The 37kDa/67kDa Laminin Receptor acts as a receptor for Aβ42 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 Aβ42 induced apoptosis. Furthermore, antibody blockade and shRNA mediated downregulation of LRP/LR significantly hampered Aβ42 internalization. These results suggest that LRP/LR is a receptor for Aβ42 internalization, mediating its endocytosis and contributing to the cytotoxicity of the neuropeptide by facilitating intra-cellular Aβ42 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, 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. It is predicted that in 2050, approximately 1 in 85 people will be afflicted by the disease 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. 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 Aβ42 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. Moreover Aβ exerts its toxicity intracellularly 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.

Although a myriad of molecular mechanisms reportedly contribute to Aβ42 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 (PrPc). PrPc is considered neuroprotective under normal physiological conditions, through the maintenance of oxidative stress homeostasis and inhibition of β-secretase cleavage of APP. In contrast, the overwhelming majority of recent
reports have demonstrated that within the AD context, PrP\textsuperscript{c} acquires a pathological role. Upon binding to Ab\textsubscript{\beta} oligomers (which it is able to do with high affinity, $k_D = 0.4 \times 10^{-10} \, \text{M}^{15,16}$) PrP\textsuperscript{c} has been shown to mediate neurotoxic signals through Fyn kinase\textsuperscript{16,17}, impair synaptic plasticity, inhibit long term potentiation and contribute to intracellular accumulation of Ab\textsubscript{\beta} by mediating the internalization of Ab\textsubscript{\beta} oligomers\textsuperscript{15}. However, owing to the glycosylphosphatidylinositol (GPI)-anchored nature of this protein\textsuperscript{14}, it is largely dependent on its receptors to mediate the aforementioned functions. One such receptor, which exhibits a high binding affinity ($k_D = 1 \times 10^{-10} \, \text{M}$) for PrP\textsuperscript{c}, is the 37 kDa/67 kDa laminin receptor (LRP/LR) (also known as LamR, RPSA and p40)\textsuperscript{15}. This multifunctional receptor is implicated in numerous physiological roles including translation, maintenance of cytoskeletal structure\textsuperscript{16}, cell survival, differentiation, proliferation and migration\textsuperscript{17,18}. LRP/LR is also involved in the development of numerous pathological states, including cancer\textsuperscript{18,19} and tumour angiogenesis\textsuperscript{20}, prion disorders and both viral\textsuperscript{21,24} and bacterial infections (of particular interest being bacterial meningitis as the receptor mediates translocation across the blood brain barrier)\textsuperscript{20}.

As LRP/LR serves as a PrP\textsuperscript{c} receptor we aimed to investigate whether LRP/LR is implicated in Ab\textsubscript{\beta} pathogenesis. Antibody blockade and shRNA mediated downregulation of LRP/LR was shown to significantly enhance the viability and proliferative potential of cells treated with Ab\textsubscript{\beta}\textsubscript{28,29}. In this study we aimed to further probe the mechanism underlying the role of LRP/LR in mediating Ab\textsubscript{\beta} pathogenesis.

## Results

**Försters resonance energy transfer between cell surface LRP/LR and Ab**\textsubscript{\beta}. 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 Ab\textsubscript{\beta} interact on the surface of HEK293 cells. The highly sensitive\textsuperscript{25,26} PE/APC FRET pair (donor and acceptor, respectively) was employed to immuno-label 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\textsuperscript{c}, CAT and Ab\textsubscript{\beta}) 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\textsuperscript{c}, CAT and Ab\textsubscript{\beta} 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\textsuperscript{c}, a cell surface protein to which LRP/LR binds with very affinity\textsuperscript{19} (positive control) and chloramphenicol acetyl transferase (CAT), a bacterial protein to which LRP/LR has been shown to not bind\textsuperscript{20} (negative control). Upon co-labelling of cells with PrP\textsuperscript{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 Ab\textsubscript{\beta}-APC and LRP/LR-PE (Fig. 1f), therefore suggests that FRET occurred between these proteins.

