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Probing the role of the 37kDa/67kDa Laminin Receptor in Amyloid beta mediated pathogenesis in Alzheimer's Disease

PhD Thesis

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I , Bianca Da Costa Dias (Person number: 301904), am a student registered for the degree of Doctor of Philosophy (PhD) in the academic year 2014.

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Research Outputs

Peer-reviewed Review Articles

1. V. Mbazima* , B. Da Costa Dias*, A. Omar , K. Jovanovic and Weiss SF.(2010). Interactions between PrP(c) and other ligands with the 37kDa/67kDa laminin receptor. *Frontiers in Bioscience*. 15:1150-1163
2. B. Da Costa Dias and S.F.T. Weiss. (2010). A kiss of a prion: New Implications for oral transmissibility. *Journal of Infectious Diseases.* 201(**11**): 1615-1616
3. B. Da Costa Dias, K Jovanovic and S.F.T. Weiss (2011). Alimentary prion infections: Touchdown in the intestine. *Prion*. 5(**1**):6-9
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5. B. Da Costa Dias, K. Jovanovic , D. Gonsalves and Weiss SF. (2011). Structural and Mechanistic commonlaities of amyloid β and the prion protein. *Prion.* 5(**3**):126-137
6. D. Gonsalves , K. Jovanovic , B. Da Costa Dias and Weiss S.F. (2012). Global Alzheimer Research Summit: Basic and clinical research: Present and future Alzheimer research. *Prion*. 6(**1**):7-10

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Original Publications (published/submitted)

1. 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.T. Weiss. (2010). Prion Interactions with the 37kDa/67kDa Laminin Receptor on Enterocytes as a cellular model for intestinal uptake of prions. *Journal of Molecular Biology*. 402(**2**):293-300
2. R. Khusal*, B. Da Costa Dias*, K. Moodley, U. Reusch, S. Knackmuss, M. Little and S.F.T. Weiss. (2013). Inhibition of angiogenesis by antibodies directed against the 37kDa/67kDa laminin receptor *in vitro*. *PLOS One* 8(**3**). e58888. doi:10.1371/journal.pone.0058888
3. K. Jovanovic, D. Gonsalves, B. Da Costa Dias, U. Reusch, S. Knackmuss, M. Little, M. Weinberg and S.F.T Weiss. (2013). Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's Disease. *Scientific Reports*. 3,2699; doi:10.1038/srep02699
4. B. Da Costa Dias, K. Jovanovic, D. Gonsalves, K. Moodley, U. Reusch, S. Knackmuss, C. Penny, M. Weinberg, M. Little and S.F.T. Weiss. (2013). Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity. *Scientific Reports* 3, 2702; doi:10.1038/srep02702
5. C. Chetty, T. Khumalo., B. Da Costa Dias, U. Reusch, S. Knackmuss, M. Little and S.F.T. Weiss. (2014). Anti-LRP/LR specific antibody IgG1-iS18 impedes adhesion and invasion of liver cancer cells. *PLOS One*. 9(**5**).e96268.doi:10.1371/journal.pone.0096268
6. K. Jovanovic, B. Loos, B. Da Costa Dias, C. Penny and S.F.T. Weiss (2014). 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*. 9(**6**):e100373. doi:10.1371/journal.pone.0100373
7. B. Da Costa Dias, K. Jovanovic, D. Gonsalves, K. Moodley, U. Reusch, S. Knackmuss, C. Penny, M. Weinberg, M. Little and S.F.T. Weiss. 37kDa/67kDa Laminin Receptor acts as a receptor for $A\beta_{42}$ internalization. *Scientific Reports*, 4, 556; doi:10.1038/srep05556.
8. M.G. Pinto, B. Da Costa Dias, K. Jovanovic, S. Knackmuss, U. Reusch, M. Little and S.F.T. Weiss (2014). The 37kDa/67kDa LRP/LR plays a central role in Aβ-PrP^c mediated cytotoxicity in Alzheimer's disease. *Journal of Alzheimer's Disease*, revised

*These authors contributed equally to this work.

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Authors: B. Da Costa Dias, K. Jovanovic, D. Goncalves, S. Knackmuss, U. Reusch, M. Little and S. F.T. Weiss

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Title: The 37kDa/67kDa Laminin Receptor interacts with and is implicated in Amyloid beta ($A\beta_{42}$) induced cytotoxicity in Alzheimer's Disease
Authors: B. Da Costa Dias, K. Jovanovic, D. Gonsalves, S. Knackmuss, U. Reusch, M. Little and S. F.T. Weiss

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Title: Amyloid beta (Aβ) mediates its cytotoxic effects through the 37kDa/67kDa Laminin Receptor - Advancing our understanding of Alzheimer's Disease
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5. SASBMB Congress 2014

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Title: The 37kDa/67kDa Laminin Receptor is a central player in Amyloid Beta ($A\beta_{42}$) internalization and cytotoxicity In Alzheimer's Disease
Authors: B. Da Costa Dias, K. Jovanovic, D.Gonsalves, K. Moodley, S. Knackmuss, U. Reusch, C. Penny, M.S. Weinberg, M. Little and S. F.T. Weiss

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"The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science" *-Albert Einstein*

Abstract

Alzheimer's Disease (AD) is characterized by neurofibrillary tangles, senile plaques and neuronal loss. Although the mechanisms underlying Amyloid beta 42 ($A\beta_{42}$) neurotoxicity have not been firmly established, it is proposed that the neuronal loss is elicited through associations with cell surface receptors. The cellular prion protein (PrP^c) has been identified as an $A\beta_{42}$ receptor and as a regulator of the amyloidogenic cleavage pathway. As $A\beta_{42}$ shares common binding partners with the 37kDa/67kDa laminin receptor (LRP/LR), including PrP^c, we investigated whether these proteins interact and assessed the pathological significance of this association. LRP/LR was found to co-localize with Aβ on the cell surface. The occurrence of FRET suggested that an interaction between LRP/LR and Aβ indeed exists at the cell surface. Furthermore, pull down assays and Aβ-specific ELISAs demonstrated that LRP/LR forms a physical association with endogenously shed Aβ, thereby verifying the physiological relevance of this association. Antibody blockade by IgG1-iS18 and shRNA-mediated downregulation of LRP/LR significantly enhanced cell viability and proliferation and decreased apoptosis in cells co-treated with $A\beta_{42}$ when compared to cells incubated with $A\beta_{42}$ alone. In addition, antibody blockade and shRNA-mediated downregulation of LRP/LR significantly impeded $A\beta_{42}$ internalization. These results suggest that LRP/LR acts as an internalization receptor for $A\beta_{42}$ and may thereby contribute to the cytotoxicity of the neuropeptide by facilitating intracellular $A\beta_{42}$ accumulation and aggregation - which has consequences for cell proliferation and may promote apoptosis. These findings recommend anti-LRP/LR specific antibodies and shRNAs as potential therapeutic tools for Alzheimer's Disease treatment.

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List of Abbreviations

α7nAChR	α-7 nicotinic acetylcholine receptor
aa	amino acids
AAV	Adeno-associated virus
Aβ	amyloid beta/beta amyloid peptide
Aβo	$A\beta_{42}$ oligomers
AβDP	Aβ degrading proteases
ACE	Angiotensin-converting enzyme
AD	Alzheimer's Disease
ADAM	A disintegrin and metalloproteinase
AICD	Amyloid intracellular domain
APH	Anterior pharynx defective
APOE ε4	Apolipoprotein E ε4 allele
APP	Amyloid Precursor Protein
BACE	Beta-site APP cleaving enzyme
Bax	B-cell leukemia/lymphoma-2-associated X protein
BBB	Blood brain barrier
BMEC	Brain microvascular endothelial cell
BSE	Bovine spongiform encephalopathy
CAT	Chloramphenicol acetyltransferase
Cdk	Cyclin-dependent Kinase
cGMP	Cyclic guanosine monophosphate
CJD	Creutzfeldt-Jakob Disease
CNF-1	Cytotoxic necrotizing factor-1
CNS	Central nervous system
CO_2	Carbon dioxide
CTF	Carboxyl terminal fragment
CSF	Cerebrospinal fluid
CWD	Chronic Wasting Disease
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
DR6	Death receptor 6
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders (4^{th} edition)

ECE	Endothelin-converting enzyme
ECM	Extracellular matrix
eEF 1A	Eukaryotic translation elongation factor 1A
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
EOAD	Early-onset Alzheimer's Disease
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FasL	Fas ligand
FDA	U.S. Food and Drug Administration
FFI	Fatal Familial Insomnia
*f*MRI	Functional magnetic resonance imaging
GAGs	Glycosaminoglycans
GM-CSF	Granulocyte- macrophage colony stimulating factor
GPI	Glycosyl phosphatidylinositol anchor
GSK-3β	Glycogen synthase kinase-3β
GSS	Gerstmann-Straussler syndrome
h	Hour(s)
HEK293	Human Embryonic Kidney cells
HIF-1	Hypoxia inducible factor-1
HSPGs	Heparan sulphate proteoglycans
ICD-10	International Statistical Classification of Diseases (10^{th} edition)
IDE	Insulin degrading enzyme
IgG	Immunoglobulin class G
IL	Interleukin
ITC	Isothermal titration calorimetry
JNK	c-Jun NH_2-terminal protein kinase
kDa	kilodaltons
KPI	Kunitz type serine protease inhibitor
LamR	Laminin receptor
LBR	Laminin binding protein
LBR-p40	Laminin binding protein precursor p40

LOAD	Late-onset Alzheimer's Disease
LRP1	Low density lipoprotein receptor-related protein 1
LRP	Laminin Receptor Precursor
LR	High affinity laminin receptor
LTP	Long-term potentiation
MAP	Microtubule associated protein
mGluR5	Metabotrophic glutamate receptor
µ	Micro
µg	Microgram(s)
miRNA	microRNA
MCI	Mild Cognitive Impairment
mg	Milligram(s)
mM	Millimolar
MK	Midkine
MMP	Matrix metalloprotease
MMSE	Mini Mental State Examination
MRI	Magnetic resonance imaging
MRLC	Myosin regulatory light chain
MRS	Magnetic resonance spectroscopy
MYPT1	Myosin phosphatise targeting subunit-1
nm	Nanometer(s)
NCAM	Neural cell adhesion molecule
NFT	Neurofibrillary tangle
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
Nct	Nicastin
NEP	Neprilysin
NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
NRG-1	Neuregulin-1
NSAID	Non-steroidal anti-inflammatory drug
p-Tau	Phosphorylated tau
p75NTR	p75 neurotrophin receptor

PD	Prion disorder
PET	Positron emission tomography
PHF	Paired helical fragment
PS	Presenilin
PSEN-2	Presenilin enhancer 2
PP	Phosphatase
PrP^{c}	Cellular prion protein isoform
PrP^{Sc}	Infectious prion protein isoform
PTP1B	Protein tyrosine phosphatise 1B
RAGE	Receptor for advanced glycation end products
RNAi	RNA interference
ROS	Reactive oxygen species
RPSA	Ribosomal protein SA
sAPPα	CTF Cleavage product generated from α-secretase cleavage of APP
sAPPβ	CTF cleavage product generated from β-secretase cleavage of APP
SPR	Surface plasmon resonance
SR-SIM	Super resolution structured illumination microscopy
TACE	Tumour necrosis factor-α converting enzyme
TBE	Tick-borne encephalitis
TBI	Traumatic brain injury
TGF	Tumour growth factor
TGN	Trans-golgi network
TIMAP	TGF-β inhibited membrane associated protein
TM	Transmembrane
TNF	Tumour necrosis factor
TSE	Transmissible spongiform encephalopathies
uPA	Urokinase-type plasminogen activator
VEE	Venezuelan equine encephalitis

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Chapter 1

1.1 Introduction

Alzheimer's Disease (AD), also referred to as Morbus Alzheimer's, Alzheimer's Dementia and Alzheimer's, is a multifactorial progressive neurodegenerative disease characterized clinically by the manifestation of dementia and histopathologically by neuronal degeneration[8]. The disease was first described by Alois Alzheimer in 1906 and has since become the most prevalent form of dementia (accounting for 60-80% of dementia cases) afflicting the elderly[9].

At present in excess of 35 million people globally[10] and approximately 1 in 68 South Africans are afflicted with Alzheimer's Disease. The incidence of AD increases exponentially with age, with people aged 65-69 exhibiting an incidence of 4.4%, whilst people over 90 years of age display an incidence of 22%[11]. Furthermore, owing to global increases in life expectancies, the prevalence of AD is predicted to rise to 86 million people by 2050[12].

In addition, it is reported that the annual cost per patient with dementia (€ 20,000 in Europe in 2008) exceeds that for patients with cancer and cardiovascular disease[13]. In 2013 it was reported that $203 billion was spent in providing care for AD patients in the United States of America and this is predicted to increase to $1.2 trillion annually by 2050 (The Alzheimer's Association, https://www.alz.org/). Thus, AD is not only a major social concern but also has grave economic implications.

1.2. Symptomology

AD related symptoms have been reported to occur in three stages. The initial symptoms, observable during the first stage of AD, include cognitive dysfunction, deficits in episodic memory, loss of visual-spatial skills[14] and the deterioration of language skills. This cognitive decline is a result of neuronal degeneration, cell loss and finally atrophy in the brain regions associated with these functions, namely the limbic system, neocortical regions and the basal forebrain[15]. The second symptomatic stage is characterised by personality and behavioural disturbances including paranoia, confusion, hallucinations, aggression and loss of social appropriateness[16, 17]. Ultimately, AD patients exhibit progressive motoric disturbances, first in performing complex tasks and later in performing even basic tasks as well as exhibit loss

in co-ordination[17, 18]. Disease progression, although variable in the chronology of symptom development and severity, is proposed to occur over a decade [19].

It is noteworthy to highlight that that the neuropathological alterations associated with AD are present more than 20 years before the onset of symptoms[20].

1.3 Diagnosis

A definitive diagnosis of AD is only attainable after a post-mortem biopsy, thereby allowing for the identification of the pathological hallmarks of AD - namely extracellular neuritic amyloid plaques and intracellular neurofibrillary tangles. Clinical diagnosis is currently performed employing a combination of neurological examinations, tests that assess mental status (including the Mini Mental State Examination (MMSE))[21] and neuroimaging[14] and is occasionally only reached by excluding other causes of dementia[8]. However, existing diagnostic criteria corresponds to advanced disease[13], thereby making diagnosis during Mild Cognitive Impairment (MCI) and early stages of AD challenging[14]. MCI is an intermediate between normal and demented states and is often a beneficial label to define people at risk of developing AD[22] (**Figure 1.1**). Patients with MCI are at triple the risk of developing AD compared to their normal counterparts[23]. However, it must be emphasized that patients with MCI may progress to other dementias, as opposed to just AD, including vascular, Lewy body and frontotemporal dementias[24] (**Figure 1.1**).

Clinical diagnostic criteria are outlined in several manuals including: the Diagnostic and Statistical Manual of Mental Disorders (4th edition) (DSM-IV)(Americam Psychiatric Association), the International Statistical Classification of Diseases (10th edition) (ICD-10)(World Health Organisation) and the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[25].

Yet, as previously stated, pathology precedes symptoms by decades and as such earlier diagnosis and therapeutic intervention, in the aim of halting disease progression to AD, is highly desirable. This therefore highlights the need to identify reliable and robust biomarkers of early pathological changes.

1.4 Biomarkers for Alzheimer's Disease

Biomarkers are biological measures of processes, either physiological or pathological in nature, that are employed to evaluate disease risk or prognosis. Although the value of diagnostic AD biomarkers cannot be stressed enough, biomarkers may additionally be indicative of the degree of neuroprotection, serve as tools for the detection and monitoring of the disease-modifying effects of AD therapeutics as well as serve as indicators of potential undesirable side effects[25]. Currently, neuroimaging techniques and cerebrospinal fluid (CSF) biomarkers are the best characterised and most accurate indicators of the ongoing pathophysiological processes in AD[26, 27]. Strides are currently being made with regards to the identification and validation of blood based AD biomarkers owing to the non-invasive and inexpensive procedures associated with sample collection.

1.4.1. Neuroimaging

Four such techniques are currently utilized. The first being magnetic resonance imaging (MRI) which reveals the degree of grey matter in the affected brain regions and is employed as a biomarker owing to its ability to ascertain the rate of AD-associated brain atrophy. Blood oxygen-dependent level (BOLD) functional MRI (*f*MRI) which assesses memory input and neuronal processing within different brain regions is the second technique. The third technique, magnetic resonance spectroscopy (MRS), allows for changes in biochemical compounds within the brain to be assessed- including glutamate, creatine, myoinisitol and N-acetyl aspartate - metabolites deregulated during AD pathology. The final technique is the most widely used neuroimaging AD biomarker, namely positron emission tomography (PET), specifically those employing the Aβ-binding compound- Pittsburgh compound B (^{11}C-PIB) – a thioflavin analogue. These amyloid-PET scans provide visual information regarding Aβ plaque load[25].

1.4.2 CSF biomarkers

Owing to the relative ease with which proteins may be transported between the brain and the CSF, CSF biomarkers most accurately reflect brain neurochemistry. The four validated and established CSF biomarkers are: $A\beta_{40}$, $A\beta_{42}$, total tau and phosphorylated tau (p-tau) levels[25]. The degree to which the levels of these biomarkers differ from those in cognitively normal people may be used as predictors of progression from MCI to AD.

The CSF levels of soluble $A\beta_{42}$ and the $A\beta_{42}$:$A\beta_{40}$ ratio is decreased in AD, and it has been proposed that this may be due to sequestration of the peptide into amyloid plaques[28] as well as enhanced cellular uptake and intraneuronal accumulation of $A\beta_{42}$. Conversely, there is an increase in tau (an indicator of axonal damage not specific to AD) and p-tau levels in AD patients.

Several other candidate CSF biomarkers, specifically those associated with amyloidogenic processing, the innate immune system, cholesterol metabolism and endosomal vesicle recycling [29], have been explored, however these have yet to be fully validated. These include: secreted isoforms of the amyloid precursor priotein (APP) and its cleavage products (sAPPα and sAPPβ), Aβ oligomers, Aβ degradation products, β-site APP-cleaving enzyme-1 (BACE-1) activity and concentration[25] as well as 24S-hydroxycholesterol, angiotensinogen, apolipoproteins, complement components, transthyretin, thioredoxin and vascular growth factor to name a few[22].

It must be noted that the combination of neuroimaging and CSF biomarkers provides enhanced sensitivity and specificity and is therefore more accurate than each modality alone[29].

1.4.3 Blood biomarkers

Another source of biomarkers that has been the focus of mounting investigative efforts is blood owing to the ease, enhanced frequency and cost-effectiveness with which samples may be collected. Although a number of studies have been conducted in the aim of identifying plasma-based biomarker panels, these results are often controversial and not well replicated. This is particularly true when assessing plasma Aβ levels as the relationship between Aβ levels in the brain and plasma remains unclear. Proposed candidate plasma-biomarkers include: desmosterol, clusterin, chitinase 3-like 1 protein, matrix metalloproteinase 2[29], α1-antitrypsin[14] as well as inflammation related proteins such as interleukins (IL-6, IL-1β). Doecke et al., have identified a panel of over 20 biomarkers that are differently represented in the plasma of AD patients[12].

In addition to these proteomic and metabolomic candidates, circulating microRNAs (miRNAs) have also gained interest as plasma-derived biomarkers. Numerous miRNAs,

including miR-137,-181c,-9 and-29a/b[30] as well as a novel 7-miRNA signature[14] have been identified and shown to successfully distinguish between non-demented and AD patients.

However, all the aforementioned plasma-biomarkers need to be further corroborated in additional studies and their AD-predictive potential needs to be validated.

As discussed previously, AD biomarkers are not only invaluable in diagnosis but also as tools for monitoring the therapeutic potential and side effects of AD drug candidates and may henceforth become central to drug screening and treatment decisions[25].

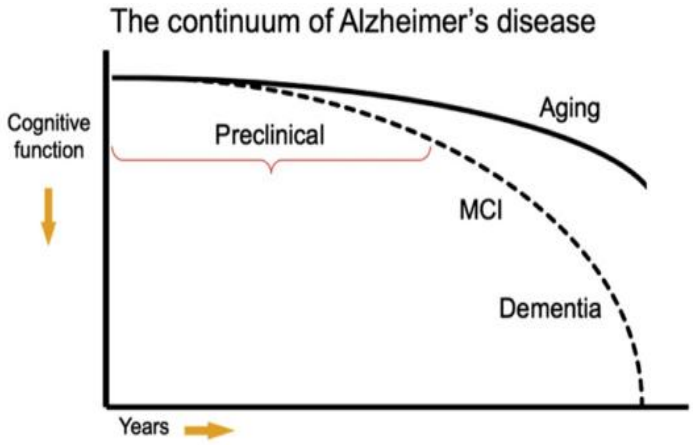


Figure 1.1| A hypothetical model of the clinical trajectory of Alzheimer's Disease. Mild cognitive impairment (MCI) precedes Alzheimer's Disease but it must be emphasized that not all individuals with MCI will progress to Alzheimer's Disease (Adopted from [1]).

1.5 Alzheimer's Disease Therapeutics

Owing to the social and economic implications of AD, the development of not only effective AD therapeutics but also potentially preventative strategies is of major importance.

There are two AD therapeutic categories namely symptomatic and disease modifying. Although there is as yet no cure for AD, five drugs have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of the disease symptoms. These include four cholinesterase inhibitors (Donepezil, Galantamine, Rivastigmine and Tacrine- although the latter is rarely prescribed due to reported adverse liver effects) – which offer benefit by reducing the enzymatic degradation of the vital neurotransmitter acetylcholine. The other

FDA approved treatment is Memantine, a *N*-methyl-D-aspartate (NMDA) receptor antagonist - as overstimulation of these receptors in AD contributes to neuronal cell death (The Alzheimer's Association, https://www.alz.org/).

Additional symptomatic treatments include non-steroidal anti-inflammatory drugs (NSAIDs) and anti-depressants[31].

While most avaliable AD therapeutics are merely palliative in nature, both academic and pharmacological research efforts over the past few decades have concentrated on developing strategies to either prevent disease onset through the sequesteration of pathogic progenitors or slow disease progression[31].

The majority of these treatment strategies target the amyloid beta (Aβ) peptide, considered the primary aetiological agent in AD. These include agents that the modulate the activity of the enzymes involved in Aβ generation namely β- and ɣ- secretases inhibitors (such as thiazolidinediones and semagacestat, respectively)[32] and α-secretase stimulators (EHT0202)[33]. However, as these enzymes have a multitude of physiological substrates (as shall be discussed below) their inhibition often results in adverse physiological effects. Thus there is a need to enhance the selectivity of such inhibitors/modulators for the amyloid precursor protein (APP) whilst not affecting the cleavage of other substrates before such strategies may be considered as AD therapeutics. Moreover, Aβ immunotherapy has received the most attention as potential AD therapeutics. This includes passive administration of antibodies directed against the neurotoxic peptide (such as Bapineuzumab and Solanezumab) and active immunisation (with the peptide itself) with the aim of inducing an endogenous polyclonal antibody response[20]. Aβ fibrillization inhibitors (i.e. metal chelators such as iodochlorohydroxyquin and cliquinol)[31] have also been designed, with those preventing the formation of Aβ oligomers (such as scyllo-inositol) believed to have the greatest potential[20].

As most therapeutics targeting Aβ have failed during clinical trials, researchers are beginning to look at therapeutic targets beyond the neurotoxic peptide. This includes targeting tau through the use of tau phosphorylating kinase inhibitors (such as valproate), aggregation inhibitors (such as Methylthioninium), microtubule stabilizing agents (such as BMS241027) as well as passive and active immunotherapy[20]. In addition, drugs targetting processes which are perturbed during AD, such as microglial-mediated inflammation, oxidative stress, cell

signalling, membrane dynamics, receptor activation and ion channel formation, are under investigation and may bode well in the search for effective disease modifying therapeutics[20].

1.6 Alzheimer's Disease Epidemiology

1.6.1 Genetic Epidemiology

AD is classified into two categories based on the age of symptom onset, namely Early onset-AD (EOAD) and Late onset-AD (LOAD).

Early onset-AD (EOAD), also referred to as Familial Alzheimer's Disease (FAD), is an autosomal dominant disease characterized by an early (<65 years old) onset of disease symptoms[11]. EOAD accounts for merely 1-5% of all AD cases, and generally results in more rapid disease progression. EOAD is the result of mutations in three genetic loci (genes) namely: *APP* on chromosome 21 and the presenilin (the enzymatically active subunit within the ɣ-secretase complex) encoding genes (*PSEN1* and *PSEN2*) on chromosomes 14 and 1, respectively[34]. These mutations result in either enhanced $A\beta_{42}$ synthesis[35] or reduced $A\beta_{40}$ generation[36].

LOAD, which accounts for >95% of all AD, is characterised by disease onset after 65 years of age. There are numerous "susceptibility" genes associated with LOAD and the difference in disease risk frequency between monozygotic and di-zygotic twins have led to the suggestion that genetic epidemiology contributes substantially (60-80%) to LOAD[22]. The ε4 allele of the apolipoprotein E (ApoE ε4), a cholesterol-binding glycoprotein, is the only unequivocally established genetic risk factor which predisposes the carrier to LOAD[37]. Numerous other candidate genes have been identified during genome-wide association studies as possible risk factors but most require additional studies to verify their reliability as susceptibility loci. Genes which have been repeatedly identified in independent studies include: *SORL1* (sortilin-related receptor, an APP transporter protein), *CLU* (apolipoprotein J, a lipid transporter protein), *BIN1* (amphiphysin II, a protein involved in cellular trafficking, actin dynamics and clathrin-mediated endocytosis), *PICALM* (phosphotidylinositol-binding clathrin assembly protein, involved in clathrin-mediated endocytosis) and *CR1* (complement receptor type I, involved in neuro-inflammation by clearing immune complexes comprising

the C3b and C4b factors). Other candidate genes have also been identified and most are involved in a handful of cellular processes such as APP processing (*CASS4, ABCA7*), tau pathology (*CASS4, FERMT2*) and immune responses (*CD33, INPP5D,CD2AP, EPHA1, MEF2C, HLA-DRB5/DRB1* as well as the *MS4A4A/MS4A4E/MS4A6E* cluster)[22]. It is important to note that the majority of these studies have been conducted on Caucasian cohorts and these may not necessarily represent susceptibility genes across other ethnic groups.

1.6.2 Environmental Epidemiology

There are several environmental factors that have been implicated in increasing AD susceptibility.

These include suffering from other health concerns, predominantly during mid-life, such as cerebrovascular disease (which contributes to brain damage, Aβ deposition and promotes tau hyperphosphorylation), hypertension (which has adverse effects on the blood-brain barrier (BBB))[22], Type II diabetes (as insulin competitively inhibits Aβ degradation and thereby facilitates Aβ deposition and tau phosphorylation), adiposity (as it leads to hyperinsulinemia and the production of cytokines)[38] and high cholesterol (influences the activity of APP cleaving enzymes and enhances Aβ deposition)[39].

Lifestyle factors also contribute to the likelihood of developing AD, with poor diet and inadequate physical activity being the primary risk factors. Excessive caloric intake and fat consumption directly contribute to oxidative stress which promotes AD pathology[40]. Consumption of diets high in fruits and vegetables (anti-oxidants and vitamins E and C) and mono- and polyunsaturated fats (fish and olive oil) are associated with reduced cognitive impairment by reducing the neuronal damage associated with reactive oxygen species (ROS) and inflammation[22]. The mechanisms underlying the neuroprotective effects, namely reduced plaque formation[41], of physical exercise are unclear but it has been proposed that exercise may enhance glucose utilization, promote cerebral blood flow and thereby facilitate oxygen extraction[22].

A history of traumatic brain injury (TBI) has also been linked to enhanced AD risk as such brain injury has been reported to promote APP overexpression and thereby result in elevated Aβ levels[42]. Furthermore, performing tasks that are cognitively stimulating has been shown to reduce the likelihood of developing dementia[22].

As previously stated the neuropathological hallmarks of AD are extracellular neuritic plaques, intracellular neurofibrillary tangles, cerebrovascular amyloidosis as well as synaptic and neuronal loss, particularly in the basal forebrain and hippocampus, these being regions of higher-order cognitive function[43].

1.7 Neuropathology

1.7.1. Neurofibrillary Tangles

The neurofibrillary tangles (NFTs) **(Figure 1.2 and Figure 1.3)** are composed of hyperphosphorylated forms of the microtubule-associated protein tau.

1.7.1.1 Tau

Tau is the predominant (>80%) microtubule associated protein (MAP)[44]. This protein, encoded on chromosome 17q21[45], exists in six isoforms [46] and is unusually hydrophilic for a cytosolic protein[47] which thereby provides an explanation for its natively unfolded state. The physiological role of tau is to promote the assembly and maintain the stability of microtubules in neurons and other cells as well as protect against microtubule length fluctuations[47]. It is important to note that the microtubule binding and stabilising activity of tau is dependent on the degree of its phosphorylation[2]. Under normal physiological conditions, 2-3 moles of phosphate are bound per mole of tau protein[48].

1.7.1.2 Hyperphosphorylated Tau

Within the AD diseased context, the degree of tau phosphorylation is thrice (3x) that of normal tau, thus 6-9moles of phosphate bind per mole of tau[48]. Three sites have been identified as distinguishers between non-diseased and AD patients and must be phosphorylated in order to induce aggregation, these are namely Thr231, Ser235 and Ser262[2]. Phosphorylation lowers the affinity of tau for microtubules resulting in their dissociation from microtubules. The microtubule binding site on tau coincides with the tau-tau interaction site, therefore dissociation of tau from microtubules consequently allows for tau aggregation[49]. Hyperphosphorylated tau consequently aggregates into paired helical filaments (PHFs) which in turn form the neurofibrillary tangles characteristic of AD[50]. It is noteworthy to add that heparan sulphate proteoglycans (HSPGs) facilitate efficient NFT formation[51].

This enhanced phosphorylation during AD may be attributable to enhanced kinase and/or reduced phosphatase activity.

The most studied tau protein kinase within the AD context is glycogen synthase kinase- 3β (GSK-3β), which is stimulated by Aβ as well as other AD associated processes such as oxidative stress, endoplasmic reticulum stress and proteasomal dysfunction[44]. Other kinases which have been reported to regulate tau phosphorylation are: cyclin-dependent kinase 5 (Cdk5), Protein Kinase A (PKA), calcium/calmodulin-dependent kinase II (CaMKII), serine kinases (Fyn, Src, Abl) and the kinases involved in the Mitogen Activated Protein Kinase (MAPK) signal cascade (ERK1/2 and JNK) (as discussed later in section **1.12.1**) to name a few[2 47] (**Figure 1.2**).

Several serine/threonine protein phosphatases have been implicated in tau dephosphorylation, these include metal-dependent protein phosphatases (PP1, PP2A, PP2B, PP4-PP7) and phosphoprotein phosphatases (PP2C). PP2A is the most abundant (>70%) and effective tau phosphatase[52]. It is important to note that GSK-3β activation promotes the accumulation of protein tyrosine phosphatase 1B (PTP1B) which consequently phosphorylates and inactivates PP2A[44]. Furthermore, specific PP2A inhibitors, PHAP-I and PHAP-II, are upregulated in AD[53].

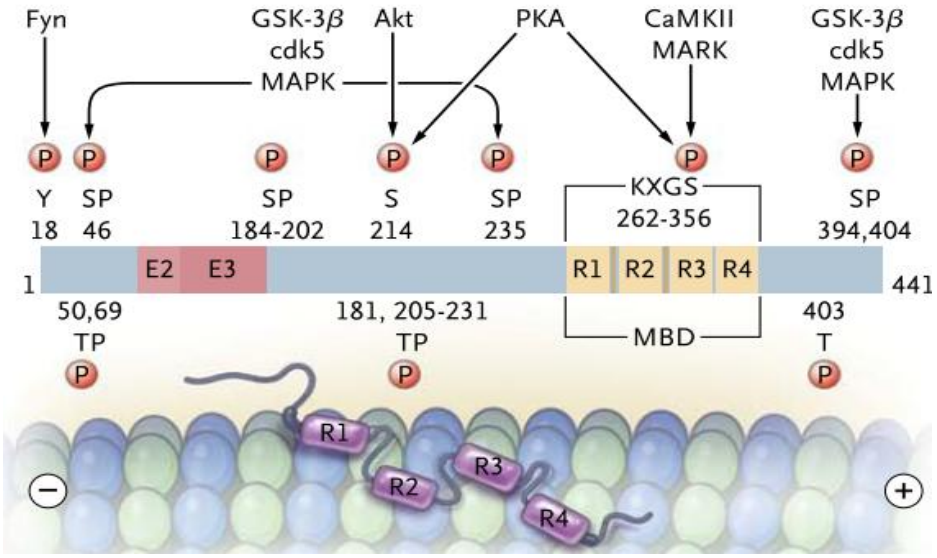


Figure 1.2| Tau phosphorylation. Graphical representation of the tau protein. The four repeat sequences which comporise the microtubule binding domain (MBD) are shown as well as sites of phosphorylation at serine (S) or threonine (T) residues. Those residues followed by Proline (P) are denoted as SP or TP, respectively. The kinases involved in tau phosphorylation at these varying sites are depicted and include: glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 5 (Cdk5), protein kinase A (PKA), calcium-calmodulin protein kinase 2 (CaMKII), mitogen activated protein kinases (MAPK), microtubule affinity regulating kinase (MARK) and Akt and Fyn kinases (Adopted from [7]).

The consequences of NFT formation, which primarily accumulate in the neuronal cell bodies and dendrites[54] (**Figure 1.3**), includes cytoskeletal destabilization which consequently has adverse effects on cellular morphology and viability as well as disrupts microtubule dynamics and intracellular trafficking of organelles, functional proteins and neurotrophins[2]. In addition, the generation of toxic intracellular aggregates reduces proteosomal efficacy[55].

Although the tangle load is reported to correlate more closely with AD severity than the plaque level[56], NFTs are additionally the central features of an assembly of neurodegenerative disorders termed "tauopathies", which include: progressive supranuclear palsy, corticobasal degeneration, Pick's disease[54] and subacute sclerosing panencephalitis[16]. The NFTs are thus not exclusively associated with AD. Conversely, Aβ neuritic plaques, although present in healthy and diseased brains (patients suffering from AD as well as Dementia with Lewy bodies (DLB)), are not the principle pathological hallmark of any other disease. Moreover, tau gene-related mutations result in the development of frontotemporal dementia as opposed to AD[57], whereas familial AD is the consequence of mutations triggering the excessive generation of $A\beta_{42}$.

Furthermore, $A\beta_{42}$ accumulation has been reported to stimulate the initiation of molecular mechanisms underlying tau hyperphosphorylation. $A\beta_{42}$ accomplishes this through kinase activation, especially GSK-3β (and consequently hinders PP2A activity) and Fyn kinase (via the cellular prion protein (PrP^{C})). In addition, Aβ induces apoptosis (to be discussed in detail in section **1.9.3** below) and the resultant proteolysis of tau by caspases promotes its aggregation[51]. It is noteworthy to add that recent reports have demonstrated that tau pathology is vital for the full extent of $A\beta_{42}$ toxicity to develop[58].

Thus, it has been highlighted that Aβ, and more specifically $A\beta_{42}$, as opposed to tau, is indeed the principal aetiological (disease causing) agent in AD.

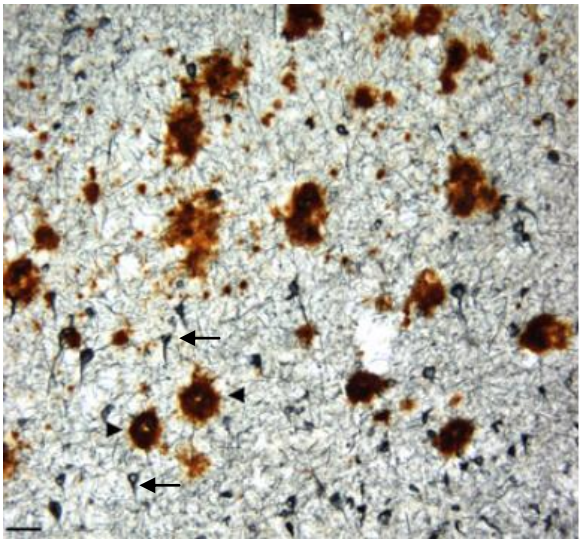


Figure 1.3| Neurofibrillary tangles (NFTs) and Amyloid beta (Aβ) composed plaque in a section of the entorhinal cortex derived from an individual with severe Alzheimer's Disease. Senile plaques, composed primarily of Aβ, are identified by arrow heads and the neurofibrillary tangles (NFTs), composed of hyperphosphorylated tau, are identified by arrows (Adopted from[59]).

1.7.2. Amyloid Plaques

The second neuropathological hallmark of AD, the extracellular senile/neuritic plaques, are largely composed of 8nm amyloid fibrils[60]. These fibrils in turn are composed of amyloid beta (Aβ) peptides **(Figure 1.3)**, predominantly the 42 amino acid isoform ($A\beta_{42}$), as it exhibits an augmented aggregation propensity[61] owing to the presence of additional hydrophobic amino acid residues, namely isoleucine and alanine[62, 63]. Although the neuritic plaques were originally regarded as the destructive agents, current hypotheses posit Aβ oligomers (Aβo), as the neurotoxic agents instead[64]. Conversely, recent studies have suggested that neuritic plaques may in fact be neuroprotective as they are able to sequester the toxic soluble Aβ oligomers, thereby diminishing their neurotoxic effects[60]. Despite the fact that plaques themselves are not the neurotoxic species, they may contribute to AD neuropathology by adversely affecting dendrite spine formation and geometry[65]. Furthermore, Aβ plaques have been demonstrated to block inter-neuronal transport and significantly impair neocortical synaptic transmission, thereby reducing the ability of neurons to integrate and propagate information[66]. Senile plaques have also been reported to deregulate calcium(Ca^{2+}) homeostasis which consequently results in structural and functional disruption of neuronal networks[67].

It must be stated that amyloid plaque load correlates poorly with the degree of cognitive impairment whilst soluble non-fibrillar Aβ oligomers are better quantitative correlates.

1.8 The molecular basis of Alzheimer's Disease

1.8.1. The Amyloid Precursor Protein (APP)

1.8.1.1 APP Structure and Trafficking

The amyloid precursor protein (APP) **(Figure 1.4)** is a type I transmembrane protein with a single transmembrane domain. It is encoded by a single gene on chromosome 21 and this thereby underlies the finding that individuals with Down Syndrome have a higher predisposition to develop AD[68]. APP, which is ubiquitously expressed but at higher levels in neurons, exists in three splice variants: APP695, APP751 and APP770[69], the expression of the former is enhanced in neuronal tissues[70]. APP may be divided into several functional domains namely (from N to C terminus): heparin and copper binding domains, an acidic domain, a Kunitz-type protease inhibitor (KPI) domain, Aβ region (which distinguishes APP from other APP-like protein APLP1 and APLP2) and the YENPTY motif which is central for clathrin-mediated endocytosis[71](**Figure 1.4**). During its transport through the secretory pathway towards the plasma membrane, APP is post-translationally modified through *N*- and *O*- glycosylation, sialylation, phosphorylation and tyrosine sulphation[72]. Moreover, it has been suggested that only approximately 10% of APP indeed reaches the plasma membrane, the remaining APP localizes primarily in the Golgi apparatus and trans-Golgi network (TGN)[72].

At the cell surface, APP may either undergo proteolytic processing or may be internalized, delivered into endosomes and consequently either degraded in lysosomes or recycled to the cell surface[73].

1.8.1.2 Physiological role of APP and its cleavage products

Several physiological roles have been ascribed to APP and its cleavage products (the generation of which will be discussed later in section **1.8.2**).

A role for APP in neuronal cell adhesion has been proposed and may be as a result of APP binding to multiple extracellular matrix components (such as laminin, collagen type I and

heparin[71] and cell surface adhesion proteins (such as neural cell adhesion molecules (NCAMs) and integrins)[71] as well as the fact that APP molecules on neighbouring cells have been demonstrated to interact[74]. Furthermore, APP and its non-amyloidogenic cleavage product sAPPα (**Figure 1.5**) have been documented to have trophic functions. APP, upon binding to extracellular proteoglycans, has been reported to induce neurite outgrowth[75, 76] and promote neurogenesis through ERK phosphorylation[77].

sAPPα similarly serves multiple physiological roles - this peptide my stimulate neuronal proliferation[78] and is required for normal brain development as well as to mediate long-term potentiation (LTP) and learning[79]. In addition, sAPPα is reportedly neuroprotective as it downregulates Cdk5 activity, thereby hindering tau hyperphosphorylation[80] and has also been documented to antagonize neuronal death triggered by proteasomal stress[81].

Cleavage of APP by ɣ-secretase yields an APP intracellular domain (AICD), which has been demonstrated to translocate to the nucleus and form a complex with Fe65 and Tip60 (a chromatin remodelling factor)[82]. A role of this transcriptional complex in activating the expression of the cellular prion protein (PrP^c) has been proposed[83]. However, the transcriptional activity and the downstream targets (supposedly GSK-3β, neprilysin, p53 and the epidermal growth factor receptor (EGFR)) of this complex have been areas of much controversy and require unambiguous confirmation[71].

APP may be metabolized via two pathways namely, a non-amyloidogenic pathway and an Aβ generating amyloidogenic pathway **(Figure 1.5)**. Both pathways occur under normal physiological conditions in healthy individuals. It is rather the disproportionate favouring of the amyloidogenic cleavage or retardation in the rate at which Aβ is cleared that results in the development AD.

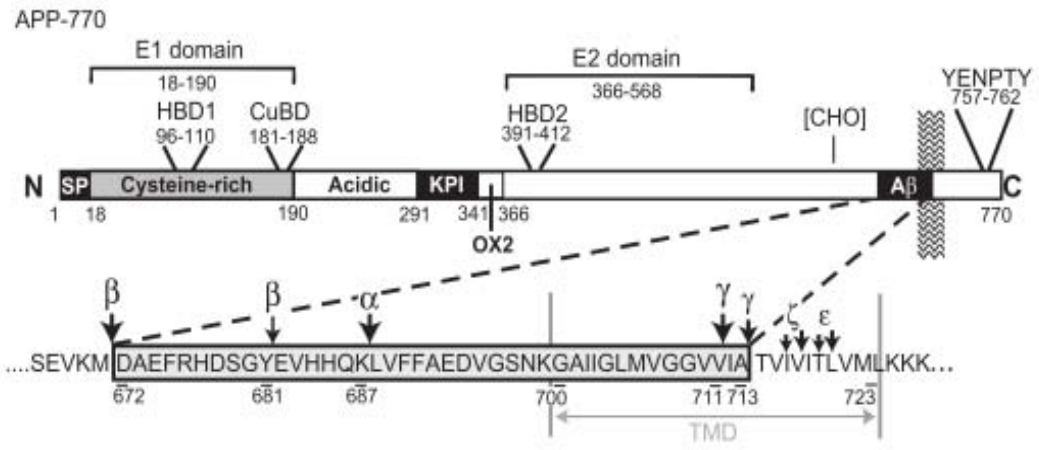


Figure 1.4| Schematic representation of the Amyloid Precursor Protein (APP) and the enzymatic cleavage sites located within the amyloid beta (Aβ) sequence. The APP770 isoform is represented here. SP represents a 17aa cell-surface N-terminal signal peptide. The protein contains an E1 domain comprising a heparin-binding domain (HBD) and a copper-binding domain (CuBD) as well as a 56aa Kunitz-type serine protease inhibitor domain (KPI), an E2 domain containing a second HBD and the Aβ region. There is a YENPTY intracellular C–terminal signal motif. The 40-42aa Aβ sequence and the enzymatic cleavage sites of β-secretase, α-secretase and ɣ-secretase are depicted.TMD = transmembrane domain (Adapted from [84]).

1.8.2 APP Processing

It is important to highlight that although APP is initially targeted to the plasma membrane via the secretory pathway, APP processing occurs at several subcellular sites.

1.8.2.1 The non-amyloidogenic pathway

This pathway involves APP proteolytic cleavage by alpha (α)-secretase between residues APP687 and APP688[85], between Lys16 and Leu17 of the Aβ sequence, and thereby precludes Aβ shedding. The released amino terminal ectodomain fragment is termed sAPPα **(Figure 1.5)**. The remaining 83aa carboxyl terminal fragment (CTF_{83}) is cleaved by gamma (ɣ)-secretase generating a soluble 3kDa p3 fragment and a 57-59aa amyloid intracellular domain (AICD).

1.8.2.1.1 α-secretase

Although several members of the disintegrin and metalloprotease (ADAM) family have been demonstrated to function as α-secretase, including ADAM9, ADAM19 and ADAM 17 (also termed tumour-necrosis factor-α converting enzyme (TACE)), it has recently been reported that ADAM10 accounts for most α-secretase activity[86]. ADAM-10 exists as a pro-enzyme and is only activated upon association with the plasma membrane[87] and thus non-

amyloidogenic processing of APP predominantly occurs at the cell surface. Moreover, non-amyloidogenic processing is the preferred pathway in non-neuronal cells but is not the predominant pathway in neuronal cells owing to their enhanced expression of β-secretase[72].

Numerous other proteins of physiological importance are similarly cleaved by α-secretase, including cadherins, other type I transmembrane proteins, Notch receptors and tumour necrosis factor α (TNFα)[72].

The activity of this enzyme may be modulated by calcium, Protein Kinase C (PKC)[87] and cholesterol levels (α-secretase activity being promoted upon cholesterol depletion)[88].

1.8.2.2 The amyloidogenic pathway

The amyloidogenic pathway is initiated by APP cleavage by beta (β)-secretase between residues APP671 and APP672[70] generating sAPPβ **(Figure 1.5)**. The resultant CTF_{99} is further cleaved by ɣ-secretase yielding a soluble 4kDa Aβ monomer and the AICD **(Figure 1.5)**. Cleavage by ɣ-secretase between APP712 and APP713 generates $Aβ_{40}$ whilst cleavage at APP714 generates $Aβ_{42}$[89].

In addition to Aβ, the sAPPβ cleavage fragment has recently been suggested to posses neurotoxic properties. It has been proposed that sAPPβ may be further cleaved into a 35kDa fragment denoted N-APP which may bind to death receptor 6 (DR6), resulting in caspase activation which consequently leads to axonal degeneration and neuronal death[90].

It must be emphasized that the amyloidogenic pathway is a normal physiological process and Aβ is therefore present in the cerebrospinal fluid (within the 3-8nM range[91]) and plasma (500pM[35]) of healthy individuals.

1.8.2.2.1 β-secretase

The aspartyl protease which catalyzes the rate limiting step in Aβ generation[92], is termed the β-secretase or more specifically β-site APP cleaving enzyme-1 (BACE-1) and is encoded by a gene on chromosome 11. This enzyme is ubiquitously expressed, with highest levels present in the pancreas and brain. It is owing to the elevated levels of BACE-1 in neuronal tissue in comparison to that of α-secretase, that amyloidogenic APP processing is favoured in

neurons[72]. BACE-1 expression is further enhanced during times of environmental[93] and cellular stress[92].

BACE-1 is transiently present on the cell surface, specifically in the lipid raft region, where it may catalyze the cleavage of APP, a process facilitated by glycosaminoglycans (GAGS)[94]. However, this enzyme is predominantly localized to the Golgi apparatus, trans-Golgi network (TGN) and endosomal compartments - which provide the ideal acidic environment for optimal enzymatic activity (pH4.5)[92]. Therefore, the majority of APP amyloidogenic processing occurs within the endosome/endocytic vesicles.

Although BACE-1 is a central target for AD therapeutics, it must be borne in mind that BACE-1 does not exclusively cleave APP but is additionally involved in the proteolytic processing of other physiological substrates, such as platelet selectin glycoprotein ligand-I, interleukin-like receptor type II as many others[95]. The most important BACE-1 substrate reported to date is Neuregulin-1 (NRG1), the cleavage of which is central to signal transduction pathways involved in myelination[72]- which is central to synaptic transmission and memory consolidation.

An explanation for the confinement of AD pathology to the brain is provided by the fact that both APP and BACE-1 are expressed at highest levels in neuronal cells.

1.8.2.2.2 ɣ-secretase

Gamma(ɣ)-secretase is also an aspartyl protease, consisting of 6-9 transmembrane domains[96, 97] and is involved in the cleavage of the C-terminal fragments (CTF) derived after either α- or β- secretase cleavage. This cleavage occurs within the transmembrane regions of these proteins. ɣ-secretase is a complex consisting of four subunits, namely Nicastrin (Nct) (a substrate receptor), Anterior pharynx defective (APH) (which serves as a scaffold protein for complex assembly), Presenilin enhancer (PSEN-2) (which facilitates proteolysis) and a catalytic subunit termed presenilin (PS), either PS1 or PS2 (which catalyze substrate cleavage)[72]. The cleavage activity of ɣ-secretase differs from that described above in that this enzyme complex catalyzes a stepwise proteolysis of its substrates - meaning that the enzyme does not cleave at a single site only but rather catalyzes sequential cleavage at multiple sites, often 3 amino acids apart. Depending on the site of initial cleavage, ɣ-secretase may result in

the formation of several Aβ isoforms ($Aβ_{37}$-$Aβ_{42}$). Enzymatically active ɣ-secretase is localized to the plasma membrane as well as the endosomal and lysosomal compartments[72].

This enzyme is of great physiological importance and is involved in the proteolysis of over 60 substrates - thereby highlighting the significance of this complex[98]. One such substrate is Notch, the resultant cleavage by ɣ-secretase is central for Notch signalling - which is central in regulating cell fate and affects cellular proliferation, apoptosis and differentiation, all of which are processes vital to normal embryonic development and homeostasis during adulthood[99, 100]. ɣ-secretase has additionally been shown to cleave N- and E-cadherins, ErbB-4 (neuregulin receptor), SREBPs (sterol regulatory element binding proteins) and it has been proposed that this enzymatic complex may play a role in protein turnover as well as in calcium homeostasis[98].

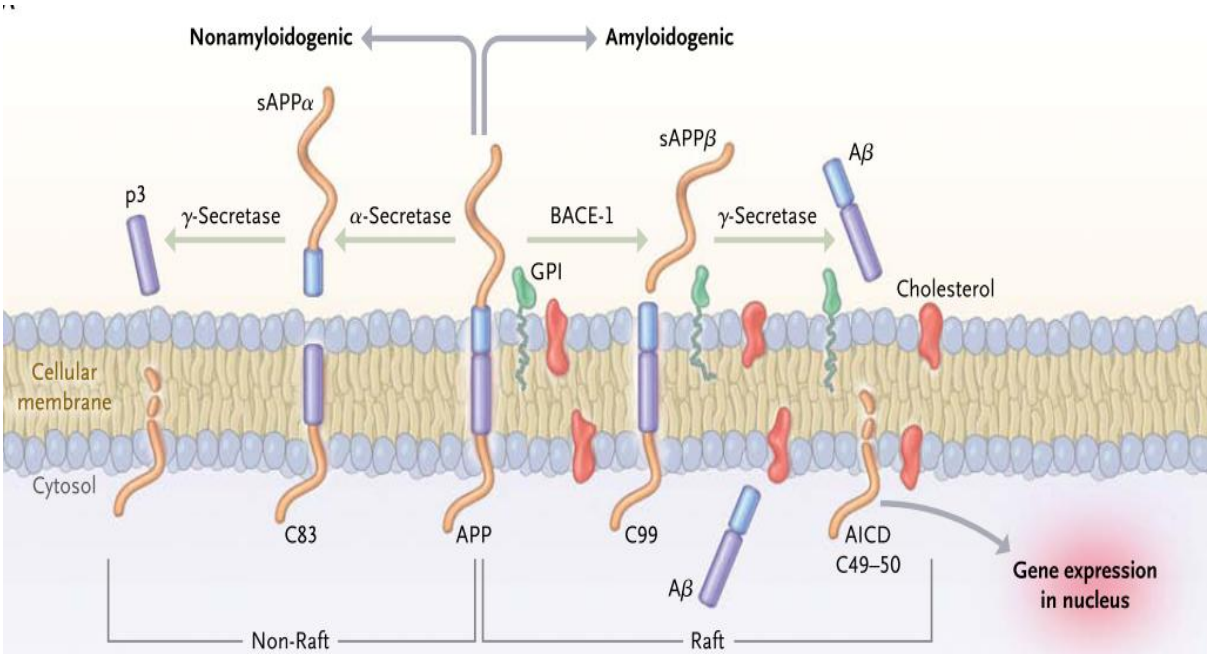


Figure 1.5| The proteolytic processing of the Amyloid Precursor Protein (APP) and its cleavage products. The non-amyloidogenic pathway (*left*) involves successive alpha(α)-secretase cleavage (after residue 687), releasing sAPPα and gamma (ɣ)-secretase cleaves the resultant 83aa C-terminal fragment CTF83 to generate p3 and the amyloid intracellular domain (AICD). The amyloidogenic pathway (*right*) entails beta (β)-secretase or β-site amyloid precursor protein-cleaving enzyme-1 (BACE-1) cleavage after residue 671, thereby releasing sAPPβ, and the subsequent CTF99 cleavage by ɣ-secretase resulting in Aβ shedding and intracellular AICD release. AICD translocates to the nucleus and stimulates transcription activation (Adopted from[7]).

1.8.3 Mutations in APP

The following missense mutations in APP are known to cause AD either by augmenting $A\beta_{42}$ production, favouring β-secretase activity or thwarting α-secretase cleavage. Approximately 40 APP missense mutations have been identified[101]. These are commonly named, with the mutated residues provided in brackets, as follows: Swedish (K670N, M671L), Flemish (A692G, E693Q), London (V717I, V717G), Indiana (V717F) and Florida (I716V), Dutch (E693Q)[96], Iowa (D694N), Artic (E693G)[102] to name a few.

1.8.4 Presenilin Mutations

More than 197 missense mutations have been identified in the *PS1* gene and 25 missense mutations in the *PS2* gene[101] and these mutations promote ɣ-secretase cleavage that produces the longer 42aa Aβ isoform[87]. Only mutations in residues conserved between PS1 and PS2 have been reported to result in a 1.5-3 fold increase in $A\beta_{42}$ production and plaque load[16] and are therefore linked to FAD/EOAD. An extensive list of such mutations is provided in the review by Cruts *et al.,*[101].

1.9 The Amyloid-beta (Aβ) peptide

The Aβ peptide, which is generated as a result of the sequential proteolytic cleavage of its parental protein APP, is amphiphilic in nature with the first 28 aa residues being polar and the remaining residues being non-polar[103] **(Figure 1.4)**. Thus, at neutral pH the peptide exhibits great differences in polarity and thus exhibits a great aggregation propensity[104, 105]. Aβ was first isolated by Glenner and Wong in 1984 and was shown exist not only as monomers (~4.4kDa) but also to associate into soluble SDS-stable dimers (~8kDa), trimers (~12kDa) and higher order oligomers (up to 20 monomers)[60, 106, 107]. It is noteworthy to add that the Aβ region of APP, which is inserted into the membrane, is largely α-helical in conformation. It is only post cleavage and release, that Aβ acquires the β-sheet conformation required for aggregate formation[60].

1.9.1 The Physiological role of Aβ

Although $A\beta_{42}$ accumulation indisputably leads to the development of AD, it must be borne in mind that amyloidogenic processing is a normal physiological process and $A\beta_{42}$ is present in the CSF and plasma of non-demented individuals throughout life[70, 108]. At physiological

concentrations (picomolar range, 1pM-1nM [109]) $A\beta_{42}$ has recently been shown to play a central role in numerous processes. Aβ is vital for neuronal cell survival, cell growth, neurite outgrowth and axonal sprouting[108] as well as differentiation (through binding to receptors for advanced glycation end products (RAGE)[110]. Furthermore, owing to its metal-binding affinity (specifically copper, zinc, aluminium and iron), $A\beta_{42}$ has been proposed to protect against metal-induced oxidation[108]. $A\beta_{42}$ serves a very important role in regulating synaptic activity upon binding to cell surface receptors (NMDA and α7 nicotinic acetylcholine receptors (α7nAChR)) by impairing long term potentiation (LTP). This thereby prevents excessive synaptic activity which would lead to excessive glutamate release and neuronal cell death as a result of excitotoxicity[111]. In addition, $A\beta_{42}$ has been reported to function in facilitating learning and regulating memory consolidation[112, 113]. The peptide has been suggested to do so by enhancing the production of acetylcholine[112] and activating signal transduction pathways involved in memory formation, namely the phosphatidylinositol-3 kinase and MAP kinase cascades[113].

Owing to the diverse physiological functions of $A\beta_{42}$, total eradication of the peptide may be detrimental to neuronal processes and perhaps should not be the aim of prophylactic and therapeutic approaches. Since it is the supraphysiological concentration (within the nanomolar range) of $A\beta_{42}$ that results in pathogenesis, which is the consequence of an imbalance between $A\beta_{42}$ production and clearance, therapeutic intervention should focus on these processes.

1.9.2 Aβ-degrading proteases

Under normal physiological conditions $A\beta_{42}$ has a relatively short half-life of approximately 1.7 h[114]. This therefore demonstrates that under physiological conditions $A\beta_{42}$ is effectively and efficiently degraded. This degradation is mediated by a host of different proteases, collectively termed Aβ-degrading proteases (AβDP), each localized to a specific cellular/subcellular compartment. These enzymes display cooperativity with regards to eliminating $A\beta_{42}$. These include: cathepsin B, matrix metalloproteases (MMP2, MMP9, MMP14), endothelin-converting enzymes (ECE1/2), angiotensin-converting enzyme (ACE), plasmin, the proteasome and BACE-1/2[115]. The enzymes identified to be of particular importance with regards to Aβ degradation are: neprilysin (NEP) and the insulin degrading enzyme (IDE).

NEP is the principal AβDP and accounts for 50% of Aβ degradation[115]. This Type-II, zinc-metalloprotease which is predominantly expressed in neuronal ER and the Golgi apparatus, is able to degrade both Aβ monomers and some Aβ oligomers[115]. It is noteworthy to add that NEP phosphorylation is mediated by the MAPKinase signal transduction pathway and results in reduced NEP activity and enhanced Aβ levels, whilst dephosphorylation, as mediated by protein phosphatase 1 (PP-1), restores NEP functionality[116]. Both MAP kinases and PP-1 are involved in the 37kDa/67kDa LRP/LR associated signalling pathways (as discussed in section **1.11.1.1** below).

IDE, also a zinc-metalloprotease, similarly degrades monomeric Aβ. This enzyme is present in the cytosol, ER, endosomes, lysosomes, peroxisomes and is also secreted into the extracellular space[115].

It must be highlighted that a cell's Aβ degradation capacity is generally reached during normal metabolism[115], and therefore these systems are unable to match the elevated Aβ production levels characterised by AD. Excessive Aβ levels may additionally serve as AβDP inhibitors, thereby augmenting the cell's Aβ degrading deficiency in AD. Furthermore, elevations in oxidative stress, which accompany aging, are detrimental to AβDP activity[117].

Moreover, cerebral Aβ levels may be reduced by transport of this peptide across the blood brain barrier (BBB) by certain proteins, such as the low-density lipoprotein receptor-related protein 1 (LRP1)[118].

1.9.3 Effects of Toxic Aβ oligomers

Interactions between $A\beta_{42}$ (usually oligomeric $A\beta_{42}$) and cell surface receptors have been reported to induce oxidative damage by stimulating the generation of reactive oxygen species (ROS), namely superoxide and hydrogen peroxide[119]. This consequently results in protein degradation and lipid (i.e. myelin) peroxidation thereby slowing the rate of signal transmission which in turn hampers the consolidation of new information, retrieval of memories and motor functions[120]. Additionally $A\beta_{42}$-receptor interactions may induce cell death pathways and thereby mediate neurotoxicity. Moreover, the interaction of $A\beta_{42}$ with metabotrophic glutamate-5 receptors (mGluR5)[121] [94] and N-methyl-D-aspartate receptors (NMDA)[122] [94]disrupts calcium (Ca^{2+}) ion homeostasis resulting in excitotoxicity, synaptic dysfunction and neuronal death[123](**Figure 1.6**). $A\beta_{42}$ interactions with cell surface receptors have also been demonstrated to result in aberrant signal transduction cascades with

consequent adverse effects on normal cellular processes – this will be discussed with regards to the cellular prion protein (PrP^c) in section **1.10.2.2** below.

The toxicity of $A\beta_{42}$ oligomers (Aβo) is not solely as a result of its cell surface associations, but is additionally attributable to intracellular $A\beta_{42}$ accumulation. $A\beta_{42}$ internalization, which is dependent on proteins located within the lipid raft region, allows for intracellular $A\beta_{42}$ accumulation which has been reported to have numerous deleterious functions including disruption of the ubiquitin-proteosomal system[124], promoting misfolding and aggregation of other functional cytosolic proteins as well as leading to organelle dysfunction including that of endocytic vesicles[125], the endoplasmic reticulum[126] and particularly that of the mitochondria[127]. Intracellular $A\beta_{42}$ has been reported to insert into mitochondrial membranes and be present within the mitochondrial matrix, distorting mitochondrial structure and leading to mitochondrial dysfunction which consequently leads to energetic dysfunctions and the overproduction of reactive oxygen species (ROS)[128]. It is important to note that this serves as a positive feedback loop since oxidative stress promotes amyloidogenic APP processing[129]. The cytotoxicity of the $A\beta_{42}$ peptide is proposed to reside between 25-35aa[130].

A protein that is celebrated within the AD research community with regards to mediating $A\beta_{42}$ toxicity at the cell surface as well as through stimulating $A\beta_{42}$ internalization is the cellular prion protein (PrP^c) - this will be discussed in detail section **1.10.2.2** below.

The $A\beta_{42}$-protein interactions, as aforementioned, may further serve as the basis for AD related neuronal loss as they may result in either aberrant signal transduction, perturbation of Ca^{2+} homeostasis[131] (**Figure 1.6**) and/or the induction of oxidative stress[132].

1.9.3.1 Neuronal cell loss

The peptide may activate a multitude of cell death modalities, the most significant being apoptosis and necrosis.

Apoptosis is the predominant cell death pathway reported in AD related neuronal loss[131]. Apoptosis is an active (requires energy), highly regulated physiological process (initiated by either the extrinsic/death receptor, intrinsic/mitochondrial or granzyme/perforin pathways) central to the maintenance of cellular homeostasis[133], but when deregulated has pathological implications. The importance of apoptosis in AD pathogenesis is evidenced by elevated

levels of pro-apoptotic proteins p53, Bax and caspase 3[134] in diseased brains as well as the fact that $A\beta_{42}$-interactions with p75NTR, FAS, TNFR1, RAGE and APP not only induce reactive oxygen species (ROS) synthesis but also result in upregulated expression of pro-apoptotic genes and enhanced mitochondrial permeability[6].

However, studies have also demonstrated that necrotic pathways as opposed to apoptosis may underlie $A\beta_{42}$ neurotoxicity[130,135]. Necrosis is a passive non-programmed, cell death mechanism resulting from severe cellular injury/insults or extreme environmental alterations. This may either be a consequence of cross-talk between the death signalling pathways, such that the interactions discussed above serve as atypical necrotic signals or as a result of the membrane perforating properties of $A\beta_{42}$[136] (**Figure 1.6**). The latter may result in Ca^{2+} influx and cause the cytosolic Ca^{2+} concentrations to rise to necrosis-inducing levels (in excess of 1μM)[137]. Furthermore, the resultant increased Ca^{2+} concentration may activate calpains (cysteine proteases) which in turn mediate lysosome rupture and the consequent release of non-specific proteases termed cathepsins which are involved in cellular destruction[138].

Thus, as has been highlighted above, the $A\beta_{42}$ peptide mediates its neurotoxicity through interactions not only with the plasma membrane but more notably through its associations with cellular proteins and receptors.

1.9.3.2 Aβ interactions and their pathological implications

Owing to their amphipatic nature, the hydrophobic C-terminus of $A\beta_{42}$ oligomers may be incorporated into lipid raft regions of the plasma membrane[139,136] (**Figure 1.6**) and/or nucleosomal, lysosomal membranes as well as those of the Golgi apparatus and endoplasmic reticulum[140]. The consequence hereof is disruption of membrane structure, function and fluidity which consequently has secondary implications for the functionality of membrane bound proteins and receptors[140] and as well as results in the formation of Ca^{2+} permeable channels[141] (**Figure 1.6**). This Ca^{2+} influx may disrupt synaptic plasticity, enhance mitochondrial permeability as well as activate both calpains and caspases - all of which contribute to cell damage and possibly death[135].

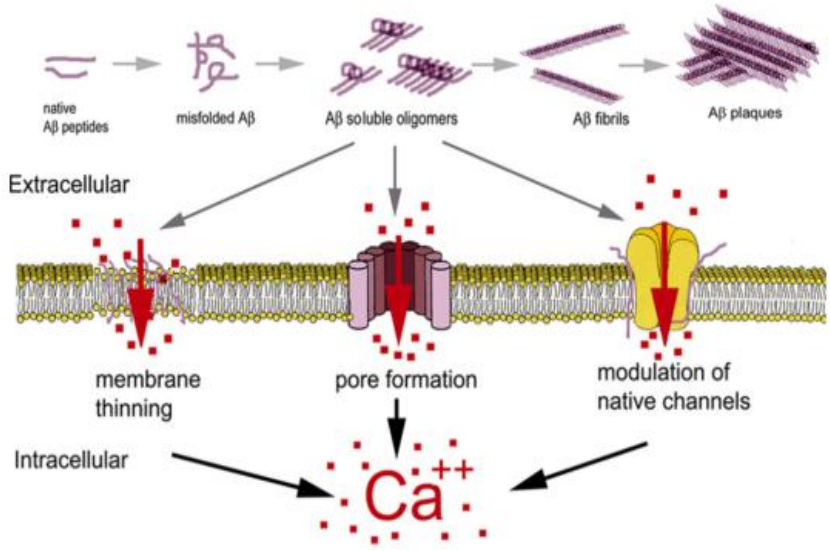


Figure 1.6| Deregulation of calcium (Ca^{2+}) homeostasis by Aβ. Aβ oligomers (Aβo) may augment intracellular Ca^{2+} levels by either: thinning the plasma membrane (PM) and thereby lowering the dielectric barrier of the PM and enhancing its ion conductivity; through insertion into the PM and the formation of Ca^{2+} permeable pores or through interactions with Ca^{2+} transporter ion-channels (Adopted from [3]).

A multitude of cell surface proteins present on neuronal and glial cells exhibit $A\beta_{42}$ binding affinities (**Table 1.1**). Whilst associations between $A\beta_{42}$ and some of these proteins are deemed neuroprotective, the majority are toxic[140]. It is noteworthy to emphasize that similar to the 37kDa/67kDa LRP/LR, the majority of these receptors (p75NTR, CLAC-P/ColXXV, RAGE, SEC-R and integrins) are transmembrane receptors[140,6].

Several of these receptors have additionally been implicated in $A\beta_{42}$ internalization processes including, α-7 nicotinic acetylcholine receptor (α7nAChR)[142], neurotrophin receptor p75 (p75NTR)[143] N-methyl-D-aspartate receptors (NMDAR), metabotrophic glutamate-5 receptor (mGluR5)[144] and LRP1[145]. It must be emphasized that the ability of the majority of these receptors (NMDAR,mGluR5 and LRP1) to induce internalization is dependent on their association with PrP^c.

Conversely, certain Aβ-protein associations may be neuroprotective in nature - one such interaction is that between Aβ and laminin. Laminin, an 850kDa glycoprotein tri-peptide composed of disulfide-bonded chains: α (5 isoforms, 200-400kDa); β (220kda) and ɣ (210kDa)[146], is a key component of the basal membrane. Laminin is central in maintaining the structure of the extracellular matrix (ECM) as well as in promoting neurite outgrowth,

cell adhesion and the formation of synapses [147-149]. The Aβ binding site (IKAV) is located on the α-chain[150] and an high affinity interaction between these proteins (K_d= 2.7x10nM) [151] has been reported to promote neurite outgrowth[152] and inhibit fibrillogenesis[151].

