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Amylin-mediated regulation of LRP1 by miR-103/107 induces cerebral microvascular dysfunction and impairs β -amyloid efflux

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Abstract

Background: The cerebral small blood vessels of individuals with Alzheimer's Disease (AD) often have deposits of amylin, an amyloid-forming protein secreted in the blood by pancreatic β -cells. To determine whether systemic pancreatic amylin dyshomeostasis impairs amyloid β (A β) efflux across the blood-brain barrier (BBB), we studied cerebral microvessels in humans and rats with pancreatic expression of amyloidogenic human amylin, and evaluated the effect of human amylin in an *in vitro* BBB model.

Methods: Brain sections from AD and cognitively unimpaired individuals were co-stained with anti-A β and anti-amylin antibodies. *In vivo* analyses of A β efflux across the BBB were carried out in aged rats that express amyloid-forming human amylin in pancreatic β -cells and littermates expressing non-amyloidogenic rat amylin. We also used an *in vitro* BBB model of A β transcytosis in which the endothelial cell monolayer was exposed to amylin-mediated stress to determine whether amylin stress downregulates LRP1, the A β efflux transporter. This allowed us to use pharmacology to rescue the endothelial LRP1.

Results: In human AD brains, A β accumulation within the perivascular space frequently co-localized with deposits of amylin in the vessel wall. In rats with pancreatic expression of amyloid-forming human amylin, the high blood levels of human amylin promoted amylin deposition in brain capillaries, increased brain A β level, lowered plasma-to-brain A β ratio and suppressed expression of LRP1 protein. *In vitro* BBB model experiment revealed that amylin-induced stress downregulates LRP1 in endothelial cells through a miRNA-based translational repression mechanism.

Conclusions: High blood human amylin levels cause cerebral microvascular dysfunction and interfere with A β efflux across the BBB through miRNA-mediated LRP1 downregulation. Lowering the blood amylin level in early AD could improve A β clearance from the brain.

Keywords: amylin, islet amyloid polypeptide, Alzheimer's disease, cerebral small vessel disease, low-density lipoprotein receptor-related protein 1

Background

Cerebral small vessel diseases are significant contributors to vascular cognitive impairment and dementia (VCID) [1] and a common pathological finding in the brains of individuals with Alzheimer's disease (AD) [2,3,4]. Mechanisms underlying small vessel-type dysfunction include cerebral amyloid angiopathy (CAA) caused by vascular deposition of amyloid β ($A\beta$) protein, arteriolosclerosis associated with aging, hypertension, and cardiovascular risk factors [5]. In addition, accumulating evidence from clinical studies demonstrates that obesity, insulin resistance and diabetes are strong risk factors for cerebral microvascular dysfunction [6] and the sporadic form of AD [7,8,9]. Correcting hyperglycemia, the hallmark of diabetic states, is not entirely effective at reestablishing vascular endothelial function [10,11] nor reducing cognitive decline [12,13,14,15]. Thus, blood glucose levels *per se* may not be the correct target for cerebrovascular disease and cognitive decline risk reduction.

Recent reports from multiple laboratories [16,17,18,19,20,21] show that vascular lesions and $A\beta$ plaques in the brains of individuals with AD have abundant deposits of amylin, a ~4 kDa hormone synthesized and co-secreted with insulin by pancreatic β -cells [22]. Amylin (islet amyloid polypeptide; IAPP) normally crosses the blood-brain barrier (BBB) [23] and participates in the central regulation of satiation [24], but it also forms pancreatic amyloid in patients with type-2 diabetes [22]. In patients with type-2 diabetes, aggregated amylin accumulates in the cardiovascular system (systemic amylin dyshomeostasis) [25,26,27] and is associated with microcirculatory disturbances and activation of hypoxia signaling in kidneys through attachment to red blood cells [27]. To uncover cerebral effects associated with systemic pancreatic amylin dyshomeostasis, we previously studied rats that express human amylin in the pancreatic β -cells [18,28], as amylin from rodents is non-amyloidogenic [29] and less prone to deposition in blood

vessels [18]. We found that human amylin-expressing rats slowly accumulate aggregated amylin in the brain microvasculature with aging (> 12-month old rats) leading to microhemorrhages [18] and late-onset behavioral changes [18,28] that are similar to those in AD rat models. At the cellular and molecular levels, accumulation of aggregated amylin in brain capillaries is associated with astrocyte activation, neuroinflammation and oxidative stress [18,28].

