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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

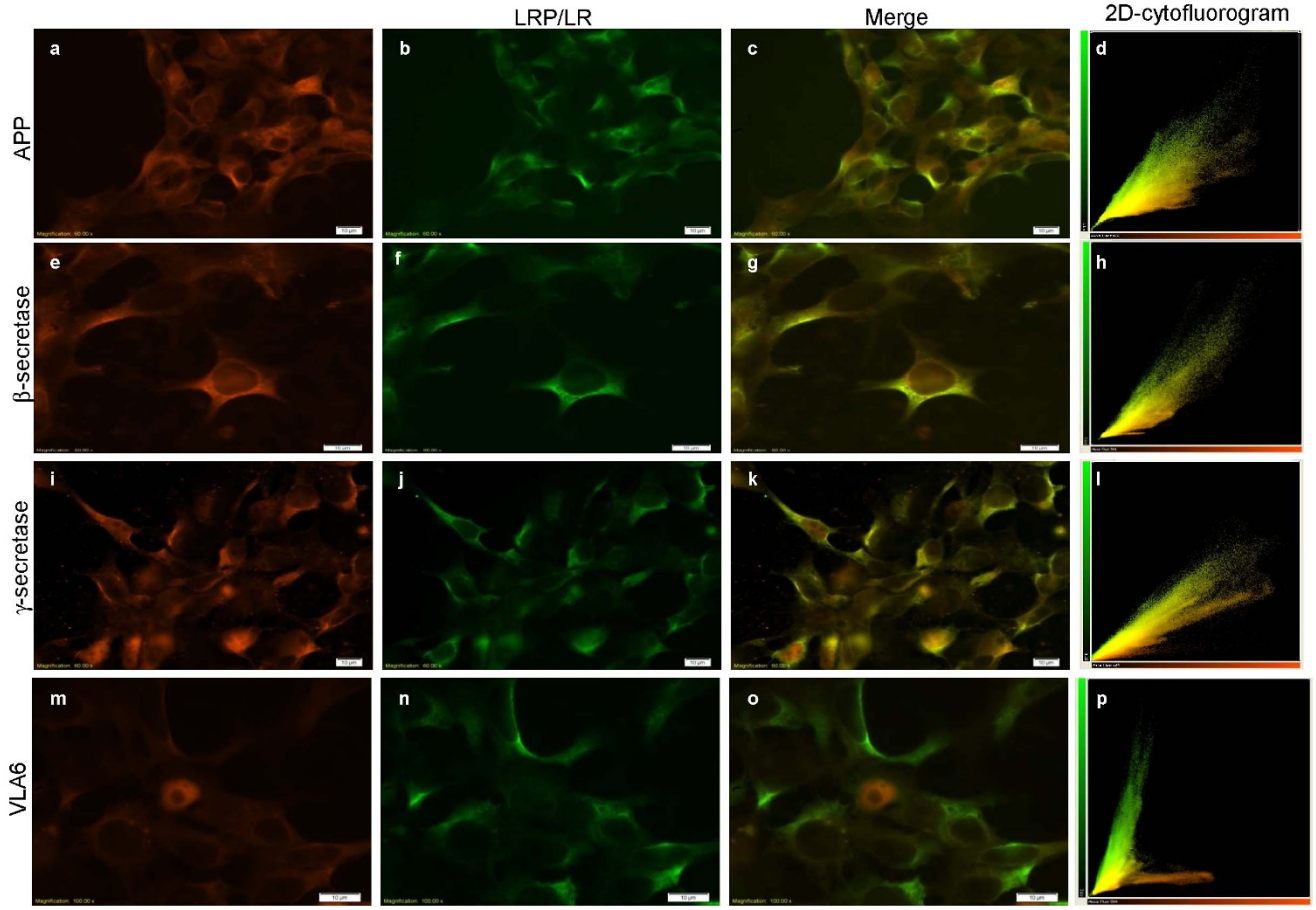

Figure 1 | Co-localisation of LRP/LR with the AD relevant proteins APP, β- and γ-secretase on the cell surace. Cell surface proteins on HEK293 cells were indirectly immunolabelled to allow for detection using the Olympus IX71 Immunofluorescence Microscope and Analysis Get It Research Software. (a) APP,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