Table 1.1: Membrane associated proteins to which amyloid beta may bind [6]

Amyloid Precursor Protein (APP)
N-methyl-D-aspartate receptor (NMDA-R)
α-7 Nicotinic Acetylcholine receptor (α7nAChR)
P75 Neurotrophin receptor ($P75^{NTR}$)
Integrins (especially $\alpha_5\beta_1$)
Receptor for advanced glycosylation end products (RAGE)
Insulin receptor
Formyl peptide receptor-like-1 (FPRL1)
Scavenger receptors – class A,B,BI
Heparan Sulfate Proteoglycans (HSPGs)

An additional interaction of pathological significance, which has been alluded to previously, is that between Aβ and PrP^c.

1.10 The Cellular Prion Protein (PrP^c) – a central factor in Alzheimer's Disease

1.10.1 Structure and physiological role of PrP^c

The cellular prion protein (PrP^c) is a ubiquitously expressed 250aa protein encoded by the *PRNP* gene located on chromosome 20. PrP^c is most highly expressed in tissues of the central nervous system including neurons, glia and cells of the spinal cord[153]. Post synthesis in the ER, PrP^c is subjected to numerous post-translational modifications, whilst on its way through the secretory system to the cell surface: the N-terminal 22aa signal sequence is cleaved, two sites (Asn-181 and Asn-197) are glycosylated through the addition of *N*-linked oligosaccharide chains, a single disulfide bond is formed and post removal of the C-terminal hydrophobic peptide, a glycosyl-phosphatidylinositol (GPI) anchor is attached to the protein[153]. The resultant 208-209aa, which is predominantly α-helical in conformation, is

subsequently anchored to the plasma membrane and may occur in non- , mono- or diglycosylated forms[154].

It is also imperative to note that PrP^c is cleaved between residues 111 and 112 to yield two neuroprotective fragments, namely the N-terminal fragment (N1) and the C-terminal fragment (C1) [153, 155, 156]. This cleavage is performed by ADAM10, the predominant α-secretase involved in non-amyloidogenic processing of APP (discussed above in section **1.8.2.1.1** above)[157]. It has been reported that N1 may block the neurotoxicity of Aβ oligomers (Aβo) – however, the neuroprotective effects thereof in the AD context are contentious owing to elevated amyloidogenic processing within the AD brain. The C1 fragment has been suggested to prevent the formation and accumulation of the infectious prion protein (PrP^{Sc}) - the aetiological agent of prion disorders (to be discussed in section **1.10.2.1** below)[157].

PrP^c is constitutively cycled between the plasma membrane and endocytic compartments[158] via clathrin coated pits. However, as these proteins lack a transmembrane domain, this process requires an association between PrP^c and a transmembrane receptor. An alternative mechanism, namely caveolae mediated internalization, has been suggested to further contribute to PrP^c internalization (**Figure 1.7**). The rate of PrP^c internalization is relatively rapid, with complete recycling reported to occur within 60min[158]. This process of internalization is of importance not only with regards to the physiological functions of the protein but also in mediating pathogenic processes involved in prion disorders (**Figure 1.7**) and Alzheimer's Disease (to be discussed in section **1.10.2.1 and 1.10.2.2** below).

The most well established physiological role of PrP^c is that of a copper-binding protein (conferred by its octarepeat region). PrP^c is central to mediating the uptake of copper ions (an essential trace metal which serves as a cofactor for many enzymes and is vital for central nervous system development and is therefore most abundant in the brain[159, 160]) from the extracellular milieu. Since free Cu^{2+} promotes ROS production, PrP^c occupies a central protective role against oxidative stress[154]. Several other physiological functions have been conferred to PrP^c including ECM adhesion, mediating signalling pathways and in the formation and generation of synapses[161]. Although PrP-null mice are viable and do not display developmental or anatomical abnormalities (which suggests that the role of PrP^c may be redundant with that of other proteins), these animals do display alterations in sleep patterns, learning and memory deficits[158] as well as neurodegeneration under oxidative stress conditions.

It must be emphasized however that many of these functions [162-164] are dependent on interactions between the PrP^c and numerous (> 70) proteins including ApoE, APP, HSPGs, RAGE, p75NTR, Aβ[6, 164] and the 37kDa/67kDa laminin receptor (LRP/LR)[165]. These include roles in synaptic transmission, neuronal and specifically hippocampal morphology, cognition and circadian rhythmns as well as in cellular adhesion, signal transduction, metal (copper) binding and in the maintenance of oxidative stress homeostasis[166].

1.10.2 Pathological roles of PrP^c

1.10.2.1 PrP^c in the development of Prion Disorders

PrP^c is imperative to the development of a group of fatal neurodegenerative diseases aptly named prion disorders (PD) also referred to as transmissible spongiform encephalopathies (TSEs)[5, 6, 167, 168]. These diseases affect humans (Kuru, Creutzfeldt-Jakob (CJD), Gerstmann-Straussler syndrome (GSS) and fatal familial insomnia (FFI)) as well as animals namely Scrapie in sheep, Bovine spongiform encephalopathy (BSE) in cattle and Chronic Wasting Disease (CWD) in ungulates[6, 167].

Prion disorders are unique in that they may be infectious, familial or sporadic in nature. The ease of transmissibility (both intra- and inter species) of the infectious agent in these diseases is of particular concern owing to the ramifications these diseases have on both live stock and population health. The causative agent of TSEs[167] is a purely proteinaceous, nucleic-acid free particle called the infectious prion protein and generally designated as PrP^{Sc}. PrP^{Sc} is generated as a result a conversion of the α-helical structure of PrP^c to a predominantly β-sheet structure[154]. Furthermore, differential proteolytic degradation of the isoforms illustrates that the tertiary structure of cellular and infectious PrP differ as well[169]. Following infection with PrP^{Sc} or a mutation-linked or spontaneous conversion of PrP^c to PrP^{Sc}, PrP^{Sc} binds to and stimulates the conversion of endogenous PrP^c to PrP^{Sc} particles. This is said to occur both at the cell surface and within the endocytic compartments during internalization[170] (**Figure 1.7**). PrP^c is vital to this pathogenesis and PrP-null mice are resistant to developing prion disorders.

The resultant intraneuronal accumulation of PrP^{Sc} as well as a possible loss of physiologically functional PrP^c have been proposed to underlie the observed neurodegeneration[154].

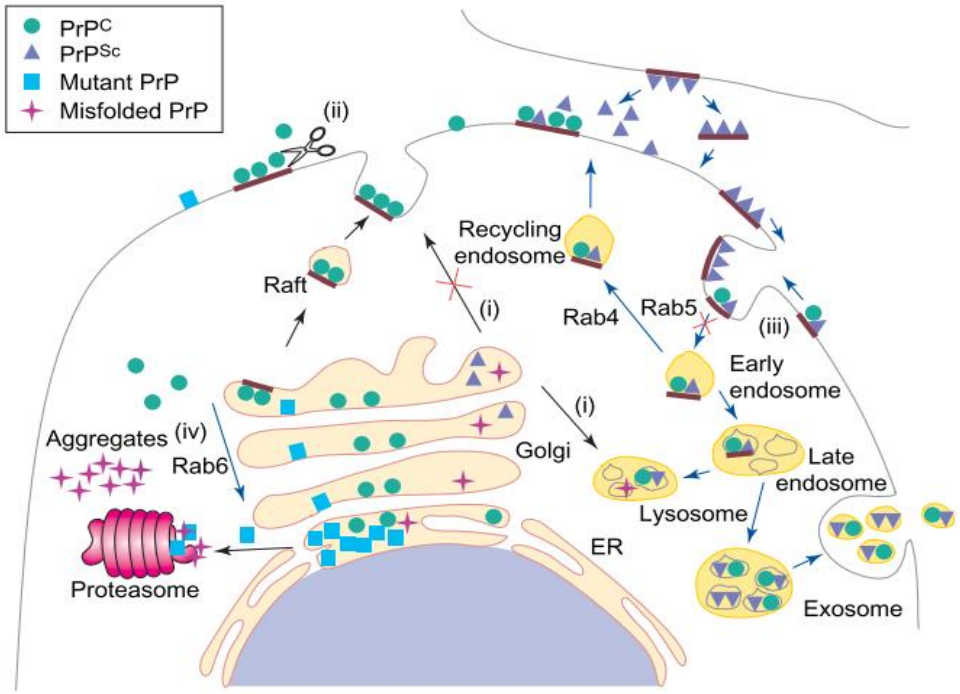


Figure 1.7| Prion protein trafficking as well as the sites of PrP^c conversion and PrP^{Sc} formation. The cellular prion protein (PrP^c) is synthesized in the ER and is transported via the Golgi apparatus to the cell surface, where it is anchored to the lipid raft region via a GPI anchor. PrP^c may be internalized by clathrin or cavoelin- dependent pathways. It is at the cell surface that the infectious prion protein (PrP^{Sc}) binds to and is internalized in association with PrP^c. PrP^{Sc} may seed the conversion of α-helical PrP^c into PrP^{Sc} at both the cell surface as well as within the endo-lysosomal system. The resultant PrP^{Sc} may form intracellular aggregates or may alternatively be recycled to the cell surface, thereby allowing for the infectious processes to persist. Inhibition of PrP^c transport to the cell membrane as well as internalization processes decreases PrP^{Sc} formation (Adopted from [4]).

The non-integrin 37kDa/67kDa laminin receptor (LRP/LR) has been identified as a cellular receptor for both PrP^{c}[165] and PrP^{Sc}[171] and is central to prion protein internalization processes[165].

1.10.2.2 The influence of PrP^c on Alzheimer's Disease

A vital role for PrP^c in AD is undeniable. The role of PrP^c was first linked to the amyloidogenic pathway, where it was proposed that PrP^c inhibits the activity of BACE-1[172], thereby inhibiting Aβ production. In line with this it was later proposed that a negative feedback loop exists between PrP^c and the amyloidogenic processing of APP. Here, the authors revealed that the AICD, upon formation of a transcriptional complex with Tip60 and Fe65 (discussed in section **1.8.1.2** above), regulates the transcription of p53 which subsequently promotes expression of *PRNP*. Thus, PrP^c inhibition of BACE-1 consequently results in reduced PrP^c expression[83, 173] (**Figure 1.8**).

Lauren *et al.*[174] revealed that PrP^c serves as a high affinity $A\beta_{42}$ receptor and specifically binds via the PrP23-27 and PrP95-110 binding sites (thus primarily through the N-terminus) to Aβ oligomers (denoted as Aβo) (not monomers nor fibrils)[175]. It is now a universal belief that the most important role of PrP^c in AD is as a consequence of its $A\beta_{42}$ binding affinity. This finding has been replicated multiple times and this interaction, specifically between $A\beta_{42}$ oligomers (Aβo) (not monomers or fibrils) and PrP^c, is indisputable through the data regarding the biological effects of this association are largely controversial. However, recent elegant studies have demonstrated that PrP^c is indeed central to mediating the toxicity of Aβo.

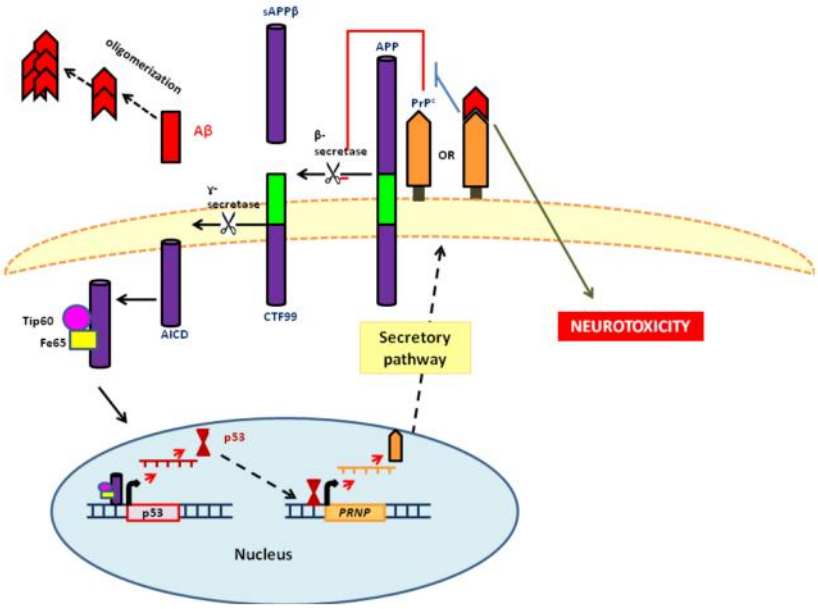


Figure 1.8| Feedback loop for the prion protein (PrP^c)-mediated regulation of β-scretase activity. The amyloid intracellular domain (AICD) amyloidogenic APP cleavage product, indirectly upregulates prion protein (PrP^c) expression through p53 gene activation. PrP^c consequently hampers beta (β)-secretase (BACE-1) activity thereby reducing APP amyloidogenic processing and Aβ synthesis. Binding of Aβ to PrP^c prevents the protein from inhibiting β-secretase activity, compromising the regulatory control exerted on the amyloidogenic process and potentially increasing toxic Aβ levels (Adopted from[6]).

It has recently been suggested that PrP^c is essential for Aβ oligomer (Aβo) induced neuronal loss, loss of long term potentiation and synaptic toxicity[176]. As cell surface PrP^c is considered a mediator of pro-apoptotic signalling[177], it may be proposed that PrP^c may mediate pro-apoptotic signalling upon interaction with Aβ oligomers (Aβo). Furthermore, PrP^c has been shown to be central in mediating $A\beta_{42}$ induced neurotoxic signal transduction cascades. Upon

binding to Aβo, PrP^c has been reported to activate the Src kinase, Fyn[178]. Fyn Kinase activation subsequently leads not only to tau hyperphosphorylation[179] (**Figure 1.9**) but also to a significant increase in phosphorylated NR2B (a subunit of the NMDA receptor) therefore overactivating cell surface NMDA receptors which subsequently leads to excitotoxicity and finally dendritic spine loss and neuronal cell death [157]. However, as previously stated, owing to its GPI anchored character, PrP^c likely relies on its transmembrane receptors to mediate these signal transduction cascades[174]. Proteins/receptors which have recently been implicated in this regard include LRP1[145]and caveolin-1, whilst others such as integrins and non-integrin receptors, which have the necessary structural characteristics, have been put forwards as possible candidates to mediate the PrP^c-Fyn activation[2]. Thus, it is probable that PrP^c signalling to Fyn may indeed occur through multiple PrP^c receptors.

Novel roles for PrP^c within the AD context have also been proposed. A recent report suggests that PrP^c, in association with its receptor LRP1, is central in mediating the transcytosis of Aβ across the blood brain barrier (BBB)[180]. PrP^c has similarly been implicated in mediating the internalization/uptake of Aβo thereby allowing for the intracellular accumulation of Aβo and the resultant toxic effects thereof (as previously discussed in section **1.9.3** above).

It may further be suggested that the binding of $A\beta_{42}$ oligomers to PrP^c may hinder PrP^c regulation of β-secretase through steric inhibition and/or through PrP^c endocytosis augmentation and thereby thwart the neuroprotective PrP^c-β-secretase interaction[173].

It is important to note that the GPI anchor of PrP^c is considered vital for PrP^c-mediated toxicity[177], thereby revealing that it is the cell surface location and the processes therewith associated, such as triggering signal transduction cascades and endocytosis, that are central to pathogenesis and not the binding of Aβ by PrP^c alone.

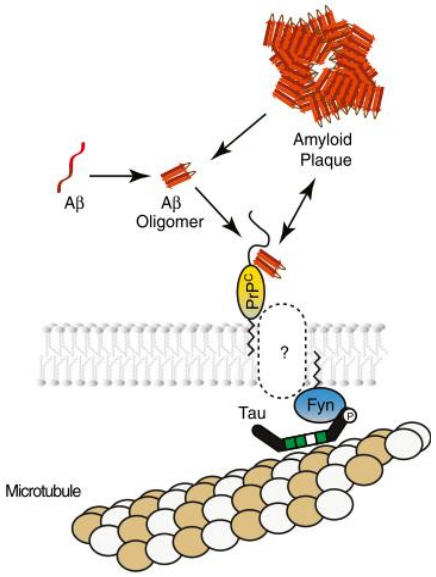


Figure 1.9| Graphical representation of the proposed model of Aβ-PrP^c induced Fyn activation. Upon binding of Aβ oligomers to the cellular prion protein (PrP^C), Fyn kinase is activated and this results in the hyperphosphorylation of tau. The consequence hereof is the subsequent microtubule dissociation and aggregation of tau into neurofibrillarly tangles (NFTs) (Adopted from [2]).

A highly conserved receptor of both physiological and pathological significance, which similarly binds to the Aβ interactors laminin and PrP^c, is the 37kDa/67kDa laminin receptor (LRP/LR). The possible role and importance of this receptor in AD has yet to be examined.

1.11 The 37kDa/67kDa Laminin Receptor (LRP/LR)

1.11.1 Structure and Function of the 37kDa/67kDa LRP/LR

The 37kDa/67kDa LRP/LR, also referred to as the 37kDa laminin binding protein (37LBP)[181], laminin receptor (LamR)[182], laminin-binding protein precursor p40 (LBP-p40) [183]and the ribosomal protein SA (RPSA)[184] is a multifunctional, multiform and multilocus protein (**Figure 1.10**). The *RPSA* gene, located on chromosome 3p21.3, is comprised of seven exons, six of which correspond to the coding sequence[185, 186]. However, the sequence does not encode a signal peptide that targets the protein to the nucleus nor the cell membrane[185, 186]- however, the protein is located within both these subcellular compartments.

The 295aa, type II receptor protein has a theoretical molecular mass of 32.854 kDa[187] but has been detected via western blotting at apparent molecular masses of both 37kDa and 67kDa[187, 188]. Although both isoforms of the protein are encoded by the same gene sequence, the mechanism through which the 37kDa LRP forms the 67kDa LR is elusive. The receptor is acylated by 3 fatty acids at Ser2 namely: palmitate, stearate and oleate[189] and the formation of the 67kDa isoform is prevented with acylation inhibition. Various isoforms of LRP/LR,

corresponding to different maturation states (44kDa,60/67kDa and 220kDa) all of which retain PrP^c binding affinities, have been identified in the murine brain[190].

This protein is divided into two domains, namely an intracellular N-terminal domain (1-209aa), which contains a palindromic LMWWML sequence which has been conserved in all metazoans[188] and an extracellular C-terminal domain (210-295aa), which is highly conserved amongst vertebrates[187, 188] (**Figure 1.10**). The amino acid sequence of this receptor is 98% identical in all mammals, thereby implying that the receptor occupies a central physiological role. The N-terminus of LRP/LR displays an overall positive charge and the C-terminus, an overall negative charge - thereby accounting for the nature of the ligands able to bind to each domain[188]. Furthermore, the N-terminus has been reported to fold according to a three-state mechanism whilst the C-terminus was shown to be intrinsically disordered [187, 188]and the two termini have been documented to weakly interact[187, 188]. Although the a crystal structure of the 37kDa LRP has been solved at a resolution of 2.15 Å[191], this structure contains residues 1-220 only. Thus, the exact structure of the C-terminus is largely unknown. It must be noted that LRP/LR is post-translationally modified by phosphorylation at multiple sites, the most critical being Tyrosine 139[192]. The phosphorylation status of the 37kDa/67kDa LRP/LR is regulated in part by TGF-β inhibited membrane associated protein (TIMAP) and protein phosphatase-1 (PP-1)[193], both of which interact with the intracellular N-terminus of the protein. It must be noted that TIMAP phosphorylation may be induced by GSK-3β [194]and LRP/LR phosphorylation as a consequence of its association with TIMAP may be involved in filopodia formation[193].

The consequence of the alterable phosphorylation status of LRP/LR requires further investigation but it may be suggested that this feature may be of particular significance with regards to the signal transduction pathways triggered by the protein.

LRP/LR is functional within multiple cellular locations, a characteristic largely attributable to its structural plasticity. The 37kDa/67kDa LRP/LR is located within the cholesterol-rich lipid raft domains of the plasma membrane, in the cytoplasm as well as in the nucleus[5, 168].

Within the nucleus, LRP/LR has been identified in association with DNA and histones H2A, H2B and H4[195], and has been suggested to play a role in the maintenance of nuclear

structures. Furthermore, the protein has been associated with the pre-ribosome in the nucleolus[196].

In the cytosol LRP/LR has been implicated in the maturation and assembly of the ribosome (through its associations with 18s rRNA and the S21 ribosomal protein)[197]. Furthermore, as LRP/LR is homologous to the ribosomal protein p40, it is not unexpected that LRP/LR forms an association with 40S ribosomal subunit and is therefore implicated in translational processes. In addition, LRP/LR has been reported to play a role in rRNA processing [196, 198]. Moreover, LRP/LR has been shown to associate with cytoskeletal proteins including actin and α-tubulin, a major component of microtubules (to which tau similarly binds as discussed in above)[199].

At the cell surface, this protein serves as a multifunctional receptor exhibiting binding sites for an array of substrates (**Figure 1.10**) including: elastin and laminin-1[185, 200, 201], heparin and heparan sulphate proteoglycans (HSPGs)[5]; viruses including Sindbis[202], Dengue[203], Venezuelan equine encephalitis (VEE) and Adeno-associated virus subtypes 2, 3, 8 and 9[5, 168] as well as the cellular (PrP^{c})[165] **(Figure 1.10)** and infectious (PrP^{Sc}) prion proteins[171]. Many of the binding sites for different ligands overlap, namely 161-180aa (termed the peptide G region) has been reported to serve as a binding site for both laminin-1 and heparin, whilst the region 205-229 may bind laminin-1 in addition to PrP^{c} [5, 204].

As has been previously stated, LRP/LR serves as a non-integrin high affinity laminin receptor, exhibiting a dissociation constant (K_d) of 5.8nM[205]. Furthermore, LRP/LR may associate with other laminin binding receptors (integrins) at the cell surface[206]. The conformation of laminin-1 is altered upon binding to the peptide G region (containing the palindromic sequence) of LRP/LR. This association has been implicated in the induction of several signal transduction cascades and thereby largely underlies the receptor's physiological roles in cellular proliferation, growth, differentiation[5], migration[207] and the remodelling of the extracellular matrix[208] through the induction of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 9 (MMP-9)[209] activity.

Cell surface associated LRP/LR also serves as a receptor for midkine (MK)[210], a growth factor which promotes gene expression as well as cellular growth, survival and migration[211].

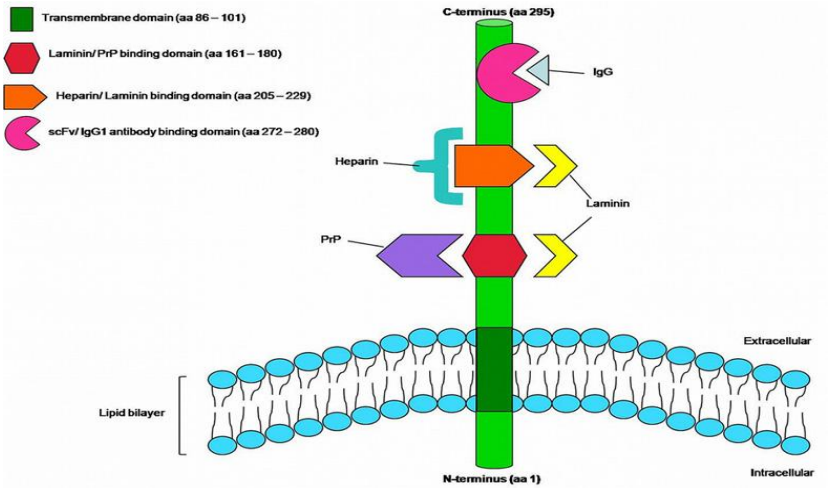


Figure 1.10| Schematic representation of the functional domains of the 37kDa/67kDa Laminin Receptor. This receptor is 295aa in length. Residues LRP86-101 represent the transmembrane domain. The receptor features three ligand-binding domains, including a PrP^c and laminin-1 binding domain (LRP161-180); a laminin-1and heparin binding domain (LRP160-180 and LRP205-229) and an IgG-antibody binding domain (LRP272-280) (Adopted from [5]).

1.11.1.1 37kDa/67kDa LRP/LR and Cellular Signalling

It is proposed that the 37kDa/67kDa LRP/LR occupies a central role in mediating the vital physiological cellular processes listed above largely as a result of its the binding to laminin-1, and the subsequent induction of the Mitogen Activated Protein Kinase (MAPK) signal transduction pathway. The 37kDa/67kDa LRP/LR has been shown to regulate expression levels of MKP1 and PAC1, MAPK phosphatases[212],which consequently dephosphorylate and deactivate ERK, p38 and JNK.

Interestingly, it has recently been reported that the LRP/LR-laminin-1 interaction, via MAPK signal transduction cascades, increases the phosphorylation status of c-Myc and thereby induces the expression of the Fas ligand (FasL)[213].

Moreover, the expression of the 37kDa/67kDa LRP/LR may in turn be regulated by the ERK and JNK MAPK signal transduction cascades, upon stimulation of these cascades by hypoxia. LRP/LR has an hypoxia response element 16bp upstream from the translational start site which is responsive to the hypoxia-inducible factor-1 (HIF-1), a transcription factor activated by a hypoxic micro-environment[209] through the ERK and JNK pathways. This is of particular relevance to cancer biology as hypoxia promotes tumour metastasis.

It has additionally been suggested that the 37kDa/67kDa LRP/LR-laminin-1 association may regulate the activity dual specific phosphatases[212], intracellular levels of Ca^{2+} [214] and calmodulin kinase II[215].

1.11.2 Pathological roles of the 37kDa/67kDa LRP/LR

1.11.2.1 The role of the 37kDa/67kDa LRP/LR in Cancer

Cancer is broadly described as the deregulation of normal cellular homeostasis resulting in uncontrolled cell proliferation. In 2012 there were an estimated 14.1 million new cancer cases globally (WCRF) and 8.2 million cancer-related deaths (GLOBOCAN, 2012), thus making cancer the 7th leading cause of death globally in 2012 (WHO).

The 37kDa/67kDa LRP/LR has been shown to be overexpressed in numerous cancer types including glioma, bile duct carcinoma, cystic carcinoma, urothelial tumours[209], colon, colorectal, breast [216], uterine, cervical, ovarian, gastric, laryngeal, oesophageal[216], liver[217], lung[218], prostate[219], acute myeloid leukaemia[220] to name a few.

The mechanism underlying the overexpression of LRP/LR in cancer is not firmly established as no gene amplification has been reported[221]. However, numerous factors have been demonstrated to regulate the expression of receptor. The 37kDa/67kDa LRP/LR – ECM associations have been suggested to result in upregulation of expression[222]. The regulatory effects of cytokines and inflammatory agents remains controversial as despite originally being reported to upregulate protein expression[223], later evidence revealed that Tumour necrosis factor alpha (TNF-α) and Interferon-gamma (IFN-ɣ) downregulate LRP/LR expression[224]. In addition, the tumour suppressor p53 has been proposed to regulate LRP/LR expression levels. The *RPSA* gene exhibits an enhancer element in intron-1 to which the transcription factor, AP-2, has been shown to bind. p53 has been shown to negatively regulate LRP/LR expression through the formation of a complex with AP-2 which consequently prevents its binding and finally results in the repression of *RPSA*[221]. As p53 is mutated and thereby functionally reduced in many cancers, this may accordingly provide an elegant explanation of the overexpression of LRP/LR in numerous cancer types. Furthermore, it must be noted that oxidative stress and Aβ peptides have been shown to conformationally alter p53 and thereby impair the activity of this transcription factor[225].

The level of LRP/LR expression has been shown to correlate directly with the invasive and metastatic potential of numerous neoplastic cells and is hence considered a marker for metastatic aggressiveness[226]. High LRP/LR expression levels are therefore a poor prognostic indicator in patients with solid tumours[227].

1.11.2.1.1 Uncontrolled Cellular Proliferation

The overriding characteristic of neoplastic cells is their unlimited proliferative potential. The mechanisms underlying the transformation of normal cells into tumorigenic cells, which are able to undergo uncontrolled proliferation, are broadly divided into six categories[228], namely: self-sufficiency in growth signals, resistance to cell cycle arrest signals, evasion of apoptosis, limitless replicative potential due to enhanced telomerase activity, the ability to induce angiogenesis as well as the ability to undergo invasion and metastasis.

The 37kDa/67kDa LRP/LR has been implicated in most of the aforementioned processes, and its roles in contributing to uncontrolled cellular proliferation have been well-established, some which are discussed below.

Overexpression of the 37kDa/67kDa LRP/LR is reportedly associated with an upregulation in the expression of cyclins A, B ,C as well as cyclin-dependant kinases (Cdk) 1 and 2, thereby allowing for cell cycle progression and irrepressible cellular proliferation and growth[229].

Furthermore, although the induction of FasL expression by LRP/LR through c-Myc, (as discussed above in section **1.11.1.1** above) may physiologically be involved in triggering apoptosis, it must be borne in mind that mutations in the Fas receptor (FasR), which are involved in both tumour development and progression both *in vitro* and *in vivo*[230], may negate this effect. Furthermore, LRP/LR induced phosphorylation activates and stabilizes c-Myc through this signalling pathway, which may thereby contribute to the neoplastic condition as c-Myc is involved in uncontrolled cellular proliferation[213].

LRP/LR has also been implicated in mediating uncontrolled proliferation in leukaemia cells. It has been reported that the receptor binds to and stimulates signal transduction cascades mediated through the granulocyte-macrophage colony stimulation factor (GM-CSF) receptor (GM-CSFR), which subsequently results in enhanced phosphorylation of the STAT5

transcription factor and ultimately stimulates proliferation (through self sufficient growth signals) and enhances resistance towards apoptosis[227].

The prominent role that this receptor occupies in mediating cell survival in cancer is emphasized by the fact that RNA interference (RNAi)-mediated downregulation of LRP/LR has been reported to induce apoptosis in neoplastic cells (liver, lung and cervical cancer respectively [183, 231]) while non-tumorigenic cells remain viable[232].

1.11.2.1.1.1 Exploitation of LRP/LR to target uncontrollable neoplastic proliferation

The overexpression of LRP/LR may conversely be exploited to mediate cancer-selective apoptosis. This is particularly true as a consequence of the binding between cell surface 37kDa/67kDa LRP/LR and the green tea polyphenol epigallocatechin-3-gallate (EGCG) (K_d= 0.4μM)[229]. This polyphenol has been ascribed anti-carcinogenic, anti-allergic and anti-inflammatory roles. This green tea component has been linked to reduced cancer risk as it has been shown to reduce neoplastic cell proliferation as well as to induce the apoptosis of tumorigenic cells without adversely affecting their non-tumorigenic counterparts. Both of these protective effects have been shown to be mediated through the 37kDa/67kDa LRP/LR.

EGCG binding to LRP/LR triggers a signal transduction pathway that consequently results in the enhanced expression of the eukaryotic translation elongation factor 1A (eEF1A)[233]. This results in the dephosphorylation of myosin phosphatase-targeting subunit-1 (MYPT1) at threonine-696, thereby promoting the activation of myosin phosphate and finally the dephosphorylation of the myosin regulatory light chain (MRLC) at threonine-18/serine-19. The consequence hereof is cytoskeletal disruption through actin rearrangement, and growth inhibition[233]. This pathway thereby underlies the tumour growth inhibitory effects of EGCG. These inhibitory effects are achieved with 1μM of EGCG, a concentration attainable by drinking two to three cups of green tea[234].

The pro-apoptotic mechanisms underlying the EGCG-LRP/LR association are varied. One study has suggested that EGCG binding to the receptor results in the activation of Akt and endothelial nitric oxide synthase (eNOS) signal transduction cascades. This would thereby induce nitric oxide (NO) production and enhance cyclic guanosine monophosphate (cGMP) levels[229], both of which would result in the growth arrest and induction of apoptosis in

tumorigenic cells[235]. This EGCG-LRP/LR association has also been suggested to contribute to caspase activation and the collapse of the mitochondrial transmembrane potential - both prerequisites for cellular apoptosis. Another study has proposed that the pro-apoptotic effects of EGCG may be as a result of lipid raft disruption and removal of LRP/LR from these regions, thereby preventing receptor mediated activation of signalling cascades implicated in uncontrolled proliferation and evasion of apoptosis[236].

1.11.2.1.2 The role of the 37kDa/67kDa LRP/LR in Metastasis and Angiogenesis

Metastasis, the dissemination of cancerous cells from a primary tumour to distal sites, causes approximately 90% of cancer deaths[237] owing to the difficulty in preventing metastasis once it has initiated as well as targeting multiple sites for treatment. Metastasis involves the adhesion of cancerous cells to the basal membrane, the subsequent degradation of the basal lamina by proteolytic enzymes which allows for tumour cell entry into either the blood or lymphatic systems and finally extravasation (again requiring adhesion and basal lamina degradation) by the neoplastic cells at a secondary site.

Tumour angiogenesis, the formation of new blood vessels to support tumour formation at a distal site through the supply of oxygen and nutrients, involves a similar process. Angiogenesis involves endothelial cell activation and enzyme-mediated proteolysis of the surrounding extracellular matrix or basal lamina[238]. Furthermore, the activated proteases subsequently release pro-angiogenic factors/peptides which stimulate endothelial cell migration towards the angiogenic signal as well as promote proliferation and differentiation into tubular structures (vessels)[238, 239]. Angiogenesis is vital for successful tumour metastasis, tumour progression and growth[240].

The 37kDa/67kDa LRP/LR has been shown to be instrumental in mediating both processes, and as previously stated, overexpression of this receptor correlates very well with the adhesive, invasive and thereby metastatic potential of numerous cancer cell types[216, 217 241]. The laminin-1 glycoprotein is a major constituent of the basal lamina and thus LRP/LR is required for cellular adhesion to the basal lamina. Furthermore, the interaction between LRP/LR and laminin-1 has been reported to induce the activity of ECM degrading enzymes, particularly matrix metalloproteases (MMPs) and type-IV collagenase[208, 242]. Additional ECM degrading enzymes, with activities enhanced by the LRP/LR–laminin-1 association, include: membrane-type I matrix metalloproteinase, MMP-2, cathepsin-L and stromelysin 3[226].

Furthermore, as described above, environmental factor such as hypoxia may induce tumour metastasis and may do so through the 37kDa/67kDa LRP/LR. Hypoxia results in ERK/JNK activation of HIF-1 which upreguates LRP/LR expression and consequently enhances the production of downstream molecules such as the matrix degrading enzymes urokinase-type plasminogen activator (uPA) and MMP-2[209]. It must be noted that hypoxia is also a particular concern in neurodegenerative disorders and has been shown to promote and contribute to Alzheimer's Disease. HIF-1 has also been shown to mediate AD neurodegeneration[243, 244].

The importance of this receptor in mediating these pathogenic processes is highlighted by the observation that, blockade the LRP/LR-laminin-1 association through the use of anti-LRP/LR specific antibodies as well as downregulation of the receptor, significantly decreases the adhesive and invasive potential of numerous cancer types[216,217,241,245-247] as well as completely abolishes angiogenic tube formation[248].

1.11.2.2 The role of the 37kDa/67kDa LRP/LR in Viral and Bacterial Infections

The 37kDa/67kDa LRP/LR serves as a cell surface receptor for many pathological agents. The receptor binds numerous viruses, including: Adeno-associated virus (AAV) (serotypes 2,3,8 and 9)[249], Dengue viruse (serotypes 1,2 and 3) [250], Sindbis virus [202], Venezuelan equine encephalitis virus (VEE)[251], tick-borne encephalitis (TBE)[252]and the West Nile Virus [253].

Furthermore, LRP/LR (C-terminus, specifically the region 263-282aa) serves as a receptor for bacterial adhesion to the blood brain barrier (BBB)[254]. As LRP/LR is highly expressed on the surface of human brain microvascular endothelial cells (BMECs)[255], cells of which the BBB is composed, the receptor is exploited by numerous bacteria for enhanced adhesion to the BBB. These include *Streptococcus pneumoniae*, *Neisseria meningitides* and *Haemophilus influenza*[256]. Upon binding to the BBB, LRP/LR facilitates the uptake of these bacteria[254]. Thus the 37kDa/67kDa LRP/LR is essential to BBB crossing and the bacterial meningitis which ensues as a consequence of these cerebral bacterial infections. In addition, LRP/LR has been reported to serve as a cell surface receptor for the cytotoxic necrotizing factor 1 (CNF1), a toxin of the *Escherichia coli* K1 strain[255].

It has therefore been revealed that the 37kDa/67kDa LRP/LR is central in mediating microbial pathogenesis and is particularly important in establishing infections of the central nervous system (CNS).

1.11.2.3 The role of the 37kDa/67kDa LRP/LR in Prion disorders

As has been previously, stated the 37kDa/67kDa LRP/LR has been shown to serve as a high affinity receptor for both cellular and infectious prion protein isoforms, PrP^{c} [165, 204] and PrP^{Sc} [171, 257] respectively. It is important to note that heparin sulphate proteoglycans (HSPGs) serve as co-receptors, facilitating the LRP/LR-PrP^{c} association[204]. Most importantly, the receptor is central to mediating the internalization of both isoforms[165, 257, 258 168, 259]. As the conversion of PrP^{c} to PrP^{Sc} is proposed to occur both at the cell surface as well as within endocytic vesicles[4,260,261] (**Figure 1.7**), and intracellular accumulation of the aggregated isoform underlies neuronal death, the fact that the 37kDa/67kDa LRP/LR is involved in the uptake mechanism[257] suggests that it is central in mediating the progression and pathogenesis of prion disorders. This has been confirmed by the fact that targeting the receptor, through the use of antibodies and decoy mutants, significantly hampers PrP^{Sc} propagation both *in vitro*[171, 262] and *in vivo*[260, 261].

Recent reports have demonstrated that LRP/LR is vital for alimentary PrP^{Sc} uptake[263] across different species (between livestock species as well as between livestock and humans). This finding possess a great threat to agriculture as well as the possibility of developing zoonotic diseases, and thus understanding the role of LRP/LR in prion pathogenesis and targeting this association has become ever more critical.

The importance of the 37kDa/67kDa LRP/LR in prion disorders has been verified by the observation that therapeutics targeting the receptor or impedance of the LRP/LR-PrP^{c} cell surface association significantly reduced PrP^{Sc} accumulation *in vitro*[171, 262] as well prolonged the preclinical (before symptom onset) phase *in vivo*[260, 261]. Such strategies included the use of an LRP/LR decoy mutant (102-295aa, thereby lacking a transmembrane domain required for cell surface anchorage)[262] as well as use of siRNA mediated downregulation of LRP/LR[264], heparin mimetics[171] and pentosane polysulfates (which interfere with the cell surface pathogenic association, thereby hindering PrP endocytosis)[168] as well as antibodies directed against LRP/LR.

1.12 Mitogen Activated Protein Kinase (MAPK) signal transduction pathway

1.12.1 The Role of MAPK in neurons

Mitogen Activated Protein Kinases (MAPK) are a family of serine/threonine kinases that, through successive phosphorylation events, modulate the activity of numerous substrates (including transcription factors and cytoskeletal proteins[265]). The MAPK cascade plays a central role in physiological processes including: gene expression, cellular proliferation, differentiation and apoptosis as well as mitosis, migration and metabolism[265].

The MAPK cascade has three mammalian subgroups. These include the extracellular signal-regulated kinases (ERKs), which are responsive to growth factors and mitogens and ultimately induce cellular proliferation, survival and differentiation [212] and inhibits apoptosis[213]. In contrast, the other two MAPK subgroups: c-Jun NH_2-terminal protein kinase (JNK)/stress-activated protein kinase and p38 MAPK, are responsive to inflammation and cellular stress (be it chemical or environmental)[212], with one of the resultant outcomes being the induction of apoptosis[266].

The MAPKinase signalling pathway is of particular importance in neuronal cells (as is evidenced by the abundance of ERK1and ERK2 in the central nervous system (CNS))[266]. In neuronal cells, neurotransmitters or neurotrophic factors stimulate either G-protein coupled receptors or ion-gated channels, triggering an increase in the intracellular Ca^{2+} concentration which ultimately activates MAPK signalling cascades.

MAPK signalling cascades are of immense physiological significance and are implicated not only in neuronal survival but also in neuronal differentiation, neurite branching and neuronal plasticity[266]. Furthermore, these signalling pathways may be involved in: the synaptic potentiation of hippocampal neuron and synaptic transmission as a result of their ability to not only phosphorylate microtubule associated proteins (MAP2) but also modulate gene expression and influence protein synthesis[266]. With particular relevance to Alzheimer's Disease, MAPK has been implicated in tau protein phosphorylation (NFTs are a consequence thereof)[266]. In addition, cross-talk between the ERK and JNK/p38 signalling pathways is crucial to the regulation of apoptosis and more specifically neurodegeneration and synaptic loss[266] and it may therefore be proposed that aberrant MAPK signalling, as arises in AD, may result in augmented neuronal loss.

1.12.2 MAPK in Alzheimer's Disease

All three aforementioned mammalian MAPK subgroups are differentially activated during the course of the Alzheimer's Disease [267]. According to reports by Zhu *et al.*,[267], non-demented patients lacking AD-associated pathology exhibit either ERK or JNK activation whilst the activation of both ERK and JNK was observed in patients displaying pathological features but not yet demonstrating dementia. However, in patients suffering from mild to severe Alzheimer's Disease, all three pathways (ERK, JNK and p38) were activated[267].

Augmented JNK signalling activity has been implicated in the phosphorylation of tau and the consequent fibrillization thereof [268]. In addition, this pathway has been implicated in Aβ-induced cytotoxicity as inhibition hereof significantly hindered apoptosis induced by Aβ[268, 269]. JNK, more specifically JNK3, has been implicated in the mitochondrial release of cytochrome c and Smac/Diablo[265], and is thereby considered pro-apoptotic and has been associated with hippocampal neuronal death.

The p38 MAPK pathway occupies a central role in AD pathogenesis and is implicated in neuroinflammation, excitotoxicity, reduced synaptic plasticity, tau hyperphosphorylation[270] and apoptosis (through increased expression of Bax proteins)[265].

ERK activation, although classically associated with cell survival, has been reported to be increased in neurons treated with Aβ peptides[269, 271]. ERK signalling has been shown to play a pro-apoptotic role, in a caspase-independent manner, in the event that the external stimulus is plasma membrane damage[265]- which is a consequence of Aβ insertion into the plasma membrane.

It must be stated that altered phosphatase activity cannot be excluded as a contributor to the MAPK signalling modulation (ERK, JNK and p38 levels and activities) observed in AD[267].

1.13 Therapeutics targeting the 37kDa/67kDa LRP/LR

The 37kDa/67kDa LRP/LR is overexpressed in numerous cancer cell types and has been shown to play a central role in the enhanced adhesive and invasive potential (key features of metastasis) of tumourigenic cells [261, 5, 168, 241]. In addition, as the receptor exhibits binding affinities for both PrP^{c} and the infectious PrP^{Sc} isoforms, LRP/LR may be implicated in either direct or indirect PrP^{Sc} uptake and consequently the establishment of prion disorders[168]. Furthermore, LRP/LR has also been considered a significant mediator in numerous viral and bacterial diseases. As a result of the wide array of pathological processes in which LRP/LR is involved, a multitude of therapeutic approaches for the modulation of the receptor have been developed. The most notable being, heparan mimetics; an LRP decoy mutants (lacking the transmembrane domain); RNA-interference strategies, including small-interfering RNAs (siRNA) for the down-regulation of the receptor and anti-LRP/LR specific antibodies [168].

1. 14 Implications of the 37kDa/67kDa LRP/LR in Alzheimer's Disease

The 37kDa/67kDa LRP/LR localizes to lipid raft domains of the plasma membrane and HSPGs have been suggested to mediate the receptor's binding interactions[5, 168]. As previously stated, $A\beta_{42}$ oligomers interact with a multitude of cell surface proteins/receptors (**Table 1.1**) and HSPGs and may additionally be directly incorporated into the lipid raft domains of the plasma membrane. Furthermore, the 37kDa/67kDa LRP/LR binds laminin-1 (to which $A\beta_{42}$ oligomers similarly bind), as well as PrP^{c} (a protein to which $A\beta_{42}$ oligomers bind with high affinity).

Therefore, $A\beta_{42}$ and LRP/LR share a similar cellular location and multiple common binding partners. Furthermore, the pathological agents of TSEs and AD share structural similarities and the resultant neurodegenerative diseases exhibit mechanistic commonalities[6]. A result hereof, a relationship between $A\beta_{42}$ and the 37kDa/67kDa LRP/LR seems to be possible. Moreover, a binding interaction between these proteins, be it direct or indirect, seems plausible and thus the possibility of such an interaction and the influence thereof on $A\beta_{42}$-mediated cell loss warranted investigation.

CHAPTER 2

2.1 Hypothesis

The 37kDa/67kDa laminin receptor (LRP/LR) forms an association with amyloid-beta (the 42aa isoform) ($A\beta_{42}$) peptides and the receptor thereby influences $A\beta_{42}$ pathogenesis.

2.2 Objectives

The objective of this study is (i) to definitively determine whether an interaction between LRP/LR and $A\beta_{42}$ exists (ii) to explore the role of LRP/LR in $A\beta_{42}$-mediated neuronal loss and (iii) to investigate the mechanism underlying the role of LRP/LR in $A\beta_{42}$ pathogenesis.

2.3Aims

2.3.1 To probe the cell surface distribution and possible co-localization of $A\beta_{42}$ and LRP/LR on both non-neuronal and neuronal cells by immunofluorescence and confocal microscopy.

2.3.2 To employ Försters resonance energy transfer (FRET) for further sensitive and reliable examination of an interaction between LRP/LR and $A\beta_{42}$ on the cell surface .

2.3.3 To employ pull down assays, western blotting and Aβ-specific ELISAs to ascertain whether a physiologically relevant physical association occurs between LRP/LR and Aβ.

2.3.4 To examine the role of LRP/LR in $A\beta_{42}$-mediated cytotoxicity by 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell viability assays.

2.3.5 To ascertain whether cell death processes (apoptosis or necrosis) and/or modulation of cellular proliferation are responsible for the observed variations in cell viability by Annexin-V-FITC/7-AAD assays as well as 5-bromo-2'-deoxyuridine (BrdU) (proliferation) assays.

2.3.6 To investigate whether LRP/LR is implicated in $A\beta_{42}$ internalization by flow cytometry and confocal microscopy.

2.3.7 To establish whether LRP/LR occupies a central role in $A\beta_{42}$ pathogenesis (objectives 2.3.4-2.3.6) by employing short hairpin RNA (shRNA) directed against LRP/LR mRNA to achieve LRP/LR downregulation.

CHAPTER 3

Peer-reviewed Review Article

I have contributed to the generation of a single review article during the course of this PhD. The manuscript was prepared in collaboration with other members of the S.F.T Weiss Research Group in the School of Molecular and Cell Biology (MCB) at the University of the Witwatersrand and reported on the proceedings of the Global Alzheimer Research Summit held in Madrid, Spain in 2011.

The review article to which I contributed during the course of this PhD is entitled:

3.1 Global Alzheimer Research Summit: Basic and clinical research: Present and future Alzheimer research.

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

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

Journal: Prion, 6(**1**):7-10

Year of Publication: 2012

Brief Overview of Article:

This meeting report briefly summarizes the central points of discussion raised during the Global Alzheimer's Summit held in Madrid, Spain in 2011. This article reports on recent advances, novel outlooks and the status of clinical trials within the broad field of Basic and Clinical Alzheimer's Disease Research. In addition to the conventional areas of investigative research, namely the molecular mechanism giving rise to Aβ and genetic risk factors, novel areas of interest were highlighted namely, the importance of oxidative stress, mitochondrial abnormalities, disruption of intracellular transport, lifestyle risk factors (such as hypertension and hypercholesterolemia) and pathogenic associations which result in neurodegeneration through aberrant cell signalling cascades (Reelin). Furthermore, the need to identify reliable biomarkers not only for disease diagnosis but also for monitoring the efficacy of treatment strategies was highlighted and the enhanced sensitivity of using both CSF and imaging biomarkers was discussed. Data regarding the progress of therapeutics undergoing clinical trials were presented and the opinion that a single AD therapeutic will not be effective in treating Alzheimer's Disease, and that rather a multitude of drugs targeting different processes is necessary, was held by most delegates.

Contribution: I contributed to the writing and editing of this review article.

*These authors contributed equally to this work.

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Global Alzheimer Research Summit

Basic and clinical research
Present and future Alzheimer research

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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.

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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.

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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.

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CHAPTER 4

Research Articles

During the course of this PhD, the results obtained in fulfilment of the aforementioned aims and the resultant novel findings were compiled into two separate scientific articles for publication in international peer-reviewed journals.

The first article, which revealed for the first time that the 37kDa/67kDa LRP/LR may occupy a central role in $A\beta_{42}$ mediated pathogenesis and thereby uncovered an as yet unidentified association between the two proteins, was published in the Scientific Reports Journal. The second article, which expanded on the results of the first paper and further provided a mechanism underlying the role of LRP/LR in $A\beta_{42}$ pathogenesis, has recently also been published in the Scientific Reports Journal.

Therefore, the scientific articles prepared in fulfilment of the requirements for a PhD degree are:

4.1 Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity.

4.2 The 37kDa/67kDa Laminin Receptor acts as a receptor for $A\beta_{42}$ internalization.

In addition, I have contributed (experimental planning, data analysis, writing and editing) to the generation, submission and publication of numerous other manuscripts throughout the course of my PhD. Some of these articles were within the context of neurodegenerative disorders whilst others were concerned with the role of LRP/LR in cancer metastasis and angiogenesis.

These articles, arranged according to relevance to data obtained during my PhD, are:

4.3 The 37kDa/67kDa LRP/LR plays a central role in Aβ-PrP^c mediated cytotoxicity in Alzheimer's Disease.

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

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

4.6 Prion Interactions with the 37kDa/67kDa Laminin Receptor on Enterocytes as a cellular model for intestinal uptake of prions.

4.7 Inhibition of angiogenesis by antibodies directed against the 37kDa/67kDa laminin receptor *in vitro*.

4.8 Anti-LRP/LR specific antibody IgG1-iS18 impedes adhesion and invasion of liver cancer cells.

4.1 Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity

Authors: B. Da Costa Dias, K. Jovanovic, D. Gonsalves, K. Moodley, U. Reusch, S. Knackmuss, C. Penny, M. Weinberg, M. Little and S.F.T. Weiss.

Journal: Scientific Reports 3, 2702; DOI:10.1038/srep02702

Year of Publication: 2013

Brief Overview of Article:

This original research article was the first to examine the possibility of an association between the 37kDa/67kDa LRP/LR and Aβ and was the first to report that this association indeed exists and has cell biological effects. It was demonstrated that the 37kDa/67kDa co-localized with $A\beta_{42}$ on the cell surface of both human embryonic kidney (HEK293) and murine neuroblastoma (N2a) cells. Furthermore, a physical association between the receptor and synthetic $A\beta_{42}$ was detected by FLAG® co-immunoprecipitation. Antibody blockade as well as shRNA-mediated downregulation of LRP/LR was shown to significantly enhance cellular viability and proliferation across all cell lines investigated - HEK293, N2a and human neuroblastoma (SH-SY5Y) cells. This led authors to propose that the cell biological effects of this association may contribute to $A\beta_{42}$-pathogenesis and LRP/LR may thereby mediate $A\beta_{42}$-induced cytotoxicity. This article recommended, for the first time, the use of tools targeted against the 37kDa/67kDa LRP/LR (such as specific antibodies and RNAi) as therapeutic tools for the treatment of Alzheimer's Disease, particularly with regards to treating the neuronal loss characteristic of the disease.

Contribution: I planned and conducted the experiments, analysed the data, wrote and edited this original research article.

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Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity

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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

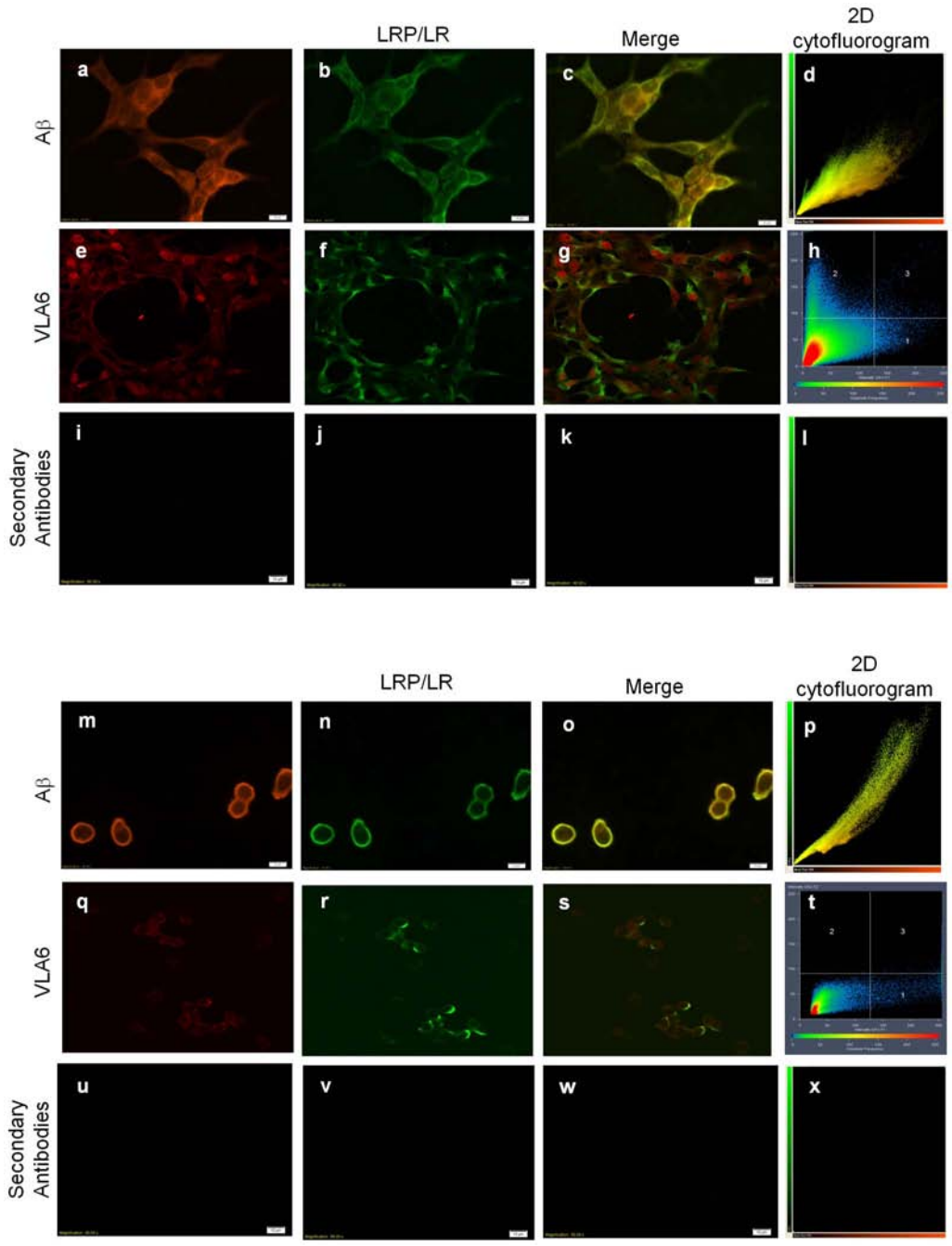


Figure 1 | Cell surface co-localisation between LRP/LR with Aβ. (a) Endogenous cell surface LRP/LR and Aβ on HEK293 (upper panel) and N2a (lower panel) cells were indirectly immunolabelled. Aβ was indirectly detected using anti-β-amyloid (22–35) (Sigma) and anti-rabbit Alexafluor 633 antibodies (Fig. 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

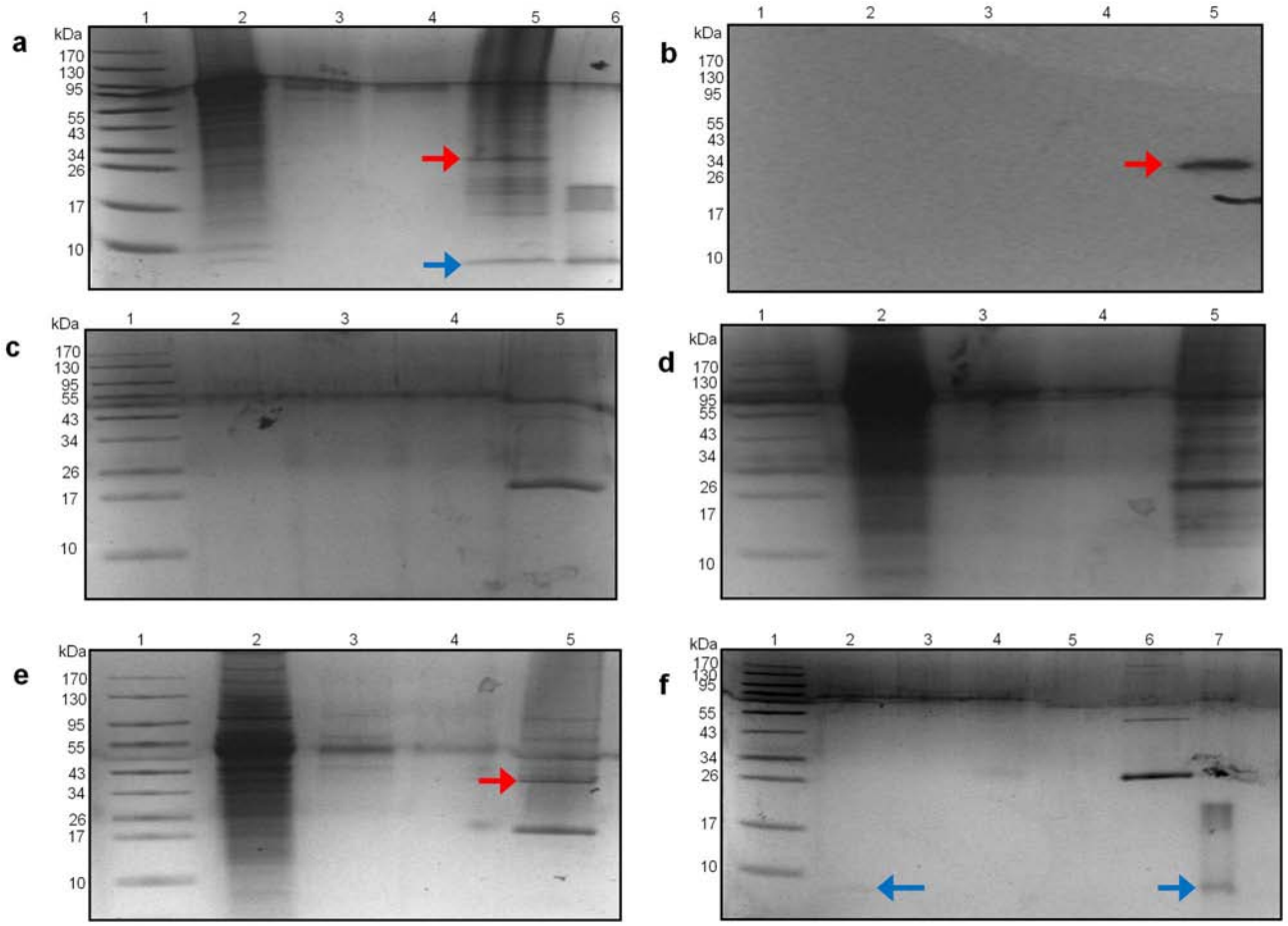


Figure 2 | LRP/LR as a potential Aβ-interacting protein. Pull down assays were employed using FLAG® Immunoprecipitation kit (Sigma Aldrich), to investigate the proteins detectable in unbound samples (lane 2), wash steps (lanes 3 and 4) and eluted samples (Fig. 2a–e: lane 5 and Fig. 2f: lane 6) and 2 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/

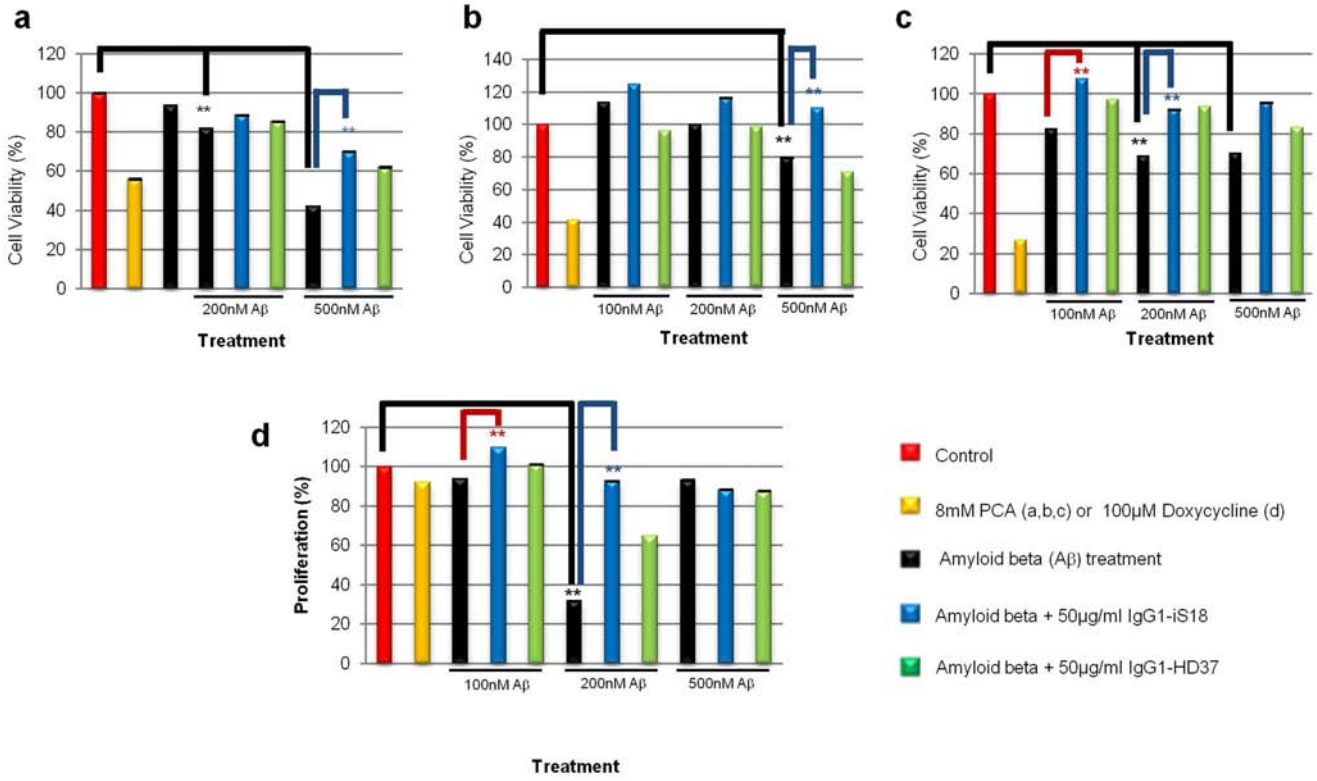


Figure 3 | Cell rescuing effects of anti-LRP/LR antibody IgG1-iS18. (a) Cellular viability of HEK293 cells, as determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) assay, post exogenous treatment with synthetic $A\beta_{42}$ and upon co-incubation with anti-LRP/LR 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

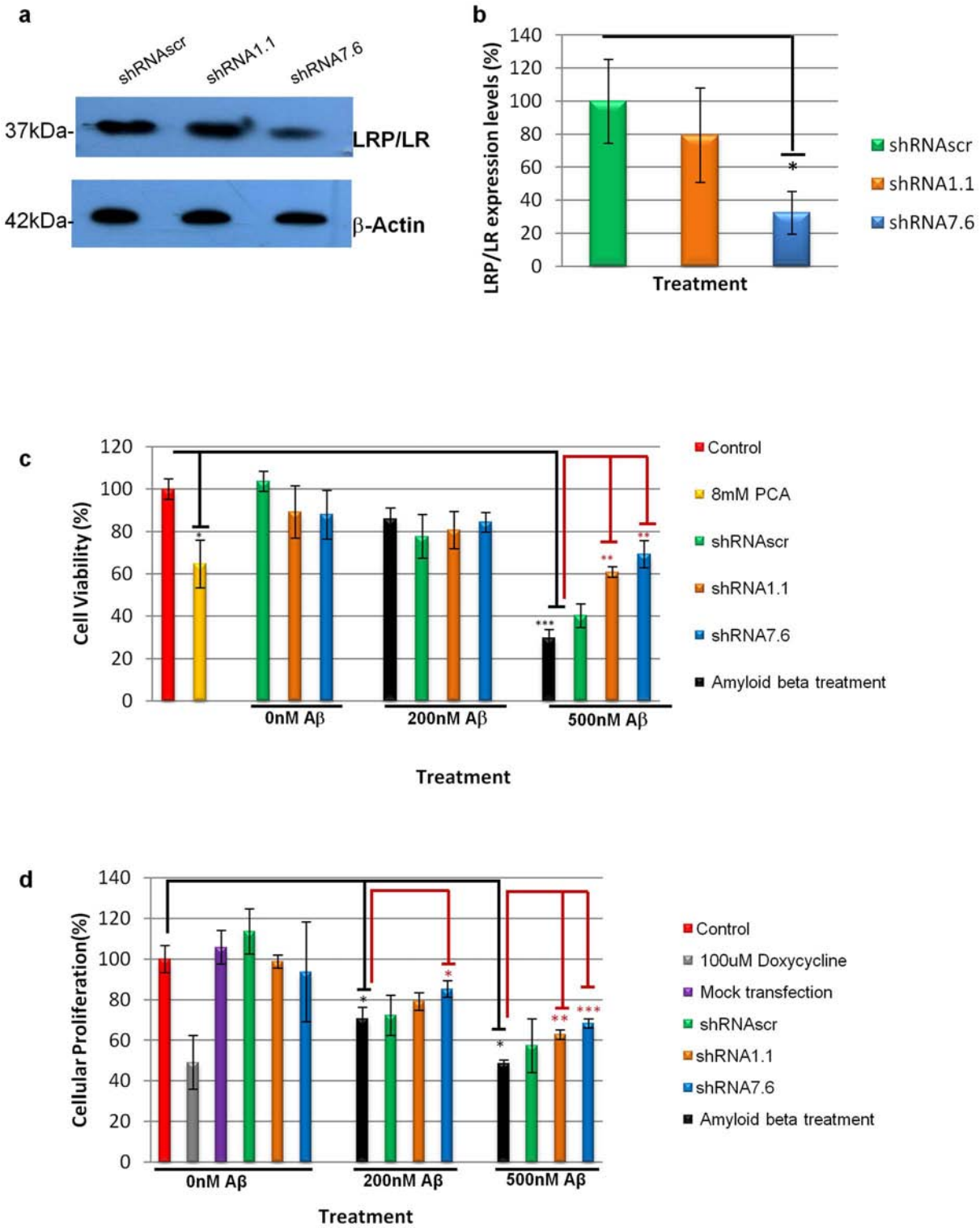


Figure 4 | shRNA-mediated downregulation of LRP/LR and the effects thereof. (a) HEK293FT cells were transfected with shRNAscr, shRNA1.1 and shRNA7.6 using the *Trans*IT®-LT1 Transfection reagent. 72 h post transfection total LRP/LR levels were assessed by Western blotting. β-actin was employed as a loading 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.

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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

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4.2 The 37kDa/67kDa Laminin Receptor acts as a receptor for $A\beta_{42}$ internalization

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Brief Overview of Article:

This original research article expanded on the findings of the first report and revealed a probable mechanism underlying the role of the 37kDa/67kDa LRP/LR in $A\beta_{42}$ pathogenesis. Owing to the low resolution of confocal microscopy employed for our preliminary co-localization studies, Försters resonance energy transfer (FRET) was employed to assess protein interactions. The detection of FRET upon immunolabelling of both proteins of interest demonstrated that LRP/LR and $A\beta_{42}$ interact on the cell surface. Furthermore, the physiological relevance of this association was verified by performing FLAG® co-immunoprecipitation studies with conditioned media (into which Aβ was endogenously shed). In addition to the cellular effects observed during the first study, it was revealed that the LRP/LR-$A\beta_{42}$ association resulted in the induction of apoptosis, which was significantly deterred upon blockade of this association with anti-LRP/LR specific antibodies. Finally, this study demonstrated that LRP/LR is central in mediating $A\beta_{42}$ internalization, as both antibody blockade and shRNA-mediated downregulation of the receptor significantly impeded $A\beta_{42}$ uptake. These results therefore demonstrated that the LRP/LR-$A\beta_{42}$ association is of physiological relevance. Moreover, it was revealed for the first time that the 37kDa/67kDa LRP/LR may serve as an internalization receptor for $A\beta_{42}$. This may thereby underlie the receptor's role in $A\beta_{42}$-induced pathogenesis. Through facilitation of $A\beta_{42}$ uptake, the receptor may promote intracellular $A\beta_{42}$ accumulation and the adverse cellular effects (induction of apoptosis and inhibition of proliferation) which are consequences thereof. These results further confirmed the potential of tools directed against LRP/LR as promising Alzheimer's Disease therapeutics.