**LRP/LR interacts with shed Ab**\textsubscript{\beta}. To confirm that LRP and Ab\textsubscript{\beta} form stable associations, pull down assays were conducted. Although similar experimental procedures have been previously reported\textsuperscript{26}, these were conducted employing exogenously administered synthetic Ab\textsubscript{\beta}28 peptide. Those reported here employed conditioned cell culture media (supernatant) from HEK293 cells into which Ab\textsubscript{\beta} was shed, thereby investigating the presence of this association within a physiological context. The averaged total concentration of Ab\textsubscript{\beta} present in the conditioned media was approximately 37.6 pg/ml, results which are consistent with the concentration of Ab\textsubscript{\beta} detected by others in the supernatant of HEK293 cells\textsuperscript{28}. 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\textsuperscript{\textregistered} 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 Ab\textsubscript{\beta} present in each pull down assay fraction required sensitive detection employing an Ab\textsubscript{\beta}-specific ELISA assay. Upon incubation of conditioned media with LRP::FLAG containing cell lysate, it was observed that Ab\textsubscript{\beta} was successfully immobilized by LRP::FLAG, as there was a significant increase (27%) (p = 0.0433) in the Ab\textsubscript{\beta} levels in the eluate sample when compared to that present in wash 3 (Fig. 2b). To account for possible binding of Ab\textsubscript{\beta} to other proteins within the cell lysate or non-specific binding to the Anti-FLAG\textsuperscript{\textregistered} M2 beads, the degree of Ab\textsubscript{\beta} 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 Ab\textsubscript{\beta} 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 Ab\textsubscript{\beta} 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 Ab\textsubscript{\beta} is unable to distinguish between the Ab\textsubscript{\beta}0 and Ab\textsubscript{\beta}42 isoforms.

**Cellular incubation with Ab\textsubscript{\beta}28 induces apoptosis.** An Annexin-V-7AAD assay was employed to assess the cellular effects of synthetic Ab\textsubscript{\beta}28 on HEK293 cells. The exogenous application of 200 nM and 500 nM Ab\textsubscript{\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 Ab\textsubscript{\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 Ab\textsubscript{\beta} 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
exogenous synthetic Aβ42 for 72 h was assessed by Annexin-V-7AAD assay. Upon assessment, cellular incubation with 200 nM Aβ42 resulted in 53.6% cell death, whilst treatment with 500 nM Aβ42 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 Aβ42 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).

**Aβ42 internalization.** The degree of cell surface Aβ42 served as a measure of the degree of receptor-mediated internalization - with lower cell surface Aβ42 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 Aβ42 administration was performed to limit internalization as receptor-mediated internalization is halted under these conditions. Thus, the cell surface levels of Aβ42 in the no internalization control (1 h, 4°C) was set to 100%. The progressive decrease in cell surface Aβ42 may therefore be interpreted as a consequence of receptor-mediated internalization of the exogenous Aβ42. Although internalization (12.05%) was observed after 5 min, the extent of Aβ42 internalization was at its highest and most evident after 15 min (66.9%) after which the level of cell surface Aβ42 remained relatively constant for a further 15 min and then increased (Fig. 5a). The significant 11.36% increase (p < 0.001) in cell surface Aβ42 at 1 h when compared to 30 min may be indicative of Aβ42 recycling to the cell surface (and ultimately exocytosis) by the cell. The internalization of the exogenously administered Aβ42 was

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**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 (PrPc, CAT and Aβ) were labelled with APC (a,c,e respectively). Co-labelling of LRP/LR-PE and PrP-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.
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 Aβ42 is located at the cell surface whilst at 15 min and more markedly at 30 min, the degree of Aβ42 labelling within the cell lumen is unmistakably enhanced. As 100 nM Aβ42 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 Aβ42 internalization.** The degree of cell surface Aβ42 served as a measure of the degree of receptor-mediated internalization. Cells were either subjected to antibody treatment (Fig. 6a) or RNA interference technology in which LRP/LR was downregulated by shRNA\(^7,6\) (Fig. 6b)\(^29\). All relevant controls were similarly incubated at 4°C, prior and post exogenously 500 nM Aβ42 administration as this halts receptor-mediated internalization processes\(^30\). The cell surface levels of Aβ42 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 Aβ42 was observed across all incubation periods when compared to untreated controls at corresponding time points (Fig. 6a). This therefore demonstrates

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**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.
that antibody blockade of LRP/LR resulted in more cell surface-associated Aβ42 across all time points (in comparison to untreated controls) and this therefore suggests that Aβ42 internalization is hampered as a result of LRP/LR antibody blockade. Cells similarly treated with the anti-CAT antibody displayed Aβ42 internalization processes analogous to those of untreated controls (Fig. 6a). Furthermore, antibody treatment alone, in the absence of Aβ42, did not significantly alter internalization processes.

