M, Georges J, McKeith IG, et al. (2007) Recommendations for the diagnosis and management of Alzheimer's disease and other disorders associated with dementia: EFNS guideline. Eur J Neurol 14: e1-26.
11. Blennow K, Hampel H, Weiner M, Zetterberg H (2010) Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. Nat Rev Neurol 6: 131-144.
12. Sunderland T, Linker G, Mirza N, Putnam KT, Friedman DL, et al. (2003) Decreased beta-amyloid1-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease. JAMA 289: 2094-2103.
13. Galasko D, Klauber MR, Hofstetter CR, Salmon DP, Lasker B, et al. (1990) The Mini-Mental State Examination in the early diagnosis of Alzheimer's disease. Arch Neurol 47: 49-52.
14. McKhann G, Drachman D, Folstein M, Katzman R, Price D, et al. (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34: 939-944.
15. Burns A, Iliffe S (2009) Alzheimer's disease. BMJ 338: b158.
16. Walsh DM, Selkoe DJ (2004) Deciphering the molecular basis of memory failure in Alzheimer's disease. Neuron 44: 181-193.
17. Birks J, Grimley Evans J, Iakovidou V, Tsolaki M (2000) Rivastigmine for Alzheimer's disease. Cochrane Database Syst Rev: CD001191.
18. Lim GP, Yang F, Chu T, Chen P, Beech W, et al. (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. J Neurosci 20: 5709-5714.

19. Tariot PN, Farlow MR, Grossberg GT, Graham SM, McDonald S, et al. (2004) Memantine treatment in patients with moderate to severe Alzheimer disease already receiving donepezil: a randomized controlled trial. JAMA 291: 317-324.
20. Kemp JA, McKernan RM (2002) NMDA receptor pathways as drug targets. Nat Neurosci 5 Suppl: 1039-1042.
21. Lipton SA (2004) Paradigm shift in NMDA receptor antagonist drug development: molecular mechanism of uncompetitive inhibition by memantine in the treatment of Alzheimer's disease and other neurologic disorders. J Alzheimers Dis 6: S61-74.
22. Shastry BS, Giblin FJ (1999) Genes and susceptible loci of Alzheimer's disease. Brain Res Bull 48: 121-127.
23. Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, et al. (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360: 672-674.
24. Goate A (2006) Segregation of a missense mutation in the amyloid beta-protein precursor gene with familial Alzheimer's disease. J Alzheimers Dis 9: 341-347.
25. Citron M, Westaway D, Xia W, Carlson G, Diehl T, et al. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. Nat Med 3: 67-72.
26. Hutton M, Hardy J (1997) The presenilins and Alzheimer's disease. Hum Mol Genet 6: 1639-1646.
27. Stritmatter WJ, Saunders AM, Schmechel D, Periack-Vance M, Enghild J, et al. (1993) Apolipoprotein E: High-avidity binding to beta-Amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci U S A 90: 1977-1981.
28. Meyer MR, Tschanz JT, Norton MC, Welsh-Bohmer KA, Steffens DC, et al. (1998) APOE genotype predicts when--not whether--one is predisposed to develop Alzheimer disease. Nat Genet 19: 321-322.
29. Reitz C, Brayne C, Mayeux R (2011) Epidemiology of Alzheimer disease. Nat Rev Neurol 7: 137-152.
30. Mayeux R (2003) Epidemiology of neurodegeneration. Annu Rev Neurosci 26: 81-104.
31. Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. Science 298: 789-791.
32. Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 120: 885-890.
33. Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, et al. (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A 82: 4245-4249.
34. Gorevic PD, Goni F, Pons-Estel B, Alvarez F, Peress NS, et al. (1986) Isolation and partial characterization of neurofibrillary tangles and amyloid plaque core in Alzheimer's disease: immunohistological studies. J Neuropathol Exp Neurol 45: 647-664.
35. Wischik CM, Novak M, Thogersen HC, Edwards PC, Runswick MJ, et al. (1988) Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. Proc Natl Acad Sci U S A 85: 4506-4510.
36. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, et al. (1986) Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A 83: 4913-4917.
37. Goedert M, Crowther RA, Garner CC (1991) Molecular characterization of microtubule-associated proteins tau and MAP2. Trends Neurosci 14: 193-199.
38. Kosik KS, Joachim CL, Selkoe DJ (1986) Microtubule-associated protein tau (tau) is a

major antigenic component of paired helical filaments in Alzheimer disease. Proc Natl Acad Sci U S A 83: 4044-4048.
39. Pimplikar SW, Nixon RA, Robakis NK, Shen J, Tsai LH (2010) Amyloid-independent mechanisms in Alzheimer's disease pathogenesis. J Neurosci 30: 14946-14954.
40. O'Brien RJ, Wong PC (2011) Amyloid precursor protein processing and Alzheimer's disease. Annu Rev Neurosci 34: 185-204.
41. Ballatore C, Lee VM, Trojanowski JQ (2007) Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci 8: 663-672.
42. Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, et al. (2005) Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci 8: 79-84.
43. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, et al. (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 416: 535-539.
44. Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, et al. (2007) Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci 27: 2866-2875.
45. You H, Tsutsui S, Hameed S, Kannanayakal TJ, Chen L, et al. (2012) Abeta neurotoxicity depends on interactions between copper ions, prion protein, and N-methyl-D-aspartate receptors. Proc Natl Acad Sci U S A 109: 1737-1742.
46. Resenberger UK, Winklhofer KF, Tatzelt J (2011) Cellular Prion Protein Mediates Toxic Signaling of Amyloid Beta. Neurodegener Dis.
47. Um JW, Nygaard HB, Heiss JK, Kostylev MA, Stagi M, et al. (2012) Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. Nat Neurosci 15: 1227-1235.
48. Crimins JL, Pooler A, Polydoro M, Luebke JI, Spires-Jones TL (2013) The intersection of amyloid beta and tau in glutamatergic synaptic dysfunction and collapse in Alzheimer's disease. Ageing Res Rev 12: 757-763.
49. Westerman MA, Cooper-Blacketer D, Mariash A, Kotilinek L, Kawarabayashi T, et al. (2002) The relationship between Abeta and memory in the Tg2576 mouse model of Alzheimer's disease. J Neurosci 22: 1858-1867.
50. Gaspar RC, Villarreal SA, Bowles N, Hepler RW, Joyce JG, et al. (2010) Oligomers of beta-amyloid are sequestered into and seed new plaques in the brains of an AD mouse model. Exp Neurol 223: 394-400.
51. Masters CL, Selkoe DJ (2012) Biochemistry of amyloid beta-protein and amyloid deposits in Alzheimer disease. Cold Spring Harb Perspect Med 2: a006262.
52. D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38: 120-134.
53. Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, et al. (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274: 99-102.
54. Gotz J, Ittner LM (2008) Animal models of Alzheimer's disease and frontotemporal dementia. Nat Rev Neurosci 9: 532-544.
55. LaFerla FM, Green KN (2012) Animal models of Alzheimer disease. Cold Spring Harb Perspect Med 2.
56. Tanzi RE, Gusella JF, Watkins PC, Bruns GA, St George-Hyslop P, et al. (1987) Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. Science 235: 880-884.

57. Lemaire HG, Salbaum JM, Multhaup G, Kang J, Bayney RM, et al. (1989) The PreA4(695) precursor protein of Alzheimer's disease A4 amyloid is encoded by 16 exons. Nucleic Acids Res 17: 517-522.
58. Neve RL, McPhie DL, Chen Y (2000) Alzheimer's disease: a dysfunction of the amyloid precursor protein(1). Brain Res 886: 54-66.
59. Giaccone G, Tagliavini F, Linoli G, Bouras C, Frigerio L, et al. (1989) Down patients: extracellular preamyloid deposits precede neuritic degeneration and senile plaques. Neurosci Lett 97: 232-238.