Contribution: I planned and conducted the experiments, analysed the data, wrote and edited this original research article.

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The 37kDa/67kDa Laminin Receptor acts as a receptor for $A\beta_{42}$ internalization

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Neuronal loss is a major neuropathological hallmark of Alzheimer's disease (AD). The associations between soluble Aβ oligomers and cellular components cause this neurotoxicity. The 37 kDa/67 kDa laminin receptor (LRP/LR) has recently been implicated in Aβ pathogenesis. In this study the mechanism underlying the pathological role of LRP/LR was elucidated. Försters Resonance Energy Transfer (FRET) revealed that LRP/LR and Aβ form a biologically relevant interaction. The ability of LRP/LR to form stable associations with endogenously shed Aβ was confirmed by pull down assays and Aβ-ELISAs. Antibody blockade of this association significantly lowered $A\beta_{42}$ induced apoptosis. Furthermore, antibody blockade and shRNA mediated downregulation of LRP/LR significantly hampered $A\beta_{42}$ internalization. These results suggest that LRP/LR is a receptor for $A\beta_{42}$ internalization, mediating its endocytosis and contributing to the cytotoxicity of the neuropeptide by facilitating intra-cellular $A\beta_{42}$ accumulation. These findings recommend anti-LRP/LR specific antibodies and shRNAs as potential therapeutic tools for AD treatment.

Alzheimer's Disease (AD), primarily identified by Austrian physician Alois Alzheimer in 1906[1], is a progressive neurological disorder characterised by extracellular neuritic plaques and intracellular neurofibrillary tangles (caused by aberrant misfolding and aggregation of amyloid beta peptides (Aβ) and the hyperphosphorylated tau protein), cerebrovascular amyloidosis as well as synaptic and neuronal loss. These neuropathological features are particularly evident in the basal forebrain and hippocampus, as these are the regions of higher-order cognitive function[2,3]. It is predicted that in 2050, approximately 1 in 85 people will be afflicted by the disease[4] owing to the global increase in aged populations due to enhanced life expectancies.

The transmembrane amyloid precursor protein (APP) is the parental protein from which Aβ is generated through sequential cleavage by β-secretase and γ-secretase. This cleavage may occur at the plasma membrane or within endosomes[5]. The resultant Aβ may consequently be shed into the extracellular space, be exocytosed or accumulate intracellularly.

Although extracellular neuritic plaques are a pathological hallmark of AD, the soluble intracellular oligomeric assemblies of Aβ, particularly the aggregation-prone $A\beta_{42}$ isoform, are largely considered the aetiological agents of this disease. They precede and may contribute to tau hyperphosphorylation and have been reported to directly cause synaptic and neuronal loss as well as vascular degeneration of the brain[6]. Moreover Aβ exerts its toxicity intracellularly[6] and the senile plaques themselves have been proposed to serve a neuroprotective role as Aβ sinks which sequester the toxic soluble intracellular oligomers- the peripheral sink hypothesis[7].

Although a myriad of molecular mechanisms reportedly contribute to $A\beta_{42}$ mediated neuropathology, the lack of effective therapeutics suggests that central role players in disease initiation and progression have yet to be identified. Until all the intricate pathological networks underlying AD are uncovered, effective therapeutic strategies may remain elusive. Thus, understanding the cellular trafficking as well as the associations between Aβ and cellular components (particularly cell surface receptors) are imperative to understanding its neurotoxicity.

A protein of immense interest with regards to Aβ pathogenesis is the cellular prion protein (PrP^c). PrP^c is considered neuroprotective under normal physiological conditions, through the maintenance of oxidative stress homeostasis and inhibition of β-secretase cleavage of APP[8]. In contrast, the overwhelming majority of recent

reports have demonstrated that within the AD context, PrP^c acquires a pathological role. Upon binding to Aβ oligomers (which it is able to do with high affinity, $k_D = 0.4 \times 10^{-9}M$[9,10]) PrP^c has been shown to mediate neurotoxic signals through Fyn kinase[11,12], impair synaptic plasticity, inhibit long term potentiation and contribute to intracellular accumulation of Aβ by mediating the internalization of Aβ oligomers[13]. However, owing to the glycosylphosphatidylinositol (GPI)-anchored nature of this protein[14], it is largely dependent on its receptors to mediate the aforementioned functions. One such receptor, which exhibits a high binding affinity ($k_D = 1 \times 10^{-7}$ M) for PrP^c, is the 37 kDa/67 kDa laminin receptor (LRP/LR) (also known as LamR, RPSA and p40)[15]. This multifunctional receptor is implicated in numerous physiological roles including translation, maintenance of cytoskeletal structure[16], cell survival, differentiation, proliferation and migration[17,18]. LRP/LR is also involved in the development of numerous pathological states, including cancer[18,19] and tumour angiogenesis[20], prion disorders and both viral[21–24] and bacterial infections (of particular interest being bacterial meningitis as the receptor mediates translocation across the blood brain barrier)[25].

As LRP/LR serves as a PrP^c receptor we aimed to investigate whether LRP/LR is implicated in Aβ pathogenesis. Antibody blockade and shRNA mediated downregulation of LRP/LR was shown to significantly enhance the viability and proliferative potential of cells treated with $A\beta_{42}$[26]. In this study we aimed to further probe the mechanism underlying the role of LRP/LR in mediating Aβ pathogenesis.

Results

Försters resonance energy transfer between cell surface LRP/LR and Aβ. Försters resonance energy transfer (FRET) is one of the most sensitive techniques employed to assess protein interactions in cellular systems. The non-radiative energy transfer from a donor to an acceptor will only occur if the fluorochromes are within 1–10 nm from each other. A cytometry-based FRET assay was employed to investigate whether LRP/LR and Aβ interact on the surface of HEK293 cells. The highly sensitive[27,28] PE/APC FRET pair (donor and acceptor, respectively) was employed to immunolabel the proteins of interest on non-permeabilised cells. As PE is maximally excited by the 488 nm argon laser and emits maximally at 575 nm it may be detected with the FL2 filter set of the Accuri C6 (BD Biosciences),whilst APC, excited by the 650 neon/helium laser and exhibiting maximal emission at 660 nm, is readily detectable with the FL4 filter set (Fig. S1). Successful labelling of the proteins of interest (LRP/LR, PrP^c, CAT and Aβ) was confirmed (Fig. S2). The presence of FRET between the proteins of interest was evaluated employing the FL3 filter set. Within this channel, excitation is achieved with the 488 nm argon laser and emission of 660 nm is detected. The APC antibody is not excited and does not exhibit fluorescence within this channel. This therefore accounts for the overlay between unlabelled cells; cells labelled solely with the APC secondary antibody as well as cells in which PrP^c, CAT and Aβ were immunolabelled with APC (Fig. 1a,c,e). However, upon the close proximity of the PE-coupled secondary antibody, APC may be indirectly excited via FRET, and this may result in the enhanced fluorescence emission of the acceptor in FL3 (Fig. S1). It is owing to this that FL3 is considered the optimal channel for FRET detection between the PE/APC pair.

The efficacy of this flow cytometry based FRET assay was investigated employing PrP^c, a cell surface protein to which LRP/LR binds with very affinity[15] (positive control) and chloramphenicol acetyl transferase (CAT), a bacterial protein to which LRP/LR has been shown to not bind[26] (negative control). Upon co-labelling of cells with PrP^c-APC with LRP/LR-PE, the fluorescence of APC was enhanced, as was observed by the rightward shift of the APC histogram along the FL3 fluorescence intensity axis (Fig. 1b). Conversely co-labelling of CAT-APC with LRP/LR-PE had no effect on the emission of APC, as was evident by the overlay between the two histograms (Fig. 1d). The augmentation of APC fluorescence intensity upon co-labelling of cells with Aβ-APC and LRP/LR-PE (Fig. 1f), therefore suggests that FRET occurred between these proteins.

LRP/LR interacts with shed Aβ. To confirm that LRP and Aβ form stable associations, pull down assays were conducted. Although similar experimental procedures have been previously reported[26], these were conducted employing exogenously administered synthetic $A\beta_{42}$ peptide. Those reported here employed conditioned cell culture media (supernatant) from HEK293 cells into which Aβ was shed, thereby investigating the presence of this association within a physiological context. The averaged total concentration of Aβ present in the conditioned media was approximately 37.6 pg/ml, results which are consistent with the concentration of Aβ detected by others in the supernatant of HEK293 cells[29]. The efficacy of this assay was confirmed by the presence of both the BAP-fusion protein (~49 kDa) and LRP::FLAG (~38 kDa) in the eluted samples, which indicates that these proteins were successfully immobilized by the Anti-FLAG® M2 beads (~17 kDa) (Fig. 2a, lane 4). The presence of CAT (~26 kDa) in the unbound sample (Fig. 2a, lane 1), reveals that this protein was not immobilised by LRP::FLAG and further confirms that CAT does not interact with LRP. Evaluation of the degree of Aβ present in each pull down assay fraction required sensitive detection employing an Aβ- specific ELISA assay. Upon co-incubation of conditioned media with LRP::FLAG containing cell lysate, it was observed that Aβ was successfully immobilized by LRP:FLAG, as there was a significant increase (27%) ($p = 0.0433$) in the Aβ levels in the eluate sample when compared to that present in wash 3 (Fig. 2b). To account for possible binding of Aβ to other proteins within the cell lysate or non-specific binding to the Anti-FLAG® M2 beads, the degree of Aβ in the eluates of samples containing conditioned media co-incubated with NT lysates or conditioned media alone, were compared to LRP::FLAG containing samples (Fig. 2c). There was a significant increase in the amount of Aβ bound to the column in the presence of LRP::FLAG when compared to that in NT lysates (34%) ($p = 0.039611287$) and conditioned media alone (19%) ($p = 0.04788224$). It is noteworthy to add that the degree of Aβ present in the eluate was not significantly different to that in wash 3 in both NT lysate (Fig. S3a) and conditioned media only samples (Fig. S3b). It must be noted that the ELISA employed to quantify the concentration of Aβ is unable to distinguish between the $A\beta_{40}$ and $A\beta_{42}$ isoforms.

Cellular incubation with $A\beta_{42}$ induces apoptosis. An Annexin-V-7AAD assay was employed to assess the cellular effects of synthetic $A\beta_{42}$ on HEK293 cells. The exogenous application of 200 nM and 500 nM $A\beta_{42}$ did not produce cytotoxic effects after 24 h (Fig. 3a) but did result in a progressive induction of apoptosis after 48 h. Apoptosis induction was concentration dependent with the degree of apoptosis detected after 72 h being approximately 30% greater in the 500 nM treatment when compared to the 200 nM treatment (Fig. 3a).8 mM PCA, an apoptosis inducing agent, was employed as a positive control and was similarly assessed over 72 h. The time-dependent induction of apoptosis was confirmed by the nuclear morphological changes observed in cells treated with 500 nM $A\beta_{42}$ (Fig. 3b). At 24 h, most nuclei appeared normally stained but 48 h post-treatment, the first stage of chromatin condensation, namely chromatin condensation around the nuclear periphery, was observed. Post 72 h treatment, apoptotic bodies were detectable (Fig. 3b).

IgG1-iS18 rescues cells from Aβ induced apoptosis. The degree of cell death (comprising early and late apoptosis as well as necrosis) induced upon cellular treatment with 200 nM and 500 nM

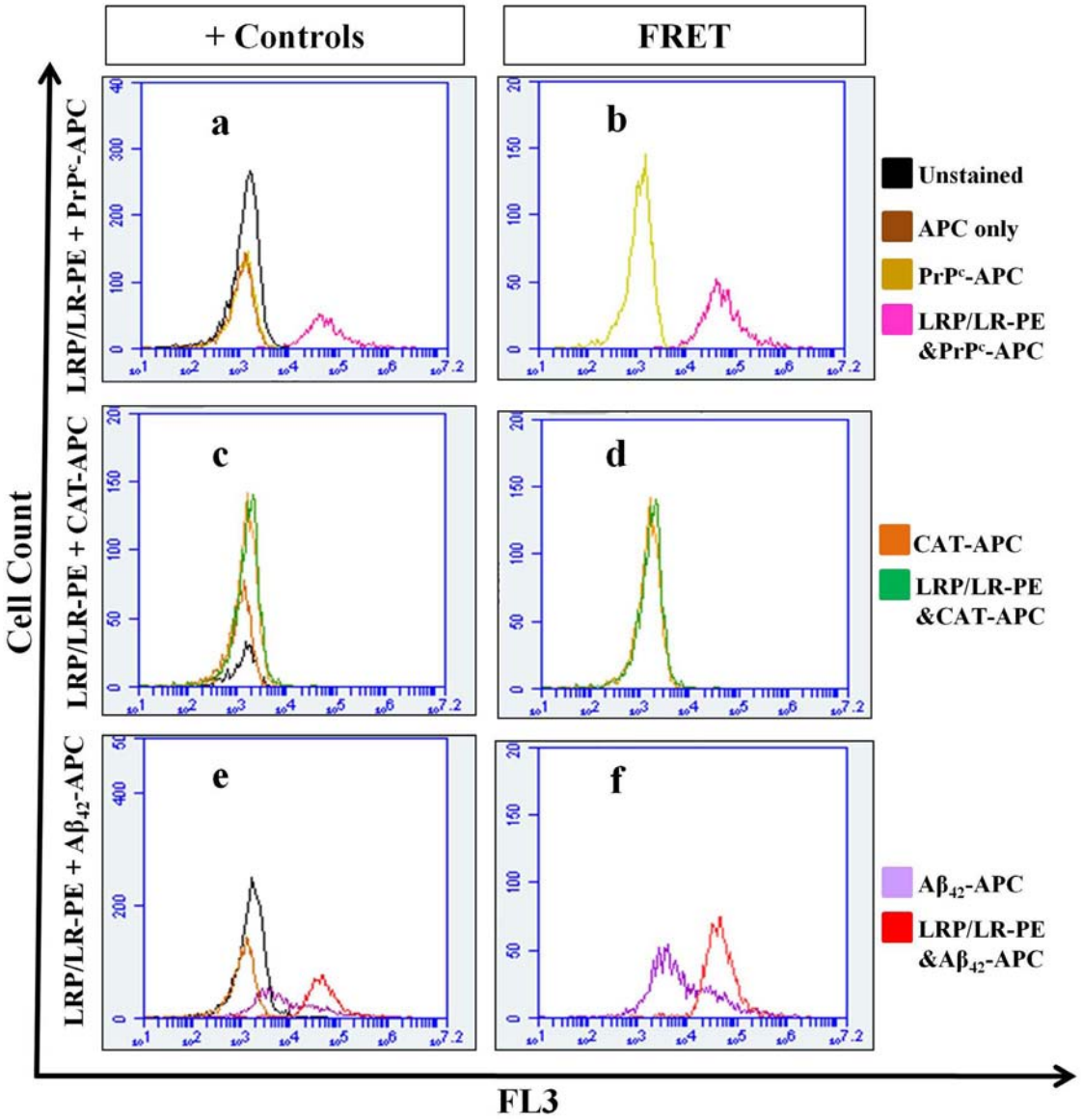


Figure 1 | Flow cytometric analysis of Försters Resonance Energy Transfer (FRET) between cell surface LRP/LR and Aβ. The fluorescence intensity histogram of the unlabelled non-permeabilised HEK293 cells (black histogram, a,c,e) was superimposed with that of cells labelled with the APC secondary antibody only (brown histogram, a,c,e) as well as with cells in which the proteins of interest (PrP^c, CAT and $A\beta_{42}$) were labelled with APC (a,c,e respectively). Co-labelling of LRP/LR-PE and PrP^c-APC (positive control) (pink histogram, b). Co-labelling of LRP/LR-PE and CAT-APC (negative control) (green histogram, d). Co-labelling of LRP/LR-PE and Aβ-APC (red histogram, f). Each panel is a representative image. Three biological replicates, each performed in triplicate, were conducted.

exogenous synthetic $A\beta_{42}$ for 72 h was assessed by Annexin-V-7AAD assay. Upon assessment, cellular incubation with 200 nM $A\beta_{42}$ resulted in 53.6% cell death, whilst treatment with 500 nM $A\beta_{42}$ resulted in 78.84% cell death (Fig. 4). In both treatments, apoptosis accounted for >85% of the detectable cell death. Co-incubation of the cells with 50 μg/ml IgG1-iS18 (anti-LRP/LR specific antibody) significantly reduced the extent of cell death induced by $A\beta_{42}$ at both concentrations by 45.35% ($p < 0.001$) and 57.39% ($p < 0.001$), respectively whilst the anti-CAT antibody (negative control) had no effect on cell death processes (Fig. 4). Protocatechuic acid (PCA), an apoptosis inducing agent, was employed as the positive control. Antibody treatment with IgG1-iS18 alone does not significantly reduce cell death when compared to the untreated control (Fig. 4).

$A\beta_{42}$ internalization. The degree of cell surface $A\beta_{42}$ served as a measure of the degree of receptor-mediated internalization - with lower cell surface $A\beta_{42}$ levels being indicative of enhanced internalization, whilst higher levels reveal reduced internalization or recycling. Cellular incubation of control cells at 4°C prior to and after exogenous $A\beta_{42}$ administration was performed to limit internalization as receptor-mediated internalization is halted under these conditions. Thus, the cell surface levels of $A\beta_{42}$ in the no internalization control (1 h, 4°C) was set to 100%. The progressive decrease in cell surface $A\beta_{42}$ may therefore be interpreted as a consequence of receptor-mediated internalization of the exogenous $A\beta_{42}$. Although internalization (12.05%) was observed after 5 min, the extent of $A\beta_{42}$ internalization was at its highest and most evident after 15 min (66.9%) after which the level of cell surface $A\beta_{42}$ remained relatively constant for a further 15 min and then increased (Fig. 5a). The significant 11.36% increase ($p < 0.001$) in cell surface $A\beta_{42}$ at 1 h when compared to 30 min may be indicative of $A\beta_{42}$ recycling to the cell surface (and ultimately exocytosis) by the cell. The internalization of the exogenously administered $A\beta_{42}$ was

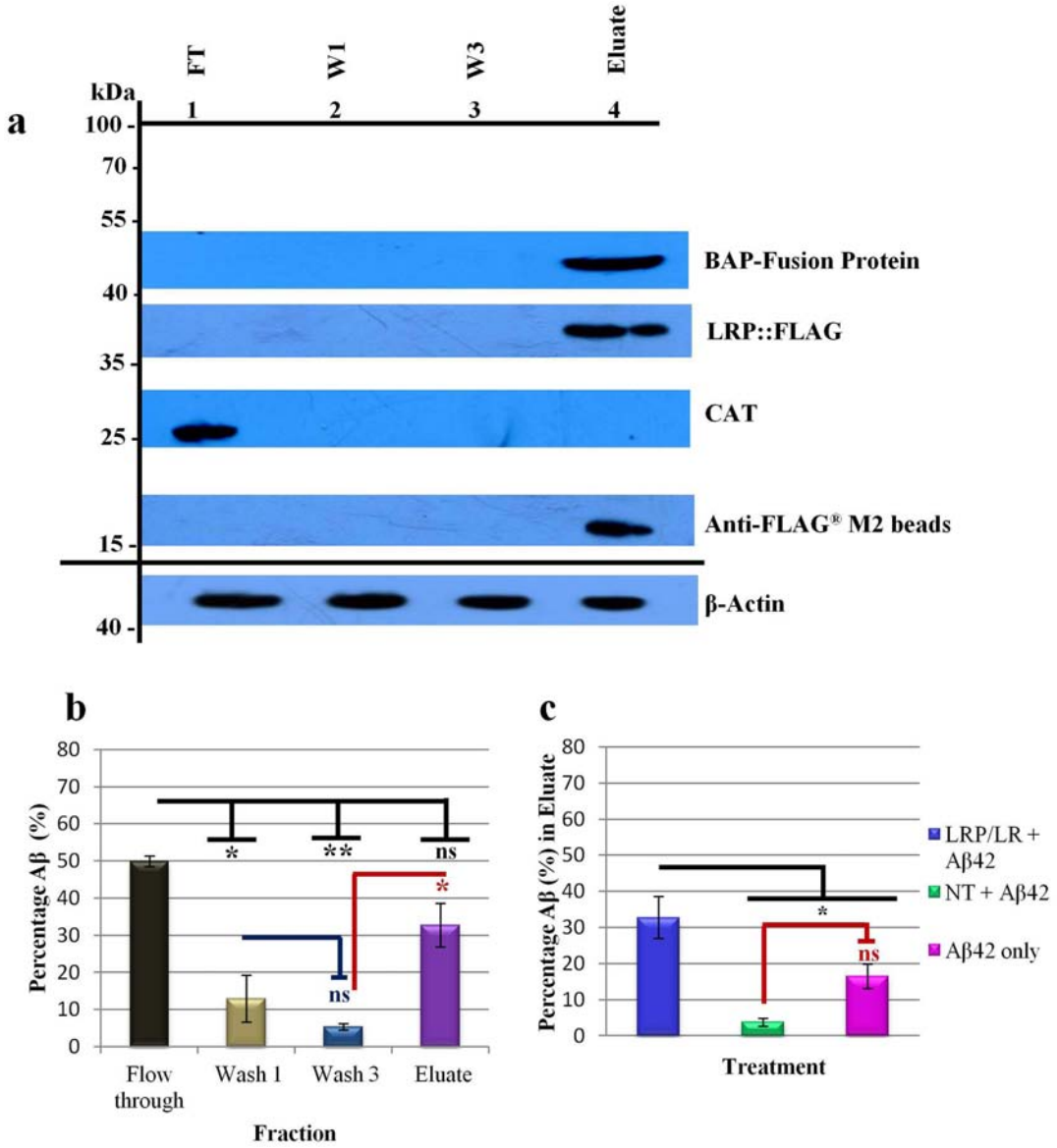


Figure 2 | FLAG® Immunoprecipitation Assays demonstrate LRP/LR-Aβ association. HEK293 cell lysates, non transfected as well as lysates containing recombinant LRP::FLAG to which either recombinant CAT lysates (negative control), BAP-Fusion protein (positive control) or conditioned tissue culture media containing Aβ was added, were subjected to pull down assays. Samples were analysed by Immunoblotting (a) Lane 1, unbound sample (flow through); lane 2, first wash step (W1); lane 3, third wash step (W3) and lane 4, eluate samples. β-actin served as the loading control. Fraction sample of pull down assays containing conditioned media were assessed by Aβ-ELISA (b & c). The Aβ levels per fractions of the LRP::FLAG pull down were detected by Aβ ELSA (b).The Aβ levels present in eluate of: LRP::FLAG lysate & conditioned media sample; NT lysate & conditioned media and conditioned media alone are depicted (c). Gels have been cropped for clarity and conciseness purposes and we all run under the same experimental conditions. Data shown are representative (mean ± s.e.m.) of three biological replicates, each performed in triplicate. *$p < 0.05$, **$p < 0.01$; Student's *t*-test.

further confirmed by confocal microscopy. Cells were previously transfected with pCFP-mem such that the plasma membrane could be readily identified. At 5 min, it is evident that most of the $A\beta_{42}$ is located at the cell surface whilst at 15 min and more markedly at 30 min, the degree of $A\beta_{42}$ labelling within the cell lumen is unmistakably enhanced. As 100 nM $A\beta_{42}$ is considered to be below the detection limit for intracellular immunostaining[6], microscopic visualization was only attained upon cellular treatment with 500 nM, to ensure dependable results were obtained. It is noteworthy to add that very high cell densities negatively affect ligand binding efficiencies and thus 70% densities were considered optimal for successful internalization. Furthermore, cell signalling and receptor-mediated internalization events were synchronized as a result of serum starvation prior to experimentation[30].

LRP/LR is a central mediator of $A\beta_{42}$ internalization. The degree of cell surface $A\beta_{42}$ served as a measure of the degree of receptor-mediated internalization. Cells were either subjected to antibody treatment (Fig. 6a) or RNA intereference technology in which LRP/LR was downregulated by shRNA7.6 (Fig. 6b)[29]. All relevant controls were similarly incubated at 4°C, prior and post exogenously 500 nM $A\beta_{42}$ administration as this halts receptor-mediated internalization processes[30]. The cell surface levels of $A\beta_{42}$ in the untreated no internalization control (1 h, 4°C) was set to 100% in both experimental sets (Fig. 6a & 6b). Upon, co-incubation of cells with 50 μg/ml IgG1-iS18 (anti-LRP/LR specific antibody), a significant enhancement in cell surface $A\beta_{42}$ was observed across all incubation periods when compared to untreated controls at corresponding time points (Fig. 6a). This therefore demonstrates

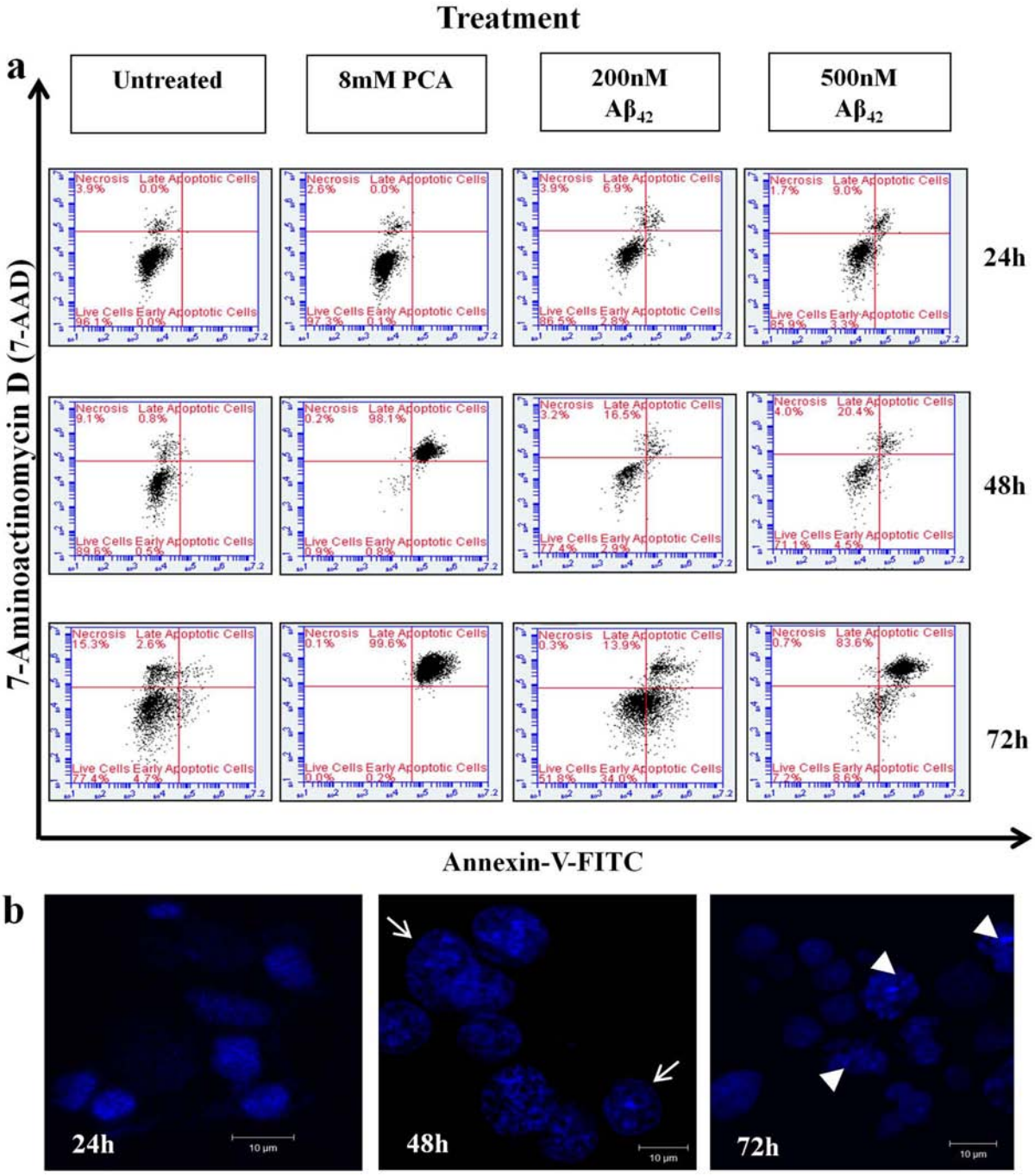


Figure 3 | $A\beta_{42}$ treatment induces apoptosis. The induction of cell death in HEK293 cells treated with 200 nM or 500 nM $A\beta_{42}$ for different incubation periods, was assessed by Annexin-V-7AAD assay (a). The induction of apoptosis was further confirmed by assessing the nuclear morphology at 24 h, 48 h and 72 h post-treatment with 500 nM $A\beta_{42}$ (b). Arrows depict chromatin condensation against the nuclear periphery, arrow heads depict apoptotic bodies. Figures shown are representative images. Three biological replicates, each performed in triplicate, were conducted per experiment.

that antibody blockade of LRP/LR resulted in more cell surface-associated $A\beta_{42}$ across all time points (in comparison to untreated controls) and this therefore suggests that $A\beta_{42}$ internalization is hampered as a result of LRP/LR antibody blockade. Cells similarly treated with the anti-CAT antibody displayed $A\beta_{42}$ internalization processes analogous to those of untreated controls (Fig. 6a). Furthermore, antibody treatment alone, in the absence of $A\beta_{42}$, did not significantly alter internalization processes.

To confirm that LRP/LR is indeed implicated in $A\beta_{42}$ internalization, and the effects observed during IgG1-iS18 treatment were not owing to steric effects of the antibody on surrounding proteins, LRP/LR was downregulated employing short hairpin RNAs (shRNAs). When compared to the shRNA scrambled (shRNA scr) controls (at corresponding incubation points), LRP/LR downregulation as mediated by shRNA7.6, significantly enhanced the degree of cell surface $A\beta_{42}$ and therefore impeded internalization (Fig. 6b). The transfection methodology itself did not adversely affect the internalization processes, as the difference in cell surface $A\beta_{42}$ levels was not significantly different between control, mock transfected and shRNAscr samples across all incubation periods (Fig. 6b).

Flow cyometric analysis confirmed that shRNA7.6 resulted in a significant 55.4% decrease ($p = 0.008$) in cell surface LRP/LR levels when compared to the shRNA scr (Fig. 6c). This was evidenced as a shift towards a lower fluorescence intensity when the fluorescence

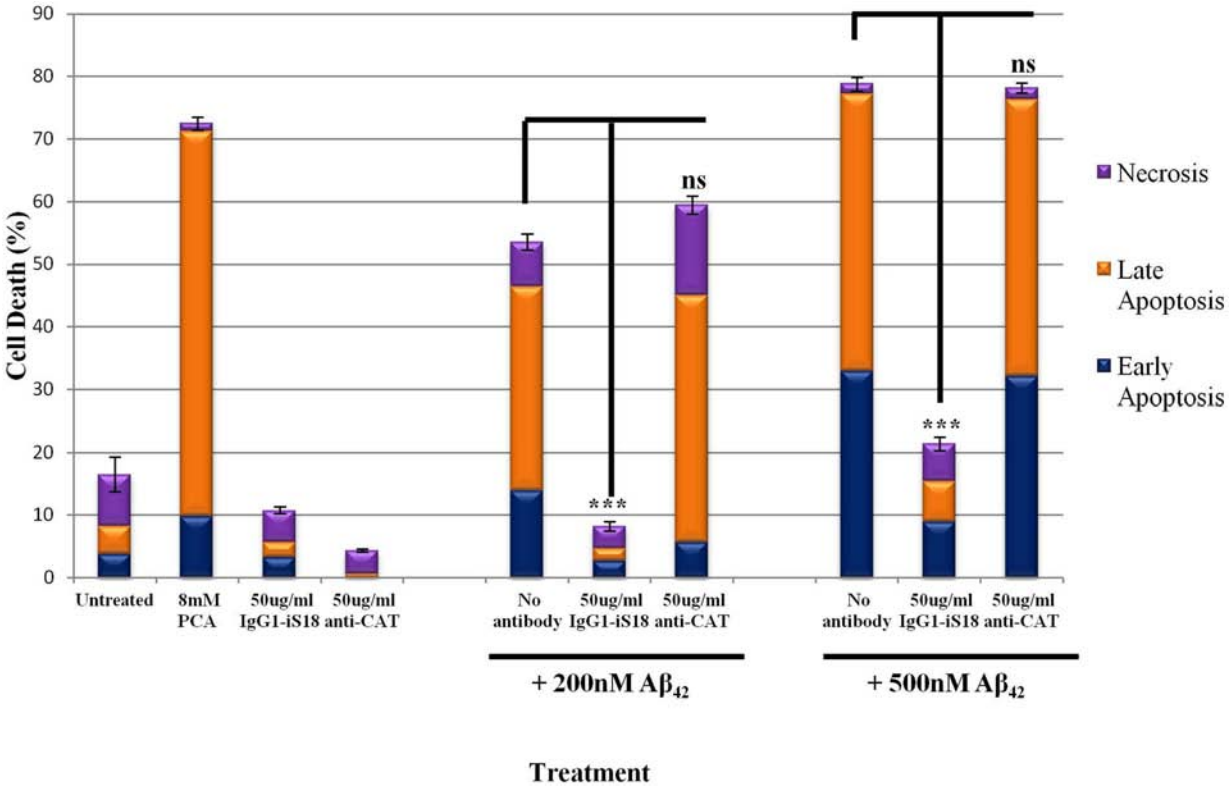


Figure 4 | Cell rescuing effects of anti-LRP/LR antibody IgG1-iS18. Cell death of HEK293 cells, assessed by Annexin-V-7AAD assay, post exogenous treatment with 200 nM and 500 nM synthetic $A\beta_{42}$ and upon co-incubation with anti-LRP/LR IgG1-iS18 or anti-CAT (negative control). Cell death was assessed 72 h post treatment. No antibody control was set to 100%. Data shown are representative (mean ± s.d.) of three biological replicates, each performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$P < 0.001$; Student's *t*-test.

intensity histograms of both treatments are overlayed (Fig. S4a). The LRP/LR fluorescence intensity histograms of control, mock and shRNA scr controls overlayed perfectly (Fig. S4b) and the median fluorescence intensities (MFI) of these treatments were not significantly different to that of the untreated control (set to 100%) (Fig. S4c). These results confirm that the transfection methodology did not alter cell surface LRP/LR expression levels. To assess whether LRP/LR downregulation may influence cell surface PrP^c levels, which would confound the observed results, the cell surface expression of PrP^c was evaluated. Flow cytometric analysis of shRNA transfected cells revealed that LRP/LR downregulation had no significant effect on cell surface PrP^c levels (Fig. 6d). Similarly the PrP^c fluorescence intensity histograms of control, mock and shRNA scr controls overlayed perfectly (Fig. S4d) and the MFIs of these treatments were again not significantly different (Fig. S4e). Thus, PrP^c cell surface expression levels were not affected by the transfection methodology.

Discussion

Ensuring that LRP/LR and $A\beta_{42}$ interact naturally preceded investigations regarding the mechanism underlying the receptor's role in AD pathogenesis. We have previously demonstrated that LRP/LR and Aβ co-localize on the surface of HEK293 and N2a cells[26], results which suggested that a potential interaction may exist between these endogenously expressed proteins. However, co-localization has a resolution limit of 200 nm and therefore positive results may not necessarily be indicative of an association, but may simply demonstrate that proteins share similar cellular locations. This would be expected as Aβ has been reported to insert into the lipid raft region of the plasma membrane, the region to which LRP/LR is localized[31]. Therefore, in order to probe the potential of such an interaction existing under normal cellular conditions, Försters resonance energy transfer (FRET) was employed. FRET is based on the principle that a donor fluorochrome (phycoerythrin (PE) in this study), within 1–10 nm of the acceptor fluorochrome (allophycocyanin (APC)), will non-radiatively transfer energy to the acceptor and this, depending on the FRET couple chosen, may result either in the enhanced fluorescence emission of the acceptor[32] or acceptor bleaching. The former is detected upon using the PE/APC FRET couple employed in this study. The PE/APC FRET pair was selected as the fluorochromes exhibit very high molar extinction coefficients (1 200 000 $M^{-1}cm^{-1}$ and 5000 $M^{-1}cm^{-1}$, respectively) and quantum yields and this makes them exceptionally sensitive when coupled to antibodies[28] and may achieve 90% FRET efficiencies[28]. Furthermore, although microscopy is classically employed to assess FRET, the tedious nature and inability to analyse large cell numbers, led researchers to apply flow cytometric detection methods instead. The efficacy and accuracy of this technique was assessed by investigating whether FRET was observed between LRP/LR and known ligands to which it either binds with high affinity (PrP^c) or has been shown not to bind (CAT). APC, a fluorochrome not excited by the 488 nm laser, either alone or employed to label a protein of interest, did not exhibit fluorescence emission in the FL3 channel (which employs 488 nm for excitation and a 660 filter set for emission detection) as the fluorescence intensity was superimposed on that obtained from unlabelled cells (Fig. 1a,c,e). This therefore demonstrates that the fluorescence detected within the FL3 channel for unstained, APC only as well as APC labelling of proteins of interest (PrP^c, CAT and $A\beta_{42}$) was owing to the native fluorescence of the cells. However, upon co-labelling of PrP^c -APC and LRP/LR-PE, the fluorescence intensity of APC was notably augmented (Fig. 1b) whilst co-labelling of CAT-APC and LRP/LR had no such effect (Fig. 1d). This therefore correctly implies that LRP/LR and PrP^c interact and this in turn allowed FRET to transpire. Since CAT and LRP/LR do not interact[26], the absence of FRET was expected. The occurrence of FRET between LRP/LR and

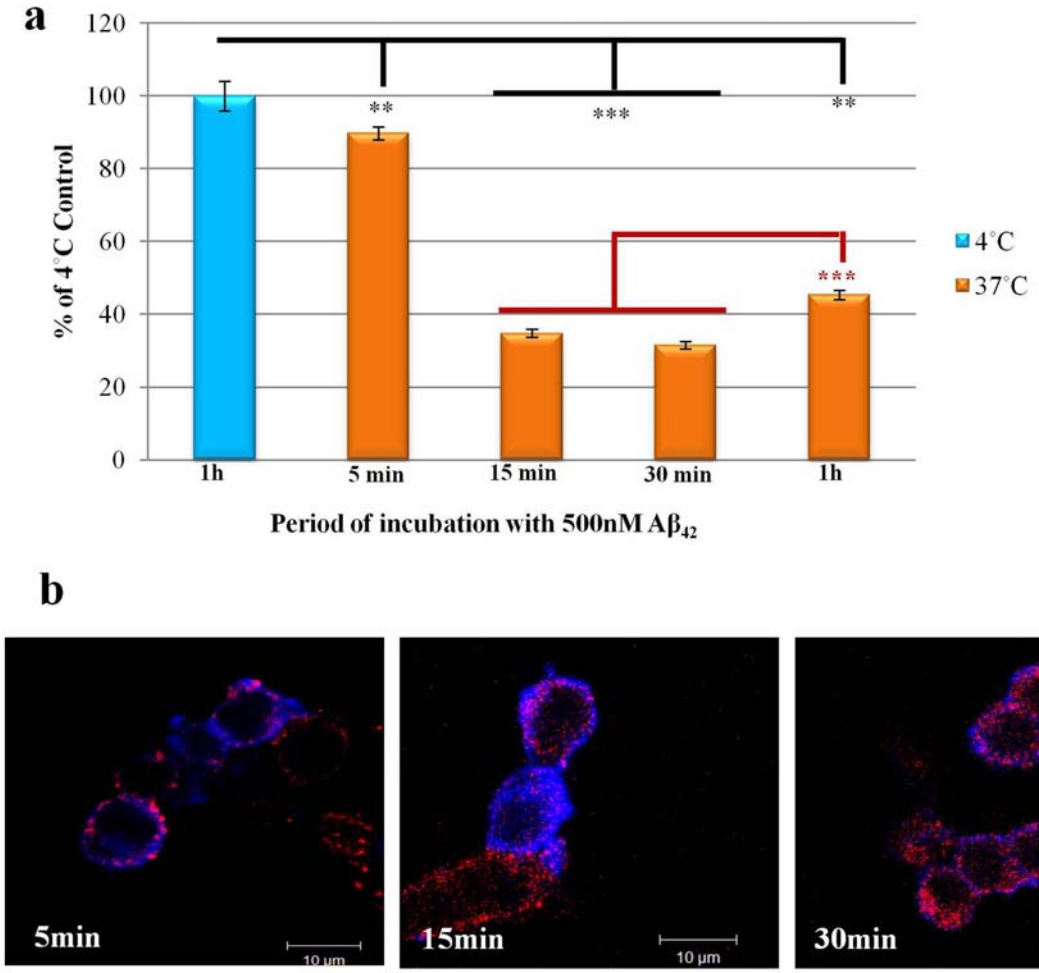


Figure 5 | $A\beta_{42}$ Internalization. The degree of cell surface Aβ served as a measure of the degree of receptor-mediated internalization. The cell surface levels of Aβ in the no internalization control (1 h, 4°C) was set to 100% (a). $A\beta_{42}$ internalization was confirmed by assessing the degree of intracellular $A\beta_{42}$ in HEK293 cells. Cells were transfected with pCFP-mem, resulting in plasma membrane labelling (depicted in blue). Intracellular Aβ was labelled with Anti-β-amyloid-APC (depicted in red) (b). Data shown are representative (mean ± s.d.) of three biological replicates, each performed in triplicate. *$p < 0.05$, **$p < 0.001$; Student's *t*-test.

Aβ (Fig. 1f) therefore suggests that these proteins are within 10 nm if each other on the cell surface and the most probable consequence thereof is that these proteins interact. Flow cytometric analysis of FRET, employing the PE/APC fluorochrome couple, has been successfully employed to identify other biologically relevant molecular interactions[28,33].

To confirm that a stable physiological association does indeed exist between LRP/LR and Aβ pull down assays were performed. Although a similar experiment has been previously performed[26], high quantities (2 μg) of synthetic $A\beta_{42}$ were utilized and thus the presence of this interaction under normal physiological conditions warranted investigation. A significantly higher proportional of shed Aβ was present in the eluate of samples containing recombinantly expressed LRP:FLAG when compared to samples containing NT cell lysate and conditioned media alone (Fig. 2c) thereby suggesting that endogenously expressed and shed Aβ was successfully immobilized by LRP/LR and that a physiologically relevant association exists between these proteins. In addition, inadequate washing and disruption of non-specific associations between Aβ and the LRP:FLAG containing column can be discounted as contributors to the high levels of immobilized Aβ as the proportion of Aβ present in the wash step 3 fraction was significantly lower than that in the eluate (Fig. 2b). Moreover, the degree of immobilized Aβ in the eluates of the control samples were not significantly different from the proportion present in the wash step 3 fraction (Fig.S3a & S3b) nor significantly different from each other (Fig. 2c), and can therefore be attributable to a low degree of non-specific binding of Aβ to the column as the results suggest that no proteins present in the NT lysate were able to mediate Aβ-column binding.

The interaction of Aβ with cell surface receptors has been repeatedly shown to lead to pathological events, including aberrant cell signalling pathways and the induction of cell death. Although apoptosis is the more common cell death modality observed, necrosis has also been suggested to underlie Aβ neurotoxicity[34] owing to the deregulation of intracellular Ca^{2+} levels which are a consequence of Aβ insertions into the plasma membrane[35] and adverse effects on cellular endoplasmic reticula and mitochondria. Therefore, the form of cell death induced upon Aβ treatment was assessed by Annexin-V-7AAD assay and the induction of apoptosis (which accounted for >85% of the cell death) was demonstrated to be both time and concentration dependent (Fig. 3a). Antibody blockade of LRP/LR, as achieved by co-incubation of the cells with IgG1-iS18 (anti-LRP/LR specific antibody) and 500 nM $A\beta_{42}$, significantly lowered the degree of apoptosis induced by the neurotoxic peptide in comparison to untreated and isotype antibody (anti-CAT) treated controls (Fig. 4). From these results it may be proposed that upon binding to Aβ, LRP/LR may stimulate/promote pro-apoptotic processes.

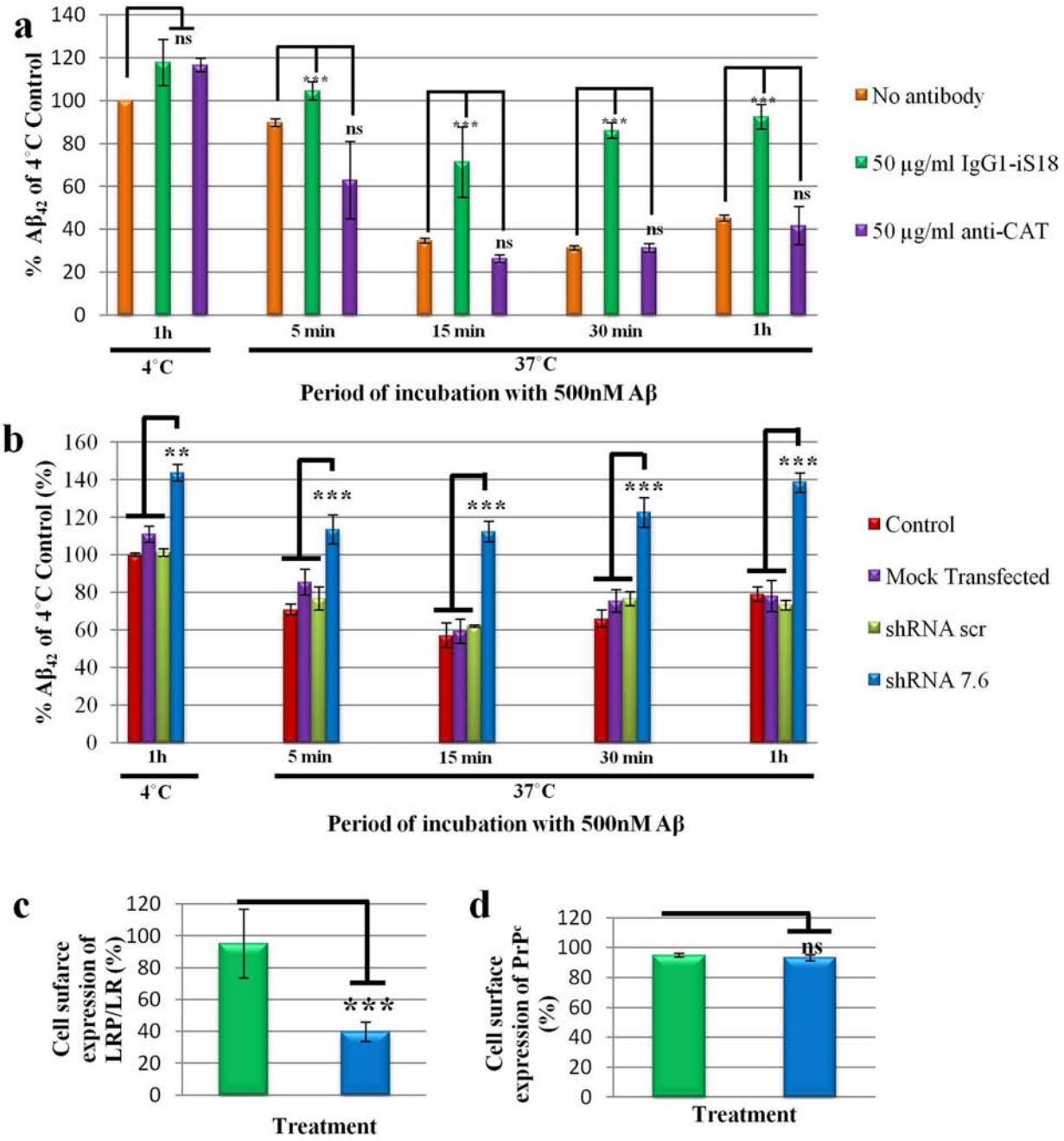


Figure 6 | Effects of antibody blockage and shRNA downregulation of LRP/LR on $A\beta_{42}$ internalization. HEK293 cells were subjected to antibody treatment: 50 µg/ml IgG1-iS18 (anti-LRP/LR specific antibody) or anti-CAT(negative control) (a) or transfected with shRNA 7.6 (against LRP/LR) (b). The degree of cell surface Aβ served as a measure of the degree of receptor-mediated internalization. The cell surface levels of Aβ in the no internalization control (1 h, 4°C) was set to 100% in both data sets. Successful downregulation of cell surface LRP/LR levels post shRNA downregulation was confirmed by flow cytometry (c). Cell surface PrP^c levels were unaffected by shRNA mediated downregulation of LRP/LR (d). Data shown are representative (mean ± s.e.m.) of three biological replicates, each performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; One way ANOVA.

This may be achieved through aberrant cell signalling. The cellular survival and proliferative role's of LRP/LR are reportedly realized through the Mitogen activated protein (MAP) kinase signal transduction pathway[36]. LRP/LR has been suggested to transduce cell survival and proliferative signals through the MAPK signalling pathway[36] upon binding to laminin-1[37]. LRP/LR regulates the expression of MAPK phosphatases (MKP1 and PAC1) and may thereby influence the activities of JNK, ERK1/2 and p38[36]. Marked MAPK deregulation ensues in AD[38]. The possibility that Aβ binding to LRP/LR may foil the receptor binding to physiologically relevant ligands such as laminin-1 and thereby perturb its normal physiological functions- which may contribute to deregulation, cannot be excluded. This would not be an unique occurrence, as epigallocatechin 3-*O*- gallate (EGCG) mediates its apoptotic activity through binding to LRP/LR[39]. Thus, despite its role in maintaining cell survival under physiological conditions, LRP/LR may gain pathological functions upon binding to certain ligands.

In addition, a multitude of research has demonstrated that Aβ binding to PrP^c leads to the induction of apoptosis through an upregulation of pro-apoptotic proteins such as Bax[40], enhanced Ca^{2+} release into the cytosol[41] and the activation of caspases- particularly caspase 8[41]. However, these pro-apoptotic signals may not be directly

transduced by PrP^c as it is not a transmembrane protein and therefore must be transduced through receptors to which PrP^c binds. We have recently demonstrated that LRP/LR does indeed contribute to PrP^c-$A\beta_{42}$ mediated cell death [Unpublished data, Pinto, M.G., Jovanovic, K., Da Costa Dias, B., Knackmuss, S., Reusch, U., Little, M. & Weiss, S.F.T. The 37kDa/67kDa LRP/LR plays a central role in Aβ-PrP^c mediated cytotoxicity in Alzheimer's disease, (2014).].

Therefore, these data suggest that LRP/LR may, either directly or indirectly, mediate $A\beta_{42}$ induced apoptosis.

It is important to note that this finding is physiologically relevant as the $A\beta_{42}$ concentrations within AD brains have been reported to be within the 200–4500 nM $A\beta_{42}$ range[42].

Furthermore, it must be noted that at nM concentrations, such as those employed in this study, $A\beta_{42}$ exists largely as low molecular weight oligomers[43]. Moreover, it has been demonstrated that even upon incubation in cell culture media, low nM concentrations of synthetic $A\beta_{42}$ (0–500 nM) do not aggregate to form higher order, less toxic fibrils[44]. Based on these findings, it may be safely proposed that the biological effects observed herein may largely be attributable to oligomeric $A\beta_{42}$, the neurotoxic species in AD.

However, as previously noted, the toxicity of Aβ is largely considered to be caused by its intracellular accumulation and aggregation- the levels of intracellular soluble Aβ are approximately 70 fold greater in AD brains compared to healthy age-matched controls[45]. Moreover it may be the ability of Aβ to incite misfolding and aggregation amongst cellular proteins which consequently leads to the deregulation of cellular processes. Therefore, it was imperative to examine whether the exogenously administered $A\beta_{42}$ was internalized into the cells. Internalization was evident from the earliest time point (5 min) and most pronounced after 15 min. However, evidence for Aβ intracellular trafficking and recycling to the cell surface (possibly along its exocytosis pathway) was apparent as the cell surface Aβ levels increased after 1 h (Fig. 5a).

LRP/LR was shown to be a central receptor in mediating the internalization of $A\beta_{42}$ as antibody blockade of the receptor significantly augmented cell surface-associated $A\beta_{42}$ and this thereby demonstrated that the amount of $A\beta_{42}$ internalized was lessened, especially at the time points 15 min, 30 min and 1 h (Fig. 6a). These results were further corroborated by shRNA mediated downregulation of LRP/LR (Fig. 6b) and thereby demonstrated that the effects observed were not due to a lack of antibody specificity. The increase in cell surface Aβ levels observed at 4°C in cells in which LRP/LR was downregulated compared to control samples (Fig.6b) may be attributable to reduced rates of PrP^c internalization, thereby allowing for enhanced Aβ binding.

This may be readily justified by the fact that PrP^c, a ligand to which LRP/LR binds with high affinity, has been firmly established as a protein required for Aβ internalization[13]. However as PrP^c lacks a transmembrane domain, its ability to mediate this internalization is dependent on its association with transmembrane receptors. LRP/LR serves a vital role in mediating PrP^c internalization into endosomes and cellular trafficking, results which have been confirmed in various neuronal cell types[46,47]. LRP/LR accounts for 25–50% of PrP^c internalization[15]. The fact that blockade of the receptor did not completely abrogate $A\beta_{42}$ internalization (Fig. 6a) may be due to the fact that only approximately 50% of $A\beta_{42}$ bound to the neuronal surface is internalized via PrP^c-dependent mechanisms[48]. It is noteworthy to add that heparan sulphate proteoglycans (HSPGs), to which LRP/LR similarly binds, have also been reported to mediate Aβ internalization[6].

Furthermore LRP/LR is not the sole PrP^c-binding protein implicated in its internalization, other such receptors include N-methyl-D-aspartate (NMDA) receptors[49], metabotropic glutamate receptor 5 (mGluR5)[50] and low-density lipoprotein receptor related protein (LRP1)[13], and these may therefore account for the internalization of $A\beta_{42}$ evident during antibody treatment.

It was due to this essential role of PrP^c, that possible downregulation of PrP^c as a consequence of LRP/LR downregulation needed to be negated (Fig. 6d). As cell surface PrP^c remained unaltered in cells exhibiting reduced LRP/LR (Fig. 6c and 6d), results which are comparable to those observed when RNAi methodologies were employed to downregulate LRP/LR *in vitro*[51], the central role of LRP/LR in Aβ internalization was validated.

Further studies are currently underway to examine whether LRP/LR mediates the internalization of Aβ directly, in the absence of PrP^c, or whether LRP/LR serves as the scaffold protein required for the internalization of the PrP^c-$A\beta_{42}$ complex.

In conclusion, it has been demonstrated that LRP/LR serves as a biologically relevant receptor of Aβ. This ubiquitously expressed receptor occupies a central role in mediating $A\beta_{42}$ internalization and may thereby contribute to the intracellular accumulation of the neurotoxic peptide and the consequent induction of apoptosis. Furthermore, specific antibodies and shRNAs directed against the 37 kDa/67 kDa laminin receptor may show promise as possible prophylactic and/or therapeutic tools for the treatment of Alzheimer's disease.

Methods

Cell Culture. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) supplemented with 10% (v/v) Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin (P/S) solution and maintained in a humidified incubator (5% CO_2, 37°C).

Transient Transfection. Upon reaching 70% confluency HEK293 cells were transfected, by calcium phosphate methodology, with pCIneo-moLRP::FLAG (Vana and Weiss, 2006) or pCDNA/3CAT (Invitrogen) plasmids and were lysed 72 h post-transfection.

Pull down Assay. Samples were composed of 200 µl of LRP::FLAG containing whole cell lysates and 200 µl of either conditioned tissue culture media (containing shed Aβ) or lysates expressing Chloramphenicol Acetyl Transferase (CAT). Conditioned tissue culture media was similarly co-incubated with non-transfected (NT) whole cell lysates (lacking LRP::FLAG) as well as subjected alone to immunoprecipitation to account for non-specific binding to the anti-FLAG® M2 beads. FLAG® Immunopercipitation Kit (Sigma-Aldrich) assays were performed as per manufacturer's instructions. Samples were subsequently subjected to both electrophoretic and western blot analysis as well as Amyloid beta Enzyme-linked Immunosorbent Assays (ELISA). Three independent experiments were performed, each in triplicate.

Immunoblotting. FLAG® Immunoprecipitation assay eluate samples were detected using murine anti-FLAG antibody (1 : 4000) (Sigma-Aldrich) or anti-Chloramphenicol Acetyl Transferase (rabbit IgG fraction) (1 : 6000) (Sigma-Aldrich). The secondary antibodies employed were goat anti-mouse HRP (1 : 10 000) (Sigma-Aldrich) and anti-rabbit-HRP (1 : 10000) (Sigma-Aldrich), respectively. The loading control, β-actin, was detected employing the rabbit anti-β-actin-HRP antibody (1 : 10000) (Sigma-Aldrich). Experiments were performed in triplicate, three times.

Amyloid beta Enzyme-linked Immunosorbent Assay (ELISA). Post FLAG® immunoprecipitation, the Aβ concentration in each fraction was assessed by Human Amyloid β (1-x) Assay kit (Immuno-Biological Laboratories Co.,Ltd) – a solid phase ELISA, performed as per manufacturer instructions. Three independent experiments were performed, each in triplicate.

Flow cytometric analysis of Försters Resonance Energy Transfer (FRET). To assess FRET between LRP/LR and CAT, HEK cells were transfected to express recombinant CAT as described above. For the rest of the samples non-transfected HEK293 cells were employed. Cells were incubated in serum-free media for 3–4 h prior to assessment. Cells were detached (5 Mm EDTA-PBS), harvested in serum free media, centrifuged at 1200 rpm (4°C, 10 min), washed thrice in ice-cold D-PBS and fixed with ice-cold 2% PFA (20 min, 4°C). These non-permeabilized cells were again washed (1x PBS) and blocked in 0.5%PBS-BSA for 10 min. The primary antibody solutions (20 µg/ml) (diluted in 0.05% PBS-BSA) employed were: human IgG1-iS18 (Affimed Therapeutics); murine anti-PrP^c (Sigma-Aldrich); rabbit anti-CAT (Sigma-Aldrich) and rabbit anti-β-amyloid (22–35) (Sigma-Aldrich). The secondary antibody solutions (20 µg/ml) (diluted in 0.05% PBS-BSA) employed were: goat-anti-human-PE (Abcam) IgG1-iS18, goat-anti-mouse-APC (Abcam) and goat-anti-rabbit-APC (Abcam). The following samples were prepared: 1) unstained cells, 2) cells stained with goat-anti-human PE only; 3) cells labelled with goat-anti-mouse-APC only; 4) cells labelled with goat-anti-rabbit-APC only; 5) cells labelled with IgG1-iS18-PE (LRP/LR detection); 6) cells labelled with anti-PrP^c-APC (PrP^c detection), 7) cells labelled with anti-CAT-APC (CAT detection); cells labelled with anti-$A\beta_{42}$-APC ($A\beta_{42}$ detection), 8) cells labelled with IgG1-iS18-PE & anti-PrP^c-

APC; 9) cells labelled with IgG1-iS18-PE & anti-CAT-APC and 10) cells labelled with IgG1-iS18-PE & anti-$A\beta_{42}$-APC. Cells were incubated in primary antibody solutions for 2 h, washed twice in 1xPBS and once in 0.5% PBS-BSA and incubated in secondary antibody solutions for 2 h. The cells were washed thrice prior to analysis. Three biological replicates, each performed in triplicate, were conducted. In each sample, 10 000 cells were analysed.

Antibody Treatment. Varying concentrations (200 nM & 500 nM) synthetic Amyloid beta peptide 42 ($A\beta_{42}$) (Sigma-Aldrich) was administered to cells in the presence or absence of 50 μg/ml IgG1-iS18 (anti-LRP/LR specific antibody) (Affimed Therapeutics) or 50 μg/ml anti-Chloramphenicol Acetyl Transferase (CAT) (rabbit IgG fraction) (Sigma-Aldrich).

Annexin-V-7Aminoactinomycin D Assay. HEK293 cells were subjected to $A\beta_{42}$ and antibody treatment as detailed above. The degree of cell death attributable to apoptosis was assessed at 24 h, 48 h and 72 h post treatment using Annexin-V-FITC/7-AAD kit (Beckman Coulter) as per the manufacturer's instructions. The effects of antibody treatment on cell death was assessed after 72 h. Three biological replicates, each performed in triplicate, were conducted. In each sample, 10 000 cells were analysed.

Nuclear staining- Assessing Nuclear Morphology. HEK293 cells were seeded onto coverslips and were subjected to 500 nM $A\beta_{42}$ or 8 mM PCA (apoptosis inducing agent served as the positive control) treatment for 72 h. Cells were subsequently fixed (4% PFA, 15 min, 4°C), rinsed thrice (1xPBS) and blocked (0.5%PBS-BSA,10 min). Hoechst 33342 (1 : 100 dilution of 2 mg/ml stock solution) (Sigma-Aldrich) was administered for 5 min (RT) to cells which were subsequently rinsed and mounted onto microscope slides using 50 μl of Fluoromount (Sigma-Aldrich). Images were acquired using Zeiss LSM780 confocal microscope and Zen 2010 imaging software.

shRNA mediated downregulation of LRP. The TransIT®–LT1 Transfection reagent (Mirus) was employed, as per manufacturer's instructions, to transfect HEK293 cells with shRNA 1.1, shRNA 7.6 and shRNA scr (scrambled shRNA, negative control). The production of the shRNA targeting LRP/LR mRNA has been described previously[26,29].

Flow cytometric analysis of cell surface levels of LRP and PrP^c. Post LRP downregulation by shRNA methodology (72 h post transfection) cell surface LRP and PrP^c levels were assessed by flow cytometry. Cells were fixed in 4% paraformaldehyde (15 min, 4°C), washed thrice (1xPBS) and blocked (0.5%PBS-BSA, 10 min). Samples were halved, one half was subjected to both primary and secondary antibody treatments whilst only secondary antibody was administered to the other sample. Cells were incubated with primary antibodies (20 μg/ml), namely human IgG1-iS18 (anti-LRP/LR specific antibody) (Affimed therapeutics) and murine anti-PrP^c 8H4 (Sigma-Aldrich) for 3 h at RT, washed thrice and were treated with 10 μg/ml goat anti-human PE (Abcam) and goat anti-mouse APC (Abcam) secondary antibodies for 2 h. The cells washed thrice prior to analysis. Three biological replicates, each performed in triplicate, were conducted. In each sample, 10 000 cells were analysed.

Internalization Assay. Flow cytometric analysis was employed to assess the degree of $A\beta_{42}$ on the cell surface during different intervals during internalization. Cells (50% confluency) were either subjected to antibody treatments (50 μg/ml of IgG1-iS18 or anti-CAT) for 48 h or transfected with shRNAs and assessed 72 h post transfection. Cellular confluency of 70% was deemed optimal for internalization analysis. Cells were incubated in serum-free media for 3–4 h prior to assessment and subsequently subjected to 500 nM $A\beta_{42}$ treatment for 5 min, 15 min, 30 min and 1 h at 37°C in a 5% CO_2 humidified environment to allow for internalization. Cells were concomitantly incubated on ice at 4°C, for 30 min, after which 500 nM $A\beta_{42}$ was administered to cells and incubated at 4°C for 1 h. Incubation at 4°C arrests cell receptor mediated internalization (Li et al., 2008). Post treatment, cells were washed thrice(ice-cold 1xPBS), detached (5 Mm EDTA-PBS), harvested (ice-cold serum free media,1200 rpm,4°C, 10 min), washed again and fixed (ice-cold 4% PFA,20 min, 4°C). These non-permeabilized cells were again washed (1x PBS) and blocked (0.5%PBS-BSA, 10 min). Samples were halved, one half subjected to both antibody treatments whilst only secondary antibody was administered to the other sample. 100 μl of primary antibody solution containing 1 : 100 anti-β-amyloid (22–35) (rabbit) (Sigma-Aldrich) was administered to the cells and incubated for 2 h. Cells were washed and were treated with 10 μg/ml goat anti-rabbit APC (Abcam) secondary antibody for 2 h. Cells washed thrice prior to analysis. Three biological replicates, each performed in triplicate, were conducted. In each sample, 10 000 cells were analysed.

Immunofluorescence Microscopy. Cells (70% confluent) were transfected with pECFP-mem (Clonetech), a plasmid encoding N-terminal 20 amino acid fragment of neuromodulin which is fused to cyan-fluorescent protein (CFP)) thereby allowing for plasma membrane visualization. Post transfection, 48 h, cells were incubated in serum-free media for 3–4 h and subsequently subjected to 500 nM $A\beta_{42}$ for 5 min, 15 min and 30 min (37°C, 5%CO_2 humidified) to allow for internalization. Post treatment, the cells were placed on ice, washed thrice (ice-cold 1xPBS), fixed (4% PFA, 20 min, 4°C), rinsed thrice (ice-cold 1xPBS) and blocked (ice-cold 0.5%PBS-BSA containing 0.25% Triton X-100,10 min). Coverslips were again washed and incubated with the primary antibody solution - 1 : 100 anti-β-amyloid (22–35) (rabbit) (Sigma-Aldrich). Post overnight incubation (4°C) coverslips were again washed thrice in 0.5% PBS-BSA and incubated with the secondary antibody- 1 : 300 goat anti-rabbit APC (Abcam) for 1 h. Coverslips were washed twice in 0.5% PBS-BSA and once in 1xPBS and mounted onto clean microscope slides using 50 μl Fluoromount (Sigma-Aldrich). Images were acquired using Zeiss LSM780 confocal microscope and Zen 2010 imaging software.

Statistical Evaluation: Student's *t*-tests and ANOVAs were used to analyse the data and obtain p values. All statistical evaluations were performed using GraphPad Prism (version 5.03) software.

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Author contributions

Conceived and designed the experiments: B.D.C.D., S.F.T.W. Design of shRNA: M.S.W. Production of shRNA: D.G., K.M. Performed experiments: B.D.C.D. Antibody (IgG1-iS18) production: U.R., S.K. and M.L. Analysed data: B.D.C.D. Wrote the manuscript: B.D.C.D. Edited the manuscript: B.D.C.D., K.J. and S.F.T.W.

Additional information

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

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4.3 The 37kDa/67kDa LRP/LR plays a central role in Aβ-PrP^c mediated cytotoxicity in Alzheimer's disease

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Brief Overview of Article:

This original research article demonstrated that the 37kDa/67kDa LRP/LR is indeed implicated in mediating PrP^c-Aβ toxicity. Our previous studies employed antibody blockade to demonstrated that LRP/LR is involved in $A\beta_{42}$-induced neuronal cytotoxicity. However, antibody steric effects may have additionally obstructed $A\beta_{42}$-PrP^c associations and thus the true significance of the receptor within this context necessitated investigation. Here we showed that overexpression of PrP^c on the cell surface (as confirmed by flow cytometry) augmented the cytotoxic effects of $A\beta_{42}$ treatment – a finding that in consistent with results obtained by others. In addition it was revealed that antibody blockade of the receptor, employing an anti-LRP/LR specific antibody IgG1-iS18, was able to significantly enhance the cell viability of cells overexpressing PrP^c when subjected to toxic $A\beta_{42}$ treatments. The degree of cell survival would not have been as greatly augmented by antibody treatment if the toxicity of the PrP^c-$A\beta_{42}$ association was independent of LRP/LR. The cell rescuing effects of the antibody in PrP^c-overexpressing and non-transfected cells were not significantly different, thereby suggesting that the toxic effects of the $A\beta_{42}$-PrP^c complex are primarily mediated through the 37kDa/67kDa LRP/LR. These results illustrated that LRP/LR is indeed implicated in $A\beta_{42}$-PrP^c mediated cytotoxicity and thereby validated the central role of LRP/LR in $A\beta_{42}$-induced pathogenesis. Thus, the promise that anti-LRP/LR specific antibodies may hold as potential therapeutic tools for Alzheimer's Disease was again validated.