Cell responses to stress conditions involve reprogramming gene expression through non-coding RNAs such as microRNAs (miRNAs) [30]. They inhibit protein synthesis by suppressing the translation of protein coding genes or by degrading the mRNA [30]. Paralog miRNAs miR-103 and miR-107 have previously been shown to be dysregulated in AD [31]. These miRNAs also appear to mediate stress-suppressed translation of the low-density lipoprotein receptor-related protein 1 (LRP1) [32], an apolipoprotein E (APOE) receptor that binds and internalizes soluble A β at the abluminal side of the BBB [33,34,35]. Because amylin deposition in the brain microvasculature affects vascular endothelial cells (ECs) [18], we hypothesized that amylin stress dysregulates miR-103/107 impairing LRP1 synthesis and A β efflux across the BBB, and that antagomirs against miR-103/107 modify the amylin-mediated stress effect on LRP1.

To determine whether amylin deposition in the brain microvasculature is associated with impaired A β efflux, we explored amylin-A β interaction in the human brain microvasculature and carried out *in vivo* analyses of A β efflux across the BBB in rats that express amyloid-forming human amylin in pancreatic β -cells versus littermates that express non-amyloidogenic rat amylin. To further define the mechanism, we used an *in vitro* BBB model of A β transcytosis in which the EC monolayer was exposed to amylin-mediated stress; antisense microRNAs were used in an attempt to rescue endothelial LRP1 expression. Our results provide a basis for targeting amylin-mediated cellular pathways at the blood-brain interface to reduce AD pathology.

Methods

The aim of this study was to determine whether elevated blood levels of human amylin impairs A β efflux across the BBB. We studied cerebral microvessels in humans and in rats with pancreatic expression of amyloidogenic human amylin versus littermates expressing non-amyloidogenic rat amylin for histological evidence of amylin-A β interaction at the BBB, and evaluated the effect of human amylin on A β efflux in an *in vitro* BBB model.

Human samples

The protocol concerning the use of autopsy tissues from patients was approved by the University of Kentucky Institutional Review Board (IRB) and informed consent was obtained prospectively. We used paraffin embedded human brain tissues (n = 9) provided by the Alzheimer's Disease Center biobank at the University of Kentucky, to explore amylin-A β interaction in the brain microvasculature. Formalin fixed dorsolateral frontal cortex (Brodmann area 9) tissue was used from six autopsied individuals >80 years of age at death and three age-matched cognitively unaffected (CU) individuals. The disease group included patients with AD without diabetes (n = 3) and AD with diabetes (n = 3). The absence/presence of diabetes was determined during life (at longitudinal clinical visits) by patient or caregiver self-report and the use of diabetic medications. Neuropathological information, neuritic amyloid plaques (Consortium to Establish a Registry for Alzheimer's Disease; CERAD), Braak NFT stage and CAA severity, along with age and sex of each individual included in the present study are summarized in the Supplementary Table 1.

Experimental animals

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

To study the impact of systemic pancreatic amylin dyshomeostasis on the A β efflux from the brain, we used rats that express human amylin in pancreatic β -cells (i.e., HIP rats) [36]. The HIP rats (non-AD rats) develop systemic amylin dyshomeostasis by ~ 10-12 months of age, which is characterized by amylin deposition in the pancreas [36] and extra-pancreatic tissues [18,25,26,27,28], including the brain microvasculature [18]. Breeding pairs were purchased from Charles River Laboratory. Wild type (WT) littermates expressing non-amyloidogenic rat amylin served as controls.