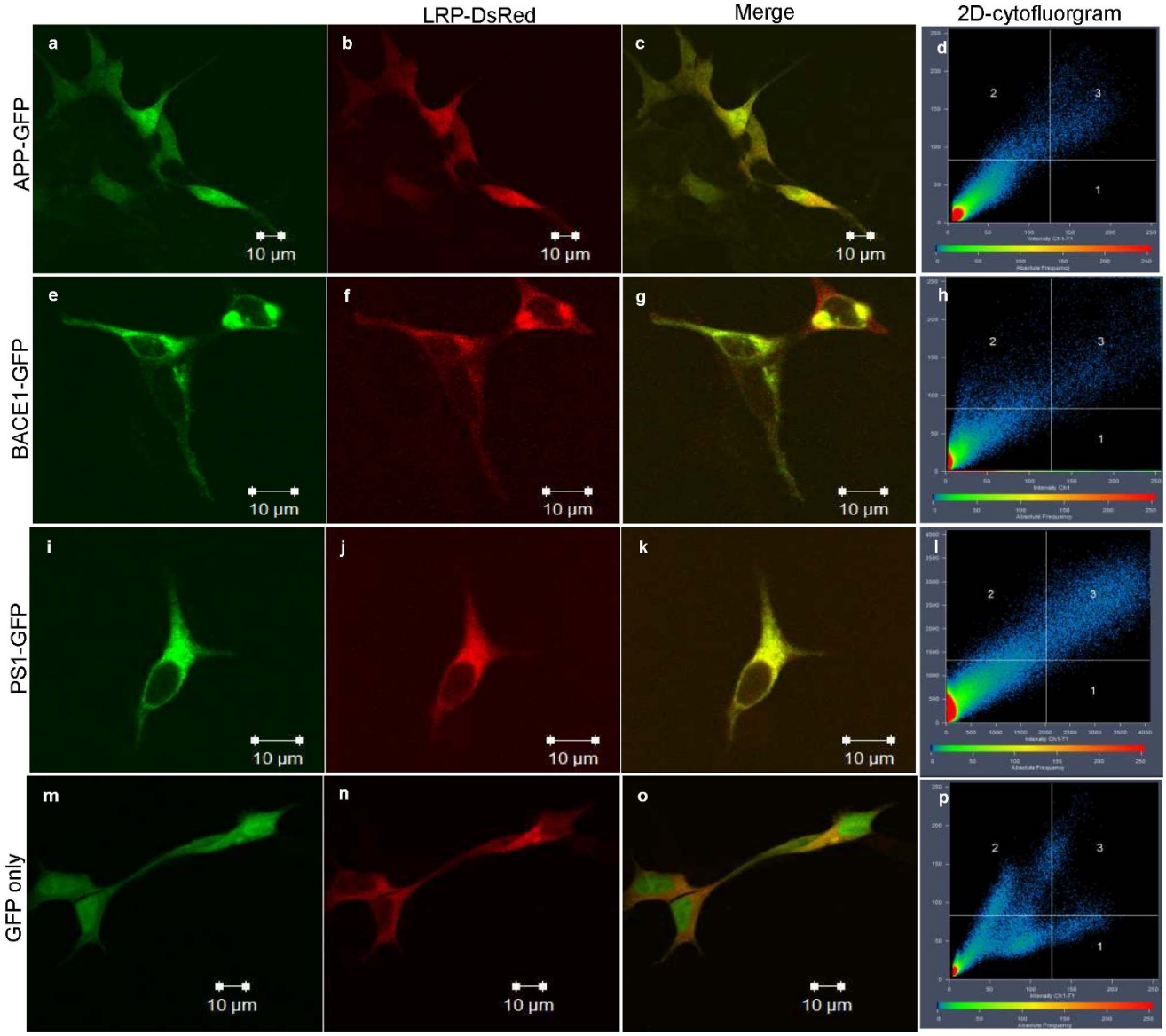

Figure 2 | Co-localisation of LRP-dsRed with APP-GFP, BACE1-GFP and PS1-GFP inside HEK293 cells. Fluorescently tagged (a) APP-GFP, (e) BACE1-GFP, (i) PS1-GFP and (m) GFP were co-expressed with LRP-dsRed (b, f, j, n) in HEK293 cells. Images were captured using the Zeiss LSM 780 confocal microscope and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