Contribution: I assisted in the planning of the experiments and the analysis of data as well as contributed significantly to the writing and editing of this original research article.

Title Page

Title

The 37kDa/67kDa LRP/LR plays a central role in Aβ-PrP^c mediated cytotoxicity in Alzheimer's disease

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Running Title

LRP/LR linked to PrP^c-Aβ induced cell death

Abstract

The neuronal perturbations in Alzheimer's disease are attributed to the formation of extracellular amyloid-beta (Aβ), neuritic plaques, composed predominantly of the neurotoxic $A\beta_{42}$ isoform. Although the plaques have demonstrated a role in synaptic dysfunction, neuronal cytotoxicity has been attributed to soluble $A\beta_{42}$ oligomers. The 37kDa/67kDa laminin receptor has been implicated in $A\beta_{42}$ shedding and $A\beta_{42}$ induced neuronal cytotoxicity. As the cellular prion protein binds to both LRP/LR and $A\beta_{42}$, the mechanism underlying this cytotoxicity may be indirectly due to the PrP^c-$A\beta_{42}$ interaction with LRP/LR. The effects of this interaction was investigated by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assays. PrP^c overexpression significantly enhanced $A\beta_{42}$ cytotoxicity *in* vitro. Anti-LRP/LR specific antibody IgG1-iS18 significantly enhanced cell viability in both pSFV-hu PrP^c 1-253 transfected and non-transfected cells treated with $A\beta_{42}$. These results suggest that LRP/LR is implicated in $A\beta_{42}$-PrP^c mediated cytotoxicity and that anti-LRP/LR specific antibodies may serve as a potential therapeutic tool for Alzheimer's disease.

Keywords

Alzheimer's Disease (AD), Amyloid-β (Aβ), Cellular prion protein (PrP^c), 37kDa/67kDa laminin receptor (LRP/LR)

Introduction

Alzheimer's disease (AD) is a progressive and devastating neurodegenerative disorder characterised symptomatically by behavioural and cognitive dysfunction [1]. As the most prevalent neurodegenerative disorder affecting the aging population, with over 38 million people being afflicted worldwide and economic costs estimated to run into millions of dollars, scientific interest remains prioritised on the development of novel therapeutic strategies [2].

The neuronal perturbations in AD are attributed to the formation of extracellular amyloid-beta (Aβ) neuritic plaques, composed predominantly of the neurotoxic $A\beta_{42}$ isoform and intracellular neurofibrillary tangles, which are aggregations of hyperphosphorylated tau protein, a microtubule associated protein[3]. $A\beta_{(40-42)}$ generation occurs via the proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase, also termed BACE1 (β-site APP cleaving enzyme-1) and the presenilin-containing γ-secretase complex thereby leading to release of the sAPPβ fragment and Aβ peptide[4]. Although this amyloidogenic pathway has a normal physiological function, it has been suggested that it is the misappropriate favouring of this pathway or the decline in $A\beta_{42}$ clearance or degradation that leads to the accumulation of $A\beta_{42}$ peptides and thus the development of AD [5].

Although the Aβ plaques and neurofibrillary tangles have been reported to play a role in synaptic dysfunction, the neuronal loss characteristic of AD has been largely attributed to the formation of soluble $A\beta_{42}$ oligomers [6]. A common thread seen in Aβ induced cell cytotoxicity is the requirement for an interaction between these toxic oligomers and cellular components[7]. Owing to their hydrophobic nature these soluble $A\beta_{42}$ oligomers are incorporated into the plasma membrane resulting in membrane distortion and ion channel formation[8] which is of particular concern with regards to the influx of Ca^{2+} ions which may

induce cytotoxicity [9]. Several factors associated with the progression of AD are believed to be attributed to the association of $A\beta_{42}$ oligomers with cell surface receptors which may lead to an increase in protein-receptor internalisation and the accumulation of intracellular $A\beta_{42}$ oligomers [10],[11],[7]. It is in turn these intracellular $A\beta_{42}$ oligomers and aggregates that lead to cellular dysfunction, aberrant cell signalling and the cellular damage which precedes cell death [7]. One such receptor to which $A\beta_{42}$ binds resulting in adverse effects is the cellular prion protein[12].

The cellular prion protein (PrP^c) is a glycosylphosphatidylinisotol (GPI) anchored cell surface receptor expressed by most tissues and in particular, at high concentrations in neuronal cells in which its physiological functions include protection against oxidative stress, synaptic transmission and copper homeostasis [13]. Little is clearly defined regarding the role of PrP^c in AD as a two-fold functionality has been demonstrated. In 2007, Parkin *et al*, demonstrated that a negative feedback loop exists between PrP^c and β-secretase [13]. PrP^c binding was shown to inhibit β-secretase cleavage of APP thus reducing Aβ production and shedding[13]. However, $A\beta_{42}$ oligomers bind with high affinity to PrP^c, the consequence of which may be the internalization of the $A\beta_{42}$-PrP^c complex. This may in turn results in lower cell surface PrP^c levels, thereby lessening the degree of β-secretase inhibition resulting in enhanced Aβ shedding. A receptor of noted physiological importance that binds to PrP^c is the 37kDa/67kDa laminin receptor (LRP/LR) [14]. The receptor is a multifunctional protein involved in cell survival, proliferation, cell adhesion, invasion and the internalization and intracellular recycling of PrP^c[15]. LRP/LR is a high affinity receptor for laminin-1 and owing to this, LRP/LR has been shown to play a central role in metastatic cancer. Anti-LRP/LR specific antibodies reduced cell adhesion and invasion as well as the induction of angiogenesis in numerous cancer cell types[16], [17], [18], [19]. An interaction between laminin-1 and $A\beta_{42}$ oligomers has been demonstrated in previous studies. As LRP/LR and

$A\beta_{42}$ share aforementioned mutual binding partners, is has been proposed that LRP/LR may be implicated in the pathogenesis of AD[20], [21] .

Recent studies have implicated LRP/LR as a key player in $A\beta_{42}$ shedding [20] and $A\beta_{42}$ induced cytotoxicity [21] but owing to the binding of PrP^c to both LRP/LR and Aβ, the mechanism by which Aβ induces neuronal cytotoxicity may be indirectly due to the PrP^c-Aβ interaction with LRP/LR. The aim of this study was to examine the effect of PrP^c in $A\beta_{42}$ induced cell cytotoxicity and to determine the role of the PrP^c-LRP/LR interaction in Aβ mediated cytotoxicity. The determination of the role played by PrP^c and more importantly the PrP^c-LRP/LR interaction in $A\beta_{42}$ induced cytotoxicity may have potentially important implications for understanding the pathogenic mechanism of AD and may thus contribute to the development of effective therapeutic strategies.

Methods and Materials

Tissue culture. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin (P/S) solution. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Protein-tagged Confocal microscopy. HEK293 cells were seeded onto microscopic coverslips as to attain 50% confluency within 24h and incubated in 5% CO_2 humidified atmosphere at 37°C. Post incubation cells were transfected with the GFP-LRP and Ds-Red-PrP^c plasmids using the TransIT©-LT1 Transfection Reagent [Mirus] and incubated in a 5% CO_2 humidified atmosphere at 37°C for 24h. The cells were fixed (4% Paraformaldehyde, 10 min, 4°C), rinsed thrice with 1xPBS and blocked with 0.5%PBS-BSA (5-10min,RT). Coverslips were then mounted using a 50µl volume of Fluoromount [Sigma Aldrich]. Slides were viewed using the Zeiss LSM 780 confocal microscope and capture using the AxioCam MRm camera. Images were subsequently analysed using Zen 2010 imaging software. The

Ds-Red and GFP-LRP/LR control and Ds-Red-PrP^c and GFP control were performed using the above protocol.

Immunofluorescence Confocal microscopy. HEK293 cells were seeded onto microscopic coverslips to a confluency of 50-70% was obtained. The cells were fixed (4% Paraformaldehyde , 10 min, 4°C), rinsed thrice with 1xPBS and blocked with 0.5%PBS-BSA (5-10mins, RT). Coverslips were additionally washed with 1xPBS and placed such that the cell free side of the coverslip lay flush with the slide. 100µl volume of primary antibody solution (diluted in 0.5%PBS-BSA) containing a 1:150 anti-PrP^c 8H4 (mouse) [Sigma Aldrich] or 1:100 anti-β-amyloid (22-35) (rabbit) [Sigma Aldrich] 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 100µl volume of secondary antibody solution containing 1:300 goat anti-mouse APC [Abcam] or 1:300 goat anti-rabbit FITC [Cell Lab] were administered to cells and incubated for 1h in the dark. Coverslips were washed thrice with 0.5%PBS-BSA and once in PBS. Coverslips were then mounted using a 50µl volume of Fluoromount [Sigma Aldrich]. Slides were viewed using the Zeiss LSM 780 confocal microscope and capture using the AxioCam MRm camera. Images were subsequently analysed using Zen 2010 imaging software. Secondary antibody controls of goat anti-mouse APC and goat anti-rabbit FITC were performed using the above protocol.

HEK293 Tranfection methodology. HEK293 cells were seeded to attain 50% confluency within 24h and incubated in 5% CO_2 humidified atmosphere at 37°C. Post incubation cells were transfected with the pSFV1-huPrP1-253 plasmid using the TransIT©-LT1 Transfection Reagent [Mirus] and incubated in a 5% CO_2 humidified atmosphere at 37°C for 48h.

Flow cytometry. pSFV1-huPrP1-253 plasmid [14] transfected and non-transfected cells were subjected to flow cytometric analysis following treatment with anti-LRP/LR antibody IgG1-

iS18 and anti-PrP^c antibody 8H4. Cells were fixed (4% Paraformaldehyde,10 min, 4°C) centrifuged at 1500 g for 10 min and resuspended in 1ml 1xPBS. Half of each cell sample was treated with 30µg/ml of either anti-LRP/LR antibody IgG1-iS18 or anti-PrP^c antibody 8H4 [Sigma Aldrich], while the other half of the cell sample was incubated in 1xPBS. Cells were then incubated for 24h at 4°C. Post incubation the cells were washed three times in 1xPBS at 1700g at RT. All cells were treated with 20µg/ml of the corresponding goat anti-human FITC coupled secondary antibody [Cell Lab] or the corresponding goat anti-mouse APC coupled secondary antibody [Abcam] and incubated for 1h at RT. The cells were washed a further three times as above. The samples were analysed using the BD Accuri C6 whereby 10000 events were recorded.

3-(4,5-dimethylthiazol-2-Yl-2,)5-diphenyltetrazolium bromide (MTT) cell viability assay. HEK293 cells were seeded in a 96 well plate as to attain 50% confluency within 24h and incubated in 5% CO_2 humidified atmosphere at 37°C. Post incubation cells were transfected with or without the pSFV1-huPrP1-253 plasmid using the TransIT©-LT1 Transfection Reagent [Mirus] and incubated at 5% CO_2 humidified atmosphere at 37°C for 24h. Post incubation, synthetic Amyloid beta ($A\beta_{42}$) peptide [Sigma Aldrich] was administered to the cells in varying concentrations (100nM, 200nM and 500nM) to determine the effect thereof on cell viability. In addition untreated controls (cells incubated in DMEM) as well as positive controls (cells incubated with 8mM protocatechuic acid (PCA)- an apoptosis inducing agent) were included. Furthermore transfected and non-transfected cells were additionally co-incubated with $A\beta_{42}$ (at 200nM and 500nM) and 50µg/ml IgG1-iS18 antibody or 50µg/ml anti- chloramphenicol acetyltransferase (CAT) antibody [Sigma Aldrich]. Treated cells were incubated for 48 hours (37°C, 5% CO_2), following which 20µl of 1mg/ml MTT was added to each well and the cells subsequently incubated (37°C, 5% CO_2) for 2h. After incubation, culture media was aspirated and 180µl of DMSO was added to each

well to lyse the cells and dissolve the formazan crystals formed within the cells. The absorbance was recorded at 570nm using an ELISA microtiter plate reader and the percentage of cell survival, relative to the untreated controls, calculated. Each experiment was performed in triplicate, three times.

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.

Results

LRP/LR and $A\beta_{42}$ co-localise with PrP^c on the cell surface. Co-localisation is regularly employed to provide evidence of potential protein-protein interactions in a cell. A close proximity between LRP/LR and $A\beta_{42}$ with PrP^c would thereby indicate a possible association between these proteins. Co-localization of fluorescent protein tagged LRP/LR and PrP^c was observed on the cell surface of non-permeabilized HEK293 cells (Fig. 1A-C). The 2D cytofluorogram represents both the green and red fluorescence and the resultant yellow diagonal (Fig. 1D) reveals that the fluorescence from both LRP/LR and PrP^c displays spatial overlap on the cell surface as seen in the yellow merged image (Fig 1C). This data is supported by the highly positive Pearson's correlation coefficient of 0.872 (Table 1) LRP/LR does not non-specifically interact with the fluorescent protein Ds-red nor does PrP^c non-specifically interact with GFP, this is evident by the lack of the yellow merge and diagonal 2D-cytofluorograms (Fig. 1G,H & Fig. 1 K,L). PrP^c does not bind non-specifically to other laminin binding receptors as is evidenced by a lack of binding between the Very Late Antigen 6 (VLA6), a laminin binding integrin [14]. Co-localisation of immunolabelled PrP^c and $A\beta_{42}$ was observed on the cell surface of non-permeabilized HEK293 cells (Fig. 1M-O). The 2D cytofluorogram represent both the magenta and blue fluorescence and the resultant diagonal (Fig. 1P) reveals that the fluorescence from PrP^c and $A\beta_{42}$ overlap on the cell

surface. Controls lacking primary antibodies were employed to account for possible secondary antibody non-specificity (Fig. 1 Q-T). Again, this data is supported by the highly positive Pearson's correlation coefficient of 0.784 (Table 2) which verifies that PrP^c and $A\beta_{42}$ co-localise on the surface of HEK293 cells.

Overexpression of PrP^c shows no effect on cell viability. PrP^c overexpression, achieved through the transfection of HEK293 cells with the pSFV1-hu PrP^c 1-253 plasmid, was confirmed using Flow cytometry. When compared to the mock transfected control, transfected cells displayed a greater fluorescent intensity (Fig. 2), thereby demonstrating that these cells displayed increased levels of PrPc on their surface (cells were non-permeabilised to allow solely for cell surface protein detection). The MTT cell viability assay was employed to assess the effects of PrP^c overexpression on the viability of HEK293 cells. The lipofection transfection methodology significantly reduced cell viability when compared to the untransfected control cells (Fig. 3A). However PrP^c overexpression did not affect cell viability when compared to the mock transfected control.

Overexpression of PrP^c significantly enhances the cytotoxicity effect of exogenous $A\beta_{42}$ treatment. The MTT viability assay was employed to assess the cytotoxic effect of synthetic $A\beta_{42}$ at various concentrations on HEK293cells (Fig. 3B). Exogenous treatment of 100nM, 200nM and 500nM of synthetic $A\beta_{42}$ significantly reduced cell viability in HEK293 cells, which confirms previous data[24]. pSFV1-hu PrP^c 1-253 transfected and mock transfected cells were subsequently treated with 100nM, 200nM and 500nM of exogenous $A\beta_{42}$. PrP^c overexpression significantly enhanced cell death induced by the $A\beta_{42}$ treatments when compared to similarly treated mock transfected cells (Fig 3C). 8mM Protocatechuic acid (PCA) solution, an apoptosis inducing agent, was employed as a positive control.

Anti-LRP/LR specific antibody IgG1-iS18 rescues cells from $A\beta_{42}$ - PrP^c mediated cytotoxicity. The MTT viability assay was employed to determine the effect of the anti-LRP/LR specific antibody IgG1-iS18 on cell viability of pSFV1-hu PrP^c 1-253 transfected and mock transfected HEK293 cells when treated with 200nM and 500nM of exogenous $A\beta_{42}$. Treatment with IgG1-iS18 at 200nM $A\beta_{42}$ in both the pSFV1-hu PrP^c 1-253 transfected and mock transfected HEK293 cells showed a significant increase in cell viability (Fig. 4A). Similar results were obtained for the 500nM $A\beta_{42}$ treatment (Fig. 4B). Antibody treatment alone, in the absence of $A\beta_{42}$ administration, did not have an effect on cell viability (Fig. 4C). 8mM Protocatechuic acid (PCA) solution, an apoptosis inducing agent, was employed as a positive control

Discussion

The co-localisation observed between LRP/LR and Aβ with PrP^c on the cell surface of HEK293 cells, as assessed by confocal microscopy, indicates that a spatial overlap occurs between the proteins (Fig 1). Although the close proximity of the proteins on the cell surface is not explicitly indicative of an interaction, however, it does imply an association between LRP/LR and Aβ with PrP^c. This data confirms interactions between LRP/LR and PrP^c as well as between PrP^c and Aβ which has been previously published [15], [21], [22]. As LRP/LR and $A\beta_{42}$ share the aforementioned mutual binding partner, PrP^c, it has been proposed that LRP/LR may be implicated in the pathogenesis of AD [20, 21]. Recent studies have implicated LRP/LR as a key player in $A\beta_{42}$ shedding [20] and $A\beta_{42}$ induced neuronal cytotoxicity [21] yet little is known regarding the exact mechanism played by PrP^c and the PrP^c-LRP/LR interaction in Alzheimer's disease.

The precise role of the prion protein in AD has recently been a topic of much debate and controversy as a two-fold functionality has been ascribed to PrP^c namely: a neuroprotective

role, demonstrated by Parkin *et al,* [13] and a cytotoxic enhancement effect demonstrated by Rushworth *et al. [22]* owing to the $A\beta_{42}$- PrP^c interaction. However, prior to assessing the role of PrP^c, it was necessary to confirm that the $A\beta_{42}$ concentrations employed were cytotoxic. MTT cell viability assays confirmed the cytotoxic effect of exogenous $A\beta_{42}$ on HEK293 cells at varying concentrations (Fig. 3B) of which the results suggest a direct correlation between $A\beta_{42}$ concentration and cell cytotoxicity once a threshold concentration has been achieved. Although the threshold of synthetic $A\beta_{42}$ has been previously documented to be a 100-200nM concentration [23], disruption of long term potentiation (LTP)[23] and significant decrease in cell viability are largely observed at 500nM Aβ concentrations [24]. The results presented here mirror those previously described, as 500nM $A\beta_{42}$ exogenous treatment resulted in greatest cell death. It is of vital importance to note that the 500nM concentration employed is below the critical concentration required for fibril formation *in vitro* [25] and it may thereby be suggested that the Aβ formed oligomers and these in turn exerted the cytotoxic effects observed. The fact that the cytotoxic effect was proportional to $A\beta_{42}$ concentration validates the notion that it is the gradual accumulation of soluble Aβ (and consequent increase in the toxic peptide's concentration), which may be initiated by the favouring of amyloidogenic process or deficits in Aβ clearance and degradation, that leads to the neuronal loss observed in AD.

Recent investigations have demonstrated that PrP^c serves as a high affinity binding partner for $A\beta_{42}$ oligomers, however, the implications of this association, be it pathological or neuroprotective, is a topic of much debate. In this study, MTT cell viability assays exploited the overexpression of PrP^c as a tool to determine the role of the $A\beta_{42}$-PrP^c interaction in $A\beta_{42}$ induced cell cytotoxicity. Cells overexpressing PrP^c displayed significantly lower cell viability upon $A\beta_{42}$ treatment when compared to similarly treated mock transfected controls (Fig. 3C). This suggests that the $A\beta_{42}$ induced cell cytotoxicity observed here is mediated

through the $A\beta_{42}$-PrP^c interaction. These results correlate with those of previous studies [22]. Cell viability was not compared to untreated controls owing to the detectable toxicity of the transfection methodology (Fig. 3A).

Rushworth *et al.* have recently demonstrated the requirement of $A\beta_{42}$-PrP^c mediated cytotoxicity, for cellular components associated with the lipid raft region of the cell membrane [22]. It is largely believed that an additional protein mediator is required to mediate the cytotoxic effect of this PrP^c-Aβ complex. Such cell surface receptors include: metabotropic glutamate receptor 5 (mGluR5)[26] and N-methyl-D-aspartate (NMDA) receptors [27] which mediate the aberrant effects on cellular signalling pathways and low density lipoprotein receptor-related protein-1 (LRP1), which induces internalization of this complex and thereby facilitates intracellular Aβ accumulation, aggregation and the resultant cellular dysfunction[22] characteristic of AD. Interestingly, the role of a key PrP^c receptor, 37kDa/67kDa LRP/LR, had until recently not been researched with regards to Aβ cytotoxicity.

Our novel findings revealed that blockade of LRP/LR rescued cells from Aβ cell death [21] .However, antibody blockade of LRP/LR may have secondarily blocked PrP^c binding to Aβ through steric effects. Therefore, it was imperative that we ascertained whether LRP/LR is indeed a key factor in Aβ pathogenesis. To accomplish, this cells overexpressing PrP^c were co-treated with anti-LRP/LR antibody IgG1-iS18 (or anti-CAT, as a negative control) in the presence of exogenously administered $A\beta_{42}$. Our results demonstrate that through the use of the anti-LRP/LR specific antibody IgG1-iS18, cells were rescued from $A\beta_{42}$-PrP^c mediated cytotoxicity (Fig. 4A & Fig. 4B) as a significant increase in cell viability was observed in both the mock transfected and pSFV-hu PrP^c 1-253 transfected samples at all three concentrations of $A\beta_{42}$ (Fig 4A & Fig 4B). The possibility that the antibodies were able to affect cellular viability in the absence of $A\beta_{42}$ treatment, in both mock and transfected cells,

was refuted as is demonstrated in (Fig. 4C). If the antibody blockade of LRP/LR indirectly enhanced cellular viability through sterical hinderance of PrP^c-Aβ and LRP/LR itself was irrelevant, antibody treatment in cells overexpressing PrP^c would not have had the significant enhanced cell survival effects demonstrated here as the PrP^c-Aβ complex would have been able to induce cell death independently of LRP/LR. The fact that the anti-LRP/LR specific antibody significantly enhanced cellular viability despite PrP^c overexpression reveals that the 37kDa/67kDa LRP/LR indeed does play a central role in $A\beta_{42}$, and more importantly PrP^c-$A\beta_{42}$ mediated, pathogenesis.

It may be proposed that the 37kDa/67kDa LRP/LR may indeed serve as an internalization mediator of the PrP^c-$A\beta_{42}$ complex, as the receptor is involved in physiological PrP^c recycling which includes PrP^c internalization through clathrin-coated pits – the mechanism largely considered the preferred endocytic pathway for PrP^c- Aβ complexes. It may therefore be suggested that the PrP^c-LRP/LR interaction may play a central role in the $A\beta_{42}$-PrP^c mediated cytotoxicity observed in AD.

Further research employing siRNAs targeting LRP/LR mRNA for LRP/LR down regulation and the design of a PrP^c-LRP/LR interaction inhibitors is currently in progress and will further elucidate the role of the PrP^c-LRP/LR interaction in $A\beta_{42}$- PrP^c mediated cytotoxicity. LRP/LR is a useful target for AD treatment, as antibody blockade of this receptor has been shown to have no adverse effects in animal trials [28], [29].

These findings are of considerable value to the AD research community as they provide evidence that LRP/LR is indeed a central player in Aβ mediated pathogenesis, thereby allowing researchers to move a step closer towards understanding the complex networks and mechanisms underlying neuronal loss. Moreover it may be suggested that the 37kDa/67kDa

LRP/LR may serve as a safe alternative target for the development of effective AD therapeutics .

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Figure legends

Figure 1. Co-localisation of LRP/LR and Aβ$_{42}$ with PrP^{c} on the cell surface of HEK293 cells. Human embryonic kidney (HEK293) cells were seeded onto coverslips and allowed to proliferate until a confluency of 70% was reached. Cells were subsequently transfected with plasmids encoding the following fluorescent proteins or fluorescent protein coupled proteins, namely **(A)** GFP-LRP/LR; **(B)** Ds-Red PrP^{c}; **(E)** GFP-LRP; **(F)** Ds-Red; **(I)** GFP; **(J)** Ds-Red PrP^{c}; and post 48h were fixed (4% paraformaldehyde, 10min, 4°C) and mounted onto coverslips. The merged images between (**A&B**), (**E&F**), (**I&J**) are shown in (**C, G, K**) respectively and the corresponding 2D-cytofluorograms have been included to confirm the degree of co-localisation **(D, H, L**). Similarly, to assess the cellular distribution of PrP^{c} in relation to Aβ$_{42}$, non-permeabilised HEK293 cells were fixed and indirectly labelled with: **(M)** anti-Aβ$_{42}$ & anti-Rabbit-FITC; **(N)** anti-PrP^{c} & anti-mouse-APC; **(Q)** anti-Rabbit-FITC only and **(R)** anti-mouse-APC only antibodies. The merged images between (**M&N**) and (**Q&R**) are shown in (**O&S**) respectively and the corresponding 2D-cytofluorograms have been included to confirm the degree of co-localisation (**P&T**). Detection of the cell surface proteins were performed using the Zeiss LSM 780 confocal microscope and captured using the AxioCam MR camera. Images were subsequently analysed using Zen 2010 imaging software.

Figure 2. Confirmation of the expression of pSFV1-hu PrP^c 1-253 plasmid in HEK293 cells. Flow cytometry was employed to confirm PrP^c overexpression on the cell surface. Non-permeabilised HEK cells (either non transfected or transfected with a PrP^c expressing plasmid) were fixed (4% paraformaldehyde, 10min, 4°C), indirectly labelled with anti-PrP^c and anti-mouse-APC antibodies. The rightward shift in fluorescence intensity of the transfected sample in comparison to the non-transfected sample, confirms an increase in PrP^c expression on the cell surface and thus the successful expression of the pSFV1-hu PrP^c 1-253 plasmid. Three technical and biological repeats were performed and ten thousand events were enumerated per analysis.

Figure 3. The effects of exogenous $A\beta_{42}$ and PrP^c overexpression on cell viability.
(A) HEK293 cells were either transfected (mock or in the presence of pSFV1-hu PrP^c 1-253 plasmid), employing the Mirus transfection reagent, or treated upon reaching 70% confluence and 72h thereafter the cellular viability of the cells was determined by MTT (1mg/ml) assay. 8mM protocatehuic acid (PCA), an apoptosis inducing agent serves as the positive control. **(B)** HEK293 cells were treated with exogenous synthetic $A\beta_{42}$ at varying concentrations (100nM, 200nM and 500nM) and the effects thereof on cell viability was assessed after 48h. **(C)** The effects of $A\beta_{42}$ treatment on mock transfected and pSFV1-hu PrP^c 1-253 transfected HEK293 cells was assessed 72h post transfection and 48h post exogenous $A\beta_{42}$ administration. pSFV-hu PrP^c 1-253 represented as pSFV-PrP in figure legend.
Data shown (Mean ± s.e.m) representative of three independent experiments (performed in triplicate). *** $p < 0.001$; Students *t*-test

Figure 4. The cell rescuing effects of anti-LRP/LR specific antibody IgG1-iS18 on cell viability. (A) The cellular viability of in both the mock transfected and pSFV-PrP^c transfected cells HEK293 cells was determined by MTT (1mg/ml) assay, post 200nM $A\beta_{42}$

treatment in the absence of antibody as well as in the presence of 50µg/ml anti-LRP/LR specific antibody IgG1-iS18 and anti-CAT antibody. **(B)** Similarly the effects of 500nM $A\beta_{42}$ treatment on non-transfected and PrPc overexpressing cells, the absence and presence of IgG1-iS18 and anti-CAT was investigated. **(C)** MTT assays were employed to assess the influence of the IgG1-iS18 and anti- CAT antibodies on cell viability in the absence of exogenous synthetic $A\beta_{42}$ treatment. pSFV-hu PrP^c 1-253 represented as pSFV-PrP in figure legend. Data shown (Mean ± s.e.m) representative of three independent experiments (performed in triplicate). *** $p < 0.001$; Students *t*-test

Tables

Table 1: Pearson's correlation co-efficient for the co-localisation between LRP/LR and PrP^c

Proteins of interest	Pearson's correlation co-efficient
GFP-LRP/LR + DsRed-PrP^c	0.872
GFP + DsRed-PrP^c	0.044
GFP-LRP/LR + DsRed	0.018

Table 2: Pearson's correlation co-efficient for the co-localisation between PrP^c and $A\beta_{42}$

Proteins of interest	Pearson's correlation co-efficient
PrPc-APC + $A\beta_{42}$ -FITC	0.789
APC + FITC	0.021

Figure 1

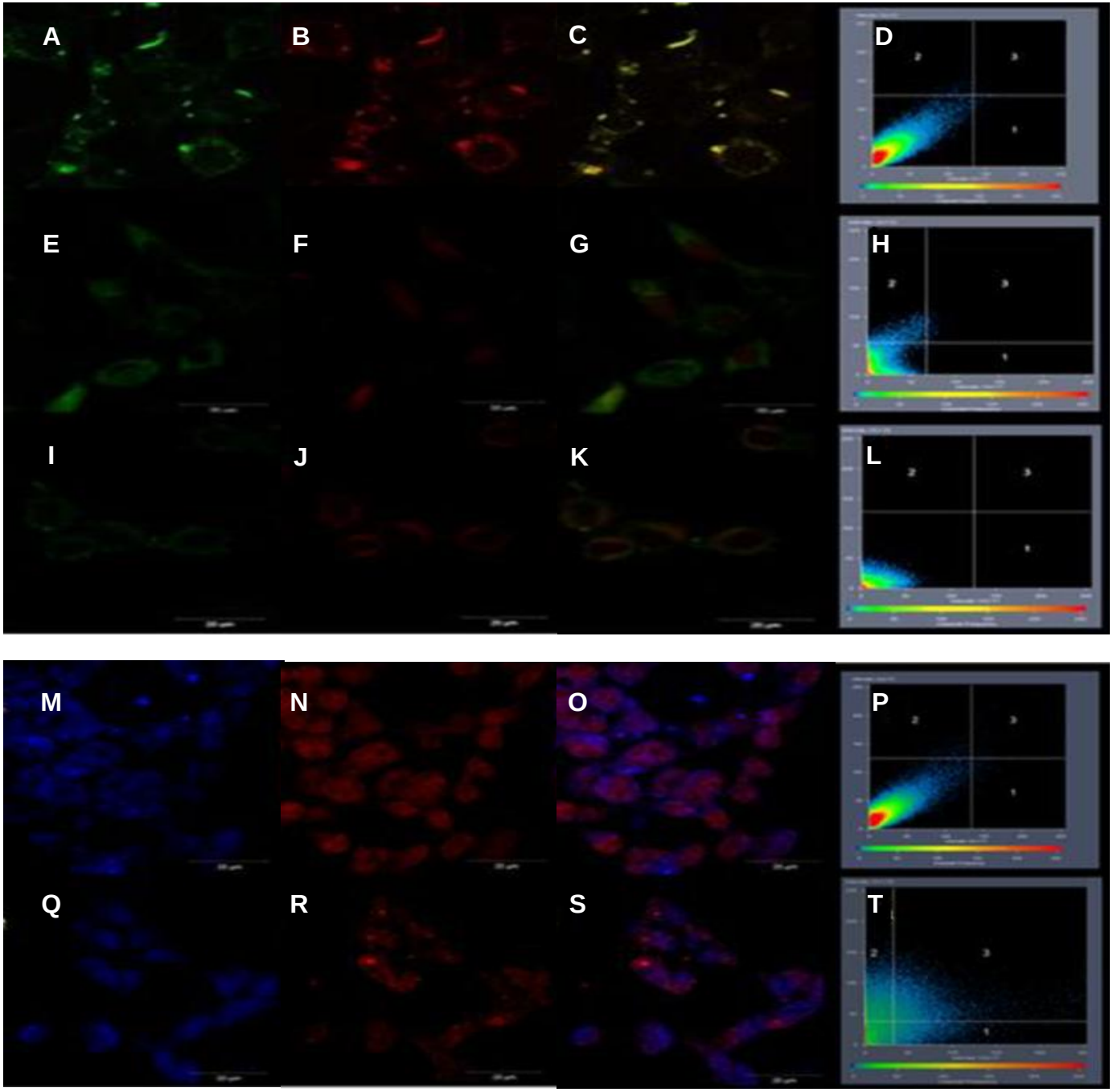
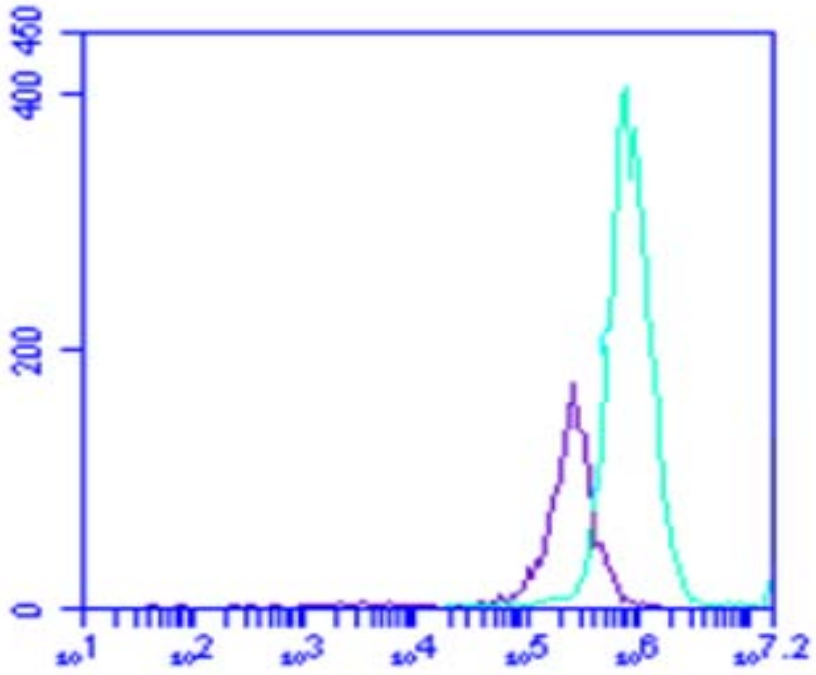


Figure 2



Non-transfected labeled with 8H4 and APC conjugated secondary antibody

Transfected with pSFV-huPrPc 1-253 and labeled with 8H4 and APC conjugated secondary antibody

Figure 3

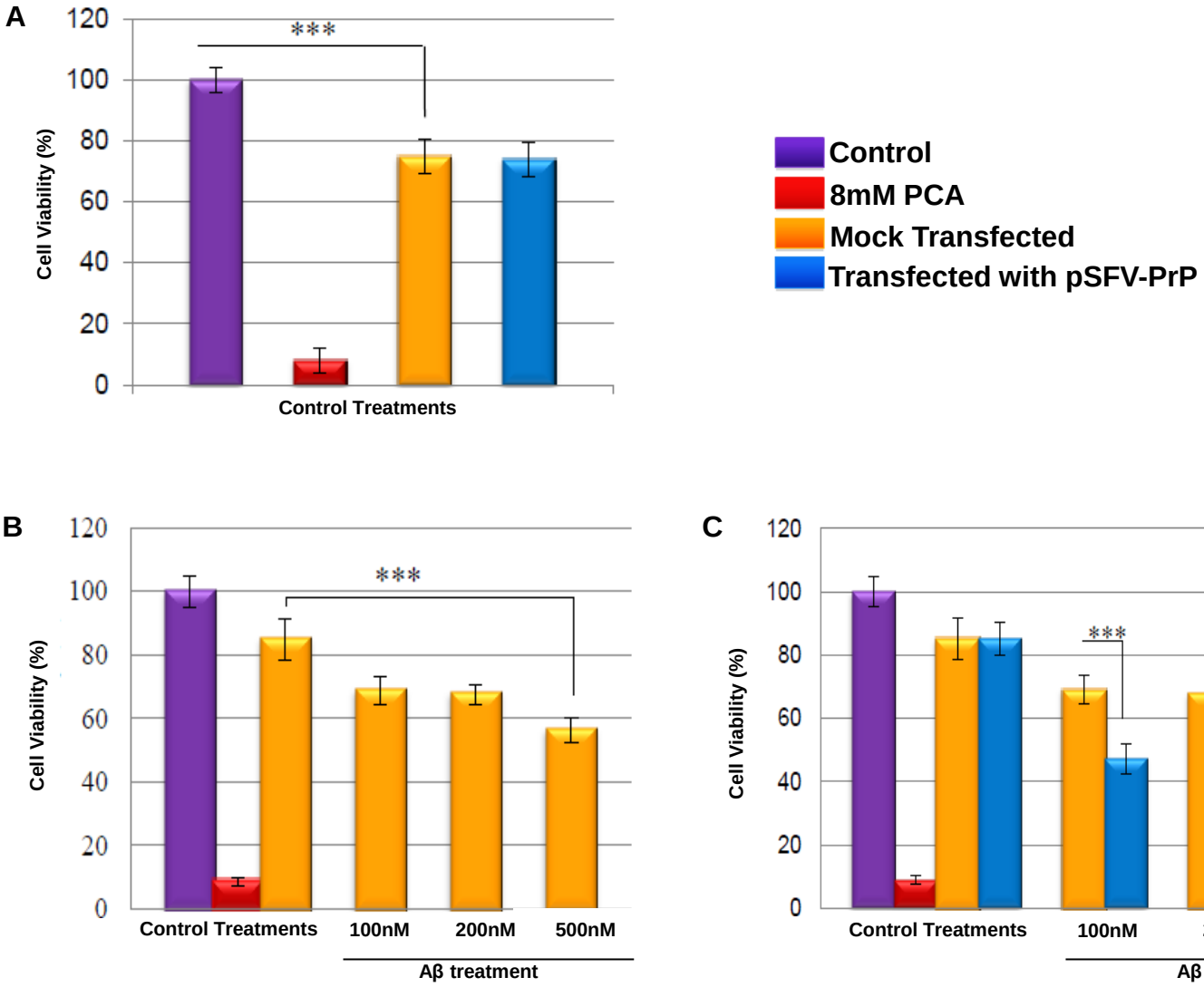
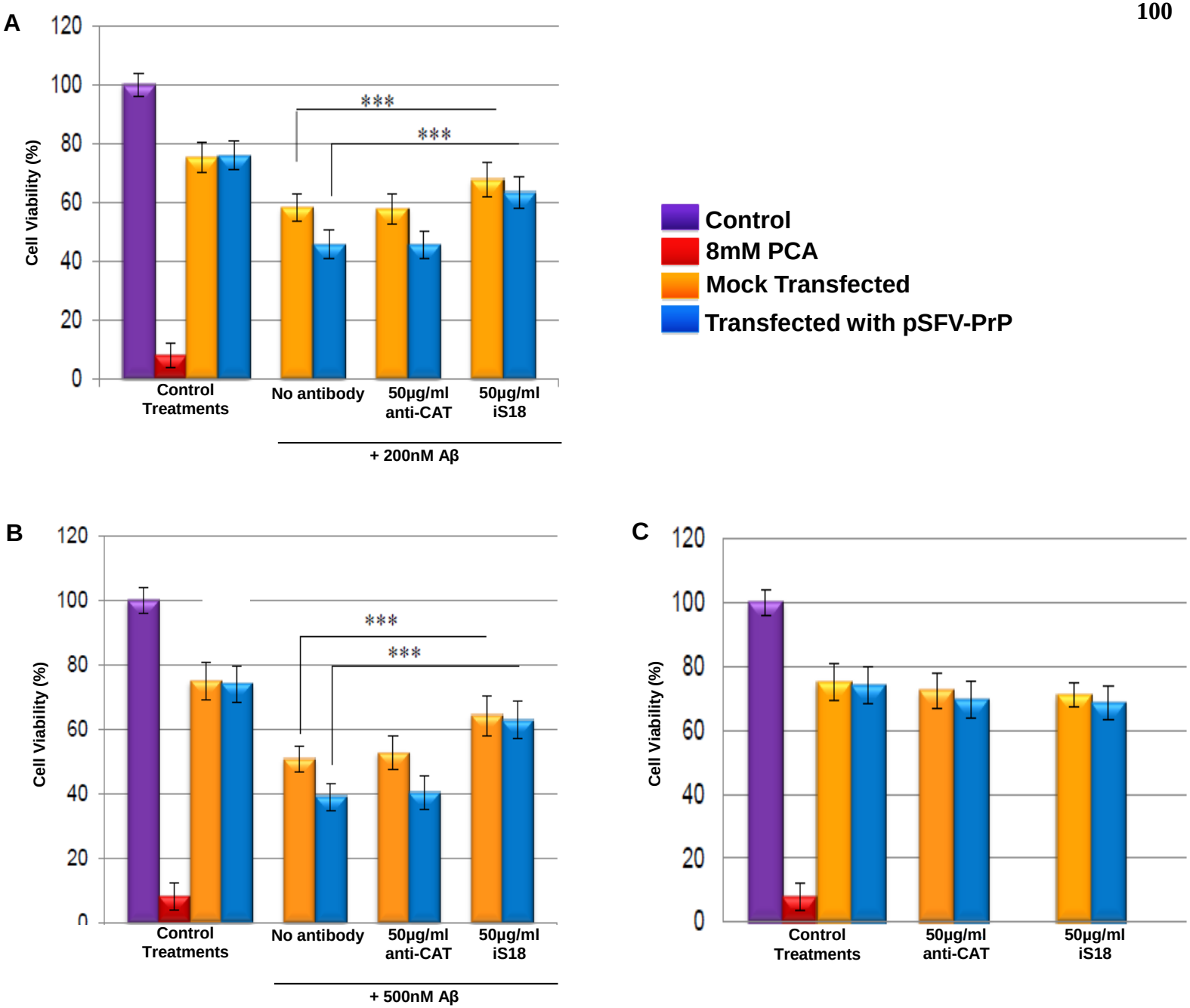

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Cell Viability (%)
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Control Treatments
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8mM PCA
Mock Transfected
Transfected with pSFV-PrP
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Cell Viability (%)
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Control Treatments
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Aβ treatment

Figure 4


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Cell Viability (%)
***
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Control
8mM PCA
Mock Transfected
Transfected with pSFV-PrP
Control Treatments
No antibody
50µg/ml anti-CAT
50µg/ml iS18
+ 200nM Aβ
B
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Cell Viability (%)
***
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Control Treatments
No antibody
50µg/ml anti-CAT
50µg/ml iS18
+ 500nM Aβ
C
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Cell Viability (%)
Control Treatments
50µg/ml anti-CAT
50µg/ml iS18

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

Authors: K. Jovanovic*, D. Gonsalves*, B. Da Costa Dias, U. Reusch, S. Knackmuss, M. Little, M. Weinberg and S.F.T Weiss.

Journal: Scientific Reports, 3, 2699; DOI:10.1038/srep02699

Year of Publication: 2013

Brief Overview of Article:

This original research article suggested for the first time that the 37kDa/67kDa LRP/LR may occupy a role in the amyloidogenic processing of APP. It was revealed by confocal microscopy that LRP/LR co-localizes with APP as well as β- and ɣ-secretases both at the cell surface and intracellularly - thereby suggesting a potential association between the receptor and these AD proteins. Pull down assays further suggested that an interaction, be it direct or indirect, exists between LRP/LR and β-secretase. Antibody blockade (employing an anti-LRP/LR specific antibody, IgG1-iS18) as well shRNA-mediated downregulation of the receptor did not significantly alter cell surface levels of APP, β- and ɣ-secretase but did significantly lower the shedding of both Aβ and sAPPβ fragments. It was therefore proposed that LRP/LR may be involved in APP processing, particularly through a possible association with β-secretase. It was also suggested that targeting this receptor by means of antibodies or RNAi methodology may hamper Aβ shedding and may thereby serve as a promising alternative approach for the treatment of Alzheimer's Disease.

Contribution: I assisted in the writing and editing of this original research article.

*These authors contributed equally to this work.

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

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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

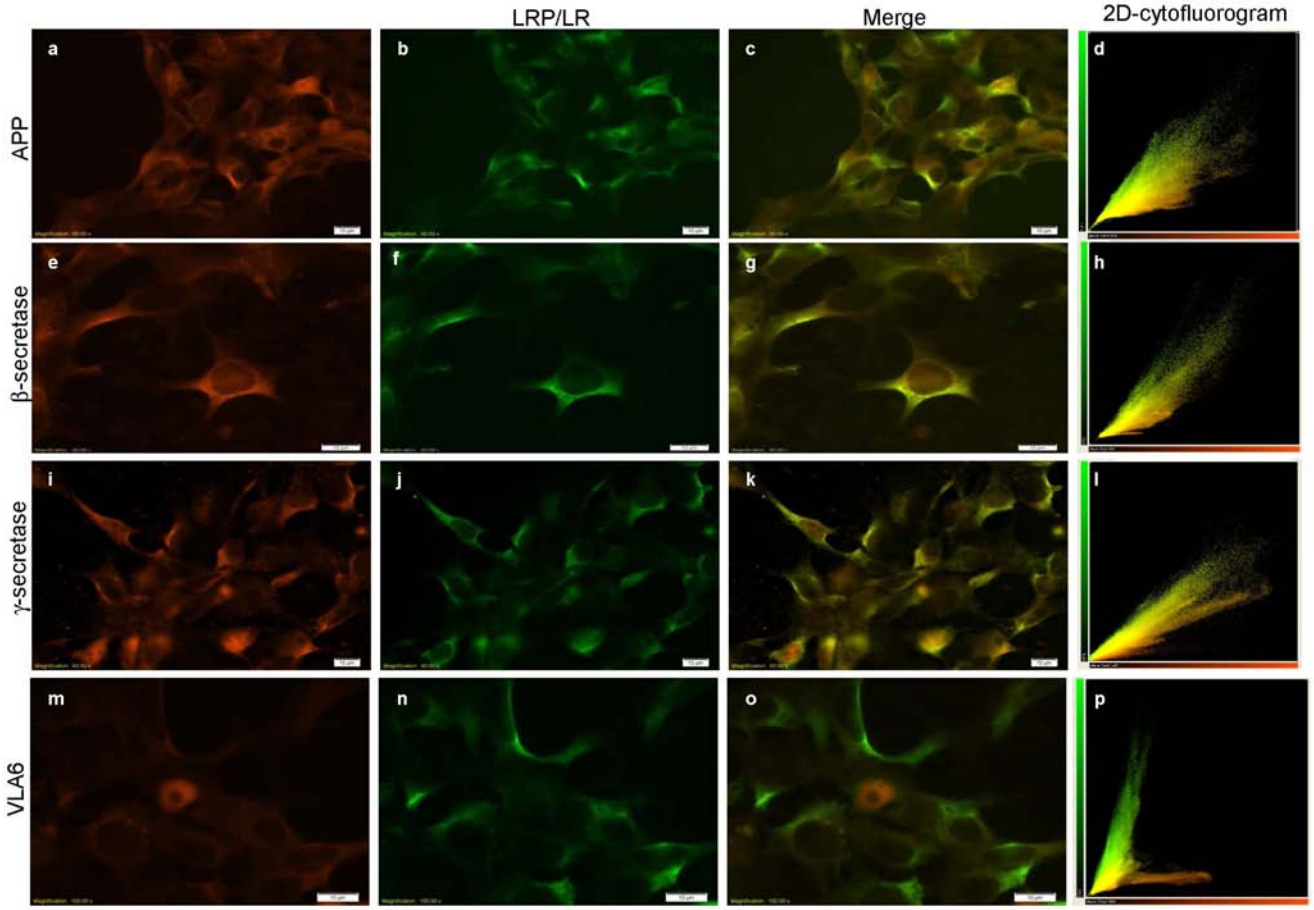


Figure 1 | Co-localisation of LRP/LR with the AD relevant proteins APP, β- and γ-secretase on the cell surace. Cell surface proteins on HEK293 cells were indirectly immunolabelled to allow for detection using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software. (a) APP, (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.

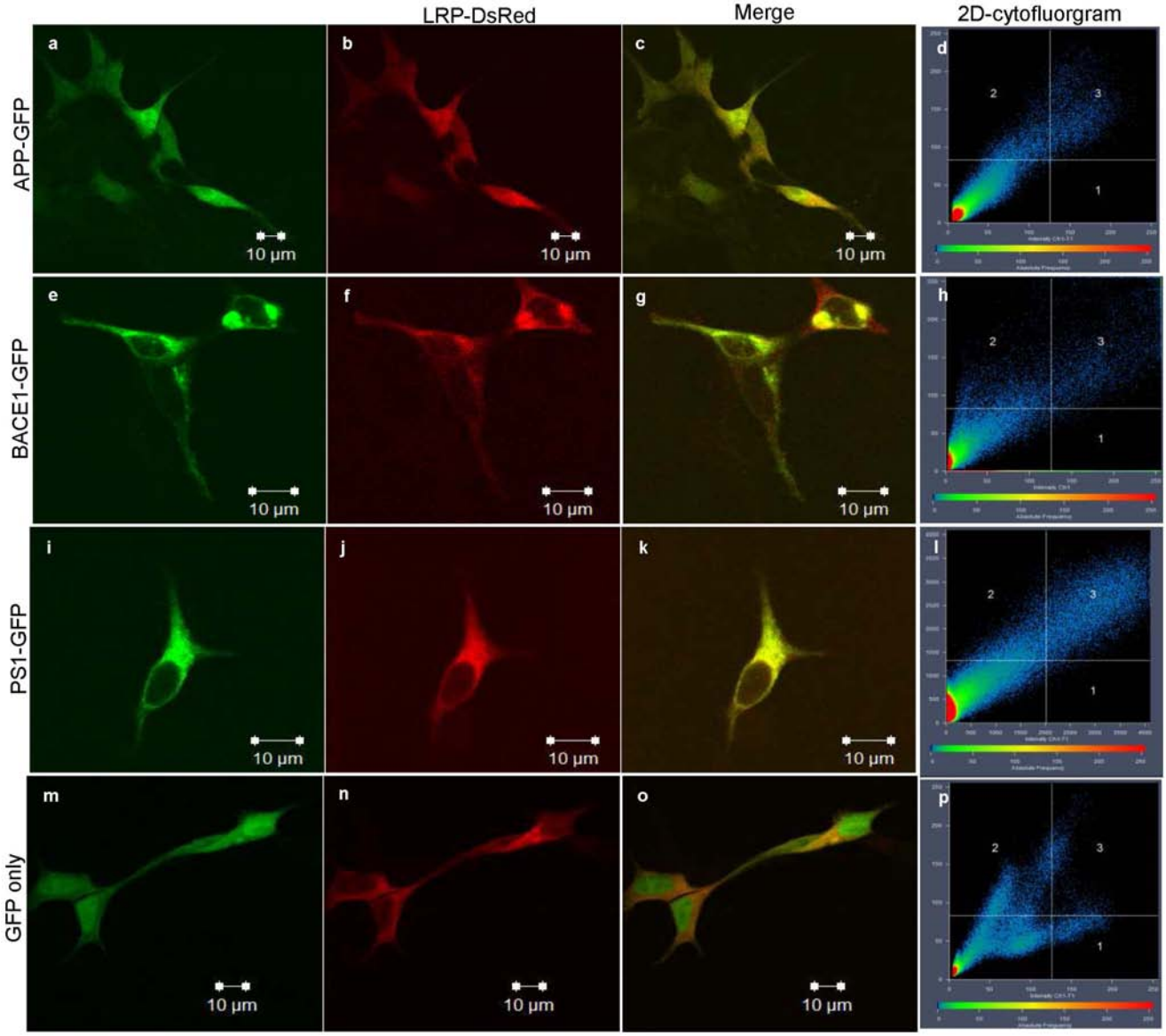


Figure 2 | Co-localisation of LRP-dsRed with APP-GFP, BACE1-GFP and PS1-GFP inside HEK293 cells. Fluorescently tagged (a) APP-GFP, (e) BACE1-GFP, (i) PS1-GFP and (m) GFP were co-expressed with LRP-dsRed (b, f, j, n) in HEK293 cells. Images were captured using the Zeiss LSM 780 confocal microscope and 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).

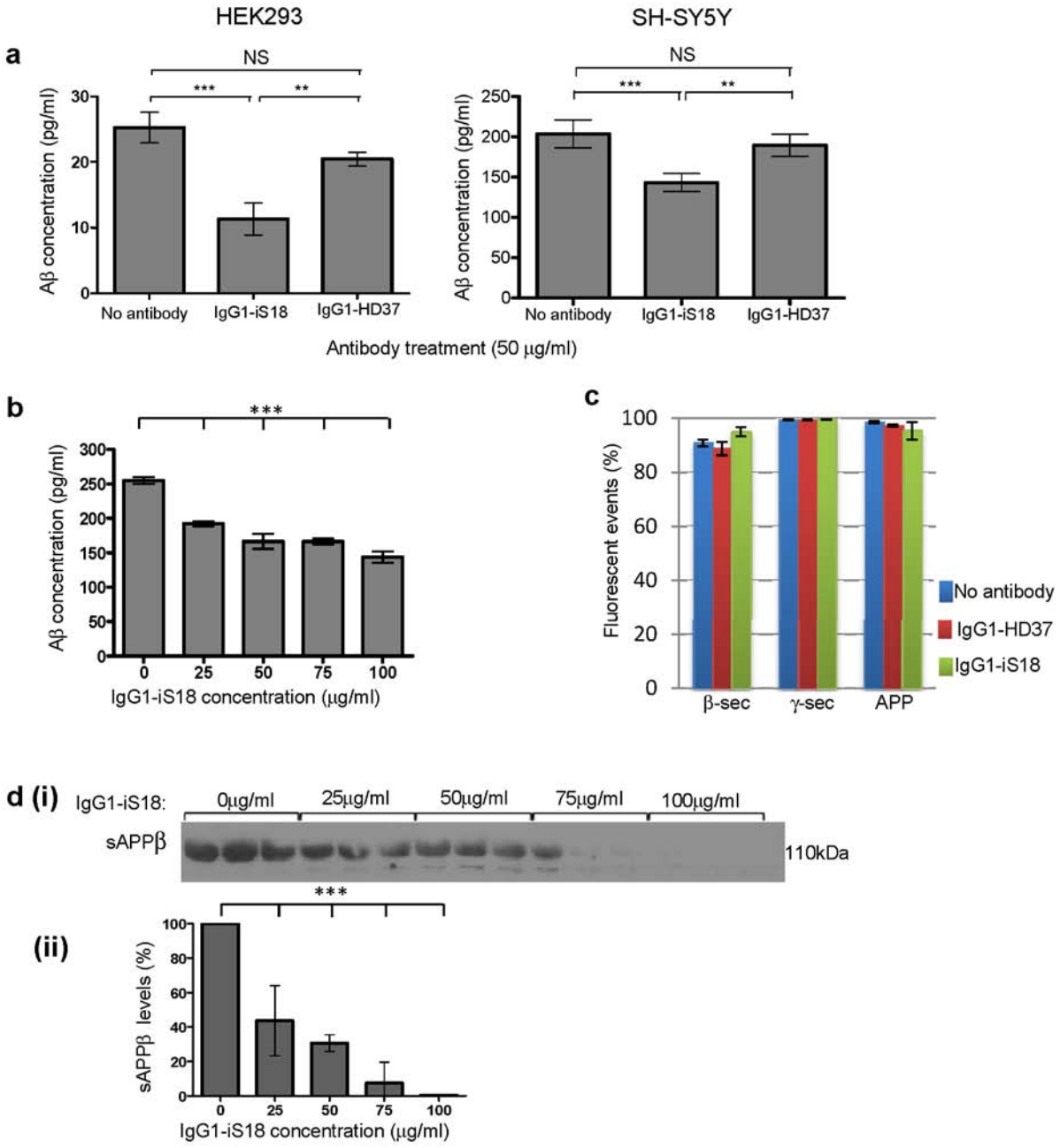


Figure 3 | Effects of IgG1-iS18. (a) Aβ levels in HEK293 and SH-SY5Y cells after treatment with IgG1-iS18 and IgG1-HD37 as detected by an Aβ ELISA after 18 hours of antibody incubation. Data shown (mean ± s.e.m) representative of three independent experiments (performed in triplicate) per cell line. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 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

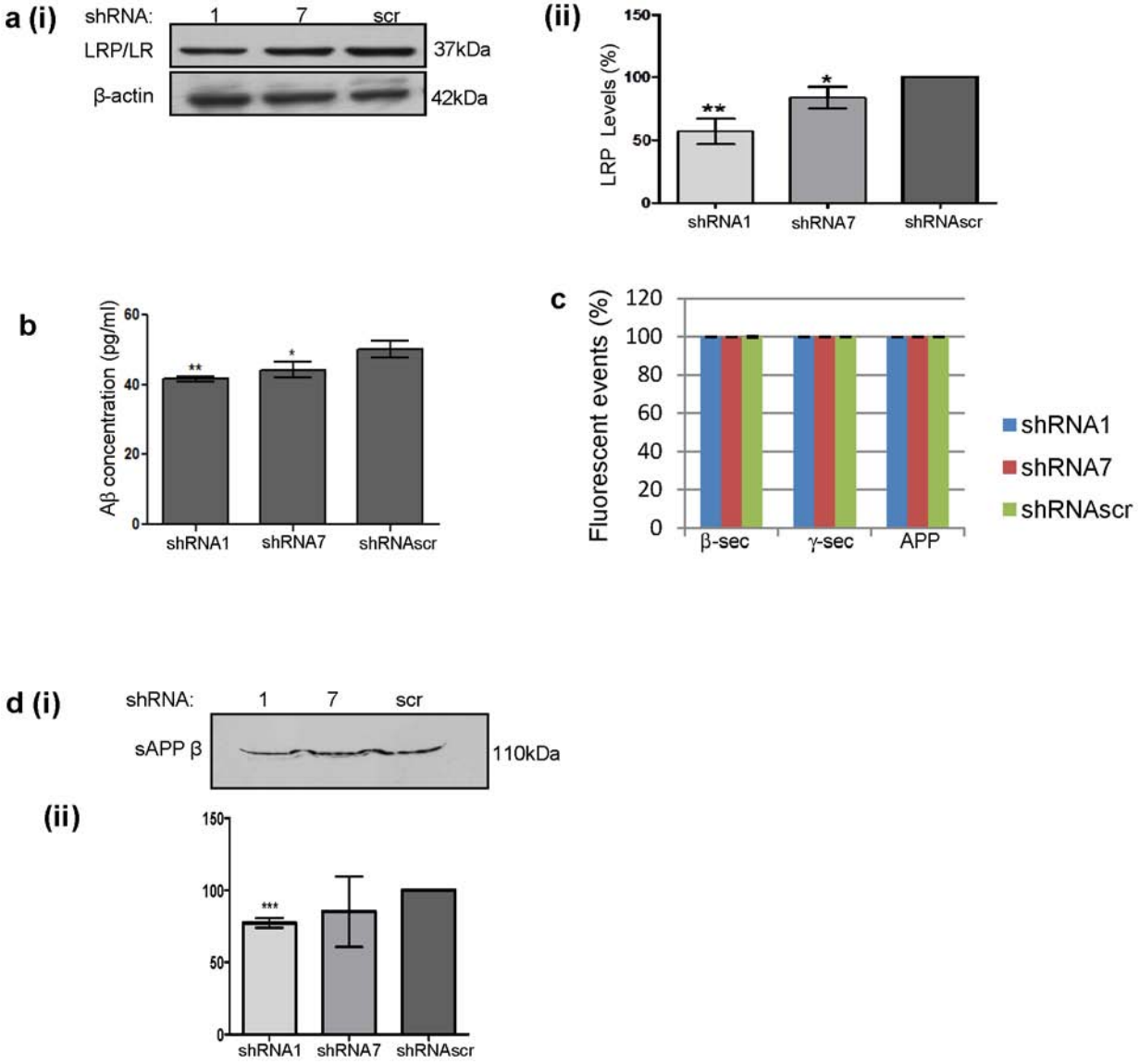


Figure 4 | Effects of LRP/LR downregulation by shRNA. (a) HEK293 cells were transfected with LRP/LR-specific shRNA1 and shRNA7 (as well as a scrambled control, shRNAscr). 72 hours post-transfection, cells were lysed and LRP/LR levels assessed by Western blotting. β-actin was used as a loading control. 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.

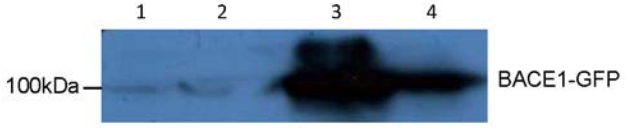


Figure 5 | LRP::FLAG Immunoprecipitation Assay Analysis. HEK293 cell lysates containing LRP::FLAG and either BACE1-GFP or CAT (Fig. S5) were subjected to Pull Down Assay analysis using Anti-FLAG M2 beads. After subsequent wash steps, eluted fractions were analysed using Immunoblotting. Lane 1 contained 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.

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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

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

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Brief Overview of Article:

This original research article expanded on the findings obtained in the original research article 4.4 (Jovanovic et al., 2013)[272] which demonstrated that the 37kDa/67kDa LRP/LR is implicated in the amyloidogenic processing of the amyloid precursor protein (APP). In this article the potential role of LRP/LR as an interaction partner for APP as well as β- and ɣ-secretases was investigated. The proteins of interest were fluorescently tagged and co-localization as well as z-stacking revealed that all the AD proteins co-localized with LRP/LR at a resolution of 200nm. However, assessment of co-localization at a higher resolution (80nm), employing super resolution structured illumination microscopy (SR-SIM), revealed that only ɣ-secretase co-localized with the receptor in multiple subcellular regions. These results were verified by Försters resonance energy transfer (FRET), a cell biological technique employed to evaluate potential protein interactions, as FRET only occurred between LRP/LR and ɣ-secretase and not with APP nor β-secretase. These findings suggest that the 37kDa/67kDa LRP/LR may influence APP processing through a direct association with ɣ-secretase and through a potential indirect interaction with β-secretase.

Contribution: I assisted in the editing of this original research article.

High Resolution Imaging Study of Interactions between the 37 kDa/67 kDa Laminin Receptor and APP, Beta-Secretase and Gamma-Secretase in Alzheimer's Disease

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Abstract

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 37 kDa/67 kDa 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, respectively, at various sub-cellular locations. Superresolution Structured Illumination Microscopy (SR-SIM) showed that interactions were unlikely between LRP/LR and APP and β-secretase, respectively, while there was strong co-localisation between LRP/LR and γ-secretase at this 80 nm 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.

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Competing Interests: S.F.T.W. is currently a PLOS ONE Editorial Board Member. Patent application PCT/IB2012/054918, published as WO 2013/042053 on 28.03.2013, in the name of the University of the Witwatersrand is entitled "Compounds for use in the treatment of Alzheimer's disease". The claims of this patent application are directed to a method of reducing Alzheimer's disease proteins using laminin receptor proteins. National applications have been filed in the US (14/345,770), Europe, China and South Africa (2014/02471). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

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 signalling upon interaction with Aβ whereby Fyn kinase is activated and leads to dendritic spine loss, lactate dehydrogenase activation and altered NMDAR expression on the plasma membrane of neurons [10,11].

The cellular receptor for both PrP^c [12] and its infectious isoform PrP^{Sc} [13] is the 37 kDa/67 KDa 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 [16,17,18,19], apoptosis [20] and angiogenesis [21].

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 both in Aβ levels [22] and Aβ induced cytotoxicity [23]. 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 [22]. 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 [22]. These findings revealed a novel role for LRP/LR 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 (FCS) (Hyclone) and 1% Penicillin/Streptomycin. IMR-32 human neuroblastoma cells were cultivated in MEM (Hyclone) supplemented with 10% FCS, 1% Penicillin/Streptomycin, 1% Non-Essential Amino Acids and 2 mM L-glutamine. Cells were incubated at 37°C with 5% CO_2. The HEK293 cell line was obtained from American Tissue Culture Collection (ATCC) while IMR-32 cells were obtained from the Fox Chase Cancer Centre. 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, HEK293 cells were seeded into 60 mm tissue culture dishes (Corning). Once the cells were 30–50% confluent, calcium phosphate transfection was performed as described previously [22] for HEK293 cells while *Trans*IT®-LT1 transfection reagent (Mirus) was used for the transfection of the IMR-32 cells (in accordance with the manufacturer's instructions). The procedure for the generation of the plasmids encoding LRP-dsRed (pLRP-dsRed) [24] and LRP::FLAG (pCIneo-moLRP::FLAG) [25] has been described previously. Plasmids for APP-GFP (pEGFPN1-APP770) and BACE1-GFP (pEGFPN1-BACE1) [26] were a generous gift from Dr. Bradley T Hyman, while pEGFP-PS1 (coding for PS1-GFP) [27] 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 into HEK293 cells in 60 mm tissue culture dishes and incubated for 72 hours, after which the cells were lysed for further experiments.

Slide preparation

24 hours post transfection, coverslips containing adherent transfected cells (LRP-dsRed with APP-GFP, BACE1-GFP or PS1-GFP) were washed with PBS 3 times and then fixed with 4% Paraformaldehyde in PBS for 15 minutes. Coverslips were washed with PBS and mounted onto clean microscope slides using 75 µl Fluoromount (Sigma Aldrich). Slides were maintained 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 ±0.4 µm (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 100×/1.46 oil DIC M27 ELYRA objective, using an ELYRA S.1 (Carl Zeiss Microimaging) microscope equipped with a 488 nm laser (100 mW), 561 nm laser (100 mW) 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 [28]. Analysis was performed on reconstructed super-resolution 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 63×/1.4 Oil DIC M27 objective. Samples were excited with a 488 nm and 561 nm laser under utilization of a MBS 488/561 beam splitter. Emission was collected for the donor-GFP channel at an emission window of 495 nm–510 nm, the FRET channel at an emission window of 586 nm–600 nm and the acceptor-dsRed channel at an emission window of 586 nm–600 nm, using the lambda setting. Appropriate controls for background, donor spectral bleed-through (DSB) and Acceptor spectral bleed-through (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. N-FRET was calculated using the Zeiss FRET plug-in for HEK293 cells, while the Image-J FRET plug-in was utilized for the IMR-32 cells, hence the slight differences in the appearance of the FRET signals in the images.

FLAG Co-immunoprecipitation

HEK293 cells were utilized for this study as they share many similarities and features with neuronal cells [29]. Only HEK293 cells were subjected to co-immunoprecipitation due to ease of transfection. Cells transfected with pCIneo-moLRP::FLAG, pCDNA3.1APPwt or 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.