60. Selkoe DJ (1994) Normal and abnormal biology of the beta-amyloid precursor protein. Annu Rev Neurosci 17: 489-517.
61. Wertkin AM, Turner RS, Pleasure SJ, Golde TE, Younkin SG, et al. (1993) Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular beta-amyloid or A4 peptides. Proc Natl Acad Sci U S A 90: 9513-9517.
62. Selkoe DJ (1998) The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. Trends Cell Biol 8: 447-453.
63. Schneider A, Rajendran L, Honsho M, Gralle M, Donnert G, et al. (2008) Flotillin-dependent clustering of the amyloid precursor protein regulates its endocytosis and amyloidogenic processing in neurons. J Neurosci 28: 2874-2882.
64. Koo EH, Squazzo SL (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J Biol Chem 269: 17386-17389.
65. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, et al. (1994) Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). Neuron 13: 45-53.
66. Jarrett JT, Berger EP, Lansbury PT, Jr. (1993) The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 32: 4693-4697.
67. Pike CJ, Overman MJ, Cotman CW (1995) Amino-terminal deletions enhance aggregation of beta-amyloid peptides in vitro. J Biol Chem 270: 23895-23898.
68. Czirr E, Cottrell BA, Leuchtenberger S, Kukar T, Ladd TB, et al. (2008) Independent generation of Abeta42 and Abeta38 peptide species by gamma-secretase. J Biol Chem 283: 17049-17054.
69. Selkoe D, Kopan R (2003) Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration. Annu Rev Neurosci 26: 565-597.
70. Vincent B, Sunyach C, Orzechowski HD, St George-Hyslop P, Checler F (2009) p53-Dependent transcriptional control of cellular prion by presenilins. J Neurosci 29: 6752-6760.
71. Young-Pearse TL, Chen AC, Chang R, Marquez C, Selkoe DJ (2008) Secreted APP regulates the function of full-length APP in neurite outgrowth through interaction with integrin beta1. Neural Dev 3: 15.
72. Saitoh T, Sundsmo M, Roch JM, Kimura N, Cole G, et al. (1989) Secreted form of amyloid beta protein precursor is involved in the growth regulation of fibroblasts. Cell 58: 615-622.
73. Clarris HJ, Key B, Beyreuther K, Masters CL, Small DH (1995) Expression of the amyloid protein precursor of Alzheimer's disease in the developing rat olfactory system. Brain Res Dev Brain Res 88: 87-95.
74. Priller C, Bauer T, Mitteregger G, Krebs B, Kretzschmar HA, et al. (2006) Synapse formation and function is modulated by the amyloid precursor protein. J Neurosci 26: 7212-7221.
75. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, et al. (1991) Physical basis of

cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 30: 572-580.
76. Meziane H, Dodart JC, Mathis C, Little S, Clemens J, et al. (1998) Memory-enhancing effects of secreted forms of the beta-amyloid precursor protein in normal and amnestic mice. Proc Natl Acad Sci U S A 95: 12683-12688.
77. Sisodia SS, Gallagher M (1998) A role for the beta-amyloid precursor protein in memory? Proc Natl Acad Sci U S A 95: 12074-12076.
78. Haass C, Hung AY, Schlossmacher MG, Teplow DB, Selkoe DJ (1993) beta-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. J Biol Chem 268: 3021-3024.
79. Mills J, Reiner PB (1999) Regulation of amyloid precursor protein cleavage. J Neurochem 72: 443-460.
80. Asai M, Hattori C, Szabo B, Sasagawa N, Maruyama K, et al. (2003) Putative function of ADAM9, ADAM10, and ADAM17 as APP alpha-secretase. Biochem Biophys Res Commun 301: 231-235.
81. Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, et al. (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc Natl Acad Sci U S A 96: 3922-3927.
82. Selkoe DJ (2004) Cell biology of protein misfolding: the examples of Alzheimer's and Parkinson's diseases. Nat Cell Biol 6: 1054-1061.
83. Jang H, Arce FT, Ramachandran S, Capone R, Azimova R, et al. (2010) Truncated beta-amyloid peptide channels provide an alternative mechanism for Alzheimer's Disease and Down syndrome. Proc Natl Acad Sci U S A 107: 6538-6543.
84. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286: 735-741.
85. Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, et al. (2003) Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. J Cell Sci 116: 3339-3346.
86. Esler WP, Wolfe MS (2001) A portrait of Alzheimer secretases--new features and familiar faces. Science 293: 1449-1454.
87. Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, et al. (2003) Reconstitution of gamma-secretase activity. Nat Cell Biol 5: 486-488.
88. Haass C, Steiner H (2002) Alzheimer disease gamma-secretase: a complex story of GxGD-type presenilin proteases. Trends Cell Biol 12: 556-562.
89. Vetrivel KS, Cheng H, Lin W, Sakurai T, Li T, et al. (2004) Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J Biol Chem 279: 44945-44954.
90. Prusiner SB (1998) Prions. Proc Natl Acad Sci U S A 95: 13363-13383.
91. Linden R, Martins VR, Prado MA, Cammarota M, Izquierdo I, et al. (2008) Physiology of the prion protein. Physiol Rev 88: 673-728.
92. Weissmann C, Aguzzi A (2005) Approaches to therapy of prion diseases. Annu Rev Med 56: 321-344.
93. Gauczynski S, Peyrin JM, Haik S, Leucht C, Hundt C, et al. (2001) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. EMBO J 20: 5863-5875.
94. Sparkes RS, Simon M, Cohn VH, Fournier RE, Lem J, et al. (1986) Assignment of the human and mouse prion protein genes to homologous chromosomes. Proc Natl Acad Sci U S A 83: 7358-7362.
95. Westergard L, Christensen HM, Harris DA (2007) The cellular prion protein (PrP(C)): its

physiological function and role in disease. Biochim Biophys Acta 1772: 629-644.
96. Vana K, Zuber C, Nikles D, Weiss S (2007) Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy. Cell Mol Neurobiol 27: 107-128.
97. Prusiner SB (1997) Prion diseases and the BSE crisis. Science 278: 245-251.
98. Prusiner SB (2001) Shattuck lecture--neurodegenerative diseases and prions. N Engl J Med 344: 1516-1526.
99. Da Costa Dias B, Jovanovic K, Weiss SF (2011) Alimentary prion infections: Touchdown in the intestine. Prion 5: 6-9.
100. Stahl N, Borchelt DR, Hsiao K, Prusiner SB (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell 51: 229-240.
101. Caughey B, Raymond GJ (1991) The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. J Biol Chem 266: 18217-18223.
102. Ostapchenko VG, Beraldo FH, Guimaraes AL, Mishra S, Guzman M, et al. (2013) Increased prion protein processing and expression of metabotropic glutamate receptor 1 in a mouse model of Alzheimer's disease. J Neurochem 127: 415-425.
103. Shyng SL, Heuser JE, Harris DA (1994) A glycolipid-anchored prion protein is endocytosed via clathrin-coated pits. J Cell Biol 125: 1239-1250.
104. Vey M, Pilkuhn S, Wille H, Nixon R, DeArmond SJ, et al. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc Natl Acad Sci U S A 93: 14945-14949.
105. Kang YS, Zhao X, Lovaas J, Eisenberg E, Greene LE (2009) Clathrin-independent internalization of normal cellular prion protein in neuroblastoma cells is associated with the Arf6 pathway. J Cell Sci 122: 4062-4069.
106. Sannerud R, Declerck I, Peric A, Raemaekers T, Menendez G, et al. (2011) ADP ribosylation factor 6 (ARF6) controls amyloid precursor protein (APP) processing by mediating the endosomal sorting of BACE1. Proc Natl Acad Sci U S A 108: E559-568.
107. Borchelt DR, Taraboulos A, Prusiner SB (1992) Evidence for synthesis of scrapie prion proteins in the endocytic pathway. J Biol Chem 267: 16188-16199.
108. Choi BR, Lee J, Kim SY, Yim I, Kim EH, et al. (2013) Prion protein conversion induced by trivalent iron in vesicular trafficking. Biochem Biophys Res Commun 432: 539-544.