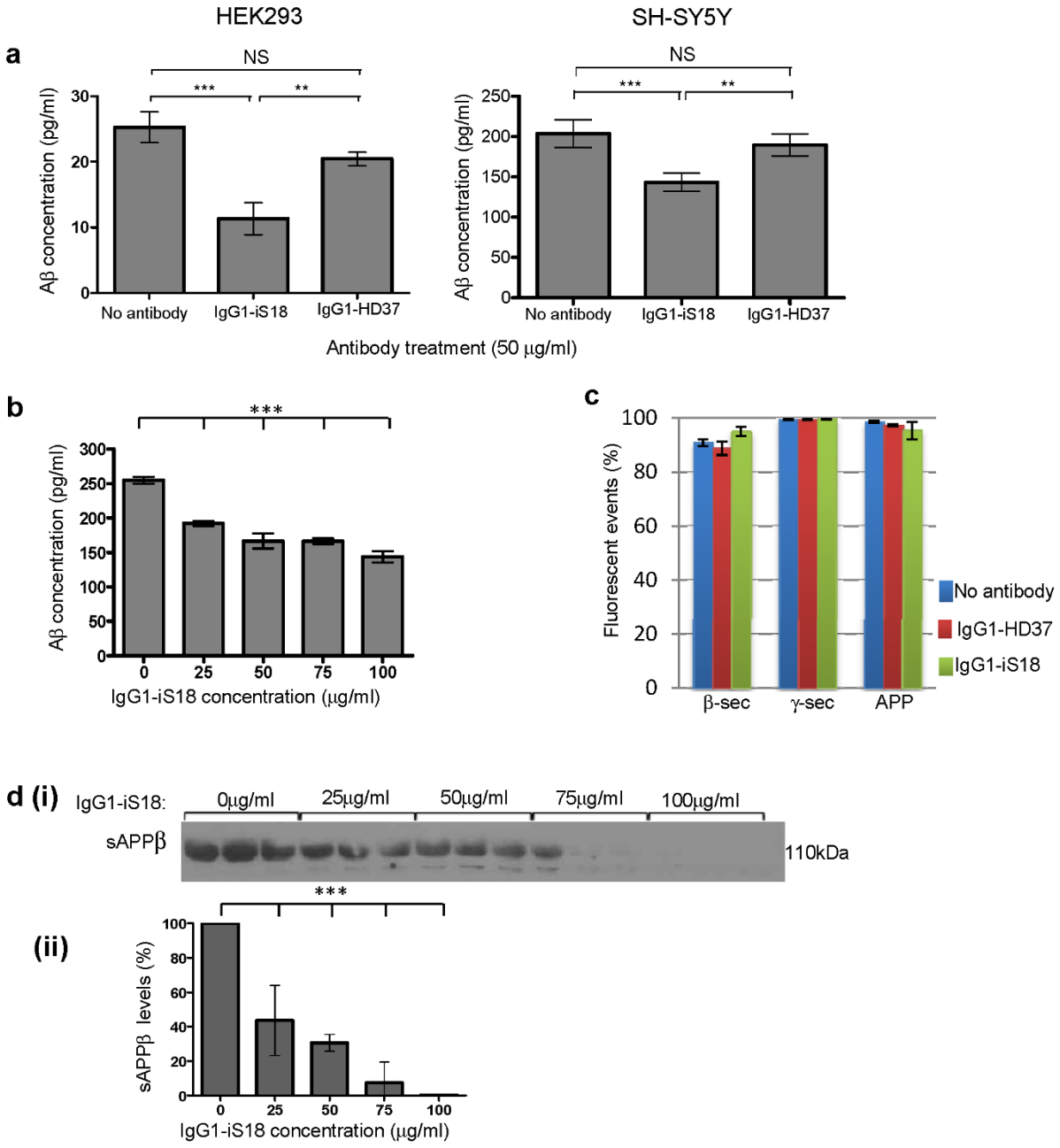

Figure 3 | Effects of IgG1-iS18. (a) Aβ levels in HEK293 and SH-SY5Y cells after treatment with IgG1-iS18 and IgG1-HD37 as detected by an Aβ ELISA after 18 hours of antibody incubation. Data shown (mean ± s.e.m) representative of three independent experiments (performed in triplicate) per cell line.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