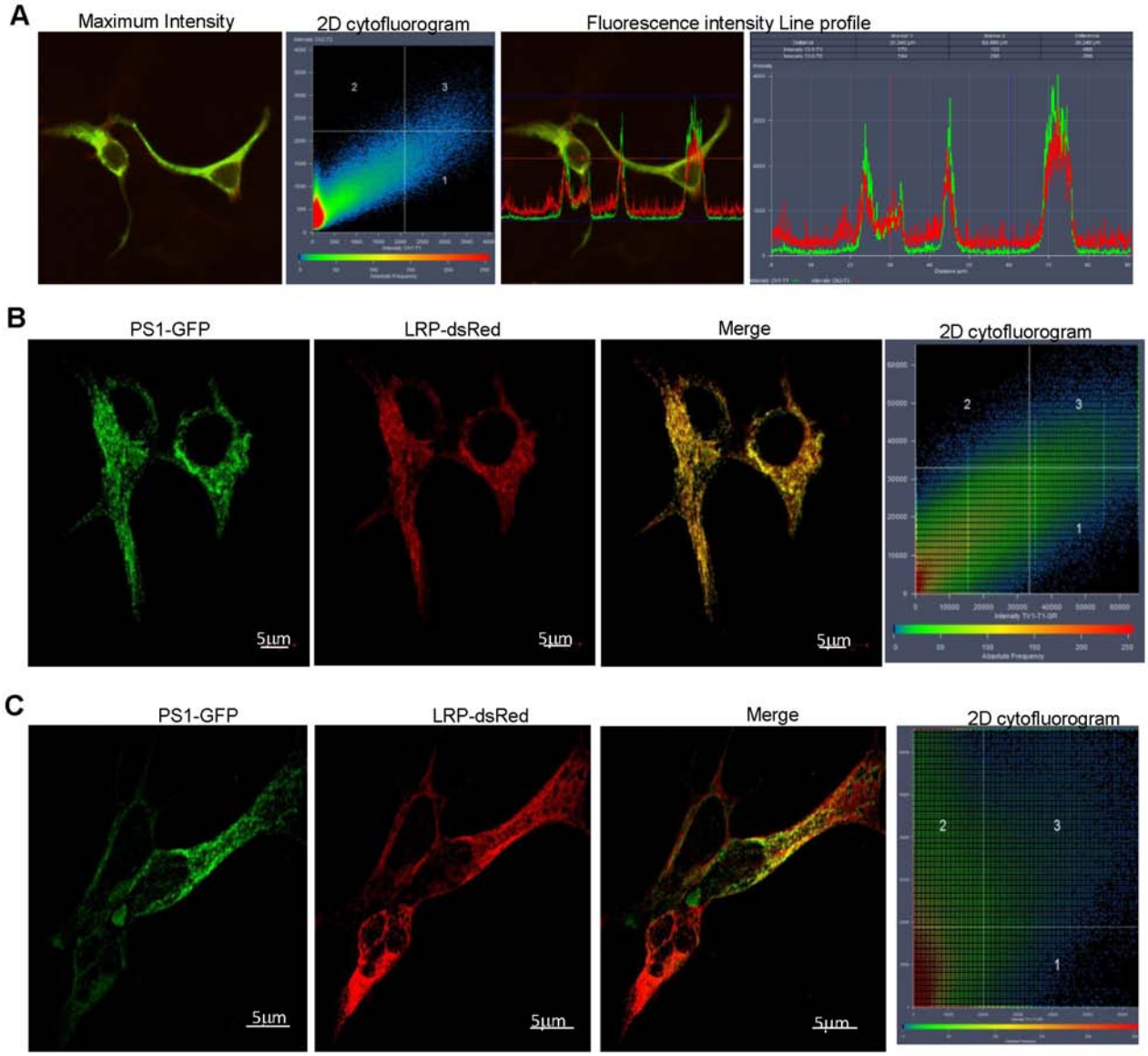


Figure 1. LRP/LR co-localises with PS1 of the γ-secretase complex. (A) Co-localisation between PS1-GFP and LRP-dsRed is shown for HEK293 cells in the maximum intensity profile for PS1-GFP and LRP-dsRed. The 2D cytofluorogram confirms co-localisation as a diagonal is observed passing through quadrant 3. A diagonal is indicative of a high degree of co-localisation. Line profile displaying the fluorescence intensities of the 2 colour channels along a line of interest and reveals co-localisation occurring specifically within the cytoplasmic regions. Line profile reveals aligned spectra suggestive of high degree of co-localisation. (B) SR-SIM reveals that PS1-GFP and LRP-dsRed show a high degree of co-localisation in HEK293 cells. 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 at this resolution (80 nm). Scale bar: 5 µm. (C) SR-SIM reveals that co-localisation between LRP-dsRed and PS1-GFP is maintained in IMR-32 cells, although the 2D cytofluorogram is skewed by the differences in transfectability with the different plasmids. Scale bars: 5 µm.
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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). APPwt 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 [30]. 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 [31]. The 4

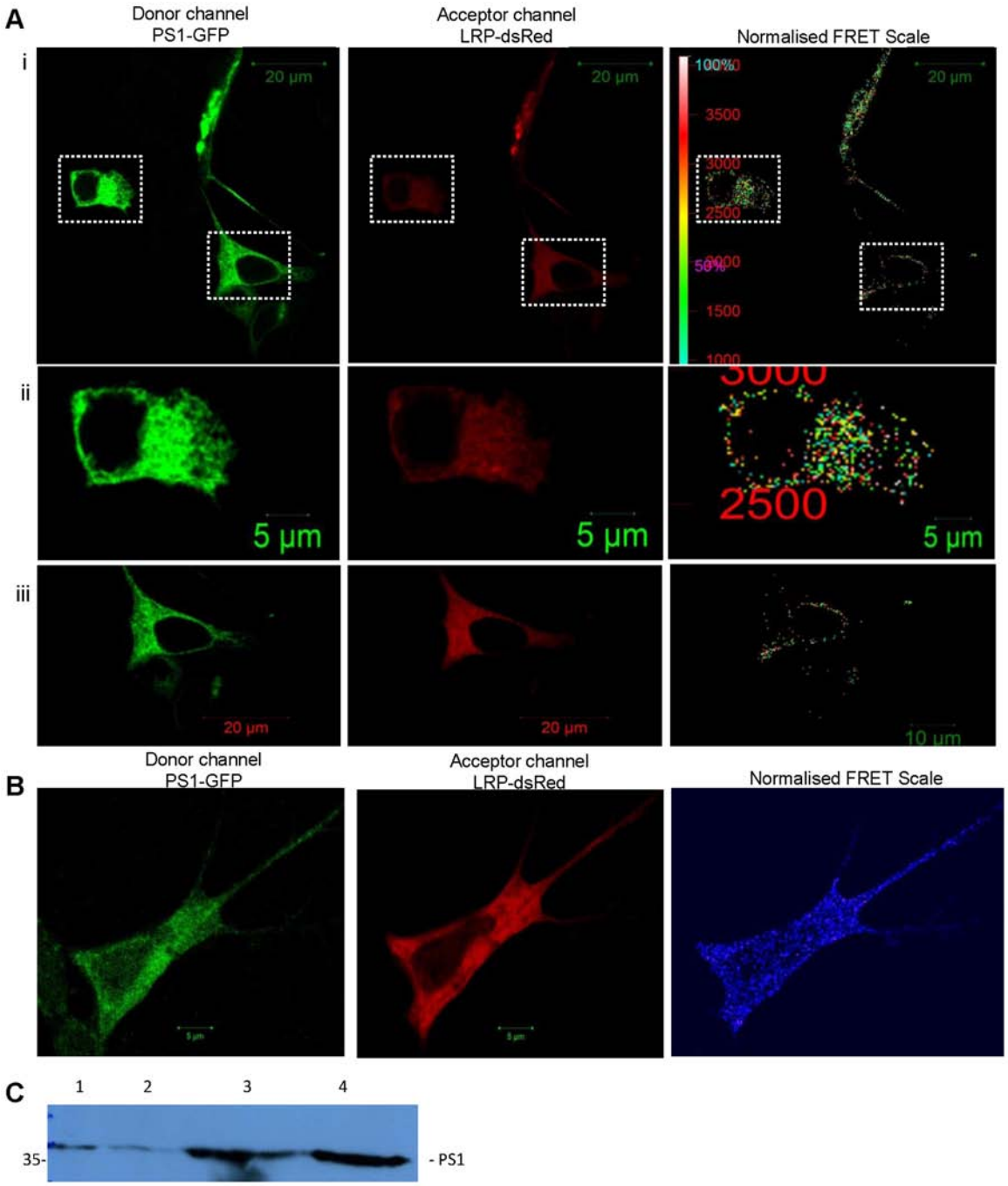


Figure 2. LRP/LR interacts with PS1 of the γ-secretase complex. (A)(i) FRET analysis with PS1-GFP donor and LRP-dsRed acceptor in HEK293 cells. Distinct FRET signal is detected on the normalized FRET scale. Scale bar: 20 µm. (ii) Enlarged view of single cell from (i) showing that energy is transferred in the cytoplasmic and plasma membrane regions of the cell. (iii) Enlarged view from cell in (i) showing FRET signal occurs in perinuclear membrane. (B) FRET analysis between PS1-GFP donor and LRP-dsRed acceptor performed on IMR-32 cells. Blue FRET signal is detected in the normalised FRET panel indicating an interaction is present between the two proteins. Scale bar: 5 µm. (C) FLAG co-immunoprecipitation assay was performed utilizing lysates of HEK293 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 36 kDa indicating PS1 dimers and not PS1-GFP.
doi:10.1371/journal.pone.0100373.g002

subunit complex is found to function predominantly within cholesterol- and sphingolipid-rich lipid raft microdomains of membranes, most notably those of the plasma membrane [32], *trans*-Golgi Network (TGN) as well as endosomes [33]. The cellular localisations of LRP/LR are limited to these same regions and the presence of this protein has also been noted in the Golgi apparatus [34]. The co-localisation observed from the maximum intensity projection between PS1-GFP and LRP-dsRed supports

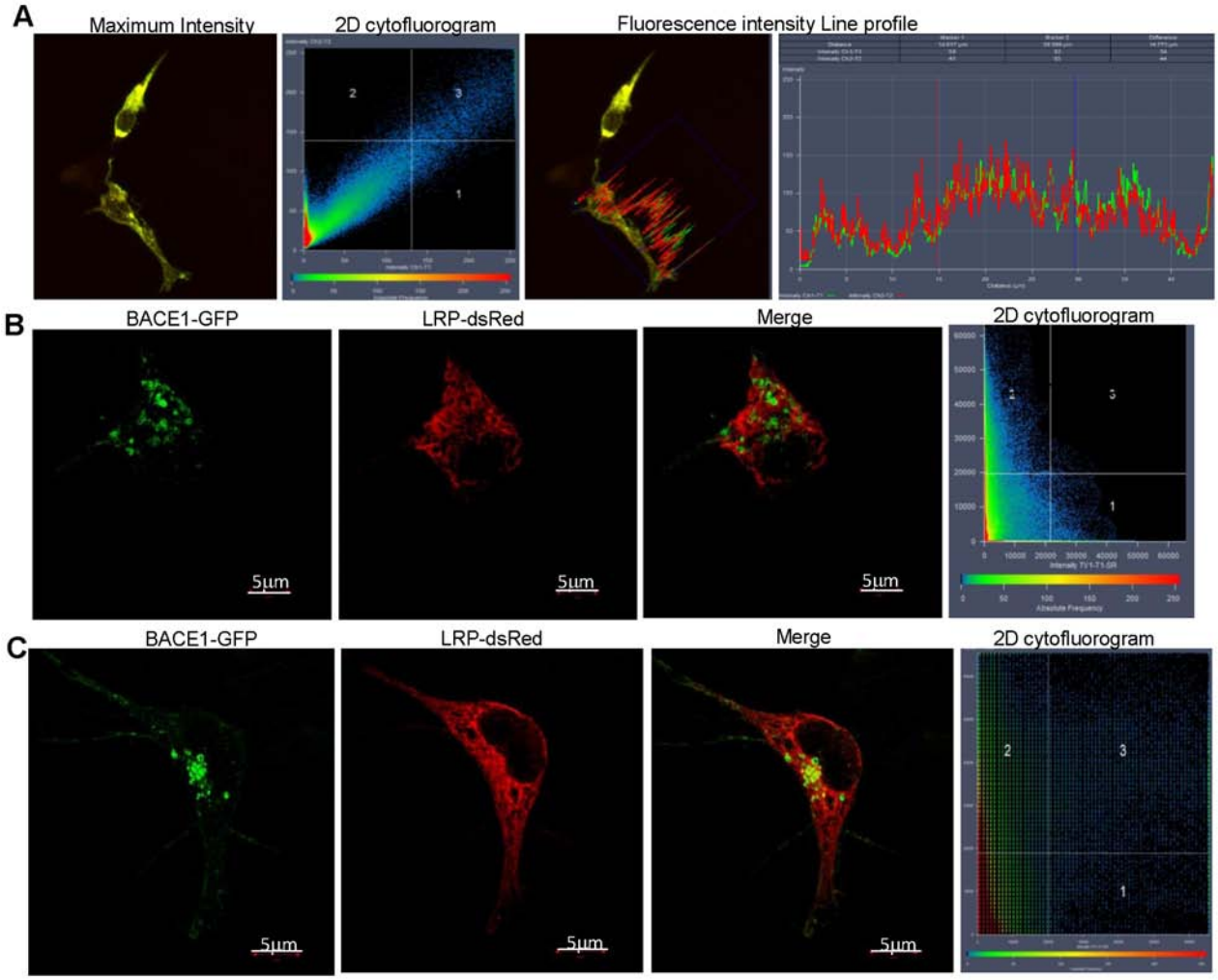


Figure 3. β-secretase fails to co-localise with LRP/LR at high resolutions. (A) Maximum intensity profile obtained from Z-stack analysis reveals strong cytoplasmic co-localisation between BACE1-GFP and LRP-dsRed in HEK293 cells. Diagonal in 2D cytofluorogram indicates high degree of co-localisation, 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: 5 µm. (C) SR-SIM employing IMR-32 cells reveals a lack of co-localisation between LRPdsRed and BACE1-GFP. Scale bars: 5 µm.
doi:10.1371/journal.pone.0100373.g003

the notion that LRP/LR and γ-secretase appear to be located in similar cellular regions and compartments in HEK293 cells (Figure 1A). The 2D cytofluorogram accompanying the maximum intensity projection shows a clear diagonal passing through quadrant 3, indicating a high degree of co-localisation between the signal in the green and red channels which results in the observed yellow pattern of co-localisation. The line profile obtained from the maximum intensity projection of the Z-stack analysis between PS1-GFP and LRP-dsRed (Figure S1 in File 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-localisation does not necessitate functional protein – protein interactions. This can in part be overcome by an SR-SIM approach, which provides a resolution limit of approximately 80 nm (compared to the 200 nm 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 in HEK293 cells. 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. Figure 1C illustrates that co-localisation between PS1-GFP and LRPdsRed is also maintained in the IMR-32 cells in similar subcellular locations as in the HEK293 cells. The lack of a clear diagonal in the 2D cytofluorogram is as a result of differing transfectability of the cells depicted in the image. As indicated, only one of the cells was successfully co-transfected with both PS1-GFP and LRPdsRed and this is where co-localisation is most evident. In order to further assess whether this co-localisation 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 a proximity of 1–10 nm to induce FRET. A well defined FRET signal is observed in the normalized FRET scale panel (Figure 2A (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

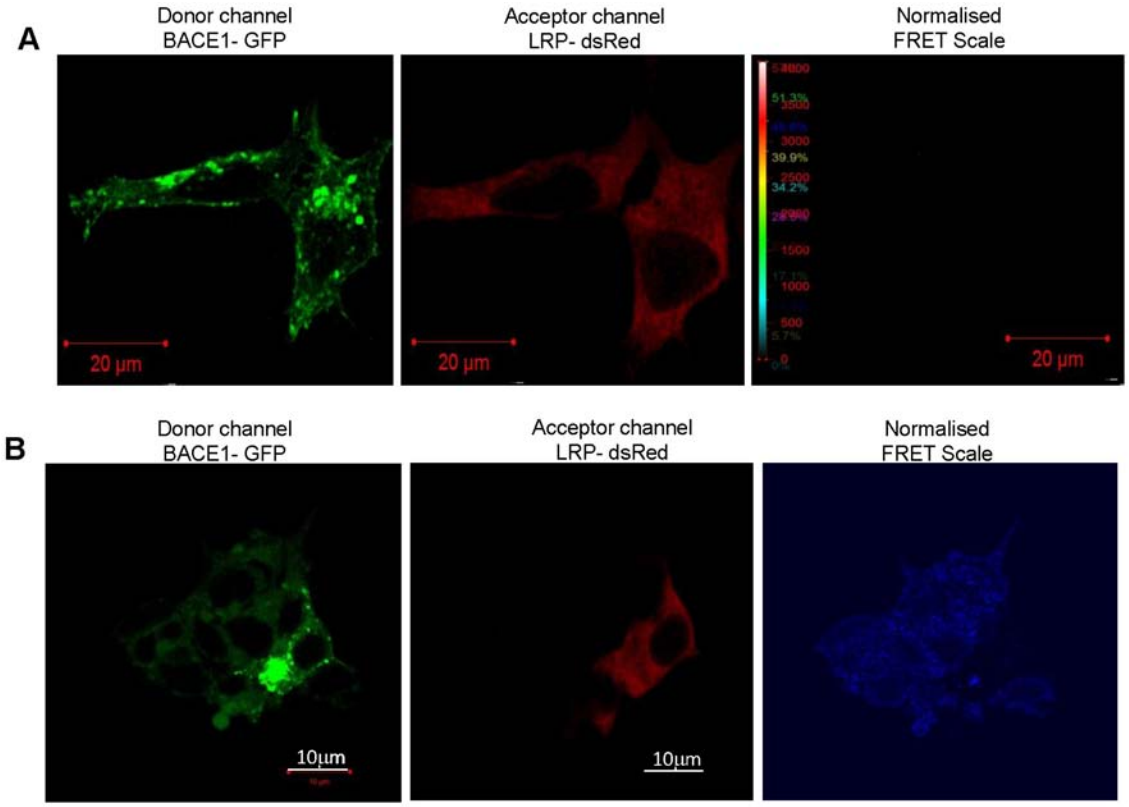


Figure 4. β-secretase doesn't interact with LRP/LR as revealed by FRET analysis. (A) FRET did not occur between the donor BACE1-GFP and the acceptor LRP-dsRed in HEK293 cells as no signal was detected on the normalized FRET scale. Scale bar: 20 µm. (B) Similarly, a lack of interaction was detected between LRPdsRed and BACE1-GFP when IMR-32 cells were used. Scale bars:10 µm.
doi:10.1371/journal.pone.0100373.g004

Figure 2A (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). Furthermore, FRET was also observed in IMR-32 cells as indicated by the blue FRET signals observed in the normalised FRET panel of Figure 2B, confirming the likelihood of an interaction between PS1 and LRP. 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 validate this, FLAG co-immunoprecipitation was performed using LRP::FLAG and PS1-GFP (Figure 1D) in HEK293 cells. 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 certainly cellular PS1 and not PS1-GFP. The band is seen at 36 kDa which confirms that the proteins detected are most likely the homo-dimer of PS1 [35,36], as the expected band size for PS1 is 18 kDa. This co-immunoprecipitation finding further substantiates the FRET data by confirming 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 [22], as these treatments may be interfering in or hampering the interaction between LRP/LR and γ-secretase. Further investigation is required to determine the binding sites involved in the interaction between these two proteins, in order to elucidate the role of the interaction between LRP/LR and PS1 in the context of AD pathology.

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 localised 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 [37]. Initial reports suggested not only co-localisation between LRP/LR and β-secretase on the cell surface, but also that these two proteins interacted, as shown by co-immunoprecipitation [22]. The findings presented in Figures 3 and 4 help to further understand the nature of the possible interactions between β-secretase and LRP/LR, where the maximum intensity projection (Figure 3A) constructed from Z-stack images (Figure S2 in File S1) shows strong co-localisation between the 2 proteins within the cellular cytoplasm – this most likely occurring 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 100–200 nm from each other in both HEK293 (Figure 3B) and IMR-32 cells (Figure 3C). 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 within both the HEK293 and IMR-32 cells (Figure 4A and B respectively). These findings infer that the interaction that was reported between LRP/

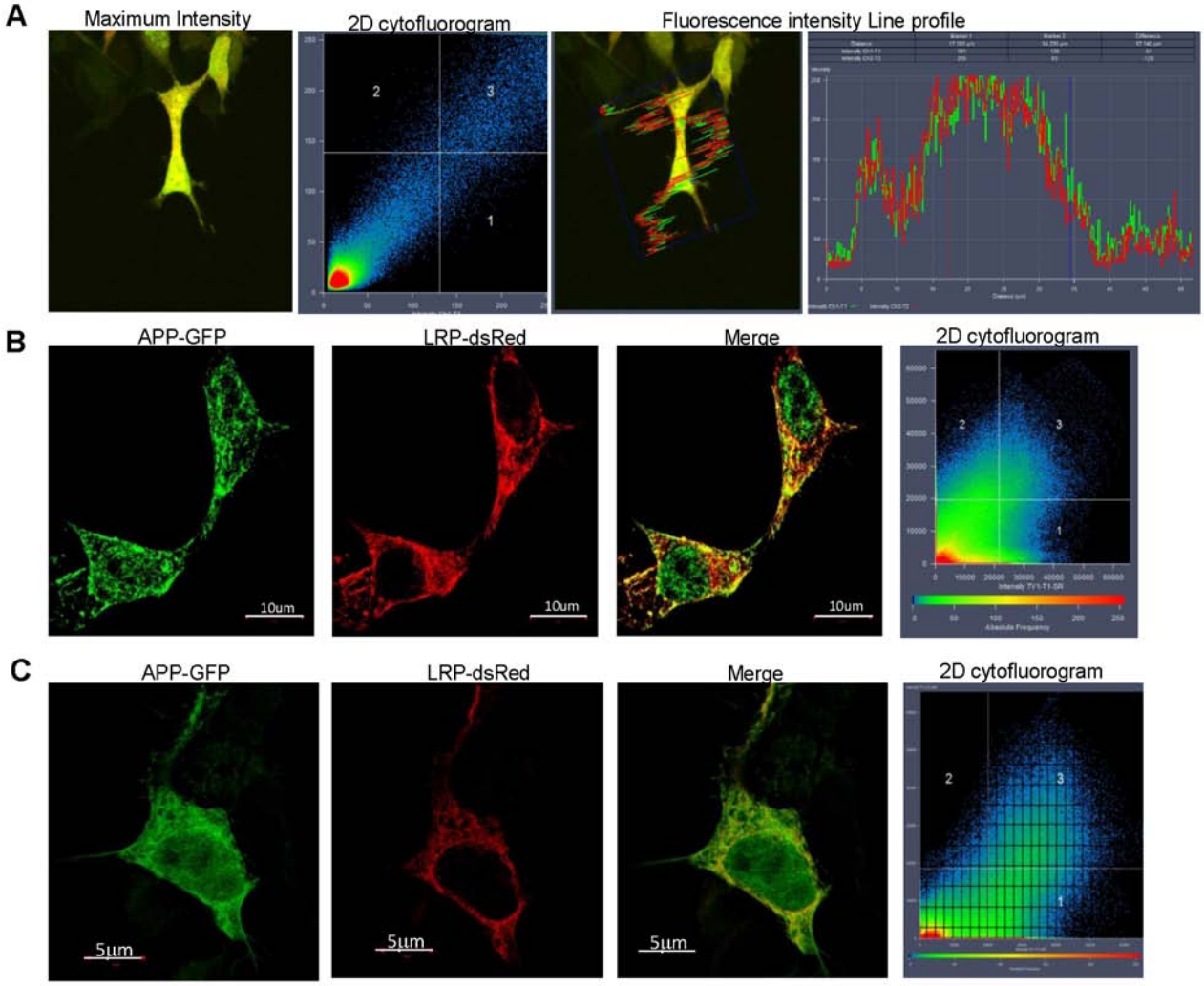


Figure 5. Co-localisation occurs between APP and LRP/LR. (A) Maximum intensity profile obtained from Z-stack analysis revealing co-localisation between APP-GFP and LRP-dsRed in the cytoplasm of HEK293 cells. 2D cytofluorogram reveals the co-distribution of green (APP-GFP) and red (LRP-dsRed) pixels. (B) SR-SIM analysis reveals that APP-GFP and LRP-dsRed co-localise to a lesser extent in HEK293 cells. Co-localisation is highest at the cell surface and in limited cytoplasmic locations. Scale bar: 10 µm. (C) Similar co-localisation is observed in IMR-32 cells. Scale bar: 5 µm.
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LR and β-secretase [22] is most likely an indirect interaction mediated by another cellular protein, as whole 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 [38,39]. It is possible that since LRP/LR is the cellular receptor for PrP^c and plays an important role in regulating endocytosis [40] and propagation [41] 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 [42]. Extensive studies have been performed on heparan sulphate, and analogues thereof, as therapeutic options for Alzheimer's Disease. Most notably this proteoglycan is seen to regulate β-secretase activity and significantly reduce the shedding of Aβ [43,44,45]. 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 [22], 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 [22] 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 [22]. Initial findings also suggested that these two proteins co-localise when detected on non-permeabilised cells (indicating cell surface co-localisation) and within the cells (indicating intracellular co-localisation) [22]. Z-stacking was performed to further verify the sub-cellular localisations of these proposed interactions and to assess whether they were maintained throughout the cell (Figure S3 in File S1). 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

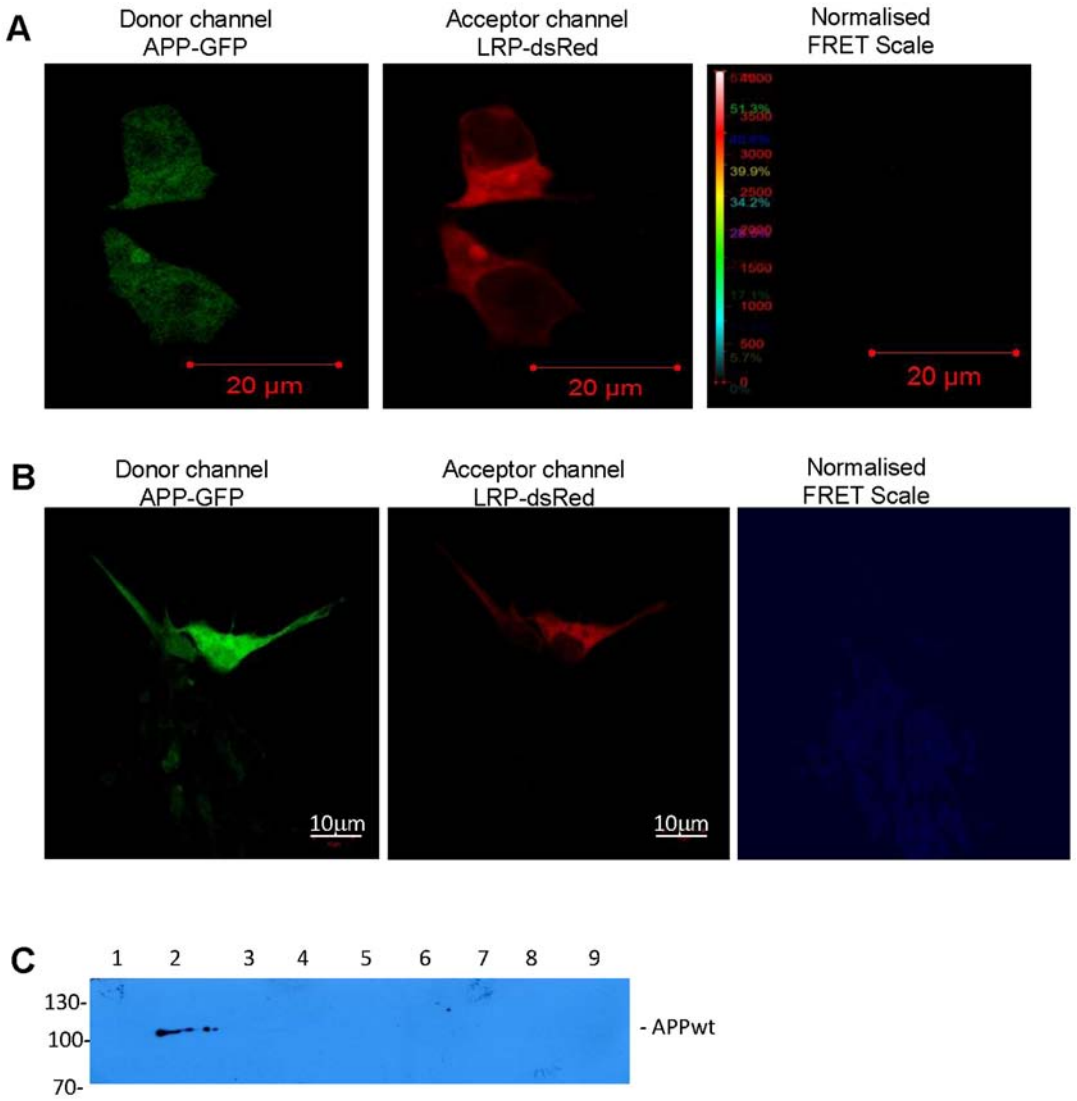


Figure 6. APP-GFP and LRP/LR fail to interact. FRET was performed using APP-GFP as donor and LRP-dsRed as acceptor in HEK293 cells. No FRET signal was observed on the normalized FRET scale indicating that no energy transfer occurred from BACE1-GFP to LRPdsRed. Scale bar: 20 µm. (B) Similarly, no FRET was observed between APP-GFP and LRPdsRed in IMR-32 cells. Scale bar: 10 µm. (C) FLAG co-immunoprecipitation analysis using lysates of HEK293 cells expressing LRP::FLAG and APPwt. Anti-APP antibody (Y188) was utilised for detection. Lane (1) cell lysates containing 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.
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merged (Figure 5A). 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 passing through the HEK293 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, where it is subsequently endocytosed from lipid raft regions into early endosomes [46,47]. Moreover, LRP/LR is known to also be located in the lipid raft [48] as well as in early endosomes [40]. Our SR-SIM data indicates a lower degree of co-localisation between APP and LRP both in HEK293 (Figure 5B) and IMR-32 cells (Figure 5C), which appears to be confined to the plasma membrane and certain cytoplasmic regions, presumably the endosomes in which both APP and LRP/LR are localised. This suggests the proteins are indeed in close proximity to one another, resulting in the distinct co-localisation 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, since signal detection was lacking on the normalized FRET scale for both HEK293 (Figure 6A) and IMR-32 cells (Figure 6B). These findings indicate that no interaction takes place between APP and LRP/LR at this 1–10 nm scale. FLAG Co-immunoprecipitation using a FLAG

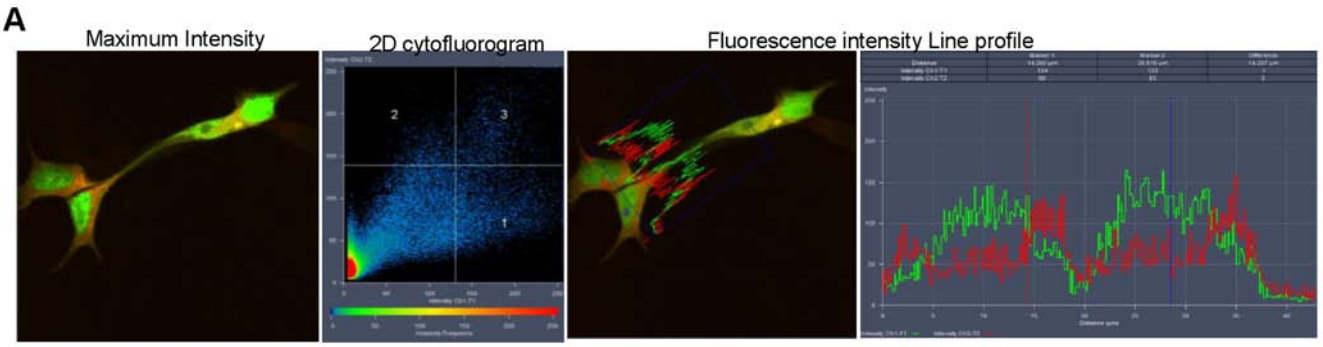


Figure 7. LRP/LR fails to co-localise with GFP. (A) Maximum intensity projection obtained from Z-stack analysis of GFP and LRP-dsRed shows poor co-localisation between the 2 proteins. This is further confirmed by the lack of a clear diagonal passing through quadrant 3 and the line profile which shows no alignment between the 2 colour channels.
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tagged LRP/LR variant (LRP::FLAG) further confirmed these results as APP was not detected in the eluate of the assay (Figure 6C, 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-localise, the proximity between them is still present when SR-SIM is employed (i.e. 80 nm), but lost at the nanometer scale required for FRET to take place, thus indicating that 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-localise with LRP/LR. This is evidenced by the maximum intensity projection (Figure 7A) obtained from Z-stack analysis (Figure S4 in File S1). 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 lacking any co-alignment of the red and green channels further confirming the low degree of co-localisation between these proteins. These findings also validate the co-localisation seen between LRP and APP, β- and γ-secretase, as the observed co-localisation is as a result of the proximity of LRP/LR to the AD proteins and not simply due to the GFP labels.

Conclusions

In light of this study, we have found that LRP/LR is seen to co-localise with APP, β- and γ-secretase both on the cell surface and intracellularly within the cytoplasm. This co-localisation is limited to 200 nm when observed for BACE1 and LRP/LR, to 80 nm between APP and LRP/LR and less than 10 nm 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 cumulatively highlight the role of LRP/LR in Alzheimer's Disease.

Supporting Information

File S1 Contains Figures S1–S4. Figure S1, Z-stack analysis of co-localisation between PS1-GFP and LRP-dsRed. Figure S2, co-localisation between BACE1-GFP and LRP-dsRed as ascertained by z-stack analysis. Figure S3, Z-stack analysis of APP-GFP and its co-localisation with LRP-dsRed. Figure S4, lack of co-localisation between GFP and LRP-dsRed as evidenced by z-stack analysis.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SFW. Performed the experiments: KJ BL. Analyzed the data: KJ BL BDD. Contributed reagents/materials/analysis tools: CP. Wrote the paper: KJ. Edited the manuscript: SFW BDD.

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4.6 Prion Interactions with the 37kDa/67kDa Laminin Receptor on Enterocytes as a cellular model for intestinal uptake of prions

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Brief Overview of Article:

This original research article demonstrated the central role of the 37kDa/67kDa LRP/LR in mediating the uptake of ingested infectious prion particles into enterocytes. More importantly, it was revealed that this uptake is not limited by species specificity and rather may indeed facilitate cross-species prion protein infection. These results have great bearing with regards to the possible oral transmissibility of prion disorders between live stock (sheep, cervids and cattle) and from live stock to humans, which may pose a zoonotic disease risk. These results are therefore important to both the agricultural and national healthcare sectors. Furthermore, this article highlights the value of employing an enterocyte model system for investigations regarding alimentary prion infections.

Contribution: I contributed to the data analysis as well as wrote and edited this original research article.

*These authors contributed equally to this work.

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Prion Interaction with the 37-kDa/67-kDa Laminin Receptor on Enterocytes as a Cellular Model for Intestinal Uptake of Prions

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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.

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Keywords: CWD; prion; laminin receptor; scrapie; variant Creutzfeldt–Jakob disease

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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.

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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}

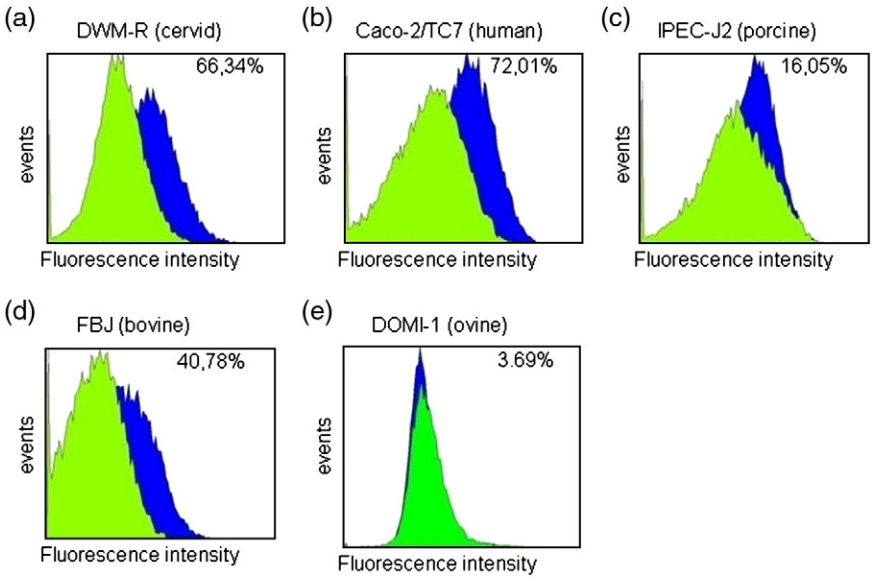


Fig. 1. Detection of cell surface LRP/LR levels on human and animal enterocytes by FACS analysis. (a) Cervid (DWM-R), (b) human (Caco-2/TC7), (c) porcine (IPEC-J2), (d) bovine (FBJ), and (e) ovine (DOMI-1) enterocytes were analyzed for LRP/LR surface level. LRP/LR levels were detected using the anti-LRP/LR 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

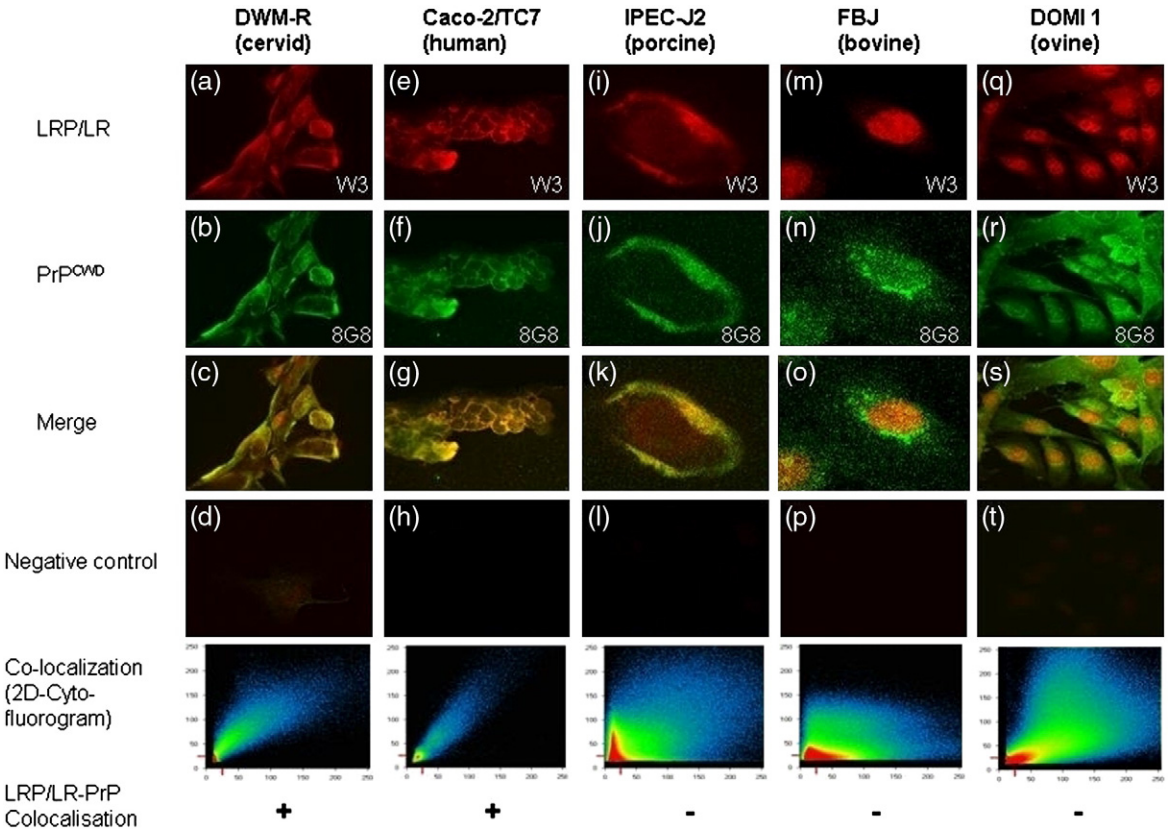


Fig. 2. Binding and colocalization of PrP^{CWD} with LRP/LR on human and cervid enterocytes. The 37-kDa/67-kDa LRP/LR was detected with the anti-LRP/LR antibody W3 on (a) cervid (DWM-R), (e) human (Caco-2/TC7), (i) porcine (IPEC-J2), (m) bovine (FBJ), and (q) ovine (DOMI-1) enterocytes. PrP^{CWD} was 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

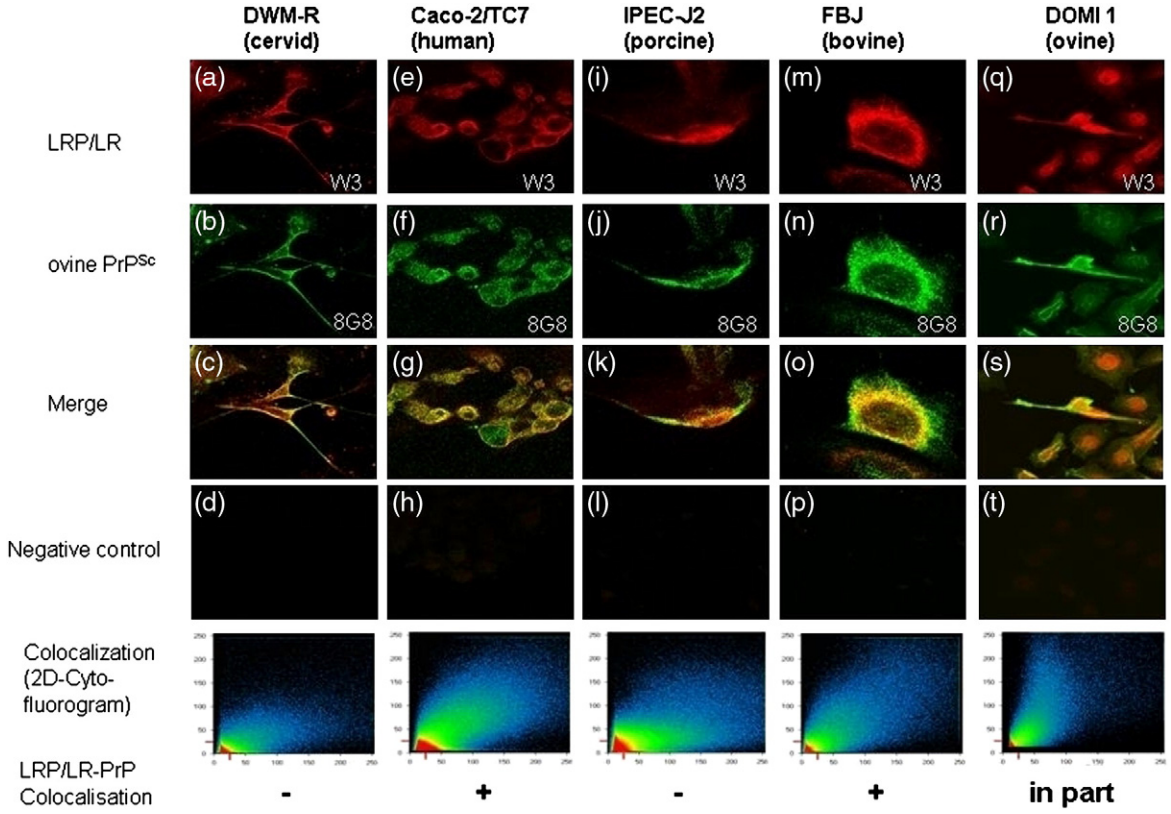


Fig. 3. Binding and colocalization of ovine PrP^{Sc} with LRP/LR on human and bovine enterocytes. The 37-kDa/67-kDa LRP/LR was detected on (a) cervid (DWM-R), (e) human (Caco-2/TC7), (i) porcine (IPEC-J2), (m) bovine (FBJ), and (q) ovine (DOMI-1) enterocytes by the anti-LRP/LR antibody W3. Ovine PrP^{Sc} 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

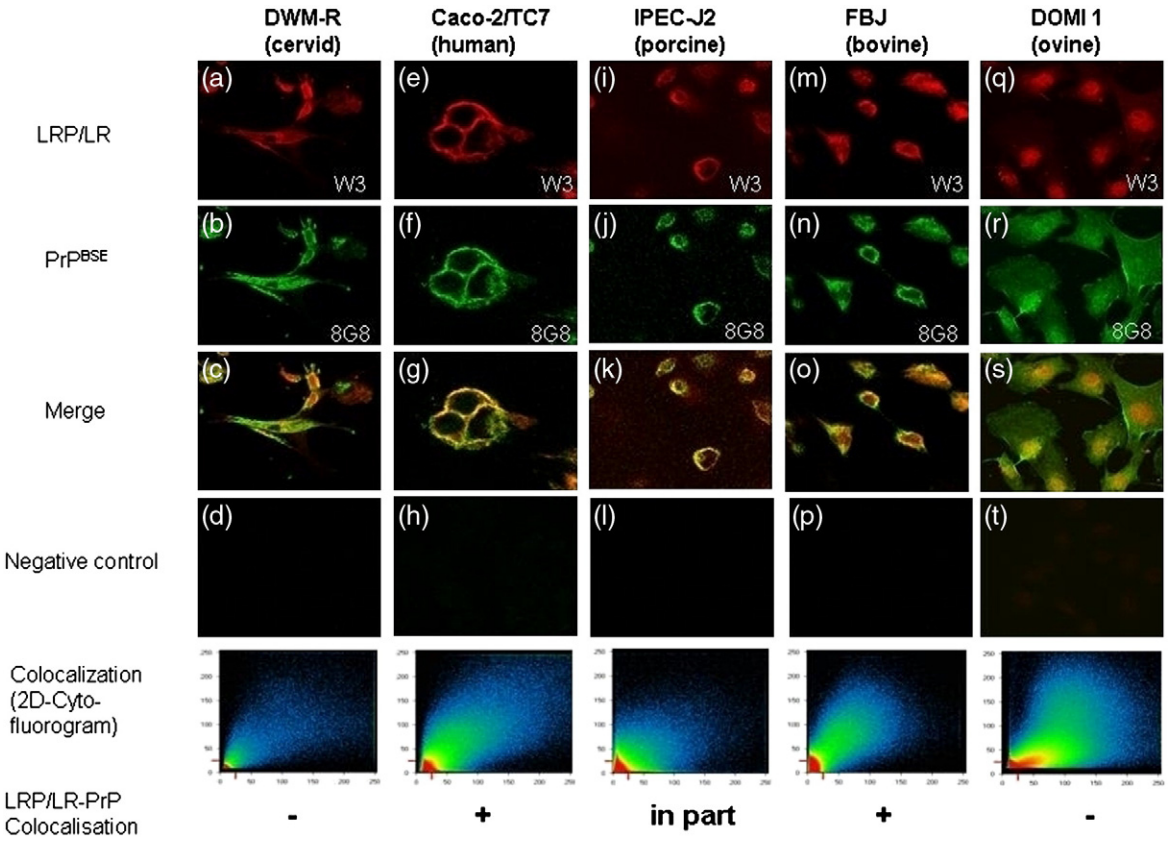


Fig. 4. Binding and colocalization of BSE prions with LRP/LR on human and bovine enterocytes. (a–d) Cervid (DWM-R), (e–h) human (Caco-2/TC7), (i–l) porcine (IPEC-J2), (m–p) bovine (FBJ), and (q–t) ovine (DOMI-1) enterocytes were incubated with BSE-infected brain homogenate (total protein concentration, 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

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4.7 Inhibition of angiogenesis by antibodies directed against the 37kDa/67kDa laminin receptor *in vitro*

Authors: R. Khusal*, B. Da Costa Dias*, K. Moodley, U. Reusch, S. Knackmuss, M. Little and S.F.T. Weiss.

Journal: PLOS One 8(**3**): e58888. doi:10.1371/journal.pone.0058888

Year of Publication: 2013

Brief Overview of Article:

This research article demonstrated for the first time that antibodies directed against the 37kDa/67kDa LRP/LR may inhibit the morphogenesis of endothelial cells into tubular structures. As the receptor has been repeatedly shown to have a central role in metastatic cancer (through promotion of cell proliferation, adhesion and migration), largely as a result of its interaction with laminin-1, this study aimed to investigate whether LRP/LR influenced tube formation in the human umbilical vein endothelial (HUVE) angiogenesis model system. Upon, application of anti-LRP/LR specific antibody, W3, angiogenic tube formation was completely abolished. These results therefore suggest that antibody blockade of the receptor ceased the differentiation of HUVE cells into tubular structures. Furthermore, since angiogenesis is vital for tumour progression, it may be proposed that anti-LRP/LR specific antibodies may show promise for the treatment of tumour angiogenesis and further confirms the potential of these tools as potential anti-cancer therapeutics.

Contribution: I contributed significantly to data analysis as well as wrote and edited this original research article.

*These authors contributed equally to this work.

In Vitro Inhibition of Angiogenesis by Antibodies Directed against the 37kDa/67kDa Laminin Receptor

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Abstract

The 37kDa/67kDa laminin receptor (LRP/LR) is a central receptor mediating interactions between tumour cells and the basement membrane and is thereby a key player in adhesion and invasion, essential processes in metastatic cancer. To affect continued tumour growth, tumours induce angiogenesis for the constant delivery of nutrients and oxygen. This study aims to determine the blocking effect of the anti-LRP/LR specific antibody, W3 on the angiogenic potential of HUVE (human umbilical vein endothelial) cells. Flow cytometric analysis revealed that 97% of HUVE cells display cell surface LRP/LR. An angiogenesis assay was conducted employing HUVE cells seeded on the basement membrane reconstituent Matrigel™ supplemented with the pro-angiogenic factor vascular endothelial growth factor (VEGF). Post 18h incubation at 37°C tubular structures, namely tube lengths were assessed. Treatment of established tubular structures with 100 μg/ml anti-LRP/LR specific antibody completely blocked angiogenesis. Our findings suggest a central role of the 37kDa/67kDa LRP/LR in tube formation and recommends anti-LRP/LR specific antibodies as potential therapeutic tools for treatment of tumour angiogenesis.

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Competing Interests: Stefan F. T. Weiss is currently a PLOS ONE Editorial Board Member. U. Reusch, S. Knackmuss, and M. Little are affiliated with or employed by Affimed Therapeutics AG, a commercial company which produces therapeutic antibodies for the treatment of cancer and inflammatory diseases. Furthermore, the anti-LRP/LR antibodies used in this study for the blockade of angiogenesis have been described in two international patents as potential therapeutic anti-cancer tools. Namely patent, EP0984987, entitled "A soluble laminin receptor precursor and methods to inhibit its interactions" has claims directed to a pharmaceutical composition comprising a soluble laminin receptor precursor or functional derivative or fragment thereof and is owned by the University of the Witwatersrand. This patent has been validated in the United Kingdom and Germany. The second patent, EP1670826, is co-owned by the University of the Witwatersrand and Affimed Therapeutics AG and is entitled "Single-chain antibody acting against 37 kda/67 kda laminin receptor as tools for the diagnosis and therapy of prion diseases and cancer, production and use thereof ". This granted European patent was validated in the United Kingdom, France, Germany, Switzerland and Austria. The claims are directed to a single chain antibody molecule specifically targeting LRP/LR for the treatment of prion diseases or cancer. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Angiogenesis, the formation of new blood vessels from pre-existing capillaries[1], is a physiologically vital process involved in embryonic development, wound healing; the female menstrual cycle, tissue growth[1] and vascular remodeling.[2] This process is highly regulated in healthy individuals. However, the de-regulation of angiogenesis has been implicated in numerous diseases including rheumatoid arthritis, ischemic heart and limb disease and retinopathy.[1] Angiogenesis is also a vital event in tumour growth and metastasis.[3]

The endothelial cells involved in the angiogenic process are responsive to two sets of cellular signals namely: soluble factors and cell signaling events transduced through the interactions with the extracellular matrix.[4,5] Soluble pro-angiogenic factors include: basic fibroblast growth factor (bFGF), transforming growth factor-α (TGFα), platelet derived endothelial cell growth factor (PDGF), insulin-like factors (IGF1 and IGF2) and tumour necrosis factor α (TNFα)[6] all of which are constituents of Matrigel™, the basement reconstituent employed in angiogenesis investigations. Furthermore, the vascular endothelial growth factor (VEGF), is the principle angiogenic inducer.[6,7,8] Angiogenesis is a multi-step process involving endothelial cell activation and subsequent degradation of the surrounding extracellular matrix or basal lamina.[1] This results in protease activation and subsequent release of pro-angiogenic factors/ peptides which in turn stimulate endothelial cell migration towards the angiogenic signal, proliferation and differentiation.[1,3]

Tumour angiogenesis involves tumour blood vessels that support continued tumour growth.[2] Once tumours exceed a certain maximal diameter, diffusion of oxygen and nutrients become limited and the resultant hypoxia and nutrient deprivation results in the secretion of growth factors and ultimately the onset of angiogenesis and subsequent tumour progression. Thus tumour cells affect vascular endothelial cells by paracrine mechanisms.[9]

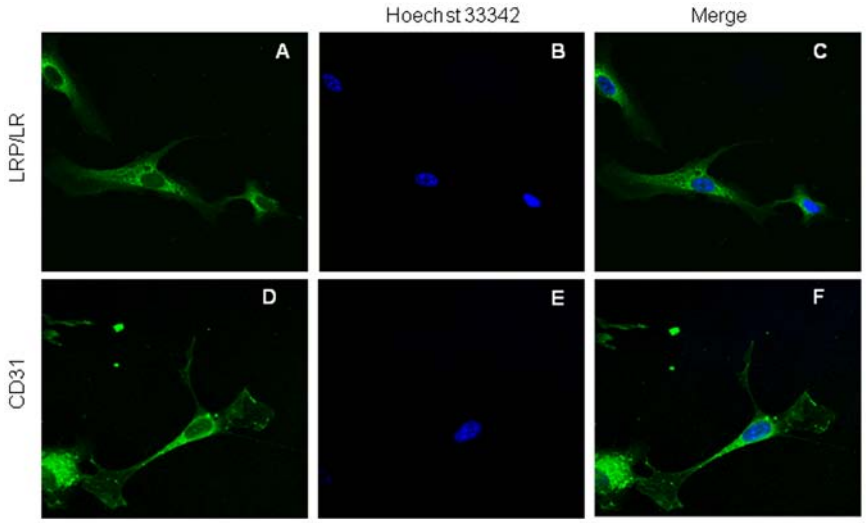


Figure 1. Detection of cell surface 37kDa/ 67kDa LRP/LR and CD31 on HUVE cells by immunofluorescence microscopy. HUVE cells were seeded on coverslips and allowed to proliferate until 30–40% confluency was reached. Non-permeabilised cells were fixed and were indirectly labeled with either an anti-human FITC (fluorescein- isothiocyanate) coupled antibody (Cell Lab) for LRP/LR detection (A) or anti-CD31-FITC antibody (Sigma-Aldrich) (D) . CD31 is an endothelial cell marker and serves as a positive control. Cells were subsequently stained with the Hoechst 33342 nuclear stain (Sigma-Aldrich) (B and E). Merged images (C and F) illustrate cell surface detection of LRP/LR and CD31 in conjunction with nuclear staining, respectively. Magnification: x63. An Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software were employed for image acquisition.
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Owing to the crucial role of angiogenesis in tumour progression and metastasis, selective inhibition of tumour angiogenesis has become a promising approach in anti-cancer therapy.[10]

As previously stated, cell-ECM interactions are imperative in angiogenesis and the basement membrane is of particular importance in this regard. Laminins are cross-shaped trimeric glycoproteins critical in the maintenance of basal membrane structure.[3,11] Of the 15 available laminin isoforms- laminin-1 (α1β1èÈ1) is of particular interest in angiogenesis as it mediates endothelial cell adhesion and differentiation[1], tube formation and furthermore modulates the activity of endostatin, an angiogenic inhibitor that blocks tube formation[12]. This laminin isoform is the major glycoprotein component of Matrigel^{TM}. [3] The α1 chain of laminin-1 contains an IKAV (isoleucine, lysine, alanine and valine) site which promotes collagenase, plasminogen and metalloprotease activity.[3,13,14] The activation of these enzymes results in matrix degradation thereby permitting cellular detachment and migration and the release of matrix-sequestered pro-angiogenic factors, all of which are central to successful tube formation.[3]

A central receptor in mediating the cell growth, movement and differentiation properties of laminin is the non-integrin 37kDa/ 67kDa laminin receptor (LRP/LR) which binds to the ECM component with high affinity.[15,16] LRP/LR possess two laminin-1 binding sites, a direct binding domain termed a peptide G sequence (161aa–180aa) and an indirect binding domain located towards the carboxyl-terminus (205aa–229aa).[15,16] This type-II transmembrane receptor is overexpressed in numerous cancers (gastric[17], breast[18], cervical[19], colon[20], colorectal[21], lung[22], ovarian, pancreatic[23] and prostate[24]) , correlates with cancer aggressiveness and it has been proposed that LRP/LR may be indicative of tumour prognosis.[23,24,25] LRP/LR downregulation has been shown to induce apoptosis and potentially hamper proliferation in cancer cell lines.[26] LPR/LR is implicated in numerous tumourigenic processes which are akin to angiogenesis namely (tumour) cell adhesion, invasion[27,28], viability, proliferation and migration.[15,16] Within classical tumour biology these processes are required for the cell invasion and the formation of metastasis.

Moreover, it is the interaction between LRP/LR and laminin-1 that results in proteolytic activation, a process central to angiogenesis, as previously discussed. Furthermore, a role for LRP/LR in tube formation has previously been proposed.[4] This study aimed to investigate the angiogenic blocking effect of anti-LRP/LR specific antibodies on the *in vitro* angiogenesis of the primary endothelial cell line, human umbilical vein endothelial (HUVE) cells.

Materials and Methods

Cell culture and conditions

HUVE cells (Invitrogen, Gibco) were cultured in Medium 200 (Invitrogen, Gibco) supplemented with Low Serum Growth supplement (LSG) (Invitrogen, Gibco) such that the resultant media consisted of: 2% (v/v) fetal bovine serum; 1 μg/ml hydrocortisone; 10 ng/ml human epidermal growth factor (EGF); 3 ng/ml basic fibroblast growth factor (bFGF) and 10 μg/ml heparin.

Reagents and Antibodies

Matrigel^{TM}, employed to induce tube formation is derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, serving as a reconstituted basement membrane, was obtained from BD Biosciences.

Polyclonal anti-LRP/LR antibody W3 was produced as described previously by Rieger et al., (1997). [29]

IgG1-HD37 was recombinantly produced in a mammalian expression system as described by Zuber *et al.*, (2008).[27] In brief, human embryonic kidney cells (HEK293 EBNA) expressing the EBNA-1 gene were transiently co-transfected, by calcium phosphate methodology, with plasmids encoding the heavy (p EU1.2 VH_HD37) and light chains (p EU4.2 VL_HD37) of the anti-

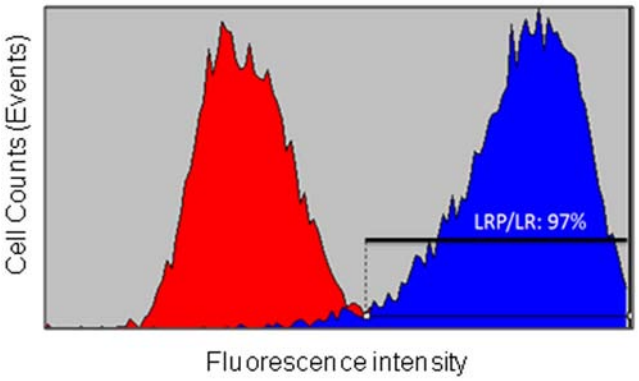


Figure 2. Flow cytometric detection of 37kDa/67kDa LRP/LR levels on the surface of HUVE cells. Cell surface LRP/LR levels on the surface of non-permeabilised HUVE cells were ascertained primarily by incubating cells with IgG1-iS18 followed by incubation with anti-human-FITC coupled secondary antibodies (Sigma-Aldrich). The red curve represents the no antibody control, whilst the blue curve represents treatment with both antibodies. The percentage represents the proportion of cells exhibiting LRP/LR on their cell surface and was calculated using a linked marker from the point of intersection between the curves and the end of the blue curve. A Coulter EPICS® XL-MCL flow cytometer was employed and ten thousand cellular events were counted.
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Cluster of differentiation 19 (CD19) antibody IgG1-HD37. Affinity chromatography employing protein A sepharose was utilized for antibody purification.

Indirect Immunofluorescence microscopy

HUVE cells were seeded on sterilised cover slips and upon attaining 30–40%, the culture media was aspirated and cells fixed. Cell surface proteins of interest were detected with the appropriate primary antibodies, anti-LRP/LR specific antibody IgG1-iS18 or anti-cluster of differentiation 31 (CD31) coupled to fluorescein isothiocyanate (FITC) (Sigma Aldrich). These proteins were detected on separate cellular samples. Antibodies were diluted in 0.5% PBS-BSA. Post overnight incubation at 4°C, secondary antibody anti-human-FITC (Beckman Coulter) was added to cells treated with IgG1-iS18 and consequently incubated for 1h (in the dark at room temperature). As the CD31 antibody is a conjugated antibody this step was not performed. Thereafter, cells were subjected to Hoechst 33342 nuclear staining. Fluorescent images were acquired using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software.

Flow cytometric Analysis

Flow cytometry was employed to determine LRP/LR levels on the surface of non-permeabilised HUVE cells as described by [28]. Control samples were re-suspended in 100 µl of sheath fluid, whilst the experimental samples were re-suspended in 100 µl anti-LRP/LR specific antibody (IgG1-iS18) solution (30 µg/ml). Post an 1h incubation at room temperature samples were subsequently incubated in the presence of 100 µl anti-human-FITC secondary antibody (20 µg/ml) for 1h. Samples incubated solely with the secondary antibody served to control for background emission and the possible non-specificity of this antibody. Post final incubation, 10 000 cells per sample were analysed employing a Beckman Coulter EPICS® XL-MCL flow cytometer. Data shown is representative of three biological replicates.

Angiogenesis Assay

To determine the endothelial tube formation potential of HUVE cells and establish the optimal vascular endothelial growth factor (VEGF) concentration required for the induction of HUVE cell tube formation, an angiogenesis assay employing varying VEGF concentrations was conducted. A volume of 50 µl of Matrigel™ (BD Biosciences) was affixed to the wells of a pre-chilled 96 well plate and incubated at 37°C for 1h to allow for Matrigel™ to polymerise. Cell suspensions, in which VEGF (Sigma Aldrich) had been exogenously applied to achieve the varying concentrations (10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml and 30 ng/ml), were prepared (using Medium 200) and 4×10^4 cells were seeded in each well. Post incubation at 37°C for 18h, tubular morphology was assessed. A Zeiss inverted microscope was employed to examine tube formation and a Canon Camera V6.0. for imaging the cultures. Remote Capture version 2.7.3.23 and AxioVision LE 4.3 software were used for tube length analysis.

To examine the role of LRP/LR in endothelial tube formation and to evaluate the efficacy of the anti-LRP/LR antibody as an angiogenic inhibitor, an angiogenesis assay (as described above) was performed. Post Matrigel™ preparation, cell suspensions containing 15 ng/ml exogenous VEGF, were employed for cell seeding and post 18h incubation at 37°C, tube length was measured. Conditioned media was gently aspirated so as to minimise tubular disruption, varying antibody concentrations (5 µg/ml, 50 µg/ml and 100 µg/ml) of polyclonal anti-LRP antibody, W3 and IgG1-HD37 (negative control) were composed in Medium 200 and administered to cells. Post 24h incubation at 37°C, cells were again examined and tubular morphology analysed. Comparisons in measurements prior to and post antibody treatment of the same cells were conducted.

Statistics

Statistical analyses were performed using a two-tailed Students' *t*-test with a 95% confidence interval. *p*-values < 0.05 were considered significant

Results

Human umbilical vein endothelial cells express LRP/LR on their cell surface

As LRP/LR is a key receptor in mediating cellular adhesion, proliferation and migration, mediating the cellular effects of laminin-1 and has previously been implicated in angiogenesis, we examined whether the receptor was expressed on the surface of the HUVE cell model employed in this study. HUVE cells displayed LRP/LR on their cell surface as is depicted by the positive staining

Table 1. Effect of varying vascular endothelial growth factor (VEGF) concentrations on *in vitro* HUVE cell angiogenesis.[a]

VEGF concentration (ng/ml)	Average Tube length (µm)
0	12.13
10	12.17
15	13.68
20	10.98
25	12.31
30	10.34

[a]Cells were seeded on Matrigel™ (BD Biosciences) at a density of 4×10^4 cells/well and incubated in 5% CO_2 humidified atmosphere (37°C) for 18h.
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Table 2. Percentage reduction[a] of endothelial tube length in HUVE cells.

		W3		IgG1-HD37	
		Percentage reduction in tube length (%)	***p*-value**	**Percentage reduction in tube length (%)**	***p*-value**
Antibody concentration (μg/ml)	5	–21.62[b]	0.2980	1.85	0.9674
	50	64.72	0.0082	50.87	0.243
	100	100	0.0024	40.10	0.0544

[a]Reductions are calculated based on comparisons between the tube lengths of antibody treatments and no antibody treatments. Average tube length of the "No antibody" treatment was set to 100%.
[b]The negative value is indicative that the average tube length was 21.62% greater than that on the " No antibody" treatment (therefore 121.62%) and therefore rather than a tube reduction an increase was observed.
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in Fig.1A. Moreover, flow cytometric analysis revealed that 97% of HUVE cells (Fig.2) exhibited LRP/LR on their cell surface further verifying the results obtained by immunofluorescence microscopy. The cluster of differentiation 31 (CD31), also called platelet endothelial cell adhesion molecule (PECAM-1), is an abundantly expressed cell surface marker of endothelial cells involved in wound healing and angiogenesis[30,31] and served as the positive control (Fig.1D).