***In vitro* BBB model of A β ₄₂ transcytosis with amylin-induced stress at the blood side**

We used an *in vitro* BBB model described previously [37] with modifications related to amylin deposition on the EC monolayer and consequent effects on the A β transport across the EC monolayer. Briefly, primary rat brain microvascular endothelial cells (Cell Applications Inc) were plated on 24-well Transwell-Clear inserts with 0.4 μ m pore polycarbonate membrane (Costar, Corning, NY, USA) and primary rat brain astrocytes (Sigma) were cultured at the bottom wells. Barrier integrity was measured from the trans-endothelial electrical resistance (TEER) as described previously [37,38] using the EVOM2 meter with STX-3 electrodes (World Precision Instruments). Maximum TEER was achieved within 8-10 days in culture. BBB permeabilities to human amylin (10 μ M; Anaspec; AS-60254-1), rat amylin (10 μ M; American Peptide) and DMSO (1mM; vehicle) were assessed using FITC-Dextran 4 kDa (Fisher Scientific) diluted in Hank's Balanced

Salt Solution (HBSS) buffer with 0.1% BSA (HBSS-BSA) as a paracellular diffusion marker. Permeability coefficients were calculated using the formula; $P = (\Delta Q / \Delta t) / (A * C_0)$, ($\Delta Q / \Delta t$) = rate of FITC-Dextran change; A= surface area of insert (0.33cm^2); C_0 = Initial FITC-Dextran input.

In the $A\beta_{42}$ transcytosis experiments, the EC monolayer was treated with a medium containing human amylin ($10\text{ }\mu\text{M}$) or vehicle (DMSO) for 24 hours. After washing, the luminal chamber was replaced with HBSS-BSA, and the abluminal chamber with $A\beta_{(1-42)}$ -FAM ($5\text{ }\mu\text{M}$; Bachem) or FITC-Dextran, respectively. $A\beta_{(1-42)}$ samples were collected from the luminal chamber for the measurement of the $A\beta_{(1-42)}$ -FAM and FITC-Dextran fluorescence intensities and $A\beta_{(1-42)}$ transcytosis quotient (TQ) as described previously [38]: $TQ = (A\beta_{(1-42)}\text{-FAM}_{\text{luminal}} / A\beta_{(1-42)}\text{-FAM}_{\text{input}}) / (\text{FITC-Dextran}_{\text{luminal}} - \text{FITC-Dextran}_{\text{input}})$.

MicroRNA mimics and antagomir transfection

To study the role of miRNA signaling in amylin-induced suppression of endothelial LRP1 expression, we used transfection of rat brain microvascular ECs with miR-103-3p (MCR01039), miR-107-3p (MCR01045) and control (MCH00000) mimics (ABMgood). Antagomir miR-103-3p (IH-320345-05-0005), miR-107-3p (IH-320348-05-0005) and negative control (IN-001005-01-05) (Dharmacon Inc.) were used in an attempt to rescue LRP1 expression. All transfections were done using RNAiMAX (Invitrogen) as per manufacturer's recommended protocol. Briefly, ECs were plated at 50% confluency in 6-well plates followed by co-transfection with either 100 nM of 103-3p and 107-3p mimics or antagomirs along with their respective negative controls. After 12-hours, antagomir-treated cell groups were further treated with $10\text{ }\mu\text{M}$ human amylin for 24-hours. After 36-hours of transfection, cells were harvested for Western blot analysis.

Real-Time Quantitative Reverse Transcription PCR

Total RNA was isolated using RNAqueous total RNA isolation kit according to manufacturer's protocol (Invitrogen, AM1914). cDNA synthesis and amplification were done using iTaq Universal SYBR Green One-Step Kit (Biorad; 1725151) with the following primer sequences: LRP1: forward (Fwd) 5'-TTGTGCTGAGCCAAGACATC-3', reverse (Rev) 5'-GGCGTGGAAGACATGTAGGT-3'; and GAPDH: Fwd 5'-GCTGCGTTTTACACCCTTTC-3', Rev 5'-GTTTGCTCCAACCAACTGC-3' (IDT, Inc, USA). For miRNA quantification, cDNA was synthesized from total RNA using miRNA cDNA synthesis kit with poly (A) polymerase (ABMgood, G902). cDNA was amplified using SYBR Green mastermix (Biorad) along with miRNA specific primers from (rno-miR-103-3p, MPR00332; rno-miR-107-3p, MPR00335; RNU6 house Keeping gene, MP-r99998) (ABMgood). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and experiments were normalized to GAPDH or U6 miRNA

MTS cytotoxicity assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega) was used to assess cytotoxicity of aggregated amylin on the EC monolayer.