109. Godsave SF, Wille H, Pierson J, Prusiner SB, Peters PJ (2013) Plasma membrane invaginations containing clusters of full-length PrPSc are an early form of prion-associated neuropathology in vivo. Neurobiol Aging 34: 1621-1631.
110. Timmes AG, Moore RA, Fischer ER, Priola SA (2013) Recombinant prion protein refolded with lipid and RNA has the biochemical hallmarks of a prion but lacks in vivo infectivity. PLoS One 8: e71081.
111. Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, et al. (1993) Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci U S A 90: 10962-10966.
112. Zuber C, Knackmuss S, Rey C, Reusch U, Rottgen P, et al. (2008) Single chain Fv antibodies directed against the 37 kDa/67 kDa laminin receptor as therapeutic tools in prion diseases. Mol Immunol 45: 144-151.
113. Vana K, Zuber C, Pflanz H, Kolodziejczak D, Zemora G, et al. (2009) LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. Infect Disord Drug Targets 9: 69-80.
114. Zuber C, Ludewigs H, Weiss S (2007) Therapeutic approaches targeting the prion

receptor LRP/LR. Vet Microbiol 123: 387-393.
115. Pflanz H, Vana K, Mitteregger G, Pace C, Messow D, et al. (2009) Microinjection of lentiviral vectors expressing small interfering RNAs directed against laminin receptor precursor mRNA prolongs the pre-clinical phase in scrapie-infected mice. J Gen Virol 90: 269-274.
116. Wong BS, Liu T, Li R, Pan T, Petersen RB, et al. (2001) Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. J Neurochem 76: 565-572.
117. Milhavet O, McMahon HE, Rachidi W, Nishida N, Katamine S, et al. (2000) Prion infection impairs the cellular response to oxidative stress. Proc Natl Acad Sci U S A 97: 13937-13942.
118. Bounhar Y, Zhang Y, Goodyer CG, LeBlanc A (2001) Prion protein protects human neurons against Bax-mediated apoptosis. J Biol Chem 276: 39145-39149.
119. Solforosi L, Criado JR, McGavern DB, Wirz S, Sanchez-Alavez M, et al. (2004) Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. Science 303: 1514-1516.
120. Da Costa Dias B, Jovanovic K, Gonsalves D, Weiss SFT (2011) Structural and mechanistic commonalities of amyloid-beta and prion protein. Prion In Press.
121. Watt NT, Hooper NM (2007) Prion proteins in Alzheimer's Disease. Future Neurology 2: 587 - 590.
122. Parkin ET, Watt NT, Hussain I, Eckman EA, Eckman CB, et al. (2007) Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. Proc Natl Acad Sci U S A 104: 11062-11067.
123. Griffiths HH, Whitehouse IJ, Baybutt H, Brown D, Kellett KA, et al. (2011) Prion protein interacts with BACE1 protein and differentially regulates its activity toward wild type and Swedish mutant amyloid precursor protein. J Biol Chem 286: 33489-33500.
124. Kellett KA, Hooper NM (2009) Prion protein and Alzheimer disease. Prion 3: 190-194.
125. Lauren J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature 457: 1128-1132.
126. Kudo W, Petersen RB, Lee HG (2013) Cellular prion protein and Alzheimer disease: link to oligomeric amyloid-beta and neuronal cell death. Prion 7: 114-116.
127. Kudo W, Lee HP, Zou WQ, Wang X, Perry G, et al. (2012) Cellular prion protein is essential for oligomeric amyloid-beta-induced neuronal cell death. Hum Mol Genet 21: 1138-1144.
128. Barry AE, Klyubin I, Mc Donald JM, Mably AJ, Farrell MA, et al. (2011) Alzheimer's disease brain-derived amyloid-beta-mediated inhibition of LTP in vivo is prevented by immunotargeting cellular prion protein. J Neurosci 31: 7259-7263.
129. Um JW, Strittmatter SM (2013) Amyloid-beta induced signaling by cellular prion protein and Fyn kinase in Alzheimer disease. Prion 7: 37-41.