Optimal VEGF concentration for in vitro angiogenesis of HUVE cells

VEGF, the major pro-angiogenic factor, is up-regulated by hypoxia and is a key soluble factor secreted by tumour cells to induce angiogenic processes in endothelial cells (paracrine signaling). Furthermore, VEGF receptors are expressed on endothelial cells such as the HUVE cells but are present on few other cell types. As exogenous VEGF administration is required for tube formation on Matrigel™, we evaluated the concentration of VEGF which would provide maximal angiogenesis, as gauged

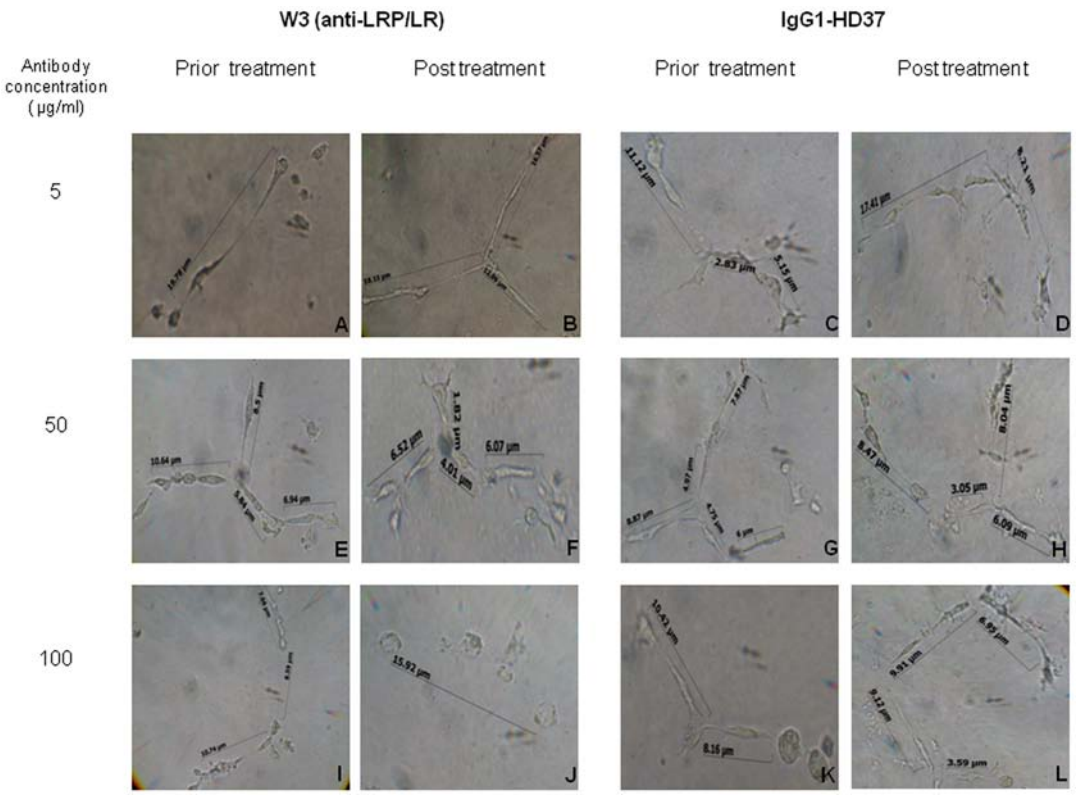


Figure 3. The anti-angiogenic effects of W3 on HUVE cell tube formation. HUVE cell suspensions were prepared with 15 ng/ml exogenously administered VEGF and plated on Matrigel™ (BD Biosciences) at a density of 4×10^4 cells/ well. Post 18h incubation, tubular structures were microscopically analysed and enumerated by Canon Camera V.6., Remote Capture Version 2.7.3.23 and Axio Vision LE 4.3 software, respectively. Post assessment conditioned media was gently aspirated to ensure minimal disruption of formed tubes, and fresh media with varying concentrations (5 μg/ml, 50 μg/ml and 100 μg/ml) of W3 (B, F, J) or IgG1-HD37 (negative control) (D, H, L) were administered the respective samples. Tubular morphology was assessed (as previously described) 24h post antibody treatment. Magnification: x40
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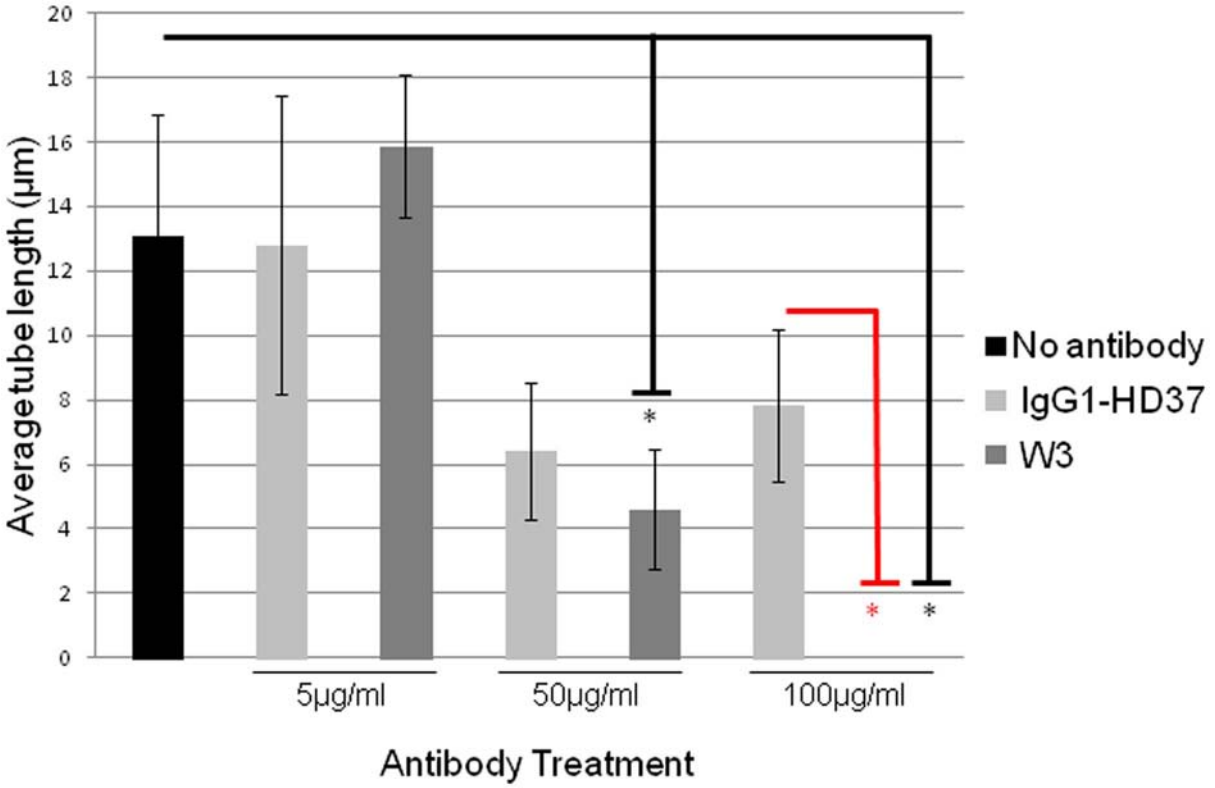


Figure 4. The effects of antibody treatment on the average tube length of HUVE cells. HUVE cell suspensions containing 15 ng/ml VEGF were prepared and plated on Matrigel™ as previously described. Post treatment with varying concentrations of (5 µg/ml, 50 µg/ml and 100 µg/ml) W3 or IgG1-HD37, tube length was enumerated. The bar graph depicts the average tube length post treatment. Error bars represent sd. *$p<0.05$; Student's *t*-test.
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according to tube length. Statistical evaluation of these results revealed no significant difference between the VEGF treatments (data not shown). However, the 15 ng/ml VEGF treatment displayed the highest average tube length (Table 1) and as such was the concentration employed for subsequent experimentation.

Anti-LRP/LR specific antibody reverses HUVE cell angiogenesis

The role of LRP/LR in the induction of angiogenesis has been proposed owing to its close association with tumourigenic processes, its interaction with laminin-1 and its role in the activation of matrix-remodeling enzymes. Thus we investigated whether impedance of the receptor by anti-LRP/LR specific antibody W3 would influence tubular morphology. Treatment of tubular structures with 50 µg/ml of W3 resulted in a significant reduction in tube length of 64.72%, whereas treatment with 100 µg/ml of W3 resulted in a significant 100% reduction in tube length (Fig.3 and Table 2). Treatment of tubular structures with IgG1 HD37 directed against CD19 did not significantly reduce tube length (Fig.3 and Table 2).

Discussion

Angiogenesis has received considerable attention over the past few decades as a possible target for pathological diseases which require vascularisation, most notably cancer.[9] Through selective inhibition of tumour angiogenesis, tumour growth and progression and the success of metastatic tumourigenic cells at distal sites, owing to oxygen and nutrient deprivation, will be halted. Thus therapeutics aimed at decreasing vascularisation are promising anti-cancer tools which may be effective against numerous cancers.

The rate-limiting step in the angiogenic process is the degradation of the basement membrane which is promptly followed by endothelial cell detachment, proliferation and re-organisation into tubular structures. A key receptor in cellular adhesion to the basal membrane is the 37kDa/67kDa LRP/LR.[28] Through interactions with the laminin-1, the major glycoprotein component of the basal lamina and Matrigel™ basal membrane reconstituent employed here, LRP/LR mediates cellular attachment and induces proteolytic activation of type IV collagenase and other matrix metalloproteases.[32,33] These in turn degrade the basal membrane, release matrix-sequestered pro-angiogenic factors and allow for cellular migration towards the angiogenic stimulus. Thus, since angiogenesis requires basal membrane degradation and LRP/LR plays a fundamental role in this process, immunofluorescence microscopy and flow cytometry analyses were performed to detect and determine the proportion of HUVE cells which expressed LPR/LR on their cell surface. Once LRP/LR was confirmed to be located on the cell surface of HUVE cells (Fig.1A), flow cytometric analysis revealed that 97% of the examined cells displayed LRP/LR on their cell surface (Fig.2). It has been reported that neoplastic cell lines express very high levels of LRP/LR on their cellular surface when compared to non-tumorigenic controls[27,28] and that these elevated levels correlate with an increased invasive potential.[27,28] Although HUVE cells are non-tumorigenic, the high LRP/LR levels correlates to the invasive role of these cells as they

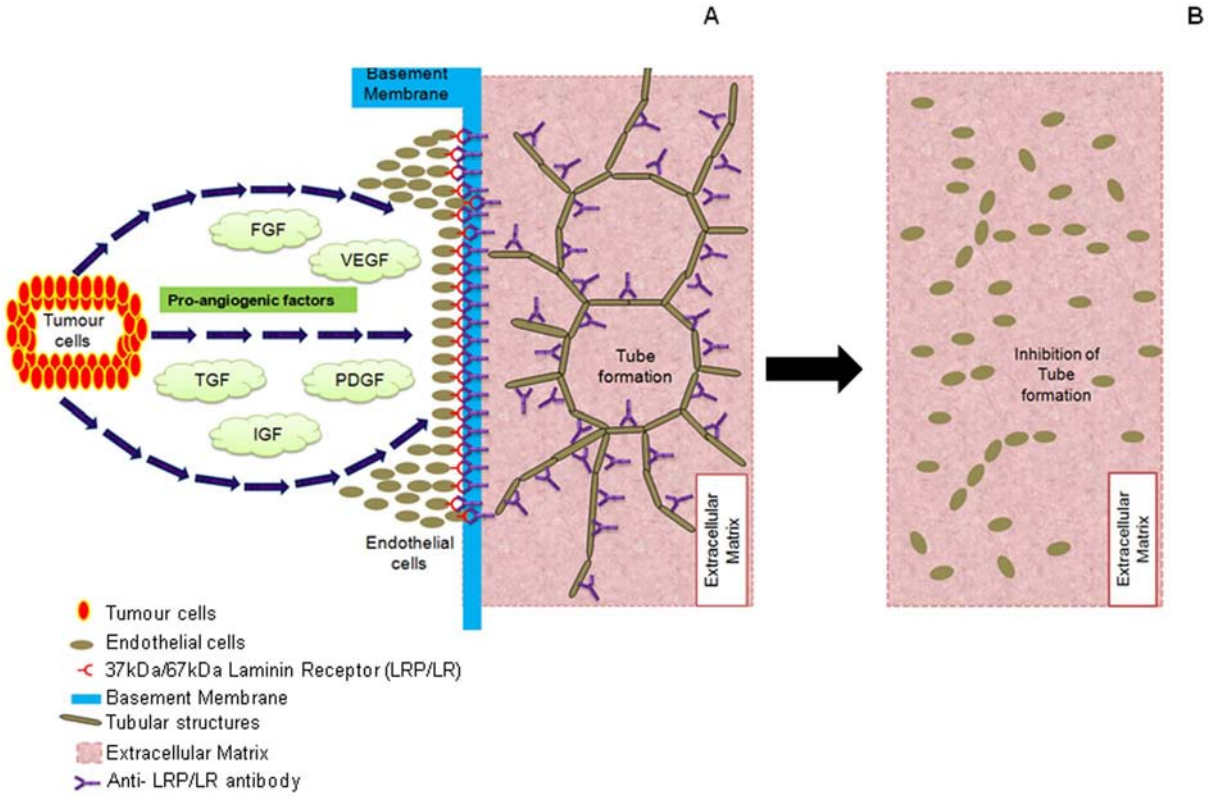


Figure 5. A schematic representation of the effect of anti-LRP/LR specific antibodies on angiogenic tube formation. (A) The administration of anti-LRP/LR antibody W3, to HUVE cells which had established tubular structures on Matrigel™, inhibited further degradation of the basement membrane, a requirement for tube formation. This halted the development for additional tubular structures. Moreover, the antibody also bound to existing tubes and thereby blocked the interaction between LRP/LR and Laminin-1, hence resulting in (B) the reversal of tube formation and cells were consequently observed as single cells on the Matrigel™.
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are required to degrade the basal membrane and migrate towards stimuli for the formation of 3D tubular structures.

Thus far, the most influential inducer of angiogenic activity is the stimulation of the VEGF molecular signaling pathway.[34] It has been reported that successful angiogenesis may be induced upon administration of VEGF within the 10 ng/ml – 30 ng/ml range.[35,36,37] However, the exogenous administration of VEGF has been shown to possess a biphasic response.[38] In this study, maximal tube length was observed at a VEGF concentration of 15 ng/ml (Table 1). Therefore, the application of 15 ng/ml exogenous VEGF in subsequent experiments was justified.

Previous studies have shown that the adhesive and invasive potential of numerous cancer types (fibrosarcoma, lung, cervical, breast, colon and prostate) is significantly reduced upon application of anti-LRP/LR specific antibodies, namely IgG1-iS18.[27,28] Other tools targeting LRP/LR, including RNA interference (RNAi) technology, the pentosan polysulfate and the heparan mimetic HM2602[16,27,28] have similarly hampered the invasion of tumourigenic cells. The mechanism of action whereby these modalities are suggested to impede invasion is through the impedance of the LRP/LR – laminin-1 interaction which subsequently thwarts cellular adhesion, this being a vital process preceding cellular invasion.

HUVE cell angiogenesis was similarly disrupted (50 μg/ml) (Fig. 3F) and completely abolished (100 μg/ml) (Fig.3J) upon administration of the anti-LRP/LR specific antibody. When compared to the no antibody control, a significant tube length reduction of 64.72% and 100% was observed upon treatment with 50 μg/ml and 100 μg/ml W3, respectively (Fig.4 and Table 2). These results therefore demonstrate that anti-LRP/LR specific antibody W3 significantly blocked tube formation by HUVE cells – thereby reiterating the fundamental role of LRP/LR in angiogenesis. This is depicted schematically in Fig.5. This is the first work to demonstrate that antibodies directed against the non-integrin laminin receptor (LRP/LR) may inhibit the morphogenesis of endothelial cells into tubular structures. It has also been reported that antibodies directed against laminin-1 under similar experimental conditions (HUVE cell induced angiogenesis on Matrigel™), did not inhibit cellular adhesion to the matrix but did preclude tube formation.[39] Therefore, it may be suggested that the anti-LRP/LR antibody W3, blocked the interaction between LRP/LR and laminin-1, thereby ceasing differentiation of HUVE cells into tubular structures.

In summary, the strikingly significant abolishment of tubular structures in the HUVE cell angiogenesis model by W3, suggests that anti-LRP/LR specific antibodies may prove a potential therapeutic tool for the treatment of tumour angiogenesis.

Author Contributions

Assisted with the immunofluorescence microscopy: CP KM. Conceived and designed the experiments: SFTW. Performed the experiments: RK. Analyzed the data: RK. Contributed reagents/materials/analysis tools: KM CP UR SK ML. Wrote the paper: BDCD.

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4.8 Anti-LRP/LR specific antibody IgG1-iS18 impedes adhesion and invasion of liver cancer cells.

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Brief Overview of Article:

In this original research article the ability of anti-LRP/LR specific antibody IgG1-iS18 to hamper the adhesion and invasion of liver cancer was demonstrated. Approximately 782000 people were diagnosis with liver cancer and 352000 people were diagnosed with leukaemia in 2012, making these the 6th and 11th most diagnosed cancers globally (GLOBOCAN and http://www.wcrf.org/cancerstatistics/worldcancerstatistics.php). In this study the influence of the 37kDa/67kDa LRP/LR in the adhesive and invasive potential of liver and leukaemia neoplastic cells was investigated. The cell surface LRP/LR expression levels were significantly higher in the examined liver cancer cell line (HUH-7) when compared to the non-invasive breast cancer cell line (MCF-7). Conversely, the leukaemia cell line (K562) revealed significantly lower cell surface LRP/LR levels in comparison to the same control. Moreover, upon antibody blockade of the receptor a significant reduction in the adhesive and invasive potential of the liver cancer cells was detected. These results therefore suggest that anti-LRP/LR specific antibodies may show promise as tools for the treatment of metastatic liver cancer.

Contribution: I contributed significantly to the data analysis as well as edited this original research article.

Anti-LRP/LR Specific Antibody IgG1-iS18 Impedes Adhesion and Invasion of Liver Cancer Cells

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Abstract

Two key events, namely adhesion and invasion, are pivotal to the occurrence of metastasis. Importantly, the 37 kDa/67 kDa laminin receptor (LRP/LR) has been implicated in enhancing these two events thus facilitating cancer progression. In the current study, the role of LRP/LR in the adhesion and invasion of liver cancer (HUH-7) and leukaemia (K562) cells was investigated. Flow cytometry revealed that the HUH-7 cells displayed significantly higher cell surface LRP/LR levels compared to the poorly-invasive breast cancer (MCF-7) control cells, whilst the K562 cells displayed significantly lower cell surface LRP/LR levels in comparison to the MCF-7 control cells. However, Western blotting and densitometric analysis revealed that all three tumorigenic cell lines did not differ significantly with regards to total LRP/LR levels. Furthermore, treatment of liver cancer cells with anti-LRP/LR specific antibody IgG1-iS18 (0.2 mg/ml) significantly reduced the adhesive potential of cells to laminin-1 and the invasive potential of cells through the ECM-like Matrigel, whilst leukaemia cells showed no significant differences in both instances. Additionally, Pearson's correlation coefficients suggested direct proportionality between cell surface LRP/LR levels and the adhesive and invasive potential of liver cancer and leukaemia cells. These findings suggest the potential use of anti-LRP/LR specific antibody IgG1-iS18 as an alternative therapeutic tool for metastatic liver cancer through impediment of the LRP/LR- laminin-1 interaction.

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Competing Interests: S.F.T.W. is currently a PLOS ONE Editorial Board Member. U.R., S.K. and M.L. are affiliated with or employed by Affimed Therapeutics A.G., a commercial company which produces therapeutic antibodies for the treatment of cancer and inflammatory diseases. Furthermore, the anti-LRP/LR antibodies used in this study for the blockade of invasion and adhesion have been described in two international patents as potential therapeutic anticancer tools. Namely patent, EP0984987, entitled "A soluble laminin receptor precursor and methods to inhibit its interactions" has claims directed to a pharmaceutical composition comprising a soluble laminin receptor precursor or functional derivative or fragment thereof and is owned by the University of the Witwatersrand. This patent has been validated in the United Kingdom and Germany. The second patent, EP1670826, is co-owned by the University of the Witwatersrand and Affimed Therapeutics AG and is entitled "Single chain antibody acting against 37 kDa/67 kDa laminin receptor as tools for the diagnosis and therapy of prion diseases and cancer, production and use thereof". This granted European patent was validated in the United Kingdom, France, Germany, Switzerland and Austria. The claims are directed to a single chain antibody molecule specifically targeting LRP/LR for the treatment of prion diseases or cancer. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Cancer is a global burden that has been shown to be the leading cause of death in economically developed countries and the second leading cause of death in economically developing countries[1]. According to the World Cancer Research Fund (WCRF), an estimated 14.1 million cases of cancer were diagnosed in the year 2012 and it is predicted that approximately 24 million new cases of cancer will be diagnosed by the year 2035, globally (http://www.wcrf.org/cancer_statistics/). Currently, lung cancer has been identified as the most commonly diagnosed cancer type, with the two cancer types central to the present study namely liver cancer and leukaemia, being ranked as sixth and eleventh most diagnosed cancer types, respectively (GLOBOCAN). It has been reported that approximately 782000 cases of liver cancer and 352000 cases of leukaemia were diagnosed in the year 2012 (http://www.wcrf.org/cancer statistics/world cancer statistics.php), thus indicating the pressing need to develop effective treatments against cancer.

Cells are largely dependent on the extracellular matrix (ECM), which is the non-cellular component of all tissues and organs that provides a physical scaffold to cellular components and also assists with initiation of essential biochemical processes needed for proper tissue differentiation, homeostasis and morphogenesis[2]. Cells adhere to the ECM via the action of ECM receptors[2]. Particularly, the non-integrin 37-kDa/67-kDa laminin receptor (LRP/LR) is a major component of the extracellular matrix, assisting in numerous physiological processes[3,4,5]. It is suggested that 37-kDa LRP is the precursor of the 67-kDa high affinity laminin receptor LR, however, the exact mechanism by which the precursor forms the receptor is unknown[6].

LRP/LR is predominantly a transmembrane receptor, however, it is also evident in the nucleus and the cytosol[7,8]. In the nucleus, LRP/LR plays a critical role in the maintenance of

nuclear structures whilst in the cytosol, it assists in translational processes[8]. As a transmembrane receptor, LRP/LR serves several functions such as cell migration[9], cell-matrix adhesion[10], cell viability and proliferation[3,4,5].

LRP/LR has been shown to have a high binding affinity for laminin-1. Laminin-1 is part of a family of laminins, which are extracellular matrix proteins that constitute several non-collagenous glycoproteins that are found in the basement membrane[11,12]. This glycoprotein is believed to play critical roles in cell attachment[11], assembly of the basement membrane[11], cell growth and differentiation[13], cell migration[11,14], neurite outgrowth[11,15] and angiogenesis[16]. Laminin-1 has also been shown to promote the invasive phenotype of tumorigenic cells[17].

LRP/LR has been found to be over-expressed on the surface of several tumorigenic cells[18]. The result of this over-expression is an increased interaction between LRP/LR and laminin-1, and this interaction has been shown to be crucial in enhancing adhesion and invasion – two key components of metastasis[19]. Essentially, laminin-1 in the basement membrane interacts with LRP/LR on the surface of tumorigenic cells leading to adhesion[19]. This, in turn, results in the secretion of proteolytic enzymes such as type IV collagenase in order to hydrolyse type IV collagen in the basement membrane, thereby allowing tumorigenic cells to invade and eventually translocate to a secondary site[19].

Since the LRP/LR-laminin-1 interaction has been identified as the crucial event in adhesion and invasion, blockage of this interaction could be deemed as an essential mechanism to treat metastatic cancer. This implicates LRP/LR as a target for the treatment of metastatic cancer. Furthermore, several studies have shown that application of anti-LRP/LR specific antibodies significantly reduces the adhesive and invasive potential of certain tumorigenic cells, such as HT1080 fibrosarcoma[18], lung[4], cervical[4], colon[4], prostate[4], breast[20] and oesophageal[20] cancer cells. Particularly, anti-LRP/LR specific antibody IgG1-iS18 has been suggested to interrupt the LRP/LR-laminin-1 interaction [4], thus IgG1-iS18 may be deemed as a possible therapeutic tool in the treatment of metastatic cancer.

In this study, the ability of anti-LRP/LR-specific antibody IgG1-iS18 to impede the adhesive and invasive potential of leukaemia and liver cancer cells was investigated. Due to the high incidence and mortality rates regarding these two cancer types, alternative therapeutic options become a necessity. It is noteworthy that similar studies have been conducted, however, it is possible that not all metastatic cancer cell types may be responsive to IgG1-iS18 treatments. It therefore becomes necessary to carry out these metastatic studies on different cancer types in order to gain insight into the use of the antibody as an alternative broad spectrum therapeutic antibody for the treatment of various cancer types. Thus, this study was conducted with the aim of determining whether IgG1-iS18 is capable of significantly reducing the adhesive and invasive potential of leukaemia and liver cancer cells, therefore providing the possibility for the antibody to be used as an alternative therapeutic tool in the treatment of these two cancer types.

Materials and Methods

Cell culture and conditions

Human breast adenocarcinoma (MCF-7), liver carcinoma (HUH7) and leukaemia (K562) cell lines obtained from ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/l) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 5% CO_2 and 37°C.

Reagents and antibodies

Matrigel used for cell invasion assays is derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma and was obtained from BD Biosciences.

Laminin-1used for cell adhesion assays was obtained from Sigma-Aldrich.

Chloramphenicol acetyl transferase (CAT) antibody was obtained from Sigma-Aldrich.

IgG1-iS18 was recombinantly produced in a mammalian expression system as reported by

Zuber et al., (2008)

Confocal microscopy

In order to visualize the location of LRP/LR on the cell surface, confocal microscopy was employed. Cells were first seeded on coverslips and allowed to reach 70% confluency. Cells were fixed in 4% paraformaldehyde in PBS for approximately 15 minutes followed by several washes with PBS. Cells were blocked in 0.5% BSA in PBS for 5-10 minutes. After one PBS wash, excess PBS was blotted off. Cover slips containing cells were placed on a glass slide (with cells facing upwards) and this was followed by addition of primary antibody IgG1-iS18 (1:100) diluted in 0.5% BSA. Post an overnight incubation at 4°C, coverslips were rinsed thrice in PBS/BSA. After addition of the FITC-coupled secondary antibody that had been diluted in 0.5% BSA, incubation in the dark was allowed for 1 hour. Followed by three washes as before, DAPI diluted in PBS was then administered for 5–10 minutes to allow for staining of the nucleus. Cells were finally washed once in PBS alone and mounted onto a clean slide using GelMount (Sigma-Aldrich). A period of 45 minutes was allocated to allow for setting to take place.

Flow cytometry

Quantification of cell surface levels of LRP/LR was conducted using flow cytometry. EDTA(5 mM) in PBS was used to facilitate detachment of adherent cells which was followed by centrifugation at 1200 rpm, 10 min. Cells were subsequently fixed by re-suspending cells in PFA for 10 min at 4°C. Cells were again centrifuged in 1X PBS which allowed for the preparation of five cell suspensions, one to which no antibody was added (thus serving as the unstained control), one to which anti-CAT antibody was added (serving as an isotype control) and one to which anti-LRP/LR specific antibody IgG1-iS18 was added. The remaining two cell suspensions were incubated only in PBS in order to be used as negative controls for the IgG1-iS18 and anti-CAT antibody. All suspensions were incubated at room temperature for 1 hour. Following three washing steps with 1X PBS, goat anti-human phycoerythrin (PE)-coupled secondary antibody (Beckman Coulter) was added to the cell suspension containing the IgG1-iS18 primary antibody as well as one of the suspensions that was incubated in PBS only. The cell suspension that was incubated with the anti-CAT antibody as well as the remaining cell suspension that was incubated only in PBS, were both supplemented with a goat anti-rabbit allophycocyanin (APC)-coupled secondary antibody followed by another 1 hour incubation period of all cell suspensions. Furthermore, three post-incubation washes were performed and cell suspensions were analysed using the BD Accuri C6 flow cytometer. Experiments were performed in triplicate and repeated at least three times.

SDS PAGE and Western blotting

Total LRP/LR levels were determined by the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

To perform the SDS-PAGE, 10 μg of total protein was used. Proteins that were separated according to size by SDS-PAGE were then identified by application of specific antibodies in the process of Western blotting. The proteins resolved on the polyacrylamide gel were transferred onto a polyvinylidene fluoride (PVDF) membrane using 1X transfer buffer (20% methanol in 192 mM glycine and 25 mM Tris) for 45 minutes at 350 mV and a semi-dry transferring apparatus. Blocking buffer (3% BSA in 1X PBS Tween) was then used in order to block the blotted membrane for 1 hour. Once blocked, the membrane was probed with anti-LRP/LR specific primary antibody IgG1-iS18 (1:10000) for 1 hour. Prior to incubation of the membrane with goat-anti-human-peroxidase (1:5000) secondary antibody, three washes with 1X PBS Tween were performed. A further three washes in 1X PBS Tween were performed after incubation in the secondary antibody, followed by the detection of HRP by use of an enhanced chemiluminescent substrate (Thermo scientific). The resulting fluorescence was developed and fixed onto an X-ray film. Experiments were executed in triplicate and repeated at least 3 times.

Adhesion assay

In order to assess the adhesive potential of the varying tumorigenic cell lines to the basement membrane *in vitro*, laminin-1 (10 μg/ml)(BD Biosciences) was used to coat 96 microwell plates, leaving uncoated wells to be used as negative controls. After the coating of the wells for 1 hour and washing with 0.1% BSA in DMEM, other protein binding sites on the microwell plate were blocked using 100 μl of 0.5% BSA in DMEM for one hour. Cells were suspended in serum-free culture medium and added to wells at a density of 4×10^5 cells/ml in order to assess the adhesion potential. Furthermore, cells that have been pre-incubated with IgG1-iS18 (0.2 μg/ml) and with anti-CAT (Sigma, 0.2 μg/ml) antibody as the negative control were added to the relevant wells in order to examine the effects of the antibodies on the adhesion potential of the cells. The plates were incubated at 37°C for 1 hour and thereafter, non-adherent cells were washed away with PBS and adherent cells were fixed with 4% paraformaldehyde for 10 minutes. Adherent cells were stained with 0.1% crystal violet. The stain was extracted using 1% SDS and the absorbance of the extracted sample at 550 nm was assayed as a measure of the adhesive potential. Experiments were performed in triplicate and repeated at least three times.

Invasion assay

In vitro analysis of the ability of the tumorigenic cell lines to invade the basement membrane was carried out using the ECM-like Matrigel. Serum-free cold culture medium (DMEM) was used in order to dilute the Matrigel and this diluted gel was dispensed onto the upper chamber of a 24 transwell plate (BD falcon, 8 μm pore size). This gel was then allowed to solidify for approximately 5 hours at 37°C. After being harvested, cells were resuspended in serum-free culture media at a density of 1×10^6 cells/ml. Antibody treatments required cells to be incubated with IgG1-iS18 (0.2 μg/ml) or the negative control anti-CAT (Sigma, 0.2 μg/ml) antibody. Cells were subsequently loaded onto the upper Matrigel-covered chamber and incubated for 18 hours. The lower chamber was filled with 500 μl of culture media containing 10% FCS (for the test) and no FCS (for the control), and incubated at 37°C for 18 hours. This was followed by aspiration of the media in the lower and upper chamber. Non-invasive cells were then removed by use of a cotton swab. The remaining invasive cells were then washed with 300 μl of PBS and fixed using 300 μl of 4% paraformaldehyde, 10 min. Cells were stained using 0.5% toluidine blue dye and after extraction of the dye using 1% SDS, absorbance was then measured at 620 nm using an ELISA reader. Experiments were carried out in triplicate and repeated at least three times.

Statistical evaluations

The two-tailed Student's t-test with a confidence interval of 95% was used in order to analyse the data, with p-values of less than 0.05 being considered significant. The extent or degree of association between LRP/LR levels on the cell surface and invasive/adhesive potential was measured using the Pearson's correlation coefficient. The Pearson's correlation coefficient was also used to measure the correlation between the adhesive and invasive potential of the cell lines. A positive coefficient was an indication of direct proportionality between the two variables, whereas a negative coefficient implied inverse proportionality.

Results

Liver cancer and leukaemia cells reveal LRP/LR on the cell surface

Pivotal to the occurrence of metastasis is the interaction between laminin-1 and LRP/LR on the cell surface. Hence, it was necessary to visualize cell surface LRP/LR as a means of confirmation that cells indeed do display LRP/LR on their surface. Both tumorigenic cell lines, as well as the poorly-invasive breast cancer control, revealed LRP/LR on the cell surface as depicted in Fig.1 a). The green fluorescence in the images below is indicative of cell surface LRP/LR as cells were non-permeabilized and the secondary antibody was shown to not bind non-specifically, as confirmed by the controls depicted in Fig.1 b) and Fig.1 c) below. Anti-CAT antibody was used as a negative control due to its ability to bind specifically to chloramphenicol acetyltransferase (CAT) which is a bacterial protein and is therefore absent in mammalian cells.

High percentages of tumorigenic cells display LRP/LR on the cell surface

Although confocal microscopy confirmed that the cell lines do indeed display LRP/LR on their cell surface, further quantification of the cell surface levels of LRP/LR was required. Flow cytometry was employed for this quantification.

As shown in Fig.2 A, C and E, all three tumorigenic cell lines revealed high percentages of cells within a specific population that display LRP/LR on the cell surface, with the shift between the two peaks in each graph being indicative of a change in fluorescence intensity due to the cell-surface staining of the cells with anti-LRP/LR specific antibody IgG1-iS18 and the fluoro-chrome-coupled secondary antibody. HUH-7 liver cancer cells displayed a higher percentage of cells exhibiting LRP/LR on the cell surface in comparison to K562 leukaemia cells as well as the poorly-invasive breast cancer (MCF-7) control cell line. Fig. 2 B, D and F additionally include the unstained control and this control served to show that the PE secondary antibody does not bind non-specifically. The shifts in fluorescence intensity of unstained, APC only and anti-CAT-APC labelled cells (the negative controls) are represented in the Fig S1. It is also noteworthy to add that cell debris and cell aggregates were excluded from analysis as they were outside the defined gate (Fig. S3).

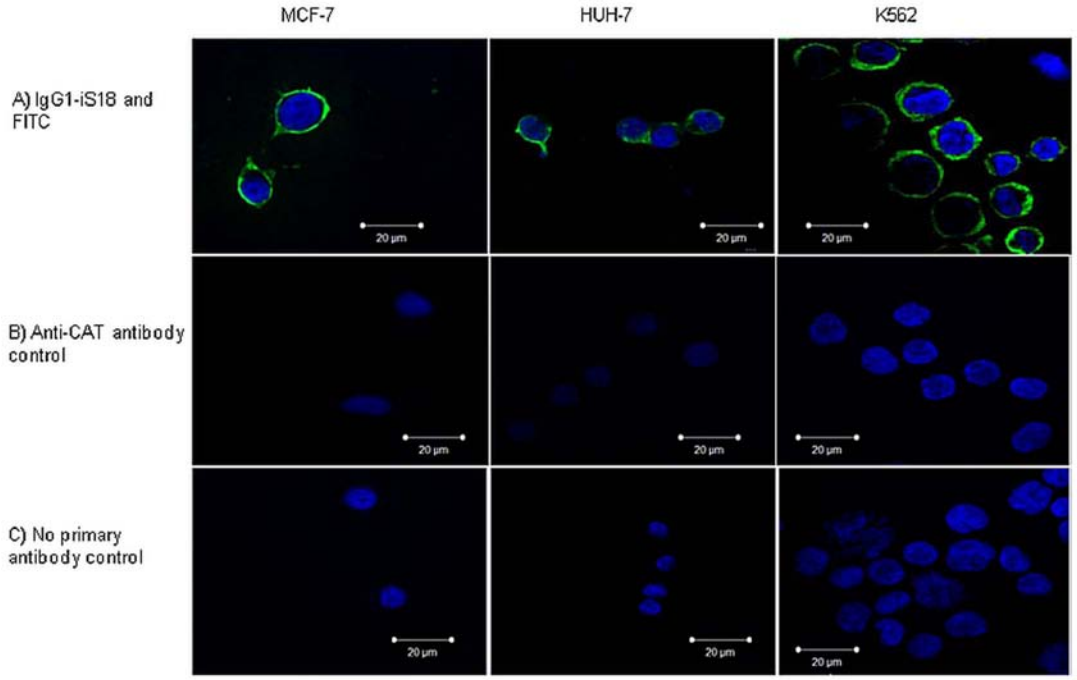


Figure 1. Visualisation of LRP/LR on the surface of liver cancer (HUH-7) and leukaemia (K562) cells. Cells were non-permeabilized in order to allow for visualisation of the cell surface. a) Cells were labelled with primary antibody IgG1-iS18 and a FITC-coupled secondary antibody. b) Cells were labelled with anti-chloramphenicol acteyltranferase (CAT) antibody as the negative control. c) Cells were labelled only with the FITC-coupled secondary antibody to confirm that the secondary antibody does not bind non-specifically.
doi:10.1371/journal.pone.0096268.g001

Liver cancer cells display significantly higher and leukaemia cells display significantly lower cell surface LRP/LR levels compared to poorly-invasive breast cancer cells

In addition to the percentage of cells exhibiting LRP/LR on their cell surface, the actual cell surface LRP/LR levels within a specific cell population was analyzed using flow cytometry. The same number of cells (20000 cells) within specific populations of the three tumorigenic cell lines were labelled with the same concentration (30 µg/ml) of previously-mentioned primary and secondary antibodies over the same time period. Thus, the more LRP/LR that is present on the surface of the tumorigenic cells, the more primary antibody IgG1-iS18 would bind to LRP/LR and subsequently, the more IgG-specifc fluorochrome-coupled secondary antibody would bind to the primary antibody. Thus, the median fluorescence intensities (MFI) would differ between the three cell lines (Table 1) and therefore can be used as an indicator of cell surface LRP/LR levels. It was observed that, in comparison to the poorly-invasive MCF-7 breast cancer control cell line, liver cancer cells (HUH-7) displayed higher levels of LRP/LR on their cell surface (Fig.3). Additionally, K562 leukaemia cells revealed lower cell surface LRP/LR levels in comparison to the MCF-7 cells (Fig.3).

The median fluorescence intensities obtained post anti-CAT labelling and detection demonstrate that there is no significant difference in the degree of cell-surface CAT staining across all three cell lines (Fig. S2)

Total LRP/LR levels do not differ significantly between the tumorigenic cell lines

As previously mentioned, LRP/LR does not exclusively occur on the cell surface but is additionally seen in the nucleus and cytosol, hence Western blot analysis was performed in order to assess total LRP/LR levels. Experiments were performed in triplicate and repeated three times. A representative blot is depicted in Fig.4. It is noteworthy to state that only the 37 kDa laminin receptor precursor could be detected by use of anti-LRP/LR specific antibody IgG1-iS18.

Following detection of LRP, quantification of total LRP levels was required and densitometry was employed to achieve this. Fig.5 depicts the densitometric analysis, which revealed that statistically, there was no significant difference observed in total LRP levels between the three tumorigenic cell lines.

IgG1-iS18 significantly impedes the adhesive potential of liver cancer cells

Pivotal to the initiation of invasion is the adhesion of a tumorigenic cell to the basement membrane through the LRP/LR-laminin-1 interaction as it allows for other interactions to occur that facilitate degradation of the basement membrane. Cells were incubated with IgG1-iS18 and anti-CAT antibodies (0.2 mg/ml) and after an hour, absorbance readings of the resultant solution were indicative of the degree of cell attachment to the laminin-1-coated plates.

As depicted in Fig.6, the no antibody control allowed for the determination of the adhesive potential of the cell lines and it was observed that both liver cancer (HUH-7) as well as leukaemia cells (K562) were more adherent than the poorly-invasive breast cancer (MCF-7) control cells. However, IgG1-iS18 was only effective at impeding the adhesive potential of liver cancer cells and no significant reduction in adhesion was observed for leukaemia cells treated with the anti-LRP/LR specific antibody IgG1-iS18. As expected, the anti-CAT

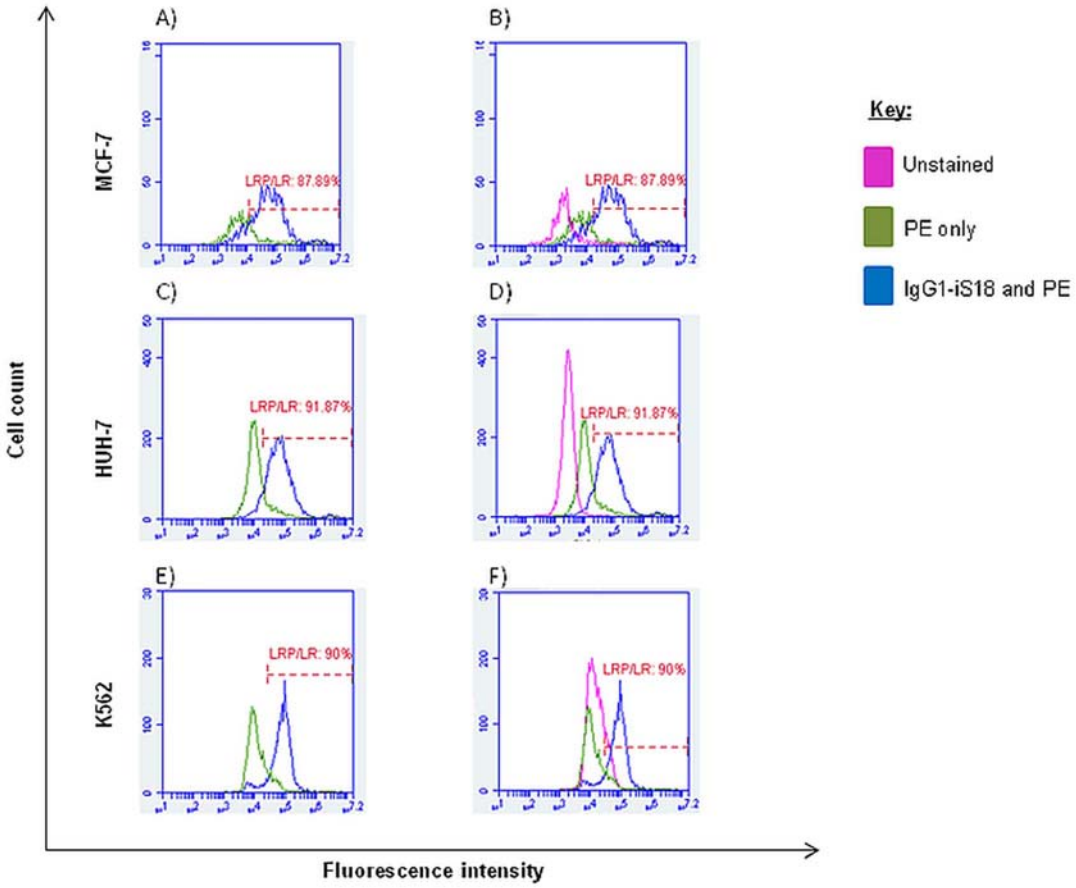


Figure 2. Quantification of liver cancer (HUH-7) and leukaemia (K562) cells within a population which exhibit LRP/LR on their cell surface. The first peak in graphs A, C and E is representative of non-labelled cells i.e. cells labelled with goat anti-human PE-coupled secondary antibody only, whereas the second peak is indicative of cells labelled with both anti-LRP/LR specific antibody IgG1-iS18 and the secondary antibody, both at a concentration of 30 μg/ml. Graphs B, D and F depict the inclusion of an unstained control to show no non-specific secondary antibody binding. Experiments were carried out in triplicate and repeated at least three times with 20 000 cells being counted per sample.
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control antibody did not have a significant effect on the adhesive potential of the tumorigenic cell lines.

Invasion of the Matrigel by liver cancer cells (HUH-7) is significantly impeded by anti-LRP/LR specific antibody IgG1-iS18

Invasion of the basement membrane is considered as a pre-requisite for the progression of a metastatic cancer, hence invasion assays using a Matrigel, which mimics the components of the basement membrane, were performed to determine the invasive potential of the tumorigenic cell lines. Similarly to the adhesion assays, the no antibody control allowed for the determination of the invasive potential of the cell lines. Furthermore, cells were treated with anti-LRP/LR specific antibody IgG1-iS18 and anti-CAT antibody (0.2 mg/ml).

As shown in Fig.7, liver cancer (HUH-7) cells are significantly more invasive in comparison to the poorly-invasive breast cancer (MCF-7) control cell line, whilst leukaemia (K562) cells showed a significantly lower invasive potential compared to the control. Moreover, IgG1-iS18 successfully hampered the invasive potential of liver cancer (HUH-7) cells whilst no significant result was observed for the leukaemia cells. As expected, the anti-CAT antibody control did not significantly affect the invasive potential of the tumorigenic cell lines.

Discussion

Several studies have revealed that, on the cell surface, various tumorigenic cell lines exhibit an overexpression of the 37 kDa/67 kDa LRP/LR, therefore suggesting that the LRP/LR-laminin-1 interaction may be pivotal for cancer cells to undergo metastasis[21]. It may therefore be useful to inhibit this interaction as a means of hampering adhesion and invasion – two events found to be crucial to the occurrence of the process of metastasis. A study conducted by Zuber *et al* has demonstrated that the IgG1-iS18 antibody is highly specific for LRP/LR[18]. Furthermore, recent research has shown that anti-LRP/LR specific antibody IgG1-iS18 significantly impedes the adhesive and invasive potential of cervical[4], lung[4], prostate[4], colon[4], breast[20] and oesophageal[20] cancer cells. The present study investigated the role of LRP/LR in the adhesion and invasion of liver cancer (HUH-7) as well as leukaemia (K562) cells, and aimed to establish whether application of anti-LRP/LR specific antibody IgG1-iS18 significantly reduces the adhesive and invasive potential of these two tumorigenic cell lines.

Initially, confocal microscopy revealed that all three tumorigenic cell lines indeed display LRP/LR on their cell surface (Fig. 1a). However, this technique is limited by the fact that it is not quantitative and further analysis was required in order to establish

Table 1. Median fluorescence intensity (MFI) values as an indicator of differential expression of LRP/LR between MCF-7, HUH-7 and K562 cell lines.

Cell lines	MFI of unstained cells	MFI of cells labelled with IgG1-iS18 and PE	(MFI of cells labelled with IgG1-iS18 and PE) – (MFI of unstained cells)
MCF-7	1623.666667	38753.16667	37129.5
HUH-7	2678.166667	49556	46877.83333
K562	13823.75	35392	21568.25

*All values are representative of an average of results obtained from experiments that were performed in triplicate and repeated three times.
doi:10.1371/journal.pone.0096268.t001

the levels at which LRP/LR is displayed on the cell surface of these tumorigenic cell lines.

Significantly high percentages (>87%) of all three tumorigenic cell lines, namely HUH-7, K562, and MCF-7 (poorly-invasive breast cancer control) cells displayed LRP/LR on their cell surface (Fig.2). Furthermore, it was observed by analysis of differences in median fluorescence intensities that, in comparison to the MCF-7 control cell line, liver cancer cells (HUH-7) revealed significantly higher and leukaemia cells (K562) revealed significantly lower cell surface LRP/LR levels (Fig.3). As previously stated, LRP/LR plays essential roles in adhesion, invasion, proliferation and migration of cells[9]. Seeing that the HUH-7 cell line is known to be invasive and the K562 is understood to be a suspension cell line (ATCC), the cell surface levels of LRP/LR that have been observed may be correlating with the invasive potential of these cell lines.

Additionally, total LRP/LR levels were analysed by Western blotting in order to account for LRP/LR in the nucleus and cytosol of the tumorigenic cell lines. Western blot analysis (Fig.4) confirmed that all three tumorigenic cell lines express the 37 kDa LRP, however densitometry analysis of these blots (Fig.5) revealed that both the invasive and poorly-invasive cell lines show similar levels of total LRP and no significant differences were observed. It is noteworthy to state that in the nucleus and cytosol, LRP/LR serves particularly to maintain nuclear structures and facilitate translational processes, respectively[8]. Hence, even though total LRP levels do not differ significantly between the invasive and poorly-invasive tumorigenic cell lines, the cell surface LRP/LR levels are of importance to the occurrence of adhesion and invasion in the invasive and poorly-invasive tumorigenic cell lines. The current study revealed differences in only cell surface levels of LRP/LR between the three tumorigenic cell lines, and this is in agreement with results obtained in previously published research [4]. However, another study contradicted these results by showing that tumorigenic cell lines differed only in total LRP/LR levels[20]. These discrepancies in the latter-mentioned study could be owing to the fact that those cancerous cells may require enhanced protein synthesis in order to carry out metastatic processes, hence increased total LRP/LR levels were observed rather than increased cell surface LRP/LR levels.

The results obtained in the present study suggests that anti-LRP/LR specific antibody IgG1-iS18 caused a significant reduction in the adhesive potential of metastatic liver cancer cells on laminin-1 and additionally hampered the invasive potential of

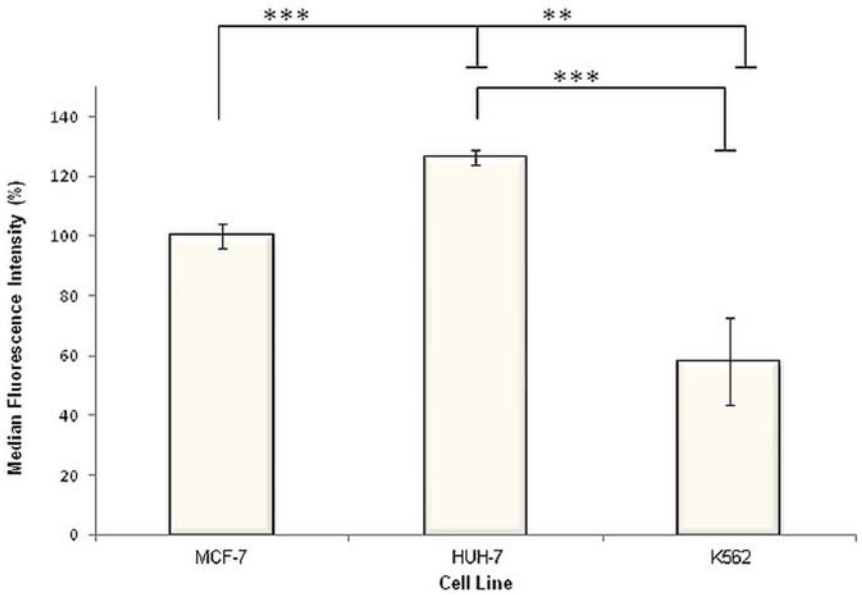


Figure 3. Quantification of cell surface LRP/LR levels on liver cancer (HUH-7) and leukaemia (K562) cells by flow cytometry. Cells were labelled with primary antibody IgG1-iS18 (1:25) and anti-human phycoerythrin (PE) secondary antibody. 20000 cells were analyzed across all three cell lines, and the median fluorescence intensities (MFI) were used as an indicator of cell surface LRP/LR levels. The MFI values indicated in the last column of Table 1 were used in order to construct this figure and it is noteworthy that the MFI value corresponding to the MCF-7 cell line was set to 100%. Experiments were performed in triplicate and repeated at least three times. **p = 0.0025, ***p<0.0001.
doi:10.1371/journal.pone.0096268.g003

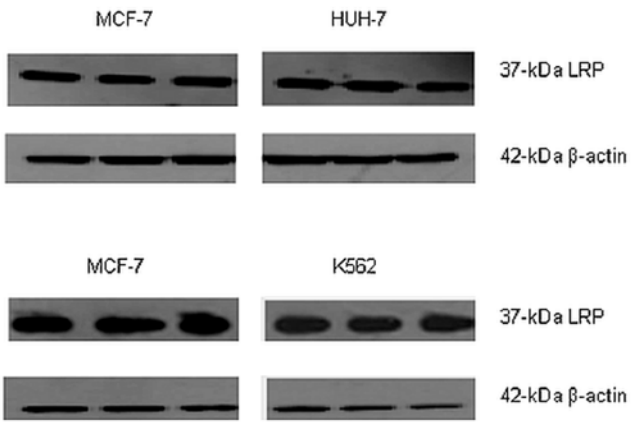


Figure 4. Detection of the relative expression of total 37 kDa LRP levels using lysates of liver cancer (HUH-7) and leukaemia (K562) cell lines. Anti-LRP/LR specific antibody IgG1-iS18 was used as the primary antibody in conjunction with a secondary HRP-coupled antibody. β-actin was employed as a loading control.
doi:10.1371/journal.pone.0096268.g004

this cancer type on the Matrigel. Figures 6 and 7 depict these significant decreases in the adhesive and invasive potential of metastatic liver cancer (HUH-7) cells upon administration of IgG1-iS18, respectively. It is noteworthy to state that leukaemia (K562) cells showed no significant reduction in the adhesive and invasive potential upon administration of IgG1-iS18. It may be suggested that variations in the adhesive and invasive potential of both HUH-7 and K562 cell lines may be attributed to variations in cell surface LRP/LR levels. Hence, lower cell surface LRP/LR levels observed for leukaemia cells (Fig.3) could be held accountable for the lower adhesive and invasive capacity observed in this cell line, and vice versa for HUH-7 cells which exhibited high cell surface LRP/LR levels (Fig.3) and resulted in the increased adhesive and invasive capacity of this cell line. The significant reduction in the invasive potential of metastatic liver cancer cells after administration of anti-LRP/LR specific antibody IgG1-iS18 may be attributed to the inhibition of adhesion (Fig.6) by the IgG1-iS18 antibody, as adhesion is understood to be a pre-requisite for the occurrence of invasion during the induction of metastasis[20].

On the contrary, the K562 leukaemia cells were observed to be more adherent (Fig.6) but less invasive (Fig.7) than the poorly-invasive MCF-7 control cell line. This observation could be due to K562 cells expressing tissue inhibitors of metalloproteinases (TIMPs) and these TIMPs could be resulting in the inhibition of type IV collagenase activity[22], thereby preventing degradation of type IV collagen in the Matrigel and preventing K562 cells from undergoing invasion *in vitro*.

Analysis of the correlation between cell surface levels of LRP/LR with the adhesive and invasive potential of liver cancer (HUH-7) and leukaemia (K562) cells, resulted in considerably high correlation coefficients (Table 2). This signifies a positive and directly proportional relationship between the two parameters. Hence, this confirms that adhesion is a pre-requisite for invasion to occur, as seen by the high correlation coefficients obtained for adhesive to invasive potential for both experimental cell lines. Furthermore, the high correlation coefficients obtained for cell surface LRP/LR levels to the adhesive and invasive potential of the cell lines suggests that the aggressiveness of these two cancer types is enhanced by high levels of cell surface LRP/LR, which is consistent with results obtained by previous studies [4,20]. It is important to note that only cell surface levels of LRP/LR were considered in the calculations of Pearson's correlation coefficients since no significant difference was observed in total LRP/LR levels between the three tumorigenic cell lines (Fig.5).

In Table 2, the high Pearson's correlation coefficients observed between the adhesive and invasive potential for both cell lines ascertains that adhesion is indeed a mandatory step for the occurrence of invasion, where lower adhesive potential in leukaemia cells subsequently resulted in a lower invasive potential as well. This finding is in line with that of previously published literature which shows that the LRP/LR-laminin-1 interaction is pivotal for adhesion as well as secretion of enzymes that degrade

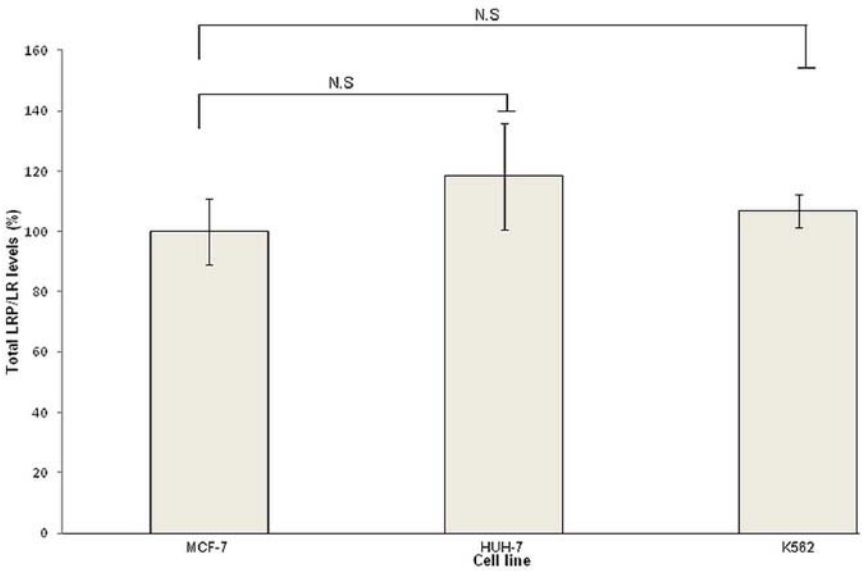


Figure 5. Total LRP levels of liver cancer (HUH-7) and leukaemia (K562) cell lines detected by Western blot analysis using primary anti-LRP/LR specific antibody IgG1-iS18 and goat anti-human HRP secondary antibody. Quantification was conducted using densitometry and data are representative of experiments carried out in triplicate and repeated three times. Non-significant (NS): $p>0.05$.
doi:10.1371/journal.pone.0096268.g005

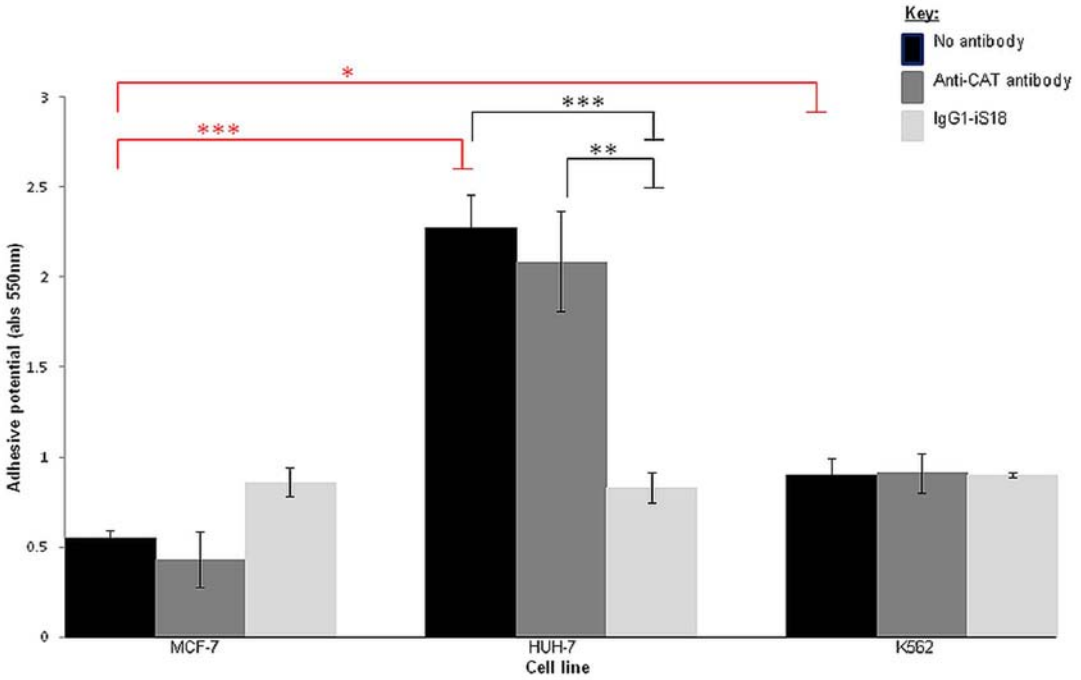


Figure 6. Effect of anti-LRP/LR specific antibody IgG1-iS18 on the adhesion of liver cancer (HUH-7) and leukaemia (K562) cells to laminin-1 determined through adhesion assays. p-values shown in red (*p = 0.0238; ***p = 0.0002) are indicative of the increase in adhesive potential of liver cancer (HUH-7) and leukaemia (K562) cells in comparison to the breast cancer (MCF-7) control cell line. p-values shown in black (**p = 0.0031; ***p = 0.0005) represent the reduction in adhesive potential after treatment of cells with appropriate antibodies. A reduction of 63.35% in the adhesive potential was observed upon administration of IgG1-iS18 to the HUH-7 liver cancer cells. Data represents experiments performed in triplicate and repeated at least three times.
doi:10.1371/journal.pone.0096268.g006

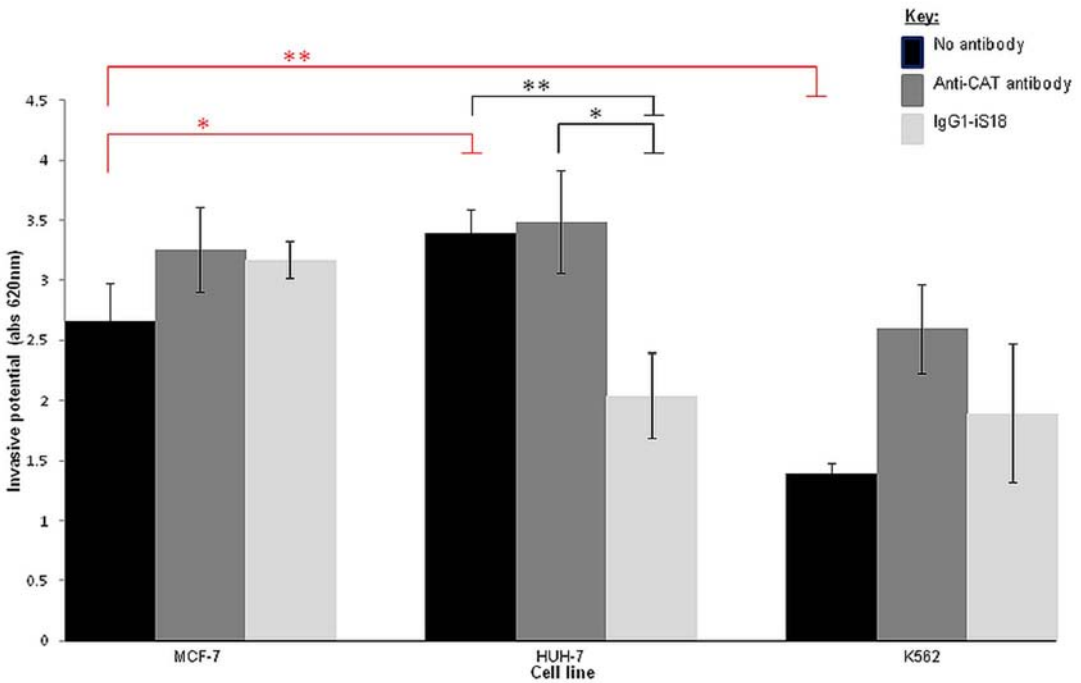


Figure 7. Effect of anti-LRP/LR specific antibody IgG1-iS18 on the invasion through the ECM-like Matrigel™ by liver cancer (HUH-7) and leukaemia (K562) cells. p-values indicated in red (*p = 0.0485; **p = 0.0053) represent the changes in invasive potential of cell lines in comparison to the MCF-7 control, whilst p-values shown in black (*p = 0.0214; **p = 0.0091) are indicative of the effect of appropriate antibodies on the invasive potential of the cell lines. Upon administration of IgG1-iS18 to the HUH-7 liver cancer cells, a reduction of approximately 39.75% was observed regarding the invasive potential of the cells. Data is representative of experiments carried out in triplicate and repeated at least three times.
doi:10.1371/journal.pone.0096268.g007

Table 2. Pearson's correlation coefficients between cell surface LRP/LR levels and the adhesive and invasive potential of liver cancer (HUH-7) and leukaemia (K562) cells.

Cell lines	LRP/LR levels to adhesive potential	LRP/LR levels to invasive potential	Adhesive to invasive potential
HUH-7	0.95	0.96	0.84
K562	0.91	0.99	0.88

doi:10.1371/journal.pone.0096268.t002

the basement membrane and therefore promote invasion through the basement membrane[23].

Several studies have suggested that anti-LRP/LR tools such as pentosan sulphates and monoclonal antibodies directed towards the laminin receptor have shown the potential to significantly impede the adhesive and invasive potential of selected cancers such as laryngeal carcinoma cells as well as human fibrosarcoma cells through the interruption of the LRP/LR-laminin-1 interaction [18,24].

Overall, it is strongly suggested that proteolytic cleavage of the basal lamina and subsequently the process of invasion is significantly enhanced through the LRP/LR-laminin-1 interaction[23]. Furthermore, anti-LRP/LR specific antibody IgG1-iS18 has been shown in the present study to significantly impact on the behaviour of metastatic liver cancer cells at the critical stages of adhesion and invasion *in vitro*, thereby suggesting the use of the antibody as an alternative therapeutic tool in the treatment of metastatic liver cancer.

Due to different cancer types exhibiting different behavioural characteristics, it cannot be assumed that anti-LRP/LR specific antibody IgG1-iS18 will have the same effect on all cancer types. Hence, the results of the current study will assist in providing the scientific community with novel aspects regarding the use of the antibody as a possible therapeutic tool for metastatic liver cancer. Furthermore, studies concerning appropriate delivery systems for the IgG1-iS18 antibody need to be conducted since LRP/LR plays critical roles in several essential physiological processes and the targeting of LRP/LR specifically in tumorigenic cells may prove to be difficult. Successful animal trials may indeed deem the antibody as a potential therapeutic tool for metastatic cancers.

Supporting Information

Figure S1 Quantification of liver cancer (HUH-7) and leukaemia (K562) cells within a population which display the CAT protein on their cell surface. The first peak in graphs A,C and E represents cells labelled with APC-coupled secondary antibody only, whilst the second peak indicates cells that are labelled with both anti-CAT antibody as well as the secondary antibody. The unstained control is included in graphs B, D and F to confirm that the secondary antibody does not significantly bind non-specifically. Experiments were performed in triplicate and repeated at least three times with 20000 cells counted per sample.
(TIF)

Figure S2 Quantification of cell surface CAT protein levels on liver cancer (HUH-7) and leukaemia (K562) cells by flow cytometric analysis. Cells were labelled with anti-CAT antibody and APC-coupled secondary antibody. An analysis was performed on 20000 cells per sample across all three cell lines. The median fluorescence intensities of the samples labelled with both anti-CAT antibody and the secondary antibody were used as an indicator of CAT expression on the cell surface (with the unstained control being taken into account). The MFI value for the MCF-7 cell line wasset to 100%. Experiments were carried out in triplicate and repeated at least three times. N.S: $p > 0.05$.
(TIF)

Figure S3 Flow cytometric gating of MCF-7 (poorly-invasive breast cancer), HUH-7 (liver cancer) and K562 (leukaemia) cell samples. Cells were gated to exclude debris and aggregated cells from the analysis. R1 and R3 indicate the gated cell population.
(TIF)

Author Contributions

Conceived and designed the experiments: SFTW. Performed the experiments: CC. Analyzed the data: CC BDCD. Contributed reagents/materials/analysis tools: UR SK ML. Wrote the paper: CC. Edited the manuscript: TK ML BDCD.

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Chapter 5

Discussion

The following discussion is based on the work published in the following two original research articles:

1. Da Costa Dias *et al.*,(2013). Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from $A\beta_{42}$ induced cytotoxicity. Scientific Reports, 3, 2702; DOI:10.1038/srep02702.

2. Da Costa Dias *et al.*,(2014). The 37kDa/67kDa Laminin Receptor acts as a receptor for $A\beta_{42}$ internalization. Scientific Reports, 4, 556; DOI:10.1038/srep05556.

It must be noted that a distinction between LRP/LR and LR will be made throughout this chapter. LRP/LR will be employed when a discrimination between the 37kDa laminin receptor precursor (LRP) and 67kDa laminin receptor (LR) isoforms could not be made-particularly with regards to expression of the laminin receptor on the cell surface. However, the pCIneoLRP::FLAG mammalian expression construct employed for the recombinant expression of the receptor employed for FLAG® co-immunoprecipitation studies exclusively encodes the 37kDa LRP isoform, and shall hereafter be denoted as LRP.

Furthermore, prior to the discussion of the results a justification for the use of human embryonic kidney (HEK) 293 cells (HEK293) throughout these studies must be provided. These cells were originally isolated from kidney tissue and should therefore biochemically most closely resemble kidney epithelial cells. However, their transformation with sheared fragments of human adenovirus type 5 (Ad5), necessary for the establishment of an immortalized cell line, has led them to display properties associated with cells of a neuronal lineage[273, 274]. Recent studies have demonstrated that HEK293 cells express four neurofilament subunits, namely: NF-L, NF-M, NF-H and α-internexin, which are normally expressed only by neurons[273]. Furthermore, 61mRNAs previously considered to be exclusively expressed by neuronal cells, have been detected in the HEK293 cells[274]. HEK293 cells have also been shown to express numerous voltage-activated ion-channels (such as Ca^{2+} channels), ligand-gated ion-channels, intracellular regulatory proteins and G-coupled receptors which are of neuronal origin[274]. Morphologically, HEK293 cells have similar proportions to neuronal soma. From this evidence, it is clear that HEK293 cells have attributes of neuronal cells. However, in contrast to neuronal cells, HEK293 cells are

relatively simple with regards to maintenance, do not have as stringent passage number limitations and are easily transfected with inexpensive and accessible methods such as calcium phosphate precipitation. It is owing to this that HEK293 cells were employed, in addition to the neuronal cell lines, throughout these studies.

Moreover, the observed trends with regards to the cell biological effects of $A\beta_{42}$ administration in the presence and absence of anti-LRP/LR specific antibody IgG1-iS18 (**Figure 3a-c, original article 4.1**) were consistent across all three cell lines (HEK293 and 2 neuronal cell lines - N2a (murine) and SHSY5Y (human)). Owing to this, later experiments conducted with the aim of identifying interactions and probing mechanisms – both of which required cellular transfection were conducted solely in the HEK293 cells. It may be suggested, owing to the biochemical similarities between the HEK293 and neuronal cells, that the results obtained in the HEK293 cells would be comparable to those obtained in neurons.

LRP/LR is highly expressed in both non-neuronal (HEK293 in this study and BHK cells[275]) and neuronal cells, including: murine Neuro-2a (N2a) and mouse neuroblastoma (MNB)[258] cells as well as ovine neuronal cells [276]. Furthermore, it may be suggested that the LRP/LR-dependent effects observed in non-neuronal cells may indeed be more pronounced in neuronal cells owing to the fact that higher levels of LRP/LR have been reported in neuronal tissues when compared to the LRP/LR levels in peripheral tissues[276].

5.1 Interactions between the 37kDa/67kDa LRP/LR and Aβ

Co-localization Studies- Co-localization studies were employed as the primary step in ascertaining whether LRP/LR and Aβ form an association on the cell surface.

Co-localization is extensively employed in cell biology to provide details regarding the spatial proximity of proteins, as sharing a similar cellular location is a prerequisite for interaction. LRP/LR and Aβ were found to co-localize on the surface of HEK293 and N2a cells (**Figure 1, original research article 4.1**) as revealed by the yellow merged image and diagonal 2D cytofluorogram (which is indicative of a spatial overlap between the fluorochromes employed to indirectly immuno-label each protein of interest). The graphical co-localization was substantiated by the highly positive (approaching +1) Pearson's correlation coefficients (**Table 1, original research article 4.1**). A laminin binding integrin,

Very-Late Antigen 6 (VLA6) (**Figure 1, original research article 4.1**), was employed as the negative control and demonstrated no co-localization with LRP/LR, results which are in accordance with that of Jovanovic et al., (2013)[272].

It is important to note that although this methodology has been widely employed to investigate potential associations between Aβ and other cellular proteins (including post-synaptic density protein 95 (PSD-95)[277], cystatin C[278], mitochondrial protein Dynamin-related protein (Drp1)[279] and lysyl oxidase[280]) additional analyses employing co-immunopreciptation, FRET and more precise biochemical methods such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) are usually undertaken to firmly establish the presence of such associations. This is owing to the fact that confocal laser scanning microscopy has a resolution limit of 200nm. As a consequence hereof, indirect immunofluorescence microscopy may only be employed as a preliminary indicator of potential interactions. Thus, these findings suggested that an association between LRP/LR and Aβ may exist, but this suggestion needed to be confirmed by more sensitive methods.