To confirm that LRP/LR is indeed implicated in Aβ42 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 Aβ42 and therefore impeded internalization (Fig. 6b). The transfection methodology itself did not adversely affect the internalization processes, as the difference in cell surface Aβ42 levels was not significantly different between control, mock transfected and shRNA scr samples across all incubation periods (Fig. 6b).

Flow cytometric 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
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 PrPc levels, which would confound the observed results, the cell surface expression of PrPc was evaluated. Flow cytometric analysis of shRNA transfected cells revealed that LRP/LR downregulation had no significant effect on cell surface PrPc levels (Fig. 6d). Similarly the PrPc 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, PrPc cell surface expression levels were not affected by the transfection methodology.

**Discussion**

Ensuring that LRP/LR and Aβ12 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, 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.

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 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⁻¹cm⁻¹ and 5000 M⁻¹cm⁻¹, respectively) and quantum yields and this makes them exceptionally sensitive when coupled to antibodies and may achieve 90% FRET efficiencies. 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 (PrPc) 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. However, upon co-labelling of PrPc-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 PrPc interact and this in turn allowed FRET to transpire. Since CAT and LRP/LR do not interact, the absence of FRET was expected. The occurrence of FRET between LRP/LR and...
Aβ (Fig. 1f) therefore suggests that these proteins are within 10 nm of 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 interactions28,33.

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 performed26, high quantities (2 mg) of synthetic Aβ42 were utilized and thus the presence of this interaction under normal physiological conditions warranted investigation. A significantly higher proportion 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 owing to the deregulation of intracellular Ca²⁺ levels which are a consequence of Aβ insertions into the plasma membrane 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 Aβ42, 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.
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. LRP/LR has been suggested to transduce cell survival and proliferative signals through the MAPK signalling pathway upon binding to laminin-1. LRP/LR regulates the expression of MAPK phosphatases (MKP1 and PAC1) and may thereby influence the activities of JNK, ERK1/2 and p38. Marked MAPK deregulation ensues in AD. 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. 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 PrPc leads to the induction of apoptosis through an upregulation of pro-apoptotic proteins such as Bax, enhanced Ca²⁺ release into the cytosol and the activation of caspases—particularly caspase 8. However, these pro-apoptotic signals may not be directly

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**Figure 6 | Effects of antibody blockage and shRNA downregulation of LRP/LR on Aβ 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 PrPc 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.
transduced by PrPα as it is not a transmembrane protein and therefore must be transduced through receptors to which PrPβ binds. We have recently demonstrated that LRP/LR does indeed contribute to PrPβ-Aβ42 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-PrPβ mediated cytotoxicity in Alzheimer’s disease, (2014)]. Therefore, these data suggest that LRP/LR may, either directly or indirectly, mediate Aβ42 induced apoptosis.

It is important to note that this finding is physiologically relevant as the Aβ42 concentrations within AD brains have been reported to be within the 200–4500 nM Aβ42 range42. Furthermore, it must be noted that at nM concentrations, such as those employed in this study, Aβ42 exists largely as low molecular weight oligomers42. Moreover, it has been demonstrated that even upon incubation in cell culture media, low nM concentrations of synthetic Aβ42 (0–500 nM) do not aggregate to form higher order, less toxic fibrils44. Based on these findings, it may be safely proposed that the biological effects observed herein may largely be attributable to oligomeric Aβ42, 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 controls45. 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 Aβ42 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 Aβ42 as antibody blockade of the receptor significantly augmented cell surface-associated Aβ42 and this thereby demonstrated that the amount of Aβ42 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 PrPα internalization, thereby allowing for enhanced Aβ binding.

This may be readily justified by the fact that PrPα, a ligand to which LRP/LR binds with high affinity, has been firmly established as a protein required for Aβ internalization46. However as PrPα 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 PrPα internalization into endosomes and cellular trafficking, results which have been confirmed in various neuronal cell types46,47. LRP/LR accounts for 25–50% of PrPα internalization48. The fact that blockade of the receptor did not completely abrogate Aβ42 internalization (Fig. 6a) may be due to the fact that only approximately 50% of Aβ42 bound to the neuronal surface is internalized via PrPα-dependent mechanisms44. It is noteworthy to add that heparan sulphate proteoglycans (HSPGs), to which LRP/LR similarly binds, have also been reported to mediate Aβ internalization49.