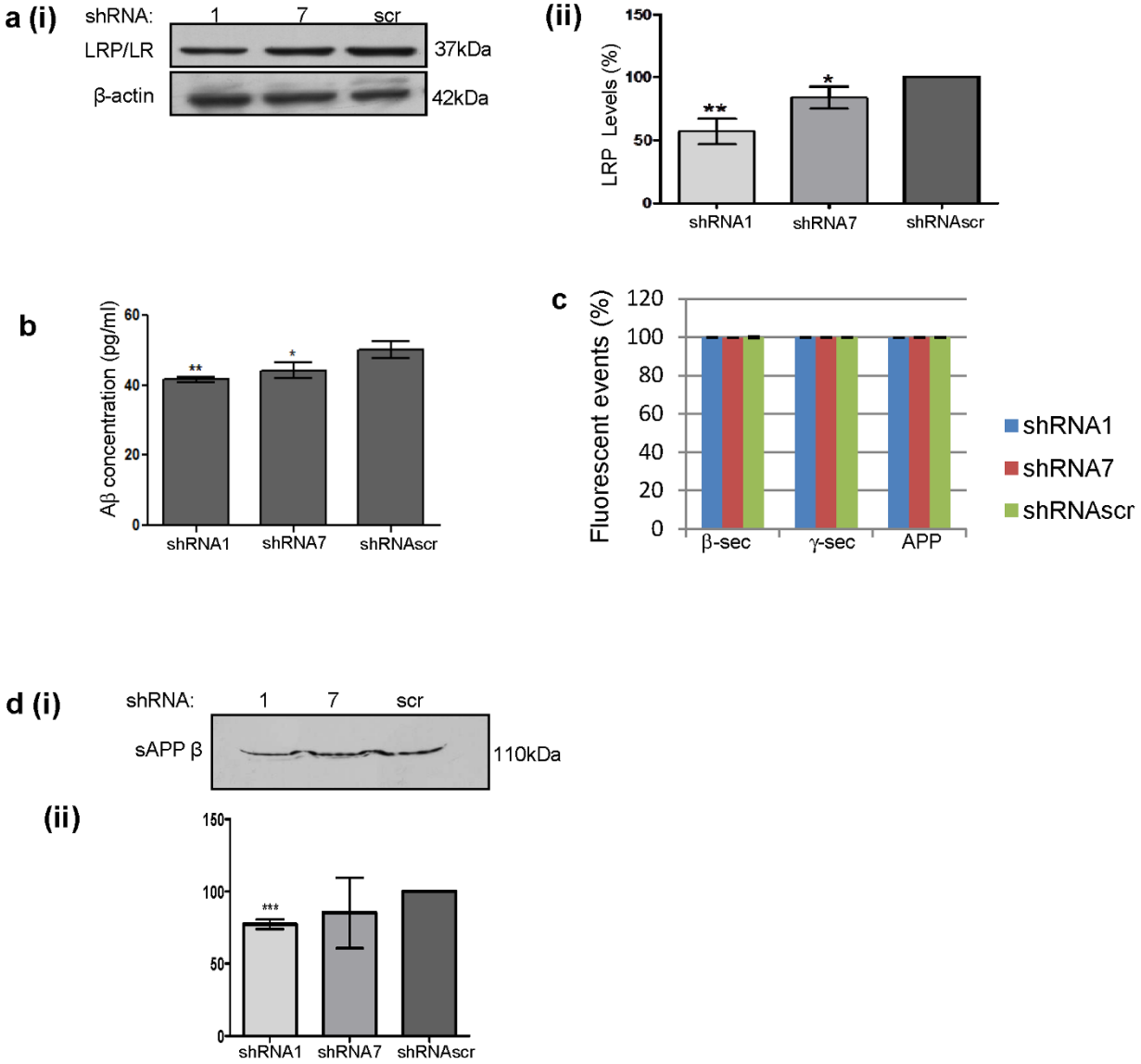

Figure 4 | Effects of LRP/LR downregulation by shRNA. (a) HEK293 cells were transfected with LRP/LR-specific shRNA1 and shRNA7 (as well as a scrambled control, shRNAscr). 72 hours post-transfection, cells were lysed and LRP/LR levels assessed by Western blotting. β-actin was used as a loading control.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

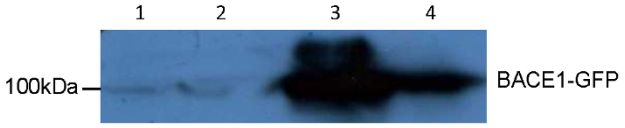

Figure 5 | LRP::FLAG Immunoprecipitation Assay Analysis. HEK293 cell lysates containing LRP::FLAG and either BACE1-GFP or CAT (Fig. S5) were subjected to Pull Down Assay analysis using Anti-FLAG M2 beads. After subsequent wash steps, eluted fractions were analysed using Immunoblotting. Lane 1 contained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