These results are not surprising as LRP/LR has been reported to localize to the lipid raft domains of the plasma membrane[281]. Similarly Aβ has been documented to insert specifically into the lipid raft regions of the plasma membrane [141, 282, 283], forming ion permeable pores which result in membrane distortion and may thereby contribute to cytotoxicity[284]. Thus, since both proteins are largely located within the lipid raft domains of the plasma membrane, their co-localization was anticipated.

The validity of this finding was further assessed by Försters resonance energy transfer (FRET) and pull down assay methodology complemented with sensitive Aβ detection employing an Aβ-specific ELISA.

Försters resonance energy transfer (FRET)- Försters resonance energy transfer (FRET) was proposed by Theodor Förster in 1946-1948 and is based on the principle that a donor fluorochrome (phycoerythrin (PE), employed in this study), within a distance of 1-10nm of the corresponding acceptor fluorochrome (allophycocyanin (APC)), will non-radiatively transfer energy to the acceptor. This will only occur if the emission spectrum of the donor and the excitation spectrum of the acceptor significantly overlap[285]. The consequence hereof depends on the FRET couple chosen, and may either result in the enhanced fluorescence emission of the acceptor, as detected in this study (**Figure 1, original article 4.2**)[286], or acceptor photobleaching.

Here a flow cytometry-based FRET assay was employed to investigate whether LRP/LR and Aβ interact on the surface of HEK293 cells. The highly sensitive PE/APC FRET pair was employed in this study[287],[285]. APC does not emit fluorescence in the FL3 channel, a conclusion reached owing to the superimposition of histograms depicting APC-labelled proteins of interest with that of unstained cells (**Figure 1 a,c,e, original article 4.2**). However, in the presence of interacting proteins, FRET indeed occurs between PE and APC, resulting in enhanced APC fluorescence emission, observed graphically as a shift towards the right (**Figure 1 b and f, original article 4.2**). The sensitivity and selectivity of this technique was validated employing proteins known either to interact (PrP^c) (**Figure 1 a and b, original article 4.2**) or not associate (chloramphenicol acetyltransferase (CAT)) (**Figure 1 c and d, original article 4.2**) with LRP/LR. Energy transfer was detected between LRP/LR and Aβ at the cell surface, thereby indicating that the proteins are within a 10nm radius of each other. This provides strong evidence for a cell surface interaction between these proteins.

It must be noted that a flow cytometric FRET approach was employed in place of the more conventional microscopic FRET analysis owing to the high throughput of the technique. Flow cytometry-based FRET allows for the fluorescence intensity of thousands of cells to be quantified and therefore significantly reduces the susceptibility of the results to investigator bias.

FRET is regularly employed to examine Aβ associations and has been successfully utilized to demonstrate that Aβ interacts with integrins and transferin[288].Moreover,
FRET is frequently exploited as a sensitive tool for investigating Aβ oligomerization mechanisms[289] and for differentiating between different Aβ aggregation states (monomers vs higher order oligomers)[290]. The frequent utilization of this technique within AD research, and specifically with regards to Aβ interaction studies, adds further confidence to the data obtained and the deductions drawn during this study.

To investigate whether LRP and Aβ form a truly stable association, pull down assays were employed.

FLAG® Pull down Assay- Pull down assays, a form of affinity chromatography, allow for the identification or confirmation of stable protein-protein interactions and was employed here to investigate the binding potential between LRP and $A\beta_{42}$.

Pull down assays were performed employing LRP::FLAG and 2µg of exogenously administered synthetic $A\beta_{42}$ and the presence of both proteins in the eluate samples (**Figure 2a, original article 4.1**) implied that a stable association between these proteins may exist. However, as the concentration of Aβ greatly exceeded that normally present in the brains of AD patients, 200-4500nM[291], the experiments were repeated employing conditioned media (media into which Aβ would be endogenously shed as a result of normal APP metabolism within the cells). The Aβ levels in each fraction were subsequently assessed and again, a significant proportion of Aβ was detected in the eluate (**Figure 2b, original research article 4.2**). To negate the possibility that Aβ immobilization may have been influenced by non-specific adhesion of other cellular components to the anti-FLAG® affinity column, both sets of experiments were conducted employing non-transfected (NT) whole cell lysate controls (**Figure 2d, original research article 4.1 and Figure 2c, original research article 4.2**). Furthermore, as consequence of the inherent "sticky" nature of $A\beta_{42}$, the ability of the peptide to bind to the affinity column in a lysate-independent manner also required investigation. In both sets of these control experiments the percentage of $A\beta_{42}$ present in eluate fractions was not significant (**Figure 2f,original article 4.1 and Figure 2c, original article 4.2**).These results therefore demonstrated that a physiologically relevant association exists between the 37kDa LRP and $A\beta_{42}$.

A direct physical association between LRP/LR and $A\beta_{42}$ is not unlikely. $A\beta_{42}$ has been reported to bind to both heparin and HSPGs, the latter interaction has been reported to promote $A\beta_{42}$ aggregation[292] and may thereby account for the presence of these cellular components within neuritic plaques[293]. The extracellular C-terminus of LRP/LR, the region to which shed and cell surface $A\beta_{42}$ would bind, mimics heparin - due to its predominantly negatively charged nature as well as the presence of 5 repeats of the E/D-W-S/T motif [187, 188]. Thus, owing to the structural and charge similarities between LRP/LR and heparin, it is probable that $A\beta_{42}$ may also be able to interact directly with the C-terminus of the receptor.

However, this interaction may be mediated by other $A\beta_{42}$ binding partners which are similarly ligands for LRP/LR such as PrP^c, laminin and HSPGs.

5.2 The cell biological effects of the 37kDa/67kDa LRP/LR – Aβ association

The exogenous application of 200nM and 500nM synthetic $A\beta_{42}$ significantly reduced cell viability and proliferation across all three cell lines, namely human embryonic kidney (HEK293), murine (N2a) and human (SH-SY5Y) neuroblastoma cells (**Figure 3, original article 4.1**). Similarly, treatment of cells with these toxic concentrations of $A\beta_{42}$ induced both time and concentration dependent apoptosis, as quantified by Annexin-V-FITC/7-AAD assays and confirmed by nuclear morphological assessment (**Figure 3, original article 4.2**). The results obtained are in accordance with those obtained in other studies with regards to the apoptotic inducing potential of $A\beta_{42}$[294] as well as with regards to the peptide's inhibitory effects on cellular proliferation[295].

The role of LRP/LR in the aforementioned $A\beta_{42}$ pathogenesis was evaluated through antibody blockade and shRNA-mediated downregulation of the receptor. Blockade of the receptor, as achieved by co-incubation of the cells with IgG1-iS18 (anti-LRP/LR specific antibody) and treatment with varying $A\beta_{42}$ concentrations, resulted in a significant enhancement of cell viability and proliferation (**Figure 3, original article 4.1**) and significantly lowered the degree of apoptosis induced by the neurotoxic peptide (**Figure 4, original article 4.2**). These results were reproduced when $A\beta_{42}$ treatments were administered to cells in which LRP/LR had been downregulated, thereby demonstrating that the cell rescuing effects of the antibody were not as a result of a lack of antibody specificity. Furthermore, the downregulation results revealed that the protective antibody effects were not owing to indirect antibody steric hindrance of other pathological associations at the cell surface. Therefore, these data suggest that LRP/LR may, either directly or indirectly, mediate $A\beta_{42}$-induced apoptosis and inhibition of proliferation.

The role of LRP/LR in $A\beta_{42}$-induced apoptosis is credible as a recent report has demonstrated that neuronal apoptosis induced by $A\beta_{42}$ may occur via the JNK pathway which consequently results in the stimultation of FasL expression[296], which is pro-apoptotic. LRP/LR is associated with both of these components (as discussed above in sections **1.11.1.1** and **1.11.2.1.1**) and it is therefore probable that LRP/LR may be implicated in $A\beta_{42}$-induced apoptosis.

It must be emphasized that these findings are of physiological relevance as the concentration of $A\beta_{42}$ within the brains of AD patients is reportedly within the 200-4500nM range[291].

Furthermore, it must be noted that at nM concentrations, such as those employed in this study, $A\beta_{42}$ exists largely as monomers or low molecular weight oligomers[297]. Moreover, it has been demonstrated that even upon incubation in cell culture media, low nM concentrations of synthetic $A\beta_{42}$ (0-500nM) do not aggregate to form higher order, less toxic fibrils[298]. Based on these findings, it may be safely proposed that the biological effects observed herein may largely be attributable to oligomeric $A\beta_{42}$, the neurotoxic species in AD.

The final aim of this study was to investigate a possible mechanism underlying the role of LRP/LR in $A\beta_{42}$-mediated pathogenesis.

Since the toxicity of $A\beta_{42}$ is largely attributable to its intracellular accumulation, understanding $A\beta_{42}$ internalization processes is of paramount importance. It must be noted that $A\beta_{42}$ internalization in neuronal cells occurs predominantly via receptor-mediated mechanisms[298].

5.3 37kDa/67kDa LRP/LR mediated $A\beta_{42}$ internalization

Our studies demonstrated that receptor-mediated uptake of exogenous $A\beta_{42}$ occurs rapidly (within 5min) and was most pronounced after 15min. The internalization and intraneuronal accumulation of exogenously administered $A\beta_{42}$ has been well documented[299-302].

Furthermore, our studies indicated that the cell surface levels of $A\beta_{42}$ increased after 1h, thereby providing evidence that the peptide may be recycled to the cell surface (**Figure 5a, original article 4.2**). This is in accordance with observations reported in previous studies which revealed that, at physiologically normal levels, both non-toxic $A\beta_{40}$ as well as $A\beta_{42}$ are readily cleared from the cell either through proteolytic degradation or efficient exocytosis[299, 303]. This accounts for the relatively low levels of intraneuronal $A\beta_{42}$ associated with organelles such as the trans-golgi network, Golgi vesicles, endosomes, lysosomes and mitochondria under normal physiological, non-disease causing $A\beta_{42}$ levels[303]. However, within the diseased state, $A\beta_{42}$ accumulation and co-localization with these organelles as well as within the cytosol is clearly evident[304]. The central role of exocytosis in maintaining homeostasis and the non-diseased state has been revealed as, even at low $A\beta_{42}$ concentrations, inhibition of exocytosis significantly augmented intracellular $A\beta_{42}$ accumulation[303]. This process of exocytosis would remain functional in diseased cells, however, owing to the higher concentration of aggregation prone $A\beta_{42}$ and its deleterious

effects on the endo-lysosomal system (as discussed briefly below) a significant proportion of $A\beta_{42}$ would be released into the cytosol with detrimental cellular effects (as discussed briefly below) and would therefore not be effectively exocytosed.

It is important to note that $A\beta_{42}$ accumulation, specifically within the endo-lysosomal system, is considered central to $A\beta_{42}$-mediated neurotoxicity (as shall be discussed below). Lysosomal accumulation of this neurotoxic peptide has been reported to be mechanistically similar to PrP^{Sc} replication[305], a process in which LRP/LR has been shown to play a central role[258, 260].

The reliance of the $A\beta_{42}$ internalization process on cell surface LRP/LR was demonstrated by the fact that antibody blockade and shRNA-mediated downregulation of the receptor significantly impeded $A\beta_{42}$ internalization (**Figure 6 a and b, original article 4.2**).Therefore, this data suggests that LRP/LR plays a central role in $A\beta_{42}$ internalization and this may be the mechanism underlying the role of the receptor in $A\beta_{42}$ pathogenesis.

This data is contradictory to studies performed by Hu *et al.,*[298] who reported that extracellular $A\beta_{42}$ is readily internalized by SH-SY5Y cells and murine neurons but not HEK293 cells. However, the degree of uptake during the Hu *et al.,*[298] study was only assessed at a single time point of 24h, and it is feasible that the majority of internalized $A\beta_{42}$ may indeed have been cycled back to the cell surface by this time. It is probable that, after multiple rounds of $A\beta_{42}$ internalization and recycling, a gradual progressive accumulation of the peptide would occur within the endo-lysosomal system and begin to "leak" into the cytosol. It is therefore highly plausible that within 24h intracellular $A\beta_{42}$ may be minimal but after 48h-72h, the degree of intracellular $A\beta_{42}$ accumulation (and the cytosolic immunodetection thereof) may be substantially elevated. This would correlate with cell biological data as both our results (**Figure 3, original article 4.2**) as well as those of others[306-308] have revealed that the induction of apoptosis is minimal after 24h but becomes more pronounced with longer periods of incubation. Furthermore, the receptors that have been reported to mediate $A\beta_{42}$ internalization are largely neuronal specific (as discussed below) and are not expressed by HEK293 cells. This may thereby account for the slower rates of internalization, and subsequent intracellular Aβ accumulation observed within the HEK293 cells by Hu *et al.,*[298].

It is noteworthy to add that HSPGs, to which LRP/LR similarly binds, have also been reported to mediate $A\beta_{42}$ internalization and toxicity[309] and HSPGs are expressed at high levels on the surface of HEK293 cells[310]. An important feature, which is universally required,

for mediating $A\beta_{42}$ toxicity, is the anchorage of the protein to the cell surface. This therefore justifies the pathological role of HSPGs compared to the neuroprotective effects of soluble heparin as heparin competes with cell surface HSPGs for $A\beta_{42}$ binding and thereby hampers $A\beta_{42}$ uptake[309].

Several cell surface receptors have been demonstrated to facilitate $A\beta_{42}$ internalization, and most are reported to do so solely as a consequence of their associations with PrP^c. PrP^c binds to approximately 50% of $A\beta_{42}$ on the neuronal cell surface and has therefore been reported to be central to the internalization of at least 50% of extracellular $A\beta_{42}$[311]. However, PrP^c lacks a transmembrane domain, a feature vital to this process, and it is therefore likely that PrP^c recruits its receptors to facilitate this uptake. Such receptors include: N-methyl-D-aspartate (NMDA) receptors[312], metabotropic glutamate receptor 5 (mGluR5)[313] and low-density lipoprotein receptor-related protein 1 (LRP1)[145].

It must be noted that although these receptors have received the most attention with regards to $A\beta_{42}$ internalization they could not have contributed to the uptake observed during this study as HEK293 cells express very low levels of LRP1[298] and do not endogenously express glutamate receptors (NMDA and mGluR5)[314].

Studies are currently underway to investigate the dependence of LRP/LR-mediated $A\beta_{42}$ internalization on the presence of cell surface PrP^c.

It is highly plausible that LRP/LR, HSPGs and PrP^c may function co-operatively in mediating $A\beta_{42}$ internalization.

5.4 The possible consequences of 37kDa/67kDa LRP/LR acting as a receptor for Aβ binding and internalization

The consequences of LRP/LR forming an association with $A\beta_{42}$ along its C-terminal domain and serving as a receptor for $A\beta_{42}$ internalization may be vast.

The most apparent outcome of such an association is that LRP/LR may facilitate intracellular $A\beta_{42}$ accumulation and aggregation. Internalization mediated by LRP/LR results in the transfer of its cargo to the endo-lysosomal pathway[257] and the fate of an $A\beta_{42}$ load would be no different. The low pH within the late endosomes and lysosomes as well as their ability to concentrate solutes promotes fibril formation and aggregation[302]. Furthermore, $A\beta_{42}$ is

notoriously resistant to lysosomal enzyme-mediated degradation[302]. $A\beta_{42}$ accumulation within the endo-lysosomal system enhances the permeability of the membranes of these organelles, resulting in the leakage of lysosomal contents into the cytosol[315]. The lysosomal enzymes released may in turn further contribute to cellular damage through the hydrolysis of vital proteins (such cytoskeletal proteins) as well as through the induction of plasma membrane blebbing - the distortion (formation of irregular bulbs) of the membrane as a consequence of local cytoskeletal decoupling from the plasma membrane [315]. In addition, the release of the internalized $A\beta_{42}$ allows for amyloid aggregates to accumulate within the cell which consequently has adverse effects on cellular processes (such as inhibition of axonal transport[316]). Moreover, cytosolic $A\beta_{42}$ may disrupt mitochondrial functioning and may promote oxidative stress, which inhibits proliferation and induces apoptosis (as discussed in section **1.9.3** above). Many AD researchers now consider the intraneuronal accumulation of $A\beta_{42}$ as the first step in the fatal cascade leading to AD establishment[305].

It has been demonstrated that LRP/LR contributes to the internalization of the neurotoxic peptide and may thereby transfer $A\beta_{42}$ to the endo-lysosomal system and this may thereby underlie the role of LRP/LR in mediating $A\beta_{42}$ pathogenesis.

In addition, there may be several secondary consequences of this internalization process. Since the binding of $A\beta_{42}$ to LRP/LR induces internalization, an increased $A\beta_{42}$ concentration would increase the rate of internalization ultimately resulting in a reduction of cell surface-associated LRP/LR. This would therefore result in fewer LRP/LR–laminin-1 interactions and thus prevent the pro-survival and pro-proliferation signals transduced as a consequence of this association. Thus, the observed inhibition of cellular proliferation caused by the LRP/LR-$A\beta_{42}$ association, may be a result of aberrations caused in the pro-survival LRP/LR-mediated signalling pathways and not intracellular $A\beta_{42}$ accumulation alone.

Furthermore, the LRP/LR-laminin-1 association has been reported to induce the activity of MAP Kinase phosphatases such as MKP1 and PAC1[212] (discussed above in section **1.11.1.1** above), which consequently dephosphorylate and deactivate ERK, p38 and JNK. However, deterring this association (through enhanced LRP/LR internalization) may consequently reduce the repression of these MAPK signalling cascades. This is deleterious as activated JNK [268, 269], p38 [265, 270] and ERK[265] have all be implicated in $A\beta_{42}$- induced apoptosis (discussed in detail in section **1.12.2** above). Thus, reduced cell surface LRP/LR levels may contribute to altered phosphatase activity which may directly modulate MAPK signalling

(ERK, JNK and p38 levels and activities) which plays a central role in $A\beta_{42}$-mediated apoptosis and therefore AD pathogenesis.

As previously discussed (in section **1.11.2.2** above) LRP/LR is expressed at high levels on human brain microvascular endothelial cells (BMECs), which constitute the BBB. LRP/LR has also been firmly established as a central receptor for mediating BBB crossing of pathogens and thereby allows for pathogenic cerebral infiltration[254]. Thus, based on the findings reported here, it is probable that LRP/LR associated with the BBB may interact with and mediate the uptake of $A\beta_{42}$ from the periphery. This uptake would contribute to the enhanced cerebral and CSF $A\beta_{42}$ levels and reduced plasma $A\beta_{42}$ levels which are indicative of AD progression (as discussed with regards to biomarkers in section **1.4** above). Augmentation of the intracerebral and CSF $A\beta_{42}$ levels would clearly promote $A\beta_{42}$ internalization and the toxic effects thereof. Thus, it is feasible that LRP/LR may play a role in AD pathogenesis by aiding the accrual of intracerebral $A\beta_{42}$.

All the aforementioned consequences are purely speculative and experimentation to investigate whether these processes may indeed contribute to $A\beta_{42}$ pathogenesis must be conducted.

5.5 Conclusion

In conclusion, the findings obtained during the course of this PhD suggest that the 37kDa/67kDa LRP/LR forms a physiologically relevant association with $A\beta_{42}$ on the cell surface. In addition, it has been demonstrated that this association is central to the apoptosis and inhibition of proliferation induced by $A\beta_{42}$. Moreover, LRP/LR has been shown to act as a receptor for $A\beta_{42}$ internalization, and may thereby contribute to AD by mediating processes leading to intraneuronal $A\beta_{42}$ accumulation and the deleterious cellular effects thereof. Antibody blockade and shRNA-mediated downregulation of LRP/LR impeded $A\beta_{42}$ internalization and rescued cells from $A\beta_{42}$-induced cell death and inhibition of proliferation. Thus, LRP/LR represents a novel target in Alzheimer's Disease. The significant cell biological rescuing effects observed during this study suggest that the aforementioned tools directed against LRP/LR, namely the anti-LRP/LR specific antibody IgG1-iS18 and shRNAs, may show promise as possible prophylactic and/or therapeutic AD tools. Since there are as yet no non-palliative AD therapeutics, a feature shared by all other neurodegenerative

disorders, these findings are of immense importance as these tools may show potential as novel disease-modifying therapeutics for the treatment of Alzheimer's Disease.

5.6 Future Work

Although this study has elucidated a novel role for LRP/LR in $A\beta_{42}$-mediated pathogenesis and a mechanism underlying the role of the receptor in this process has been identified, many other contributing factors and mechanisms may be additionally involved and therefore require elucidation. Furthermore, whether LRP/LR is dependent on PrP^c to mediate the functions revealed in this study requires investigation.

It must be noted that previous administration of single chain Fv antibodies directed against the 37kDa/67kDa Laminin receptor[261] and siRNA downregulation of LRP/LR did not have any side effects *in vivo* and thus it is presumed that IgG1-iS18 (an improved full length antibody) would similarly not result in adverse side-effects. The overall pharmacological properties of IgG1-iS18 are considered generally appropriate since IgG1-iS18 exhibits high specificity for the 37kDa/67kDa LRP/LR and binds exclusively to peptide TEDWSAAPT, which spans the region 272-280aa[246]. Furthermore, the half-life of W3, a polyclonal anti-LRP/LR antibody, in the blood is 14 days[260] and it is presumed that IgG1-iS18 woud have a comparable half-life. However, the large size of full-length IgG antibodies (150kDa) would limit the blood-brain barrier permeability of this treatment strategy.

The blood-brain barrier permeability of IgG1-iS18 as well as the safety, pharmacokinetics and pharmacodynamics of the antibody must be investigated prior to the initiation of animal trials.

Thereafter, the efficacy of the antibody will be validated in animal trials. Transgenic AD mice harbouring 5 EOAD/FAD mutations (3 mutations in *APP* and 2 mutations in the *PSEN1* encoding genes) develop pathological features mimicking the human condition within 4 months, namely: plaque deposition, synaptic and neuronal loss as well as cognitive deficits. These transgenic mice will be the most suitable to assess whether IgG1-iS18 is able to slow/prevent the neuronal cell loss and consequently prevent or prolong the period prior to the cognitive decline associated with AD progression.

In the event that the animal trials are successful, phase I clinical trials will be initiated to assess the efficacy of IgG1-iS18 treatment in human patients.

Chapter 6

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Annexure I

Patent Application

Patent Title: Compounds for use in the treatment of Alzheimer's disease

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Applicant: University of the Witwatersrand

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Description:

The Patent application PCT/IB2012/054918 (Weiss 3), entitled "compounds for use in the treatment of Alzheimer's disease" was published as WO 2013/042053 on 28 March 2013, in the name of the University of the Witwatersrand. The claims of this patent application involve methods employing tools targeting the laminin receptor (LRP/LR) as a means of reducing Alzheimer's Disease proteins and thereby possibly as treatment strategies for Alzheimer's Disease. National applications have been filed in the US (14/345,770), Europe, China and South Africa (2014/02471).

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(54) Title: COMPOUNDS FOR USE IN THE TREATMENT OF ALZHEIMER'S DISEASE

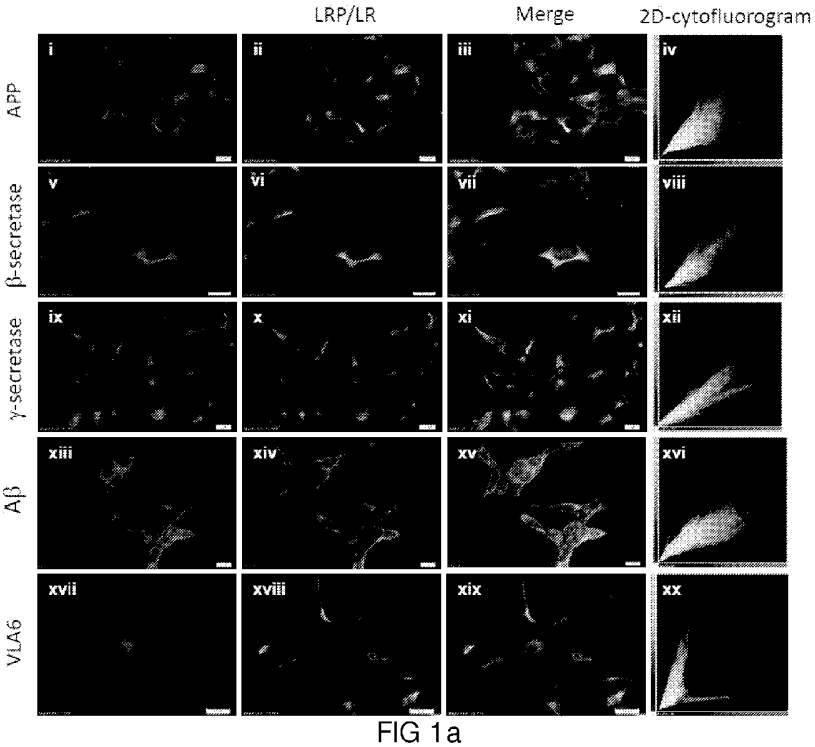


FIG 1a

(57) Abstract: The invention relates to a method of modulating concentration of Alzheimer's Disease (AD) relevant proteins amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), and also relates to a method of reducing Aβ shedding. Furthermore, this invention extends to a compound for use in the treatment of AD, and also to a method of treating AD.

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COMPOUNDS FOR USE IN THE TREATMENT OF ALZHEIMER'S DISEASE

FIELD OF INVENTION

This invention relates to a compound for the modulation of 37 kDa/67 kDa laminin receptor in humans and/or animals. The invention extends to a method of modulating concentration of Alzheimer's Disease (AD) relevant proteins amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ). Furthermore, this invention extends to a compound for use in the treatment of AD, and also to a method of treating AD.

BACKGROUND

Alzheimer's disease (AD) is notably the most prevalent form of dementia afflicting the elderly and is associated with a multitude of genetic, environmental, epigenetic, dietary and lifestyle risk factors[1]. AD is said to affect in excess of 37 million people globally[4].

The neuropathological hallmarks of AD include intracellular neurofibrillary tangle formation and extracellular amyloid beta peptide (Aβ) plaque deposition[5]. The sequential cleavage of the amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases[2] results in the shedding of the 4kDa Aβ which aggregates to form amyloid plaques. Aβ, as a soluble oligomer, as well as plaque-incorporated aggregate, is the predominant focus of investigative efforts to treat AD.

Aβ and more specifically the 42 amino acid isoform ($A\beta_{42}$), is largely considered the primary disease causing agent in AD (as Aβ accumulation is a pre-requisite for tau hyperphosporylation, another AD-associated protein)[6]. Specifically, Aβ is generated through the proteolytic cleavage of the type I transmembrane protein APP by β- and γ- secretase. The mechanisms underlying Aβ induction of neuronal loss (one of the key pathophysiological features of AD) are yet to be firmly established. It is proposed that the neurotoxicity of Aβ is partially mediated through its interactions with cellular receptors[3]. These interactions may include binding of Aβ to a surface receptor on a neuron thereby changing its biochemical structure, which negatively affects neuronal communication. It is proposed that Aβ may affect neuronal communication by eliciting alterations in signal transduction pathways

through direct binding to cell surface receptors, (such as N-methyl-d-aspartate (NMDA) receptors, insulin receptors, α-7 nicotinic receptors)[3,7]. Alternatively, Aβ may alter signal transduction pathways indirectly via incorporation into lipid membranes of the plasma membrane and, to a lesser extent, cellular organelles[8]. This is thought to induce structural and functional alterations in lipid bound receptors and consequently results in aberrant signal transduction pathways[8].

There is a need for compounds which in use modulate the production and concentrations of APP, (β) and (γ) secretases and Aβ in a human or animal in order to treat AD. There is a further need for compounds that modulate intracellular neurofibrillary tangle formation and extracellular Aβ plaque deposition in order to treat AD.

SUMMARY

According to a first aspect of this invention there is provided a method for reducing concentration of at least one Alzheimer's Disease (AD) relevant protein selected from the group including, but not limited to, amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), the method comprising contacting a cell surface protein, preferably an extracellular matrix glycoprotein, with a cell surface protein specific antibody, preferably a monoclonal antibody, or any fragment of the aforementioned, such that binding occurs between a surface epitope of the cell surface protein and the cell surface protein specific antibody causing a decrease in the concentration of the at least one AD relevant proteins.

The reduction in Aβ concentration may be a reduction relative to Aβ concentration in a normal healthy human or animal, or it may be a reduction relative to Aβ concentration in a human or animal suffering from AD.

It is to be understood that the binding between the surface epitope of the cell surface protein and the cell surface protein specific antibody at least hinders, preferably prevents, binding of the at least one AD relevant proteins to the cell surface protein.

The cell surface protein may be a laminin receptor protein. In a preferred embodiment of the invention the laminin receptor is 37 kDa/67 kDa laminin receptor (LRP/LR) of a human

and/or animal. LRP/LR is also known as LAMR, RPSA and p40. The cell surface protein may also be a protein showing at least 80% or greater homology to the laminin receptor protein, preferably showing at least 80% or greater homology to LRP/LR.

In a preferred embodiment of the invention the AD relevant protein whose concentration is reduced via the method of this invention is Aβ. The reduced amount of Aβ causes reduced intracellular neurofibrillary tangle formation and/or reduced extracellular Aβ plaque deposition in human and/or animal cells, preferably neuronal cells, therein treating and/or preventing AD.

The cell surface protein specific antibody may be any antibody, or fragment thereof, raised against the cell surface protein. In a preferred embodiment the antibody is raised against LRP/LR or against a protein having 80% or greater homology with LRP/LR. The antibody, or fragment thereof, may be a F(ab')2 fragment, a Fab fragment scFv, a bi-specific scFv, a tri-specific scFv, a single chain or tandem diabody, a single domain antibody (dAb), a minibody or a molecular recognition unit (MRU). Furthermore, the antibody, or fragment thereof, may be monovalent, bivalent or multivalent. The antibody, or fragment thereof, may additionally comprise at least one further antigen-interaction site and/or at least one further effector domain.

In a preferred embodiment of the invention, the antibody or fragment thereof may be an anti-laminin receptor specific antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18.

In a preferred embodiment of the invention the cell surface protein and/or the cell surface protein specific antibody is a human or animal cell surface protein and/or cell surface protein specific antibody. The cell surface protein may be located on murine neuronal cells (N2a), human neuronal cells (SH-SY5Y), baby hamster kidney cells (BHK) and human embryonic kidney cells (HEK293 and/or HEK293 FT).

In a preferred embodiment of the invention, the method for reducing concentration of at least one AD relevant protein selected from the group including, but not limited to, APP, beta (β) and gamma (γ) secretases and Aβ, the method comprises contacting LRP/LR with IgG1-iS18,

or any fragment thereof, such that binding occurs between LRP/LR and IgG1-iS18, or any fragment thereof, causing a decrease in the concentration of Aβ.

According to a second aspect of this invention there is provided a method for reducing amyloid beta peptide (Aβ) shedding caused by the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases, the method comprising contacting a cell surface protein, preferably an extracellular matrix glycoprotein, with a cell surface protein specific antibody, preferably a monoclonal antibody, or any fragment of the aforementioned, such that binding occurs between a surface epitope of the cell surface protein and the cell surface protein specific antibody therein hindering the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases.

The reduction in Aβ shedding may be a reduction relative to Aβ shedding in a normal healthy human or animal, or it may be a reduction relative to Aβ shedding in a human or animal suffering from AD.

It is to be understood that the binding between the surface epitope of the cell surface protein and the cell surface protein specific antibody at least hinders, preferably prevents, binding of at least one of the AD relevant proteins APP, beta (β) and gamma (γ) secretases and Aβ to the cell surface protein. It is to be understood that this binding causes a reduction in Aβ shedding.

The cell surface protein may be a laminin receptor protein. In a preferred embodiment of the invention the laminin receptor is 37 kDa/67 kDa laminin receptor (LRP/LR) of a human and/or animal. LRP/LR is also known as LAMR, RPSA and p40. The cell surface protein may also be a protein showing at least 80% or greater homology to the laminin receptor protein, preferably showing at least 80% or greater homology to LRP/LR.

The cell surface protein specific antibody may be any antibody, or fragment thereof, raised against the cell surface protein. In a preferred embodiment the antibody is raised against LRP/LR or against a protein having 80% or greater homology with LRP/LR. The antibody, or fragment thereof, may be a F(ab')2 fragment, a Fab fragment scFv, a bi-specific scFv, a tri-specific scFv, a single chain or tandem diabody, a single domain antibody (dAb), a minibody or a molecular recognition unit (MRU). Furthermore, the antibody, or fragment thereof, may

be monovalent, bivalent or multivalent. The antibody, or fragment thereof, may additionally comprise at least one further antigen-interaction site and/or at least one further effector domain.

In a preferred embodiment of the invention, the antibody or fragment thereof may be an anti-laminin receptor specific antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18.

In a preferred embodiment of the invention the cell surface protein and/or the cell surface protein specific antibody is a human or animal cell surface protein and/or cell surface protein specific antibody. The cell surface protein may be located on murine neuronal cells (N2a), human neuronal cells (SH-SY5Y), baby hamster kidney cells (BHK) and human embryonic kidney cells (HEK293 and/or HEK 293 FT).

In a preferred embodiment of the invention, the method for reducing Aβ shedding caused by the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases, the method comprises contacting LRP/LR with IgG1-iS18, or any fragment thereof, such that binding occurs between LRP/LR and IgG1-iS18, or any fragment thereof, causing a reduction in Aβ shedding.

According to a third aspect of this invention there is provided a method for reducing concentration of at least one Alzheimer's Disease (AD) relevant protein selected from the group including, but not limited to, amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), the method comprising contacting a cell surface protein, preferably an extracellular matrix glycoprotein, with a nucleotide sequence, preferably an RNA sequence, further preferably a short hairpin RNA (shRNA) sequence or a short interfering RNA (siRNA) sequence or a micro RNA (miRNA) sequence, such that binding occurs between mRNA of the cell surface protein and the nucleotide sequence causing a downregulation of the cell surface protein which in turn causes a decrease in the concentration of the at least one AD relevant proteins.

It is to be understood that the binding between the mRNA of the cell surface protein and the nucleotide sequence downregulates the cell surface protein such that there are fewer cell

surface proteins present on the cell when compared to regular physiological functioning. Therefore, there are fewer binding sites available for the at least one AD relevant proteins to bind to. A reduction in binding sites leads to reduced concentrations of the at least one AD relevant proteins APP, beta (β) and gamma (γ) secretases and Aβ.

The cell surface protein may be a laminin receptor protein. In a preferred embodiment of the invention the laminin receptor is 37 kDa/67 kDa laminin receptor (LRP/LR) of a human and/or animal. LRP/LR is also known as LAMR, RPSA and p40. The cell surface protein may also be a protein showing at least 80% or greater homology to the laminin receptor protein, preferably showing at least 80% or greater homology to LRP/LR.

Preferably, when binding between the nucleotide sequence and the mRNA occurs, such binding is between the nucleotide sequence and LRP mRNA.

In a preferred embodiment of the invention the AD relevant protein whose concentration is reduced via the method of this invention is Aβ. The reduced amount of Aβ causes reduced intracellular neurofibrillary tangle formation and/or reduced extracellular Aβ plaque deposition in human and/or animal cells, preferably neuronal cells, therein treating and/or preventing AD. The reduction in Aβ concentration may be a reduction relative to Aβ concentration in a normal healthy human or animal, or it may be a reduction relative to Aβ concentration in a human or animal suffering from AD.

The nucleotide sequence is preferably shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

In a preferred embodiment of the invention the cell surface protein and/or the nucleotide sequence is a human or animal cell surface protein and/or nucleotide sequence. The cell surface protein may be located on murine neuronal cells (N2a), human neuronal cells (SH-SY5Y), baby hamster kidney cells (BHK) and human embryonic kidney cells (HEK293 and/or HEK 293 FT).

In a preferred embodiment of the invention, the method for reducing concentration of at least one AD relevant protein selected from the group including, but not limited to, APP, beta (β)

and gamma (γ) secretases and Aβ, the method comprises contacting LRP mRNA with shRNA1 and/or shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively, such that binding occurs between the LRP mRNA and shRNA1 and/or shRNA7 causing a decrease in the concentration of Aβ.

According to a fourth aspect of this invention there is provided a method for reducing Aβ shedding caused by the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases, the method comprising contacting a cell surface protein, preferably an extracellular matrix glycoprotein, with a nucleotide sequence, preferably an RNA sequence, further preferably a short hairpin RNA (shRNA) sequence or a short interfering RNA (siRNA) sequence or a mirco RNA (miRNA) sequence, such that binding occurs between mRNA of the cell surface protein and the nucleotide sequence causing a downregulation of the cell surface protein which in turn causes a decrease in the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases.

The reduction in Aβ shedding may be a reduction relative to Aβ shedding in a normal healthy human or animal, or it may be a reduction relative to Aβ shedding in a human or animal suffering from AD.

It is to be understood that the binding between the mRNA of the cell surface protein and the nucleotide sequence downregulates the cell surface protein such that there are fewer cell surface proteins present on the cell when compared to regular physiological functioning. Therefore, there are fewer binding sites available for the at least one AD relevant proteins to bind to. A reduction in binding sites leads to a reduction in Aβ shedding.

The cell surface protein may be a laminin receptor protein. In a preferred embodiment of the invention the laminin receptor is 37 kDa/67 kDa laminin receptor (LRP/LR) of a human and/or animal. LRP/LR is also known as LAMR, RPSA and p40. The cell surface protein may also be a protein showing at least 80% or greater homology to the laminin receptor protein, preferably showing at least 80% or greater homology to LRP/LR.

Preferably, when binding between the nucleotide sequence and the mRNA occurs, such binding is between the nucleotide sequence and LRP mRNA.

The nucleotide sequence is preferably a shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

In a preferred embodiment of the invention the cell surface protein and/or the nucleotide sequence is a human or animal cell surface protein and/or nucleotide sequence. The cell surface protein may be located on murine neuronal cells (N2a), human neuronal cells (SH-SY5Y), baby hamster kidney cells (BHK) and human embryonic kidney cells (HEK293 and/or HEK 293 FT).

In a preferred embodiment of the invention, the method for reducing Aβ shedding caused by the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases, the method comprises contacting LRP mRNA with shRNA1 and/or shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively, such that binding occurs between LRP mRNA and shRNA1 and/or shRNA7 reducing LRP/LR cell surface levels causing a reduction in Aβ shedding.

According to a fifth aspect of this invention there is provided a method for reducing concentration of at least one Alzheimer's Disease (AD) relevant protein selected from the group including, but not limited to, amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), the method comprising contacting a cell surface protein of the first aspect of the invention with the antibody of the first aspect of the invention and the nucleotide sequence of the third aspect of the invention.

According to a sixth aspect of this invention there is provided a method for reducing Aβ shedding caused by the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases, the method comprising the method comprising contacting a cell surface protein of the second aspect of the invention with the antibody the second aspect of the invention and the nucleotide sequence of the fourth aspect of the invention.

According to a seventh aspect of this invention there is provided for use of an anti-laminin receptor specific antibody in the manufacture of a pharmaceutical composition to treat Alzheimer's Disease (AD).

In a preferred embodiment of the invention the anti-laminin receptor specific antibody may be anti-37 kDa/67 kDa laminin receptor (LRP/LR) specific antibody, further preferably the anti-LRP/LR specific antibody may be IgG1-iS18.

According to an eighth aspect of this invention there is provided an anti-laminin receptor specific antibody for use in treating Alzheimer's Disease (AD). In a preferred embodiment of the invention the anti-laminin receptor specific antibody may be anti-37 kDa/67 kDa laminin receptor (LRP/LR) specific antibody, further preferably the anti-LRP/LR specific antibody may be IgG1-iS18.

According to a ninth aspect of this invention there is provided for use of a nucleotide sequence in the manufacture of a pharmaceutical composition to treat Alzheimer's Disease (AD).

In a preferred embodiment of the invention the nucleotide sequence may be a shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively. The use may further include an anti-laminin specific receptor antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18.

According to a tenth aspect of this invention there is provided a nucleotide sequence for use in treating Alzheimer's Disease (AD).

In a preferred embodiment of the invention the nucleotide sequence may be a shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively. There is provided for the nucleotide sequence to be used together with an anti-laminin specific receptor antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18.

According to an eleventh aspect of this invention there is provided a method of treating Alzheimer's Disease (AD) comprising administering an anti-laminin specific receptor antibody, preferably an anti-37 kDa/67 kDa laminin receptor (LRP/LR) specific antibody, further preferably the anti-LRP/LR specific antibody may be IgG1-iS18 to a human or animal in need thereof.

According to a twelfth aspect of this invention there is provided a method of treating Alzheimer's Disease (AD) comprising administering a nucleotide sequence, preferably shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively, to a human or animal in need thereof. The method may further include administering an anti-laminin specific receptor antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18, to the human or animal in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is now described, by way of example only, with reference to the accompanying diagrammatic drawings, in which

Figure 1a shows co-localisation of 37 kDa/67 kDa laminin receptor (LRP/LR) with the Alzheimer's Disease (AD) relevant proteins amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), on the surface of human embryonic kidney cells (HEK293) cells via immunofluorescence microscopy;

Figure 1aa shows black and white line drawings of Figure 1a;

Figure 1b shows co-localisation of LRP/LR with the AD relevant proteins APP, beta (β) and gamma (γ) secretases and Aβ, on the surface of murine neuroblastoma (N2a) cells via immunofluorescence microscopy;

Figure 1bb shows black and white line drawings of Figure 1b;

Figure 2a shows Aβ concentrations in HEK293 and human neuronal cells (SH-SY5Y) cells after treatment with antibodies IgG1-iS18 and IgG1-HD37 as detected by an Aβ ELISA (Human Amyloid β(1-x) Assay Kit (IBL)) after 18 hours of antibody incubation;

Figure 2b shows Aβ concentrations after SH-SY5Y cells were treated with varying doses of IgG1-iS18 for 18 hours, as determined by an Aβ ELISA;

Figure 2c shows flow cytometric analysis of APP, β-secretase and γ-secretase levels on the surface of human embryonic kidney cells (HEK293FT) and SH-SY5Y cells post treatment with IgG1-iS18;

Figure 2d shows a Western blot analysis depicting sAPPβ (SAPPβ is a shedded cleavage product of APP generated by the action of β-secretase) levels from cell culture medium after SH-SY5Y cells were treated with varying concentrations (0- 100 μg/ml) of IgG1-iS18 for 18 hours;

Figure 3 shows LRP/LR target sequences and structure of short hairpin RNA1 (shRNA1) and short hairpin RNA7 (shRNA7);

Figure 4a shows a Western Blot analysis of HEK293 cells transfected with LRP-specific shRNA1 and shRNA7 (as well as a scrambled control, shRNAscr);

Figure 4b shows the effects of shRNA on downregulation of LRP/LR wherein the Aβ concentration of the cell culture medium of shRNA-transfected HEK293 cells was analysed using an Aβ ELISA;

Figure 4c shows flow cytometric analysis of APP, β-secretase and γ-secretase levels on the surface of shRNA-transfected HEK293 cells;

Figure 4d shows sAPPβ levels in shRNA-transfected HEK293 cells were analysed by Western blotting;

Figure 4e shows Aβ concentration of the cell culture medium of shRNAscr-transfected and mock-transfected HEK293 cells;

Figure 5a shows flow cytometry histogram overlay plots for β-secretase, γ-secretase and APP after antibody treatment to HEK293 cells;

Figure 5b shows flow cytometry histogram overlay plots for β-secretase, γ-secretase and APP after antibody treatment to SH-SY5Y cells;

Figure 5c shows flow cytometry histogram overlay plots for β-secretase, γ-secretase and APP after shRNA treatment to HEK293 cells were transfected with either shRNA1, shRNA7 or shRNAscr;

Figure 6a(i) shows a pull down assay of cell lysates containing recombinantly expressed LRP/LR::FLAG co-incubated with exogenous Aβ;

Figure 6a (ii) shows an immunoblot employed to validate the position of LRP::FLAG (~38kDa);

Figure 6b shows 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);

Figure 6c shows cellular viability of SH-SY5Y cells;

Figure 6d shows cellular viability of N2a cells;

Figure 6e shows cellular proliferation of N2a cells as determined by colorimetric 5-bromo-2'-deoxyuridine (BrdU) non-isotopic immunoassay (Calbiochem®), allowing 4h for BrdU incorporation into cultured cells; and

Figure 7 shows post pull down assay protein detection of control samples.

DETAILED DESCRIPTION OF THE DRAWINGS

According to a first aspect of this invention there is provided a method for reducing concentration of at least one Alzheimer's Disease (AD) relevant protein selected from the group including, but not limited to, amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), the method comprising contacting a cell surface protein, preferably an extracellular matrix glycoprotein, with a cell surface protein specific antibody, preferably a monoclonal antibody, or any fragment of the aforementioned, such that binding occurs between a surface epitope of the cell surface protein and the cell surface protein specific antibody causing a decrease in the concentration of the at least one AD relevant proteins.

The reduction in concentration of the at least one AD relevant protein is a reduction relative to the amount of AD relevant proteins in a normal healthy human or animal, or relative to a human or animal suffering from AD.

It is to be understood that the binding between the surface epitope of the cell surface protein and the cell surface protein specific antibody at least hinders, preferably prevents, binding of at least one of the AD relevant proteins to the cell surface protein.

The cell surface protein may be a laminin receptor protein. However, in a preferred embodiment of the invention the laminin receptor is 37 kDa/67 kDa laminin receptor (LRP/LR) of a human and/or animal. LRP/LR is also known as LAMR, RPSA and p40. The cell surface protein may also be a protein showing at least 80% or greater homology to the laminin receptor protein, preferably showing at least 80% or greater homology to LRP/LR.

In the examples illustrated and/or exemplified below the AD relevant protein whose concentration is reduced via the method of this invention is Aβ. The reduced amount of Aβ causes reduced intracellular neurofibrillary tangle formation and/or reduced extracellular Aβ plaque deposition in human and/or animal cells, preferably neuronal cells, therein treating and/or preventing AD. When exercising this method of reducing Aβ concentration in an effort to treat and/or prevent AD, the contacting typically takes place in vivo.

The cell surface protein specific antibody may be any antibody, or fragment thereof, raised against the cell surface protein. Typically, the antibody is raised against LRP/LR or against a protein having 80% or greater homology with LRP/LR. The antibody, or fragment thereof, may be a F(ab')2 fragment, a Fab fragment scFv, a bi-specific scFv, a tri-specific scFv, a single chain or tandem diabody, a single domain antibody (dAb), a minibody or a molecular recognition unit (MRU). Furthermore, the antibody, or fragment thereof, may be monovalent, bivalent or multivalent. The antibody, or fragment thereof, may additionally comprise at least one further antigen-interaction site and/or at least one further effector domain. Preferably, the antibody or fragment thereof an anti-laminin receptor specific antibody, further preferably anti-LRP/LR specific antibody, still further preferably IgG1-iS18.

The cell surface protein and/or the cell surface protein specific antibody is typically a human or animal cell surface protein and/or cell surface protein specific antibody. The cell surface protein may be located on murine neuronal cells (N2a), human neuronal cells (SH-SY5Y), baby hamster kidney cells (BHK) and human embryonic kidney cells (HEK293 and/or HEK 293 FT).

In a preferred embodiment of the invention, the method for reducing concentration of at least one AD relevant protein comprises contacting LRP/LR with IgG1-iS18, or any fragment thereof, such that binding occurs between LRP/LR and IgG1-iS18, or any fragment thereof,

causing a decrease in the concentration of Aβ. From a practical point of view, when exercising this method of reducing Aβ concentration in an effort to treat and/or prevent AD, the antibody (typically IgG1-iS18, or any fragment thereof) is formulated into a pharmaceutical composition and further formulated into a pharmaceutical dosage form to be administered to a human or animal in need of treatment. The pharmaceutical composition may include excipients. The dosage form may be formulated to deliver the pharmaceutical composition via oral and/or parenteral means.

According to a second aspect of this invention there is provided a method for reducing Aβ shedding caused by the proteolytic cleavage of APP by beta (β) and/or gamma (γ) secretases, the method comprising contacting the cell surface protein described above, with the cell surface protein specific antibody described above, such that binding occurs between a surface epitope of the cell surface protein and the cell surface protein specific antibody therein hindering the proteolytic cleavage of APP by beta (β) and/or gamma (γ) secretases.

The reduction in Aβ shedding may be a reduction relative to Aβ shedding in a normal healthy human or animal, or it may be a reduction relative to Aβ shedding in a human or animal suffering from AD.

It is to be understood that the binding between the surface epitope of the cell surface protein and the cell surface protein specific antibody at least hinders, preferably prevents, binding of at least one of the AD relevant proteins APP, beta (β) and gamma (γ) secretases and Aβ to the cell surface protein. It is further to be understood that it is this binding that causes a reduction in Aβ shedding.

As mentioned in the first aspect of the invention, in a preferred embodiment of the invention the cell surface protein is a laminin receptor, preferably the laminin receptor is LRP/LR of a human and/or animal. The cell surface protein may also be a protein showing at least 80% or greater homology to the laminin receptor protein, preferably showing at least 80% or greater homology to LRP/LR.

The cell surface protein specific antibody may be any antibody, or fragment thereof, raised against the cell surface protein. In a preferred embodiment the antibody is raised against

LRP/LR or against a protein having 80% or greater homology with LRP/LR. The antibody, or fragment thereof, may be a F(ab')2 fragment, a Fab fragment scFv, a bi-specific scFv, a tri-specific scFv, a single chain or tandem diabody, a single domain antibody (dAb), a minibody or a molecular recognition unit (MRU). Furthermore, the antibody, or fragment thereof, may be monovalent, bivalent or multivalent. The antibody, or fragment thereof, may additionally comprise at least one further antigen-interaction site and/or at least one further effector domain.

In a preferred embodiment of the invention, the antibody or fragment thereof may be an anti-laminin receptor specific antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18.

In a preferred embodiment of the invention the cell surface protein and/or the cell surface protein specific antibody is a human or animal cell surface protein and/or cell surface protein specific antibody. The cell surface protein may be located on murine neuronal cells (N2a), human neuronal cells (SH-SY5Y), baby hamster kidney cells (BHK) and human embryonic kidney cells (HEK293 and/or HEK 293 FT).

In a preferred embodiment of the invention, the method for reducing Aβ shedding caused by the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases, the method comprises contacting LRP/LR with IgG1-iS18, or any fragment thereof, such that binding occurs between LRP/LR and IgG1-iS18, or any fragment thereof, causing a reduction in Aβ shedding. As described in the first aspect of the invention above, the IgG1-iS18 is typically formulated into a pharmaceutical composition which is formulated into a pharmaceutical dosage form which is administered to a human or animal in need of AD treatment.

According to a third aspect of this invention there is provided a method for reducing concentration of at least one AD relevant protein selected from the group including, but not limited to, APP, beta (β) and gamma (γ) secretases and Aβ, the method comprising contacting a cell surface protein, preferably an extracellular matrix glycoprotein, with a nucleotide sequence, preferably an RNA sequence, further preferably a short hairpin RNA (shRNA) sequence or a short interfering RNA (siRNA) sequence or a micro RNA (miRNA) sequence, such that binding occurs between mRNA of the cell surface protein and the nucleotide

sequence causing a downregulation of the cell surface protein which in turn causes a decrease in the concentration of the at last one AD relevant proteins.

The reduction in concentration of the at least one AD relevant protein is a reduction relative to the amount of AD relevant proteins in a normal healthy human or animal, or relative to a human or animal suffering from AD.

The cell surface protein may be a laminin receptor protein and preferably LRP/LR as described above in the first and second aspects of the invention. Preferably, when binding between the nucleotide sequence and the mRNA occurs, such binding is between the nucleotide sequence and LRP mRNA.

In a preferred embodiment of the invention the AD relevant protein whose concentration is reduced via the method of this invention is Aβ as described above.

The nucleotide sequence is preferably shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

In a preferred embodiment of the invention, the method for reducing concentration of at least one AD relevant protein selected from the group including, but not limited to, APP, beta (β) and gamma (γ) secretases and Aβ, the method comprises contacting LRP/LR with shRNA1 and/or shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively, such that binding occurs between LRP mRNA and shRNA1 and/or shRNA7 causing a decrease in the concentration of Aβ.

According to a fourth aspect of this invention there is provided a method for reducing Aβ shedding caused by the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases, the method comprising contacting a cell surface protein, preferably an extracellular matrix glycoprotein, with a nucleotide sequence, preferably an RNA sequence, further preferably a short hairpin RNA (shRNA) sequence or a short interfering RNA (siRNA) sequence or a mirco RNA (miRNA) sequence, such that binding occurs between mRNA of the cell surface protein and the nucleotide sequence causing a

downregulation of the cell surface protein which in turn causes a decrease in the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases.

The cell surface protein may be a laminin receptor protein and preferably LRP/LR as described above in the first and second aspects of the invention. Preferably, when binding between the nucleotide sequence and the mRNA occurs, such binding is between the nucleotide sequence and LRP mRNA.

The reduction in Aβ shedding may be a reduction relative to Aβ shedding in a normal healthy human or animal, or it may be a reduction relative to Aβ shedding in a human or animal suffering from AD.

The nucleotide sequence is preferably a shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

In a preferred embodiment of the invention, the method for reducing Aβ shedding caused by the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases, the method comprises contacting LRP/LR with shRNA1 and/or shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2 respectively, such that binding occurs between LRP mRNA and shRNA1 and/or shRNA7 causing a reduction in LRP/LR cell surface levels and hence a reduction in Aβ shedding.

According to a fifth aspect of this invention there is provided a method for reducing concentration of at least one AD relevant protein selected from the group including, but not limited to, APP, beta (β) and gamma (γ) secretases and Aβ, the method comprising contacting a cell surface protein of the first aspect of the invention with the antibody the first aspect of the invention and the nucleotide sequence of the third aspect of the invention. Essentially, the LRP/LR is downregulated by the shRNAs (either shRNA 1 or 7 or both) and is substantially blocked by the anti-LRP/LR specific antibody, in so doing, further reducing the concentration of Aβ.

According to a sixth aspect of this invention there is provided a method for reducing Aβ shedding caused by the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases, the

method comprising the method comprising contacting a cell surface protein of the second aspect of the invention with the antibody the second aspect of the invention and the nucleotide sequence of the fourth aspect of the invention. Essentially, the LRP/LR is downregulated by the shRNAs (either shRNA 1 or 7 or both) and is substantially blocked by the anti-LRP/LR specific antibody, in so doing, further reducing Aβ shedding.

According to a seventh aspect of this invention there is provided for use of an anti-laminin receptor specific antibody in the manufacture of a pharmaceutical composition to treat AD. Typically, the anti-laminin receptor specific antibody is IgG1-iS18.

According to an eighth aspect of this invention there is provided an anti-laminin receptor specific antibody for use in treating AD. Typically, the anti-laminin receptor specific antibody is IgG1-iS18.

According to a ninth aspect of this invention there is provided for use of a nucleotide sequence in the manufacture of a pharmaceutical composition to treat AD. Typically, the nucleotide sequence may be RNA, preferably a shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively. The use may further include an anti-laminin receptor specific antibody described above.

According to a tenth aspect of this invention there is provided a nucleotide sequence for use in treating AD. Typically, the nucleotide sequence may be RNA, preferably a shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively. There is provided for the nucleotide sequence to be used together with an anti-laminin receptor specific antibody described above for use in treating AD.

According to an eleventh aspect of this invention there is provided a method of treating AD comprising administering an anti-laminin receptor specific antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18 to a human or animal in need thereof.

According to a twelfth aspect of this invention there is provided a method of treating AD comprising administering a nucleotide sequence, preferably a RNA sequence, further preferably shRNA, further preferably at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively, to a human or animal in need thereof. The method may further include administering an anti-laminin receptor specific antibody, preferably an anti-LRP/LR specific antibody, further preferably IgG1-iS18 to the human or animal.

EXAMPLES

A representative example of the invention is described and/or illustrated and/or exemplified below and should not be viewed as limiting to the scope of the invention. The sequence listing attached hereto is incorporated by reference.

In the examples of the invention illustrated and exemplified below, it is shown that 37kDa/67kDa laminin receptor (LRP/LR) is associated with the Alzheimer's Disease (AD) relevant proteins amaloid precursor protein (APP), β- and γ-secretase as well as amaloid beta peptide (Aβ). It was found that Aβ binds to LRP/LR and this interaction may contribute to Aβ-induced cytotoxicity. Furthermore, antibody blockage (or substantial blockage) and shRNA downregulation of LRP/LR was shown to reduce 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 may be implicated in AD pathogenesis and could lead to novel therapeutic interventions for use in modulating LRP/LR and/or modulating the concentration of AD relevant proteins APP, β- and γ-secretases and Aβ in a human or animal in the treatment of AD.

The extracellular matrix glycoprotein, laminin, exhibits an Aβ binding site, namely the IKAV peptide sequence located on the alpha (α) chain of the tri-peptide[15]. However, the association between laminin and Aβ is reported to promote neurite outgrowth[16] and inhibit fibrillogenesis[15] and thereby thwart Aβ pathogenesis. Prior research does not suggest LRP/LR functioning being important in the determining concentrations of the abovementioned AD relevant proteins, specifically Aβ, nor does the prior art suggest that LRP/LR could play any role in impeding β-secretase activity.

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[17]. Associations between LRP/LR 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[18].

The experimental protocols described hereunder show a nexus between LRP/LR and the amyloidgenic pathway in AD, more specifically a nexus between LRP/LR and Aβ shedding into the extracellular space.

To explore the above mentioned nexus indirect immunofluorescence microscopy was employed to assess the cellular distribution of AD relevant proteins, namely the APP, β- and γ-secretases and Aβ. LRP/LR was shown to co-localise with APP (Figure 1a, i-iv), β-secretase (Figure 1a, v-viii), γ-secretase (Figure 1a, ix-xii) and Aβ (Figure 1a, xiii-xvi) on the surface of non-permeabilised human embryonic kidney cells (HEK293). An alternative laminin binding receptor, Very Late Antigen 6 (VLA6), was employed as a negative control (Figure 1a, xvii-xx). Analogous results were obtained for murine neuroblastoma (N2a) cells (Figure 1b).

In general, Figure 1a shows co-localisation of LRP/LR with the AD relevant proteins APP, β-secretase, γ-secretase and Aβ on the surface of human embryonic kidney cells (HEK293 and/or HEK 293 FT) cells. Figure 1a shows cell surface receptors on HEK293 cells having been indirectly immunolabelled to allow for detection using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software. In particular, Figure 1a shows (i) APP (detected by anti-APP (rabbit polyclonal IgG) (Abcam), (v), β-secretase (detected using anti-BACE (M-83) (rabbit polyclonal IgG) (Santa Cruz Biotechnology)), (ix), γ-secretase (detected by anti-PEN-2 (FL-101) (rabbit polyclonal IgG) (Santa Cruz Biotechnology)), (xii), Aβ (detected using anti-β-amyloid (22-35) (Sigma)) and (xvii), VLA6 (detected by anti-very late antigen-6 (VLA6) CD49-f (rabbit monoclonal IgG) (Immunotech) were indirectly labelled with Alexaflour 633, while an anti-human FITC coupled antibody (Cell Lab) was used to label LRP/LR (ii, vi, x, xiv, xviii).

The merges between LRP/LR and AD relevant proteins are shown (iii, vii, xi, xv, xix) in Figure 1a and the corresponding 2D-cytofluorograms (acquired using CellSens Software) have been included to confirm the degree of co-localisation (iv, viii, xii, xvi, xx).

Figure 1b shows the same as Figure 1a above, but as seen on the surface of N2a cells. Scale bars on the figures are 10μm.

The proximity of the AD relevant proteins on the cell surface thereby suggested that an association/interaction between LRP/LR and AD relevant proteins is feasible and that the receptor may indeed be implicated in AD pathogenesis. 2D-cytofluorograms (Figure 1a and b, iv, viii, xii, xvi) show the joint distribution of the red and green fluorescence, with a diagonal indicating co-localisation between the cell surface proteins of interest. Figure 1aa and 1bb show a black and white reproducible version of Figure 1a and 1b, where a diagonal again indicates co-localisation between the cell surface proteins of interest. Pearson's Correlation co-efficients for co-localisation were employed to further confirm the observed results as shown in Table 1 below.

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

	human embryonic kidney cells (HEK293)	Murine neuronal cells (N2a)
LRP/LR+APP	0.862	0.948
LRP/LR+Aβ	0.926	0.969
LRP/LR+β-secretase	0.915	0.900
LRP/LR+γ-secretase	0.938	0.914
LRP/LR+VLA6	0.583	0.563

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 0 is indicative of no co-localisation between proteins of interest. The co-efficient was calculated for LRP/LR and AD relevant proteins APP, Aβ, β- and γ-secretase respectively, as well as the negative control VLA6.

To investigate whether the 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[21] and anti-cluster of differentiation (CD19) antibody IgG1-HD37[21] (negative control).

Essentially, cellular incubation with IgG1-iS18 resulted in a significant reduction (47.6% in human embryonic kidney cells (HEK293FT) and 28.5% in human neuronal cells (SH-SY5Y)) in Aβ concentration when compared to the no antibody control (Figure 2a). To assess the optimal concentration of IgG1-iS18 for Aβ shedding impairment, dose dependency assays were conducted and a noteworthy reduction in Aβ concentration was observed for 25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml (Figure 2b). The distinction between 50 μg/ml - 100 μg/ml was nominal and thus the of choice 50 μg/ml IgG1-iS18 for further experimental procedures was warranted.

In general, Figure 2 shows the effects of IgG1-iS18 on Aβ concentration. Figure 2a shows Aβ concentrations in HEK293 and SH-SY5Y cells after treatment with IgG1-iS18 and IgG1-HD37 as detected by an Aβ ELISA (Human Amyloid β(1-x) Assay Kit (IBL)) after 18 hours of antibody incubation. Data shown (mean± s.e.m) are 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.

Figure 2b shows 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), ***$p<0.001$; n=3; Student's t-test.

Figure 2c shows flow cytometric analysis of APP, β-secretase and γ-secretase levels on the surface of HEK293FT and SH-SY5Y cells post treatment with IgG1-iS18 (mean±s.d., NS not significant, n=3, Student's t-test).

Figure 2d shows a Western blot analysis depicting sAPPβ (the shedded cleavage product after cleavage of APP by β-secretase) 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.

Owing to the ability of IgG1-iS18 to decrease Aβ concentration, it is thought that LRP/LR mediates this process. To further confirm this role in the amyloidogenic pathway, RNA interference technology, specifically short hairpin RNA (shRNA) (see Figure 3), was employed to downregulate LRP/LR expression as shown in Figure 4.

Figure 3 shows LRP/LR target sequences and structure of shRNA1 and shRNA7. It is to be understood that the references to thymine should are to be read as uracil since the nucleotide sequences are RNA. The sequence data for both shRNA1 and shRNA7 are attached hereto as SEQ ID NO: 1 and 2 respectively. 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 polymerase chain reaction (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.

In general, Figure 4 shows the effects of shRNA on downregulation of LRP/LR. Figure 4a shows a Western Blot analysis of HEK293 cells that were transfected with LRP-specific shRNA1 and shRNA7 (as well as a scrambled control, shRNAscr). 72 hours post-transfection, cells were lysed and LRP 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.

Figure 4b shows the Aβ concentration of the cell culture medium of shRNA-transfected HEK293 cells analysed using an Aβ ELISA. Data shown (Mean±s.d.) compare Aβ levels of shRNA1 and shRNA7 to shRNAscr, *$p<0.05$, **$p<0.01$; n=3; Student's t-test. Thus Figure 4b shows the effects of shRNA on downregulation of LRP/LR.

Figure 4c shows 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.

Figure 4d shows sAPPβ levels in shRNA-transfected HEK293 cells analysed by Western blotting.

These experiments, illustrated by Figures 4a-d, showed that 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) (Figure 4e). This downregulation correlated

to a significant 16.88% and 11.95% decrease in Aβ shedding in HEK293 cells (for shRNA1 and shRNA7 respectively) (Figure 4b).

No significant difference was observed between mock-transfected and shRNAscr control transfected HEK293 cells (as can be seen in Figure 4e). Figure 4e shows 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; $p>0.05$.

To investigate whether the receptor influences the amyloidogenic pathway through altering cell surface protein expression levels of APP, β-secretase and γ-secretase, flow cytometric analysis of the cell surface levels of APP, β-secretase and γ-secretase was performed post antibody (Figure 2c) and shRNA treatment (Figure 4c). Blockage and/or downregulation of LRP/LR did not significantly alter cell surface expression levels of the aforementioned proteins in comparison to controls as shown in Figure 5. This suggests that the involvement of LRP/LR in the amyloidogenic process may be independent of gene expression modulation and possibly entails receptor interactions with the AD relevant proteins.

Figure 5 shows flow cytometry histogram overlay plots for β-secretase, γ-secretase and APP after antibody or shRNA treatments. In particular, Figure 5a shows flow cytometry histogram overlay plots after 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). β-secretase levels was detected using anti-BACE (M-83) (rabbit polyclonal IgG) (Santa Cruz Biotechnology) and goat anti-rabbit FITC secondary antibody (Cell labs). γ-secretase levels on the surface of the cells was detected by a primary antibody directed against the PEN-2 subunit of the γ-secretase complex (anti-PEN-2 (FL-101) (rabbit polyclonal IgG) (Santa Cruz Biotechnology)), and the corresponding goat anti-rabbit FITC secondary antibody. Cell surface APP levels were ascertained using an anti-APP (rabbit polyclonal IgG) (Abcam) and the corresponding goat anti-rabbit FITC secondary antibody. Images shown are averages of 3 independent experiments.

Figure 5b shows the same as above in Figure 5a but with SH-SY5Y cells.

Figure 5c shows flow cytometry histogram overlay plots after HEK293 cells having been 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.

The fact that blockage and/or downregulation of LRP/LR did not significantly alter cell surface expression levels of the AD relevant proteins suggests that the involvement of LRP/LR in the amyloidogenic process may be independent of gene expression modulation and possibly entails receptor interactions with the said proteins.

In an attempt to elucidate the mechanism by which LRP/LR influences the amyloidogenic pathway, sAPPβ levels were assessed post antibody (Figure 2d) and shRNA treatment (Figure 4d). Upon a dose dependent administration of IgG1-iS18, a significant reduction in sAPPβ levels was observed across all antibody concentrations (56.29%, 69.35%, 92.42% and 99.76% for 25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml respectively). Similar results were obtained for shRNA1 mediated LRP/LR downregulated HEK293 cells (Figure 4d).

Following the above, pull down assays were conducted to examine whether LRP/LR (recombinantly expressed fused to a FLAG tag) and 100ng/ml exogenously applied synthetic $A\beta_{42}$ (Sigma-Aldrich) (which mimics augmented concentrations of soluble $A\beta_{42}$ present in AD brains) form stable interactions. The presence of both proteins in eluted samples (Figure 6a, i - lane 6) implies that such an association exists. The identity of LRP/LR was further confirmed by immunoblotting (Figure 6a, ii). Relevant controls are shown in Figure 7 discussed below.

In general, Figure 6 shows LRP/LR as an Aβ interacting protein and the cell rescuing effects of anti-LRP/LR antibody IgG1-iS18. Figure 6a (i) shows that a FLAG® Immunoprecipitation kit (Sigma Aldrich) was employed to perform a pull down assay of cell lysates containing recombinantly expressed LRP/LR::FLAG co-incubated with exogenous Aβ. Lane 1: Molecular weight marker; lane 2: unbound sample; lanes 3-4: washes; lane 5: eluted sample and lane 6: 2μg of synthetic Aβ42 (positive control).

Figure 6a (ii) shows experimental results where Immunoblot was employed to validate the position of LRP::FLAG (~38kDa).

Figure 6b shows 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 48h post treatment and the no antibody control was set to 100%. SH-SY5Y (as shown in Figure 6c) and N2a cells (as shown in Figure 6d) were exposed to similar treatments. It can be seen that the co-incubation with anti-LRP/LR IgG1-iS18 significantly enhanced cell viability.