130. Rao NC, Barsky SH, Terranova VP, Liotta LA (1983) Isolation of a tumor cell laminin receptor. Biochem Biophys Res Commun 111: 804-808.
131. Ludwig GV, Kondig JP, Smith JF (1996) A putative receptor for Venezuelan equine encephalitis virus from mosquito cells. J Virol 70: 5592-5599.
132. Wang KS, Kuhn RJ, Strauss EG, Ou S, Strauss JH (1992) High-affinity laminin receptor is a receptor for Sindbis virus in mammalian cells. J Virol 66: 4992-5001.
133. Tio PH, Jong WW, Cardosa MJ (2005) Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3. Virol J 2: 25.
134. Hundt C, Peyrin JM, Haik S, Gauczynski S, Leucht C, et al. (2001) Identification of

interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. EMBO J 20: 5876-5886.
135. Leucht C, Simoneau S, Rey C, Vana K, Rieger R, et al. (2003) The 37 kDa/67 kDa laminin receptor is required for PrP(Sc) propagation in scrapie-infected neuronal cells. EMBO Rep 4: 290-295.
136. Morel E, Andrieu T, Casagrande F, Gauczynski S, Weiss S, et al. (2005) Bovine prion is endocytosed by human enterocytes via the 37 kDa/67 kDa laminin receptor. Am J Pathol 167: 1033-1042.
137. Auth D, Brawerman G (1992) A 33-kDa polypeptide with homology to the laminin receptor: component of translation machinery. Proc Natl Acad Sci U S A 89: 4368-4372.
138. Makrides S, Chitpatima ST, Bandyopadhyay R, Brawerman G (1988) Nucleotide sequence for a major messenger RNA for a 40 kilodalton polypeptide that is under translational control in mouse tumor cells. Nucleic Acids Res 16: 2349.
139. Sato M, Kinoshita K, Kaneda Y, Saeki Y, Iwamatsu A, et al. (1996) Analysis of nuclear localization of laminin binding protein precursor p40 (LBP/p40). Biochem Biophys Res Commun 229: 896-901.
140. Kinoshita K, Kaneda Y, Sato M, Saeki Y, Wataya-Kaneda M, et al. (1998) LBP-p40 binds DNA tightly through associations with histones H2A, H2B, and H4. Biochem Biophys Res Commun 253: 277-282.
141. Gauczynski S, Nikles D, El-Gogo S, Papy-Garcia D, Rey C, et al. (2006) The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. J Infect Dis 194: 702-709.
142. Kolodziejczak D, Da Costa Dias B, Zuber C, Jovanovic K, Omar A, et al. (2010) Prion interaction with the 37-kDa/67-kDa laminin receptor on enterocytes as a cellular model for intestinal uptake of prions. J Mol Biol 402: 293-300.
143. Zuber C, Knackmuss S, Zemora G, Reusch U, Vlasova E, et al. (2008) Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. J Mol Biol 378: 530-539.
144. Khumalo T, Reusch U, Knackmuss S, Little M, Veale RB, et al. (2013) Adhesion and Invasion of Breast and Oesophageal Cancer Cells Are Impeded by Anti-LRP/LR-Specific Antibody IgG1-iS18. PLoS One 8: e66297.
145. Omar A, Reusch U, Knackmuss S, Little M, Weiss SF (2012) Anti-LRP/LR-specific antibody IgG1-iS18 significantly reduces adhesion and invasion of metastatic lung, cervix, colon and prostate cancer cells. J Mol Biol 419: 102-109.
146. Khusal R, Da Costa Dias B, Moodley K, Penny C, Reusch U, et al. (2013) In vitro inhibition of angiogenesis by antibodies directed against the 37kDa/67kDa laminin receptor. PLoS One 8: e58888.
147. Moodley K, Weiss SF (2013) Downregulation of the non-integrin laminin receptor reduces cellular viability by inducing apoptosis in lung and cervical cancer cells. PLoS One 8: e57409.
148. Rieger R, Edenhofer F, Lasmezas CI, Weiss S (1997) The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. Nat Med 3: 1383-1388.