Furthermore LRP/LR is not the sole PrPα-binding protein implicated in its internalization, other such receptors include N-methyl-D-aspartate (NMDA) receptors50, metabotropic glutamate receptor 5 (mGlur5)51 and low-density lipoprotein receptor related protein (LRP1)52, and these may therefore account for the internalization of Aβ42 evident during antibody treatment.

It was due to this essential role of PrPα, that possible downregulation of PrPα as a consequence of LRP/LR downregulation needed to be negated (Fig. 6d). As cell surface PrPα 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 vitro42, 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 PrPα, or whether LRP/LR serves as the scaffold protein required for the internalization of the PrPα-Aβ42 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 Aβ42 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 Calff Serum (FCS) and 1% Penicillin/Streptomycin (P/S) solution and maintained in a humidified incubator (5% CO2, 37°C).

Transient Transfection. Upon reaching 70% confluency HEK293 cells were transfected, by calcium phosphate methodology, with pcNeo-molLR-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 head. FLAG® Immunoprecipitation 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: 10 000) (Sigma-Aldrich), respectively. The loading control, β-actin, was detected employing the rabbit anti-β-actin-HRP antibody (1: 10 000) (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 (1-3) Assay kit (Innovo-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 (Affimun Therapeutics); murine anti-PrP (Sigma-Aldrich); rabbit anti-α-Tubulin (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) uninfected 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-APC (PrPα detection), 7) cells labelled with anti-CAT-APC (CAT detection); cells labelled with anti-β-amyloid-APC (β-amyloid detection), 8) cells labelled with IgG1-iS18-PE & anti-PrP-
Hydrox et al. (2007). Flow cytometric analysis of cell surface levels of LRP and PrPc

Flow cytometric analysis of cell surface levels of LRP and PrPc. Post LRP
downregulation by shRNA methodology (72 h post transfection) cell surface LRP
and PrPc levels were assessed by flow cytometry. Cells (50%
confluency) were either subjected to antibody treatments (50
μg/ml of IgG1-iS18 or 8 mM PCA (apoptosis inducing
agent served as the positive control) treatment for 72 h. Cells were subsequently fixed (4%
FPA, 15 min, 4°C), rinsed thrice (1xPBS) and blocked (0.5%PBS-BSA,10 min).

Hoechst 33342 (1:100 dilution of 2 mg/ml solution) (Sigma-Aldrich) was
administered for 5 min (RT) to cells which were subsequently rinsed and mounted onto
microscope slides with 50 μl of Fluormount (Sigma-Aldrich). Images were
acquired using Zeiss LSM780 confocal microscope and Zen 2010 imaging software.

Annexin-V-7-Aminoactinomycin D Assay. HEK293 cells were subjected to ApoA
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.

Internalization Assay. Flow cytometric analysis was employed to assess the degree of
Apob on the cell surface during different intervals during internalization. Cells (50%
confluency) were either subjected to antibody treatments (50 μg/ml of IgG1-siRNA or
anti-CAT) for 48 h or transfected with shRNAs and assessed 72 h post transfection.
Cellular confinement of 70% was deemed optimal for internalization analysis. Cells were
incubated in serum-free media for 3 h prior to assessment and subsequent
lysis; Apob was subjected to 500 nM Apob treatment for 5 min, 15 min and 30 min
and 1 h at 37°C in a 5% CO2, humidified environment to allow for internalization. Cells were
concomitantly incubated on ice at 4°C, for 30 min, after which 500 nM Apob 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, pH 7.4, 1xPBS, 4°C, 10 min), washed again and fixed (ice-cold 4% paraformaldehyde,20 min, 4°C). These non-permeabilized cells were again washed (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-siRNA (anti-LRP/LR specific antibody) (Affimated Therapeutics) and
murine anti-PrPc 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.

Immunofluorescence Microscopy. Cells (70% confluent) were transfected with
eFCFP-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 Apob for 5 min,
15 min, 30 min and 1 h at 37°C in a 5% CO2, humidified environment 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-beta-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
gold 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
Fluormount (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
determine 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.

Additional information

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