Figure 6e shows cellular proliferation of N2a cells as determined by colorimetric 5-bromo-2'-deoxyuridine (BrdU) non-isotopic immunoassay (Calbiochem®), allowing 4h for BrdU incorporation into cultured cells. Error bars represent sd. **$p<0.01$; Student's t-test.

Figure 7 shows the results of pull down assay protein detection of control samples. Pull down assays were employed to investigate the proteins detectable in unbound samples (lane 2), wash steps (lanes 3 and 4) and eluted samples (Figure a-c, lane 5, Figure d, lane 6). Figures represent anti-FLAG® M2 beads incubated with (a) lysis buffer, (b) non-transfected HE293 cell lysates, (c) HEK293 cell lysates of cells transfected with pCIneo::FLAG as well as (d) pure synthetic $A\beta_{42}$ in the absence of cell lysate. Lane 7 of (d) represents 2µg of synthetic $A\beta_{42}$ which serves as a positive control. Samples were resolved on 16% Tris-tricine SDS PAGE gels and stained with Coomassie Brilliant Blue. Blue, red and green arrows are indicative of $A\beta_{42}$, LRP::FLAG and anti-FLAG M2 agarose beads, respectively.

In summary, exogenous application of 200 nM and 500 nM $A\beta_{42}$ significantly reduced cell viability in HEK293 cells (Figure 6b). Co-incubation of cells with 50µg/ml anti-LRP/LR specific antibody IgG1-iS18 and 500nM $A\beta_{42}$ (the concentration at maximal cytotoxic effects were observed) significantly enhanced cell viability (Figure 6b). Similar results, albeit at different $A\beta_{42}$ concentrations were observed for SH-SY5Y (Figure 6c) and N2a (Figure. 6d) cells. The reduction in cell viability observed in N2a cells (Figure 6d) was later shown to be as a result of reduced cellular proliferation (Figure 6e). It is shown that LRP/LR may be implicated in $A\beta_{42}$ mediated cytotoxicity and that the identified association (direct or indirect) may be pathological in nature.

Animal Trials for validation of anti-LRP/LR specific antibodies in the treatment Alzheimer's Disease (AD)

LRP/LR plays a definitive role in the in Aβ mediated pathogenesis in AD, as proposed by the above in vitro data above. It has been shown in vitro that IgG1-iS18 (LRP/LR specific antibody) plays an important role in modulation of LRP/LR and the modulation of the concentrations of Alzheimer's Disease (AD) relevant proteins amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ). It is further proposed that the antibodies and/or shRNAs described above may rescue neurons from Aβ mediated cell death or impede their proliferation.

Animal trials probing the potential of these antibodies and/or shRNAs as an AD therapeutic will be initiated and conducted. Transgenic AD mice harbouring human transgenes with 5 AD related mutations (3 mutations in the APP protein and 2 in the PSEN1 enzymatic subunit of γ-secretase) (The Jackson Laboratory, strain: B6SJL-Tg (APPSwFlLon,PSEN1*M146L*L286V)6799Vas|Mmjax) will be employed. Transgenic animals (caged in individual cages in a temperature controlled environment) will be divided into six groups (5mice/group). Ten wild-type mice per treatment will serve as phenotype controls.

These 5x-Tg-AD mice develop pathological features mimicking the human condition within 4 months, namely: plaque deposition, synaptic and neuronal loss as well as cognitive deficits.

The antibodies and shRNA described above will be utilized in any such animal experimentation. Particularly, anti-LRP/LR antibody, IgG1-iS18, or IgG1-HD37 (negative control) (50μg/ml) will be stereotaxically administered as a single intracerebroventricular (ICV) injection either prior to plaque deposition (< 4months) or post plaque deposition (> 4 months). Antibodies will be administered into the third ventricle (due to its proximity to the hippocampus). Age matched transgenic mice receiving ICV injections of the vehicle shall serve as controls. At varying weekly time intervals, groups of mice will be tested for deficits in spatial learning by means of the Morris Water Maze Test. Two days prior to euthanization, the final Morris Water Maze Test shall be performed. Mice will be euthanized by transcardial perfusion with ice-cold saline followed by 4% buffered paraformaldehyde (in saline

solution). Approximately 18-20 hippocampal sections (35µm thickness) per animal (from both hemispheres) will be collected and immunohistochemical methods (anti-Aβ antibodies) employed to assess total Aβ levels and Congo Red staining used to detect plaque deposits on these sections (as detailed by Chauhan and Siegel, 2003). To ensure antibody administration did not cause cerebral damage and haemorrhaging sections shall be stained for haemosiderin using Prussian blue.

CONCLUSION

The results for the in vitro experiments indicate that LRP/LR co-localises with all the relevant AD proteins (APP, β and γ-secretase as well as Aβ) and consequently implies that an association between these proteins and the receptor may exist, as was further validated by pull down assay methodology with respect to the neurotoxic $A\beta_{42}$ peptide. In addition, receptor blockage and/or downregulation of LRP/LR effectively impeded Aβ shedding affirming the importance of the receptor in the amyloidogenic process. Interestingly, LRP/LR blockage did not result in modulation of cell surface proteins central to the amyloidogenic process, thereby inferring that the influence of LRP/LR may rather be as a result of protein interactions. The observed decrease in sAPPβ levels post antibody and shRNA treatment suggests that LRP/LR exerts its affects via β-secretase. LRP/LR was further implicated in Aβ induced cytotoxicity and the interaction may possibly result in aberrant proliferative cell signalling pathways. In conclusion, our findings suggests that the LRP/LR is implicated in AD's pathogenesis and recommends anti-LRP/LR specific antibodies and shRNAs as possible alternative therapeutic tools for AD treatment.

While the invention has been described and/or illustrated and/or exemplified in detail with respect to specific embodiments and/or examples thereof, it will be appreciated that those skilled in the art, upon attaining an understanding of the foregoing, may readily conceive of alterations to, variations of and equivalents to these embodiments. Accordingly, the scope of the present invention should be assessed as that of the appended claims and equivalents thereto.

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CLAIMS

1. A method for reducing concentration of at least one Alzheimer's Disease (AD) relevant proteins selected from the group consisting of amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), the method comprising:

   contacting a cell surface protein with a cell surface protein specific antibody, or antibody fragment, such that binding occurs between a surface epitope of the cell surface protein and the cell surface protein specific antibody causing a decrease in the concentration of the at least one AD relevant proteins.

2. The method according to claim 1, wherein the cell surface protein is a laminin receptor protein.

3. The method according to claim 2, wherein the laminin receptor protein is 37 kDa/67 kDa laminin receptor (LRP/LR) or a protein having at least 80% or greater homology to LRP/LR.

4. The method according to any one of claims 1 to 3, wherein the antibody or fragment thereof is raised against the cell surface protein.

5. The method according to claim 4, wherein the antibody or fragment thereof is an anti-laminin receptor specific antibody or fragment thereof.

6. The method according to claim 5, wherein the anti-laminin receptor specific antibody or fragment thereof is an anti-LRP/LR specific antibody or fragment thereof or an antibody or fragment thereof having at least 80% or greater homology to anti-LRP/LR specific antibody or fragment thereof.

7. The method according to claim 6, wherein the anti-LRP/LR specific antibody or fragment thereof is IgG1-iS18 or a fragment thereof.

8. The method according to claim 1, wherein the AD relevant protein is Aβ, the cell surface protein is 37 kDa/67 kDa laminin receptor (LRP/LR) and the cell surface protein specific antibody is IgG1-iS18 or a fragment thereof.

9. A method for reducing amyloid beta peptide (Aβ) shedding caused by the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases, the method comprising:

contacting a cell surface protein with a cell surface protein specific antibody, or any fragment thereof, such that binding occurs between a surface epitope of the cell surface protein and the cell surface protein specific antibody therein hindering the proteolytic cleavage of APP by beta (β) and gamma (γ) secretases.

10. The method according to claim 9, wherein the cell surface protein is a laminin receptor protein.

11. The method according to claim 10, wherein the laminin receptor protein is 37 kDa/67 kDa laminin receptor (LRP/LR) or a protein having at least 80% or greater homology to LRP/LR.

12. The method according to any one of claims 9 to 11, wherein the antibody or fragment thereof is raised against the cell surface protein.

13. The method according to claim 12, wherein the antibody or fragment thereof is an anti-laminin receptor specific antibody or fragment thereof.

14. The method according to claim 13, wherein the anti-laminin receptor specific antibody or fragment thereof is an anti-LRP/LR specific antibody or fragment thereof or an antibody or fragment thereof having at least 80% or greater homology to anti-LRP/LR specific antibody or fragment thereof.

15. The method according to claim 6, wherein the anti-LRP/LR specific antibody or fragment thereof is IgG1-iS18 or a fragment thereof.

16. The method according to claim 9, wherein the AD relevant protein is Aβ, the cell surface protein is 37 kDa/67 kDa laminin receptor (LRP/LR) and the cell surface protein specific antibody is IgG1-iS18 or a fragment thereof.

17. A method for reducing concentration of at least one Alzheimer's Disease (AD) relevant protein selected from the group consisting of amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), the method comprising:

contacting a cell surface protein with a nucleotide sequence, such that binding occurs between mRNA of the cell surface protein and the nucleotide sequence causing a downregulation in the cell surface protein which in turn causes a decrease in the concentration of the at least one AD relevant proteins.

18. The method according to claim 17, wherein the nucleotide sequence is RNA.

19. The method according to claim 18, wherein the RNA is short hairpin RNA (shRNA), alternatively small interfering RNA (siRNA), further alternatively micro RNA (miRNA), or any combination of the aforementioned.

20. The method according to claim 19, wherein the RNA is a shRNA and is at least one of the shRNA 1 and shRNA 7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

21. The method according to any one of claims 17 to 20, wherein the cell surface protein is a laminin receptor protein.

22. The method according to claim 21, wherein the laminin receptor protein is 37 kDa/67 kDa laminin receptor (LRP/LR) or a protein having at least 80% or greater homology to LRP/LR.

23. The method according to claim 17, wherein the cell surface protein is 37 kDa/67 kDa laminin receptor (LRP/LR) and the nucleotide sequence is at least one of the shRNA 1 and shRNA 7 having sequence listing as set forth in SEQ ID NO: 1 and 2,

respectively, and binding occurs between LRP mRNA and at least one of shRNA 1 and shRNA 7.

24. A method for reducing amyloid beta peptide (Aβ) shedding caused by the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases, the method comprising:

contacting a cell surface protein with a nucleotide sequence, such that binding occurs between mRNA of the cell surface protein and the nucleotide sequence causing a downregulation in the cell surface protein which in turn causes a decrease in the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases.

25. The method according to claim 24, wherein the nucleotide sequence is RNA.

26. The method according to claim 25, wherein the RNA is short hairpin RNA (shRNA), alternatively small interfering RNA (siRNA), further alternatively micro RNA (miRNA) or any combination of the aforementioned.

27. The method according to claim 26, wherein the RNA is a shRNA and is at least one of the shRNA 1 and shRNA 7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

28. The method according to any one of claims 24 to 27, wherein the cell surface protein is a laminin receptor protein.

29. The method according to claim 28, wherein the laminin receptor protein is 37 kDa/67 kDa laminin receptor (LRP/LR) or a protein having at least 80% or greater homology to LRP/LR.

30. The method according to claim 24, wherein the cell surface protein is 37 kDa/67 kDa laminin receptor (LRP/LR) and the nucleotide sequence is at least one of the shRNA 1 and shRNA 7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively, and binding occurs between LRP mRNA and at least one of shRNA 1 and shRNA 7.

31. A method for reducing concentration of at least one Alzheimer's Disease (AD) relevant protein selected from the group consisting of amyloid precursor protein (APP), beta (β) and gamma (γ) secretases and amyloid beta peptide (Aβ), the method comprising:

contacting the cell surface protein according to any one of claims 1 to 8 with the antibody according to any one of claims 1 to 8 and the nucleotide sequence according to any one of claims 17 to 23.

32. A method for reducing amyloid beta peptide (Aβ) shedding caused by the proteolytic cleavage of amyloid precursor protein (APP) by beta (β) and gamma (γ) secretases, the method comprising:

contacting a cell surface protein according to any one of claims 9 to 16 with the antibody according to any one of claims 9 to 16 and the nucleotide sequence any one of claims 24 to 30.

33. Use of an anti-laminin receptor specific antibody in the manufacture of a pharmaceutical composition to treat Alzheimer's Disease (AD).

34. The use of claim 33, wherein the anti-laminin receptor specific antibody is anti-37 kDa/67 kDa laminin receptor (LRP/LR) specific antibody.

35. The use of claim 34, wherein the anti-LRP/LR specific antibody is IgG1-iS18.

36. An anti-laminin receptor specific antibody for use in treating Alzheimer's Disease (AD).

37. The anti-laminin receptor specific antibody of claim 36, wherein the anti-laminin receptor specific antibody is anti-37 kDa/67 kDa laminin receptor (LRP/LR) specific antibody.

38. The anti-laminin receptor specific antibody of claim 37, wherein the anti-LRP/LR specific antibody is IgG1-iS18.

39. Use of a nucleotide sequence in the manufacture of a pharmaceutical composition to treat Alzheimer's Disease (AD).

40. The use according to claim 39, wherein the nucleotide sequence is an RNA sequence.

41. The use according to claim 40, wherein the RNA sequence is a small hairpin RNA (shRNA) sequence, alternatively small interfering RNA (siRNA) sequence, further alternatively micro RNA (miRNA) sequence, or a combination of the aforementioned.

42. The use according to claim 41, wherein the RNA sequence is a shRNA sequence and is at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

43. The use according to any one of claims 39 to 42, which further includes an anti-laminin specific receptor antibody.

44. The use according to claim 43, wherein the anti-laminin specific receptor antibody is an anti-LRP/LR specific antibody.

45. The use according to claim 44, wherein the anti-LRP/LR specific antibody is IgG1-iS18.

46. A nucleotide sequence for use in treating Alzheimer's Disease (AD).

47. The nucleotide sequence according to claim 46, wherein the nucleotide sequence is a RNA sequence.

48. The nucleotide sequence according to claim 47, wherein the nucleotide sequence is a short hairpin RNA (shRNA) sequence, alternatively small interfering RNA (siRNA) sequence, further alternatively micro RNA (miRNA) sequence, or a combination of the aforementioned.

49. The nucleotide sequence according to claim 48, wherein the RNA sequence is a shRNA sequence and is at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

50. The nucleotide sequence according to any one of claims 46 to 49, wherein the nucleotide sequence is used together with an anti-laminin specific receptor antibody.

51. The nucleotide sequence according to claim 50, wherein the anti-laminin specific receptor antibody is an anti-LRP/LR specific antibody.

52. The nucleotide sequence according to claim 51, wherein the anti-LRP/LR specific antibody is IgG1-iS18.

53. A method of treating Alzheimer's Disease (AD) comprising administering an anti-laminin specific receptor antibody to a human or animal in need thereof.

54. The method according to claim 53, wherein the anti-laminin specific receptor antibody is an anti-37 kDa/67 kDa laminin receptor (LRP/LR) specific antibody.

55. The method according to claim 54, wherein the anti-LRP/LR specific antibody is IgG1-iS18.

56. A method of treating Alzheimer's Disease (AD) comprising administering a nucleotide sequence to a human or animal in need thereof.

57. The method according to claim 56, wherein the nucleotide sequence is a RNA sequence.

58. The method according to claim 57, wherein the RNA sequence is a shRNA sequence, alternatively small interfering RNA (siRNA) sequence, further alternatively micro RNA (miRNA) sequence, or a combination of the aforementioned.

59. The method according to claim 58, wherein the RNA sequence is a shRNA sequence and is at least one of shRNA1 and shRNA7 having sequence listing as set forth in SEQ ID NO: 1 and 2, respectively.

60. The method according to any one of claims 56 to 59, further including administering an anti-laminin specific receptor antibody to the human or animal in need thereof.

61. The method according to claim 60, wherein the anti-laminin specific receptor antibody is an anti-LRP/LR specific antibody.

62. The method according to claim 61, wherein the anti-LRP/LR specific antibody is IgG1-iS18.

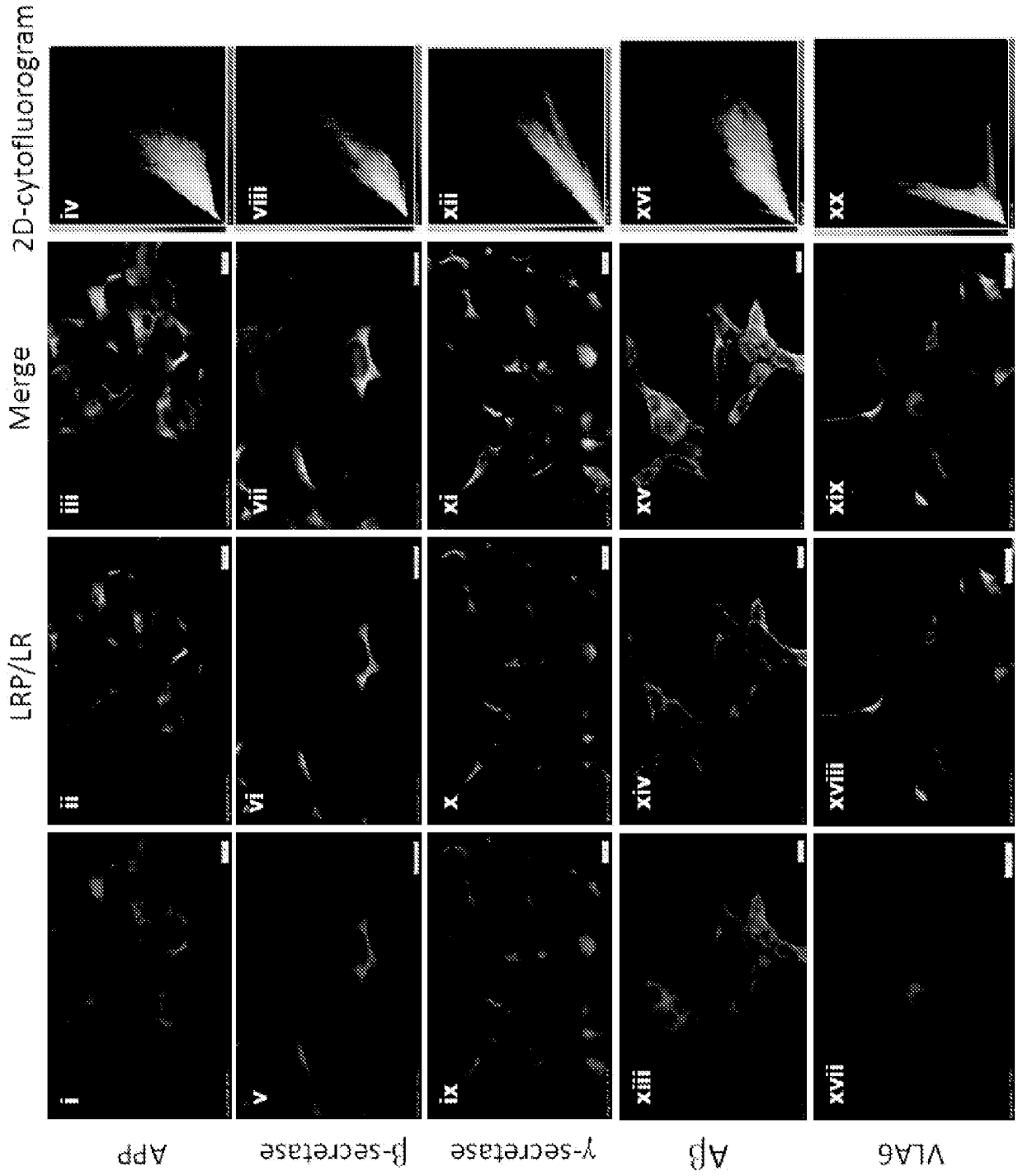

LRP/LR
Merge
2D-cytofluorogram
APP
β-secretase
γ-secretase
Aβ
VLA6
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
xiv
xv
xvi
xvii
xviii
xix
xx

FIG 1a

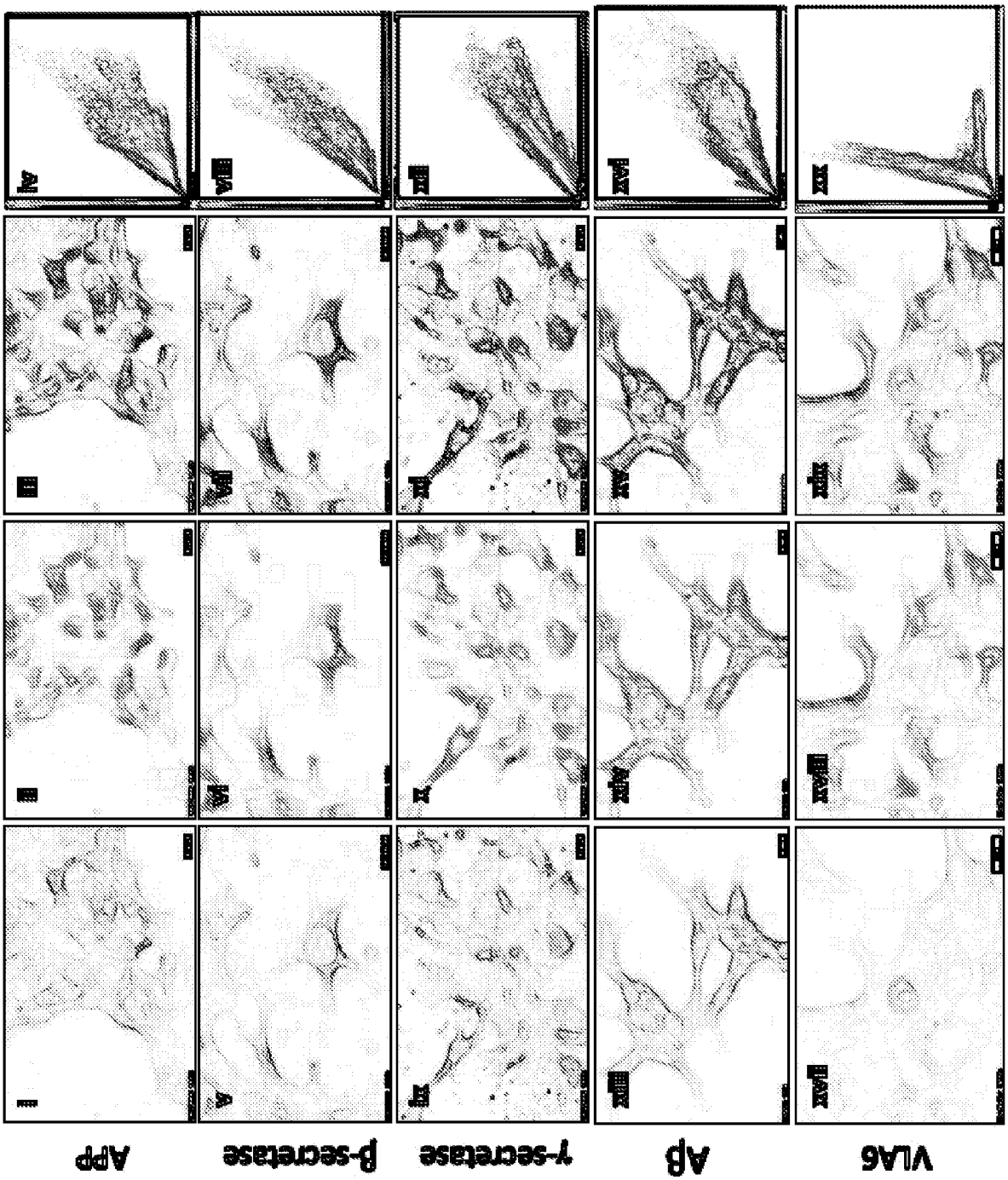


FIG 1aa

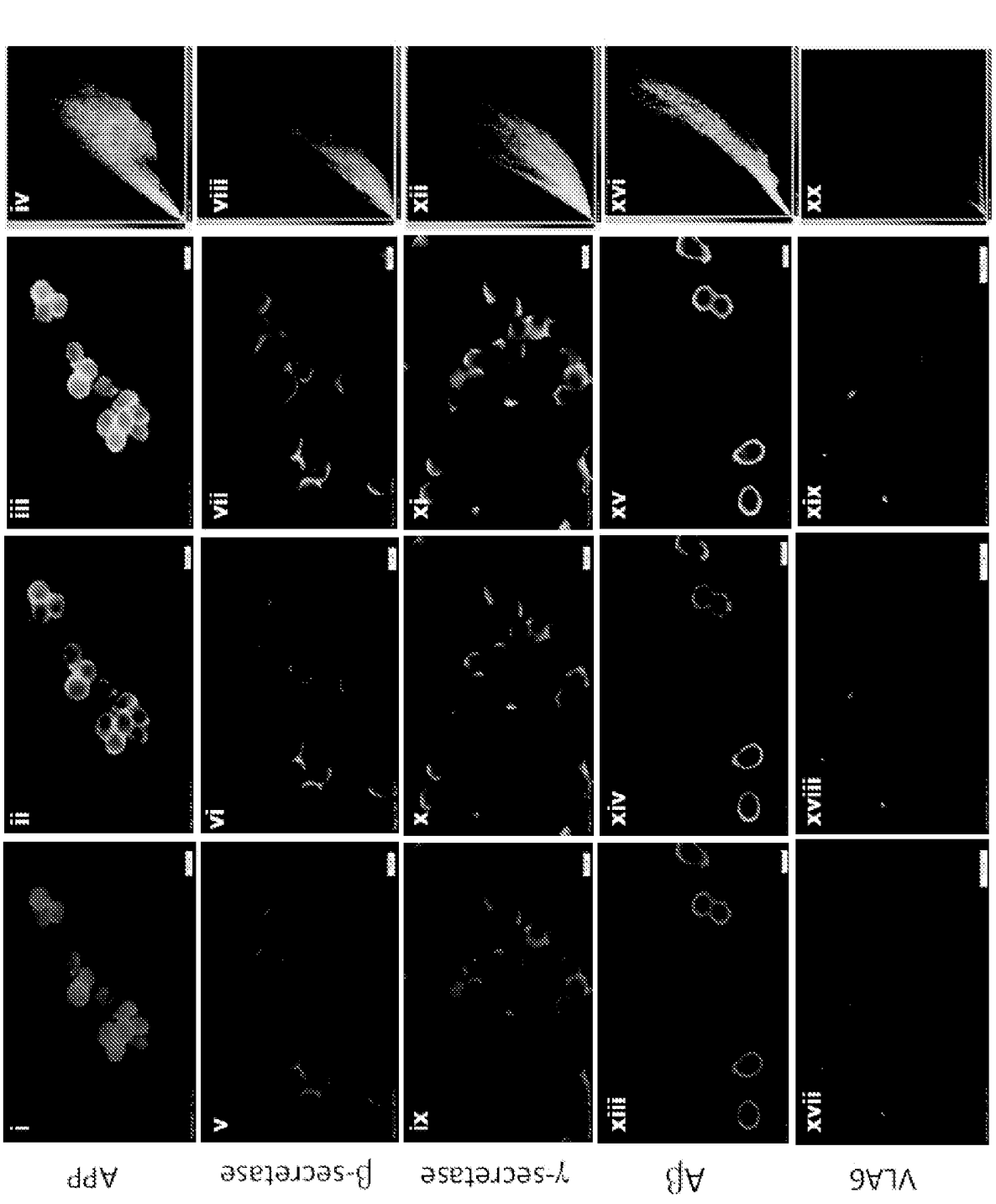

LRP/LR
MERGE
2D-cytofluorogram
APP
β-secretase
γ-secretase
Aβ
VLA6
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
xiv
xv
xvi
xvii
xviii
xix
xx

FIG 1b

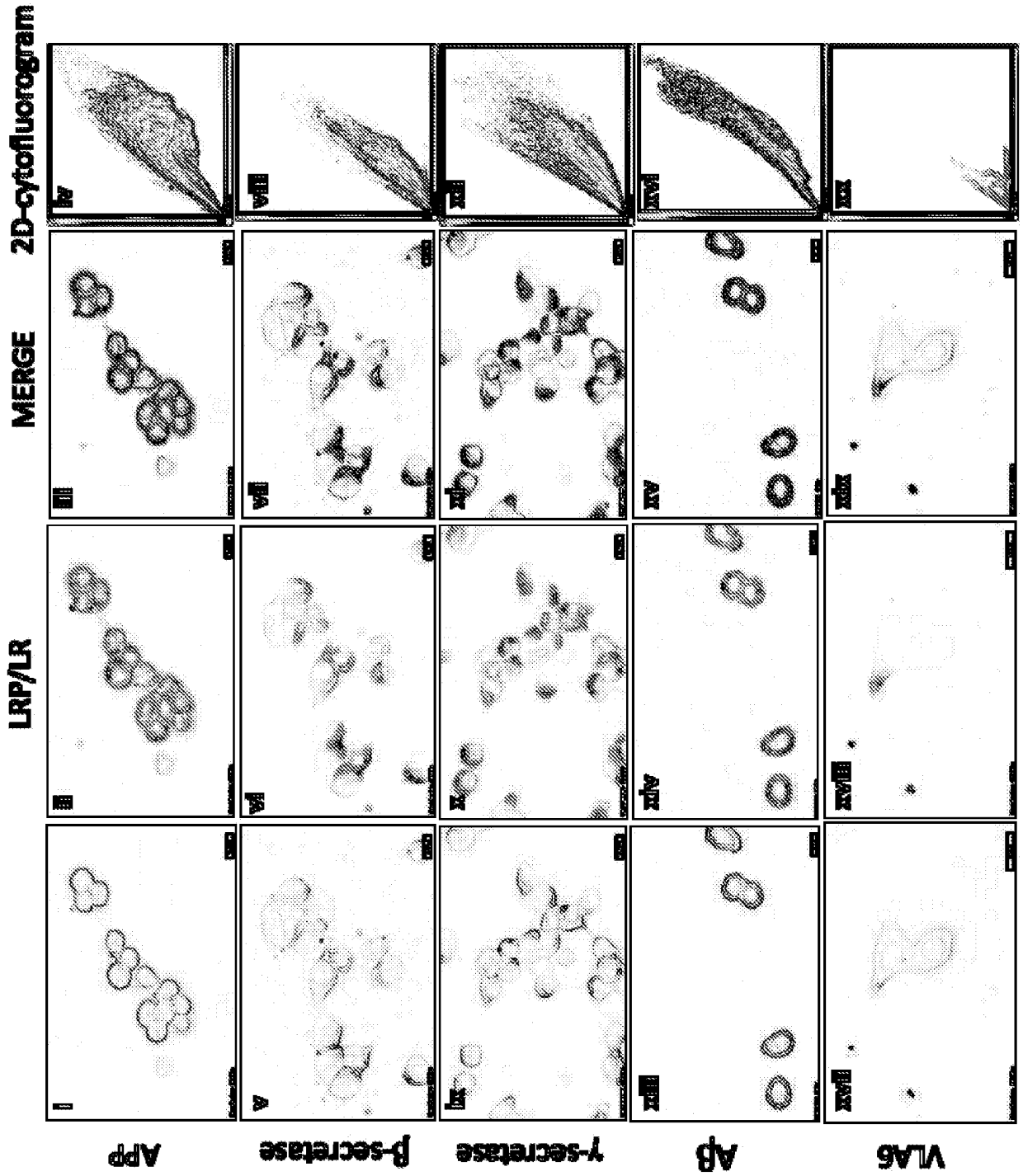

LRP/LR
MERGE
2D-cytofluorogram
APP
β-secretase
γ-secretase
Aβ
VLA6

FIG 1bb

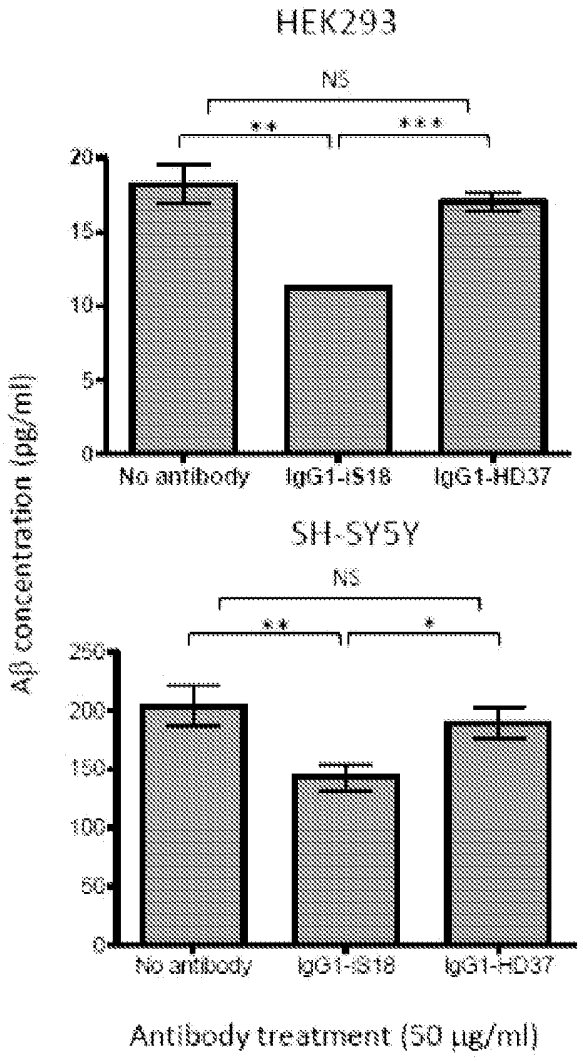

HEK293
NS
**
***
20
15
10
5
0
No antibody
IgG1-iS18
IgG1-HD37
SH-SY5Y
NS
**
*
250
200
150
100
50
0
No antibody
IgG1-iS18
IgG1-HD37
Aβ concentration (pg/ml)
Antibody treatment (50 µg/ml)

FIG 2a

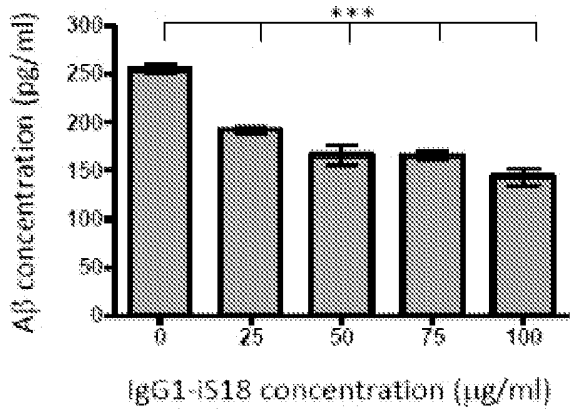

***
300
250
200
150
100
50
0
0
25
50
75
100
Aβ concentration (pg/ml)
IgG1-iS18 concentration (µg/ml)

FIG 2b

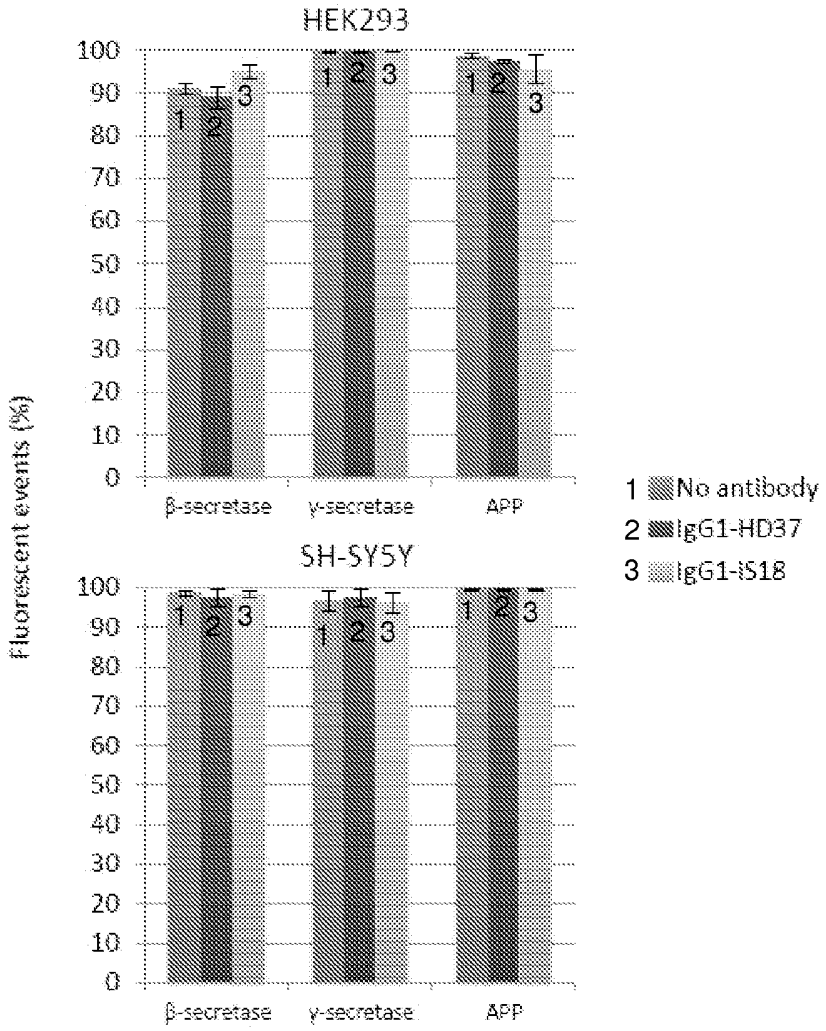

HEK293
SH-SY5Y
Fluorescent events (%)
100
90
80
70
60
50
40
30
20
10
0
β-secretase
γ-secretase
APP
1 No antibody
2 IgG1-HD37
3 IgG1-iS18

FIG 2c

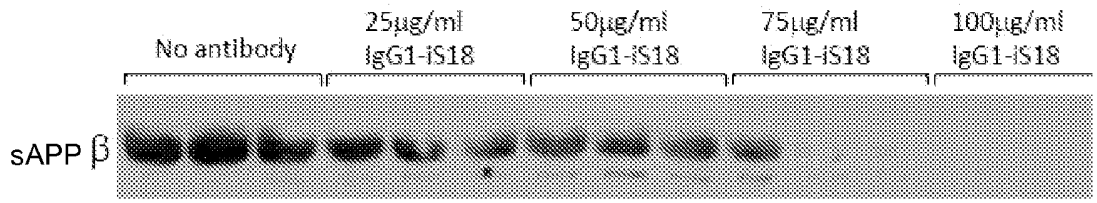

No antibody
25µg/ml IgG1-iS18
50µg/ml IgG1-iS18
75µg/ml IgG1-iS18
100µg/ml IgG1-iS18
sAPP β

FIG 2d

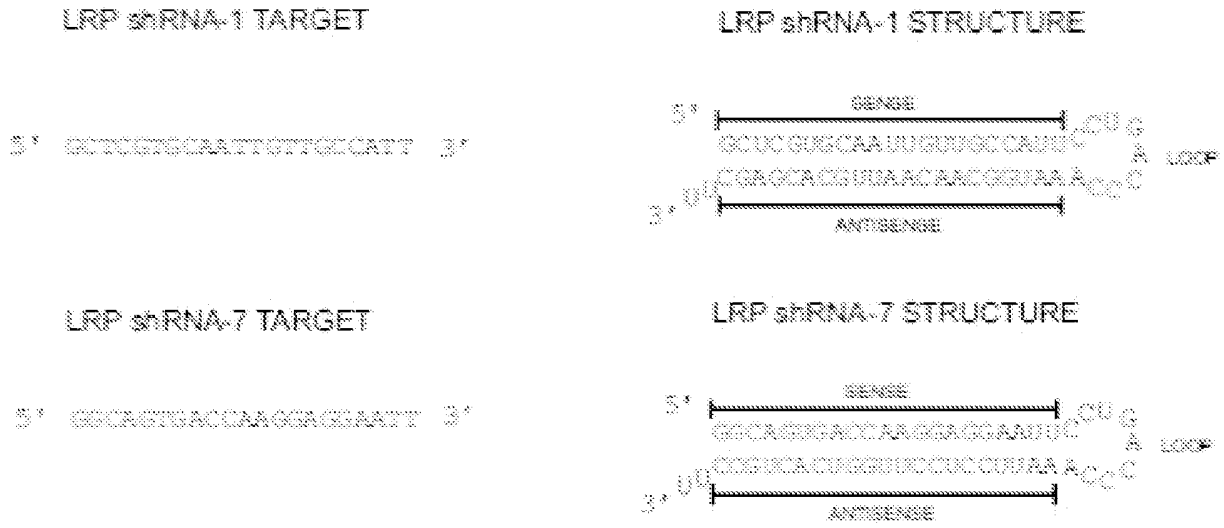


FIG 3

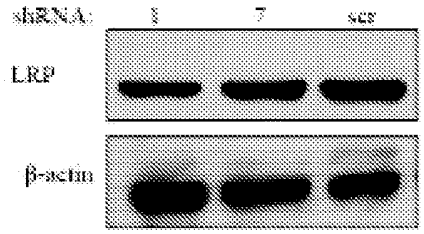


FIG 4a

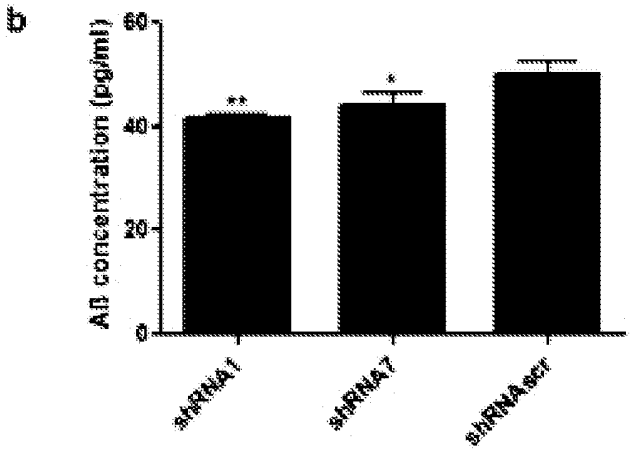


FIG 4b

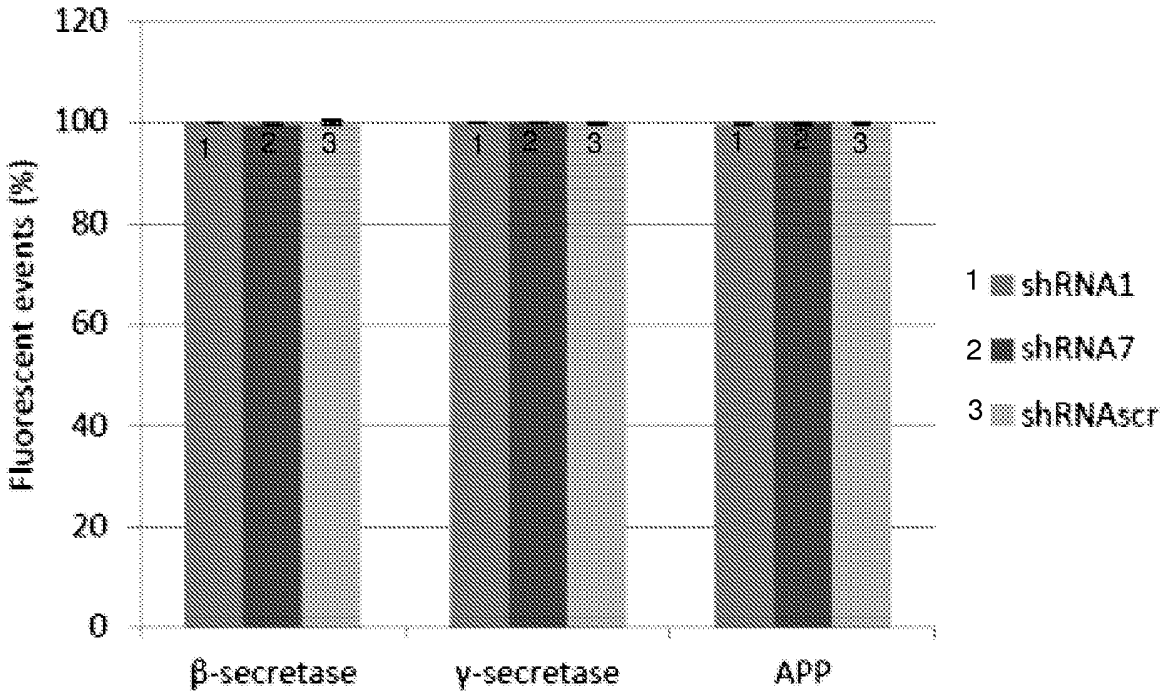

120
100
80
60
40
20
0
Fluorescent events (%)
1
2
3
β-secretase
γ-secretase
APP
1 shRNA1
2 shRNA7
3 shRNAscr

FIG 4c

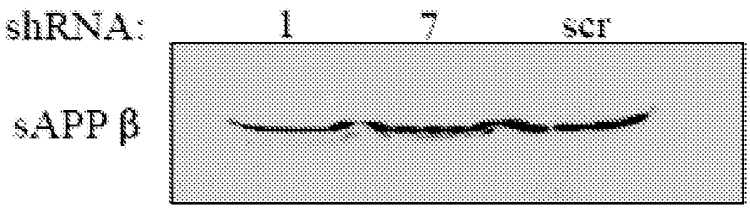

shRNA:
1
7
scr
sAPP β

FIG 4d

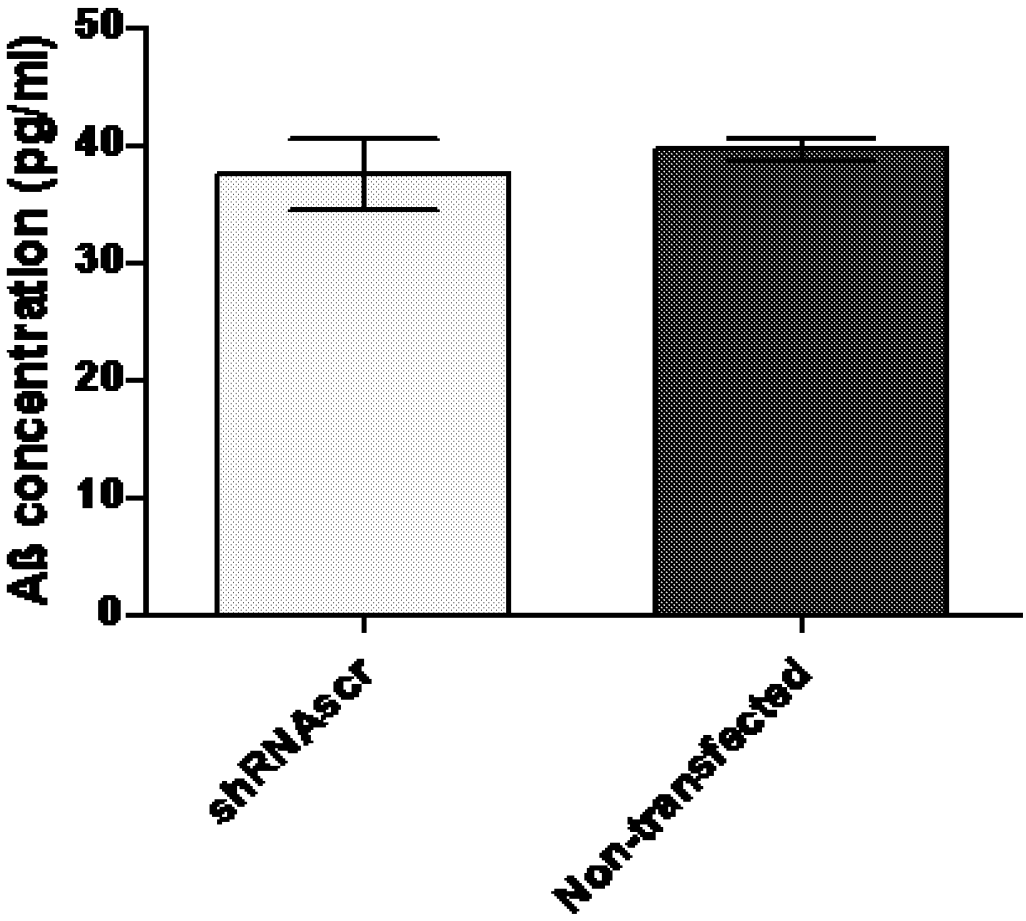

Aß concentration (pg/ml)
50
40
30
20
10
0
shRNAscr
Non-transfected

FIG 4e

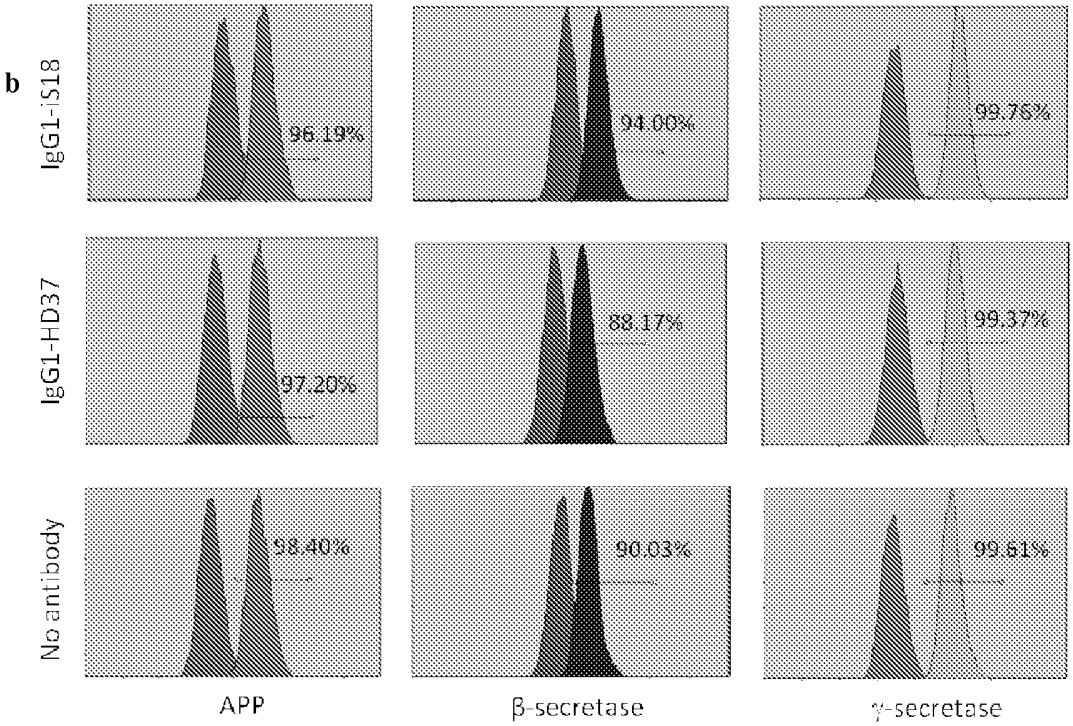

b
IgG1-i518
IgG1-HD37
No antibody
96.19%
94.00%
99.75%
97.20%
88.17%
99.37%
98.40%
90.03%
99.61%
APP
β-secretase
γ-secretase

FIG 5a

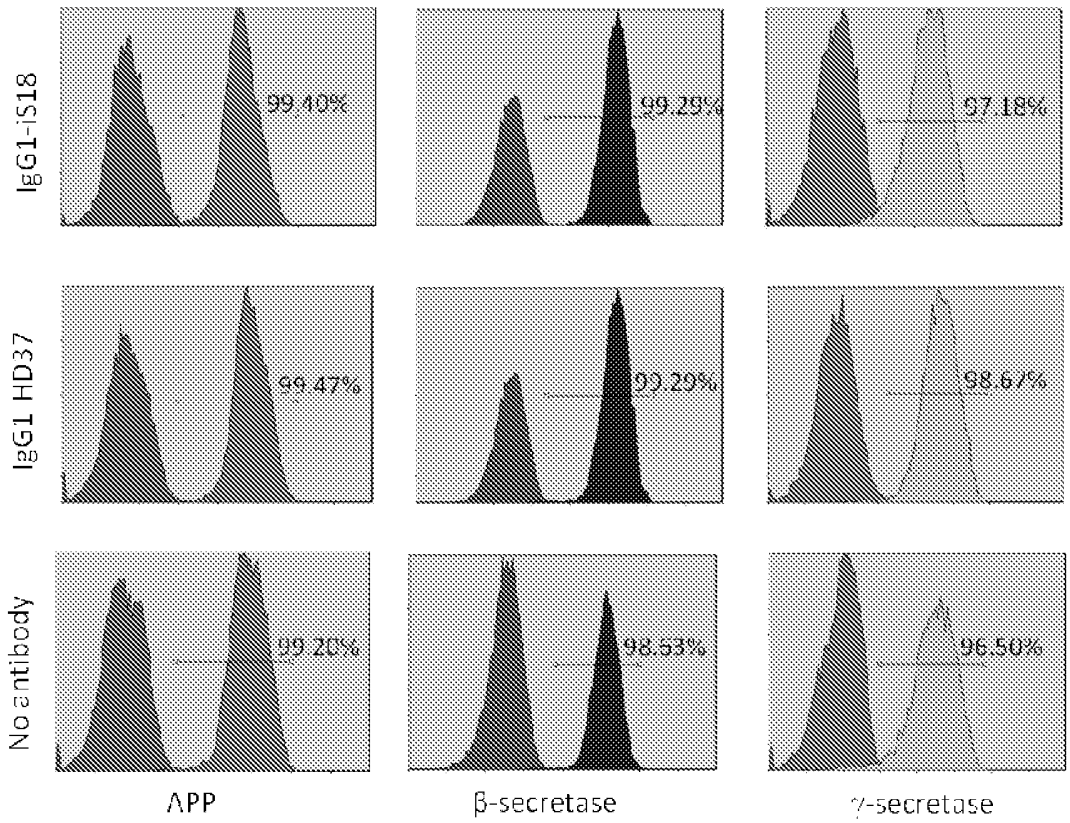

IgG1-i518
IgG1 HD37
No antibody
99.40%
99.29%
97.18%
99.47%
99.29%
98.67%
99.20%
98.53%
96.50%
APP
β-secretase
γ-secretase

FIG 5b

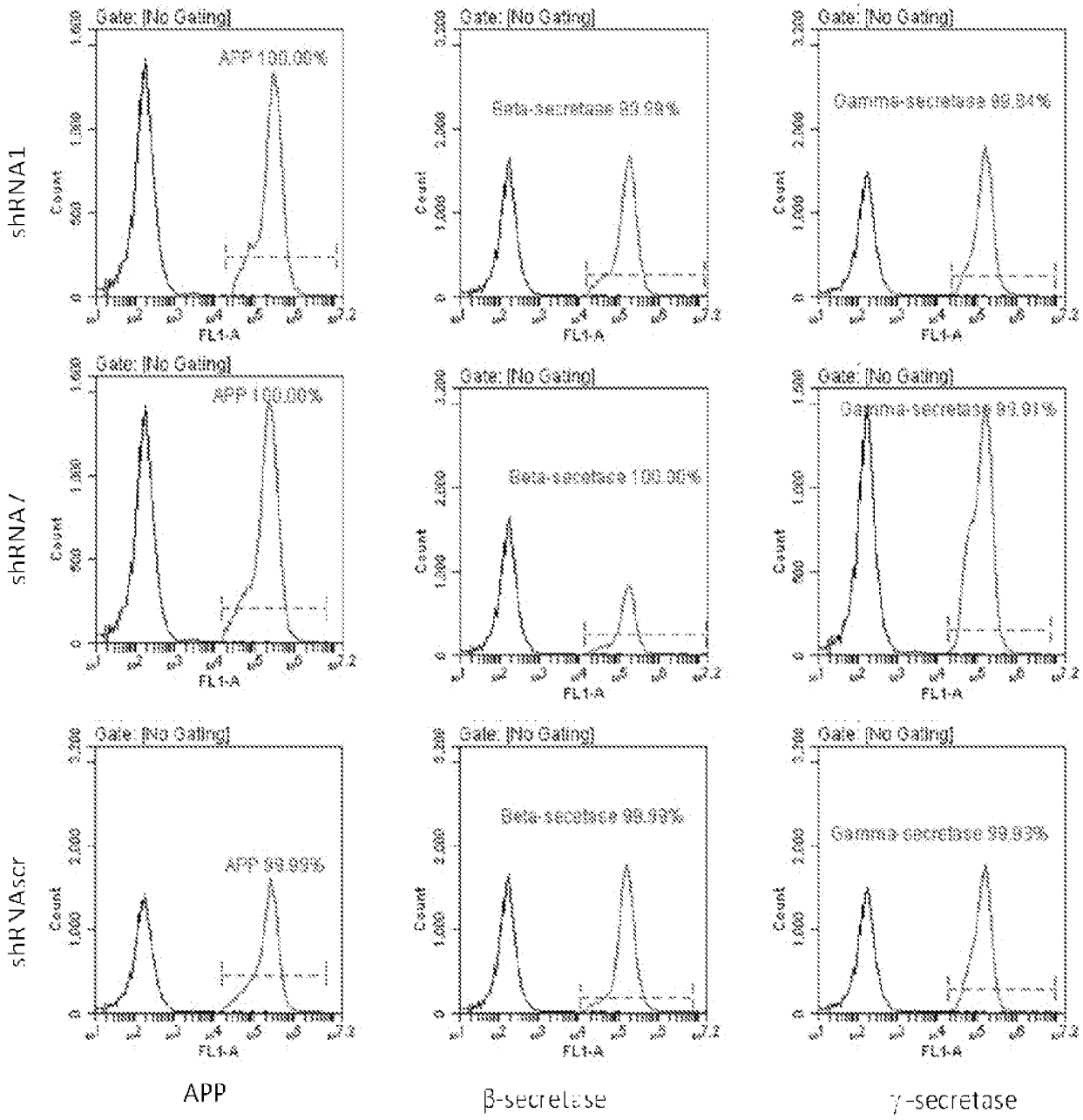

shRNA1
shRNA /
shRNAscr
Gate: [No Gating]
APP 100.00%
Beta-secretase 99.98%
Gamma-secretase 99.94%
APP 100.00%
Beta-secretase 100.00%
Gamma-secretase 99.97%
APP 99.99%
Beta-secretase 99.99%
Gamma-secretase 99.83%
Count
FL1-A
APP
β-secretase
γ-secretase

FIG 5c

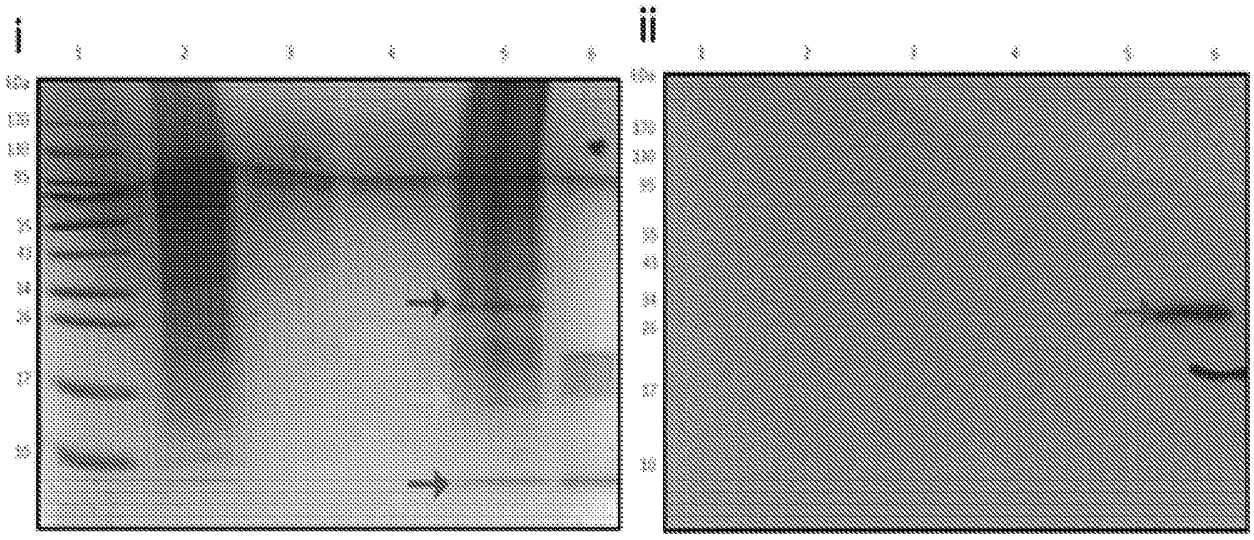


FIG 6a

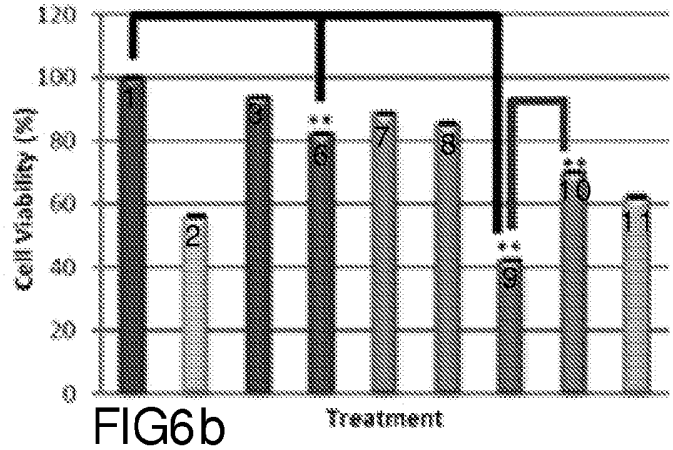


FIG6b

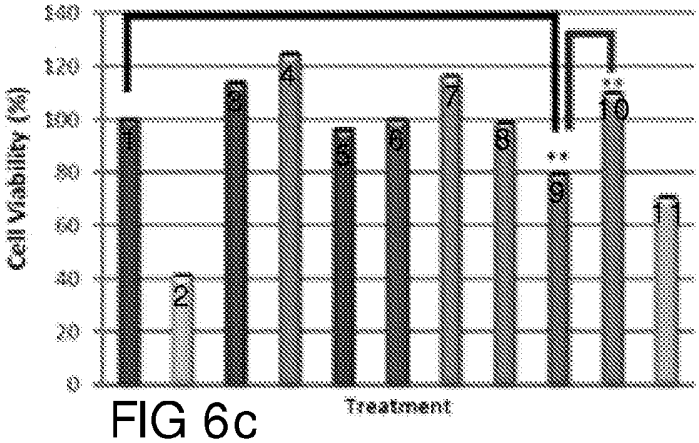


FIG 6c

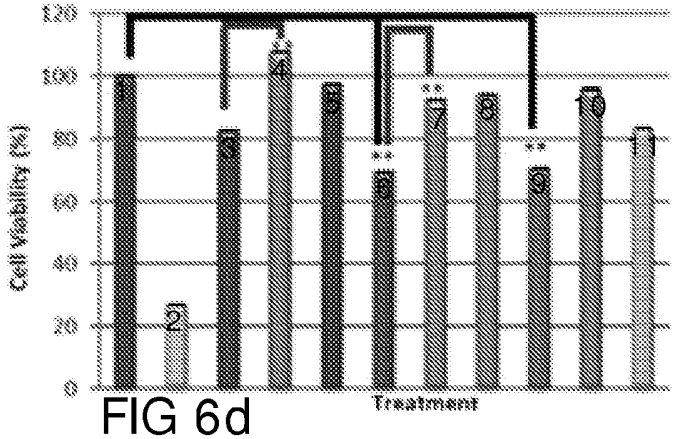


FIG 6d

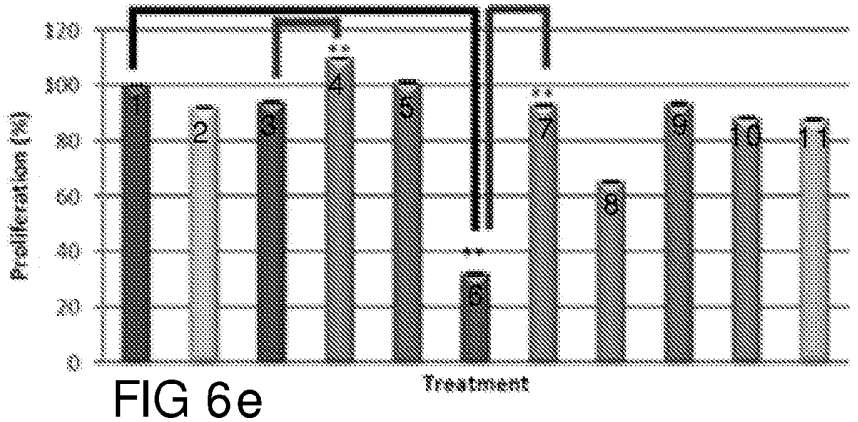


FIG 6e

1 Control
2 8mM PCA
3 100nM Amyloid beta
4 100nM Amyloid beta + 50ug/ml IgG1-IS18
5 100nM Amyloid beta + 50ug/ml IgG1-HD37
6 200nM Amyloid beta
7 200nM Amyloid beta + 50ug/ml IgG1-IS18
8 200nM Amyloid beta + 50ug/ml IgG1-HD37
9 500nM Amyloid beta
10 500nM Amyloid beta + 50ug/ml IgG1-IS18
11 500nM Amyloid beta + 50ug/ml IgG1-HD37

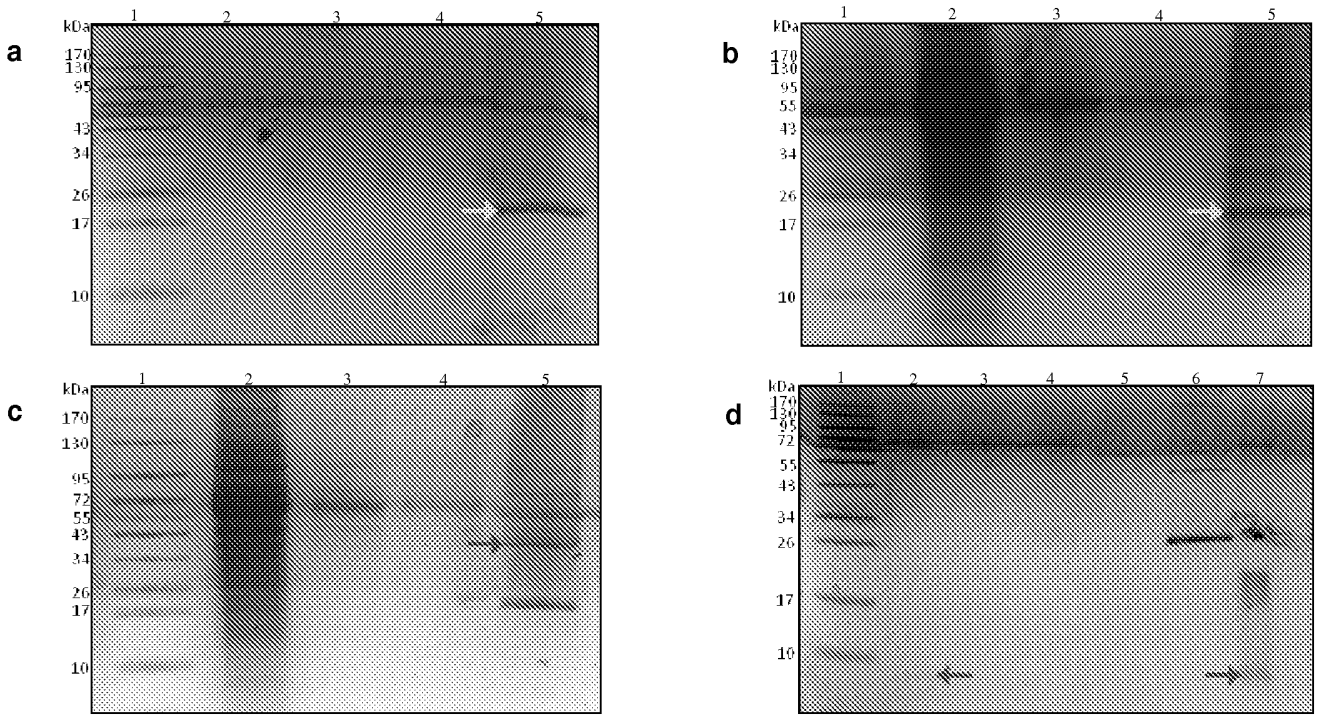


FIG 7