149. Patra SK, Rizzi F, Silva A, Rugina DO, Bettuzzi S (2008) Molecular targets of (-)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts. J Physiol Pharmacol 59 Suppl 9: 217-235.
150. Ehehalt R, Keller P, Haass C, Thiele C, Simons K (2003) Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol 160: 113-123.

151. Haass C, Lemere CA, Capell A, Citron M, Seubert P, et al. (1995) The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. Nat Med 1: 1291-1296.
152. Rajendran L, Annaert W (2012) Membrane trafficking pathways in Alzheimer's disease. Traffic 13: 759-770.
153. Annaert W, De Strooper B (1999) Presenilins: molecular switches between proteolysis and signal transduction. Trends Neurosci 22: 439-443.
154. Pasternak SH, Callahan JW, Mahuran DJ (2004) The role of the endosomal/lysosomal system in amyloid-beta production and the pathophysiology of Alzheimer's disease: reexamining the spatial paradox from a lysosomal perspective. J Alzheimers Dis 6: 53-65.
155. Kaether C, Schmitt S, Willem M, Haass C (2006) Amyloid precursor protein and Notch intracellular domains are generated after transport of their precursors to the cell surface. Traffic 7: 408-415.
156. Rajendran L, Honsho M, Zahn TR, Keller P, Geiger KD, et al. (2006) Alzheimer's disease beta-amyloid peptides are released in association with exosomes. Proc Natl Acad Sci U S A 103: 11172-11177.
157. Rechards M, Xia W, Oorschot VM, Selkoe DJ, Klumperman J (2003) Presenilin-1 exists in both pre- and post-Golgi compartments and recycles via COPI-coated membranes. Traffic 4: 553-565.
158. Chyung JH, Raper DM, Selkoe DJ (2005) Gamma-secretase exists on the plasma membrane as an intact complex that accepts substrates and effects intramembrane cleavage. J Biol Chem 280: 4383-4392.
159. Jares-Erijman EA, Jovin TM (2003) FRET imaging. Nat Biotechnol 21: 1387-1395.
160. Scholefield Z, Yates EA, Wayne G, Amour A, McDowell W, et al. (2003) Heparan sulfate regulates amyloid precursor protein processing by BACE1, the Alzheimer's beta-secretase. J Cell Biol 163: 97-107.
161. Patey SJ, Yates EA, Turnbull JE (2005) Novel heparan sulphate analogues: inhibition of beta-secretase cleavage of amyloid precursor protein. Biochem Soc Trans 33: 1116-1118.
162. Beckman M, Holsinger RM, Small DH (2006) Heparin activates beta-secretase (BACE1) of Alzheimer's disease and increases autocatalysis of the enzyme. Biochemistry 45: 6703-6714.
163. Nikles D, Vana K, Gauczynski S, Knetsch H, Ludewigs H, et al. (2008) Subcellular localization of prion proteins and the 37 kDa/67 kDa laminin receptor fused to fluorescent proteins. Biochim Biophys Acta 1782: 335-340.
164. Sperling R, Salloway S, Brooks DJ, Tampieri D, Barakos J, et al. (2012) Amyloid-related imaging abnormalities in patients with Alzheimer's disease treated with bapineuzumab: a retrospective analysis. Lancet Neurol 11: 241-249.
165. Galasko DR, Graff-Radford N, May S, Hendrix S, Cottrell BA, et al. (2007) Safety, tolerability, pharmacokinetics, and Abeta levels after short-term administration of R-flurbiprofen in healthy elderly individuals. Alzheimer Dis Assoc Disord 21: 292-299.
166. Doody RS, Raman R, Farlow M, Iwatsubo T, Vellas B, et al. (2013) A phase 3 trial of semagacestat for treatment of Alzheimer's disease. N Engl J Med 369: 341-350.
167. Farlow M, Arnold SE, van Dyck CH, Aisen PS, Snider BJ, et al. (2012) Safety and biomarker effects of solanezumab in patients with Alzheimer's disease. Alzheimers Dement 8: 261-271.