Immunohistochemistry, immunofluorescence and immunocytochemistry

For immunohistochemistry, formalin-fixed, paraffin-embedded brain tissues from humans and rats were processed as described before [16,18,28,39]. Antibodies against amylin (1:200; clone E5; SC-377530; Santa Cruz) and A β (1:300; CST2454; Cell Signaling Technology) were the primary antibodies. Biotinylated IMPRESS horse anti-rabbit-AP conjugated (2 drops/slide; MP-5401; Vector), biotinylated horse-anti mouse (1:300, BA-2000, Vector) were secondary antibodies. The specificity of the amylin antibody in both human and rat brain tissues was established in previous

studies [16,18,28,29]. Pancreatic tissue from rats with deleted amylin gene (AKO rats) was the negative control for amylin. The generation of AKO rat model was described previously [18].

In immunofluorescence experiments, we used formalin-fixed, paraffin-embedded human brain tissue processed as previously described [18,18,39], brain capillaries from HIP and WT rats isolated as previously described [18]. and cultured ECs. Anti-amylin (1:200; clone E5; SC-377530; Santa Cruz), anti-collagen IV (1:500; ab6586; abcam), anti-alpha smooth muscle actin-Alexa Fluor 405 (1:200; ab210128, abcam), anti-caveolin-1 (1:100; sc-894; Santa Cruz, TX), anti-LRP1 (1:500; sc-57351; Santa Cruz), anti-4HNE (1:200; ab46545; abcam) were the primary antibodies. Secondary antibodies were: Alexa Fluor 488 conjugated anti-mouse IgG (1:300; A11029; Invitrogen), Alexa Fluor 568 conjugated anti-rabbit IgG (1:200; A11036; Invitrogen), Alexa Fluor 568 conjugated anti-mouse IgG (1:300; A11004; Invitrogen). Nuclei were counterstained with DAPI mounting media. For triple staining of human brain tissues, smooth muscle actin-Alexa Fluor 405 antibody was added after staining with human amylin and collagen IV; DAPI free mounting media was used. For Thioflavin S staining, after secondary antibody incubation, brain slides were incubated in 0.5% Thioflavin S for 30 minutes at room temperature. Slides were then incubated for 3 minutes in 70% ethanol, 5 minutes in 0.2% Sudan black before washing and mounting. Immunocytochemistry was performed as described previously [39,40].

Immunoprecipitation

To immunoprecipitate rat A β from brain homogenates and plasma, a previously published protocol (16) was used. Briefly, 1000 μ g of protein was incubated with anti-rat and human A β (2 μ g; CST2454; Cell Signaling Technology) overnight with end-over-end rotation, at 4°C. All of the elution was used for Western blot analysis.

Western blot and enzyme-linked immunosorbent assay (ELISA)

Western blot analysis was performed on isolated brain capillaries, brain tissue homogenate and plasma from rats. Tissues were processed as described previously [16,18,28,39]. RIPA buffer with 2% SDS was used to retrieve A β monomers from frozen brain samples [41]. The lysate was centrifuged at 17,000 xG for 30-minutes. The supernatant was separated from pellet after centrifugation and was then used for Western blotting. Total protein levels were estimated using a BCA kit (23225, ThermoFisher). Anti-LRP1 antibody recognizing the β -subunit of LRP1 (1:1,000; clone 5A6; sc-57351; Santa Cruz), rabbit anti-amylin polyclonal (1:2,000; T-4157, Bachem-Peninsula Laboratories, CA), anti-rat and human A β (1:1,000; 2454; Cell Signaling Technology), mouse anti- β actin (1:10,000; clone BA3R; MA5-15739; ThermoFisher), mouse monoclonal anti-GAPDH (1:10,000; clone 6C5; ab8245; Abcam), anti-rabbit IgG HRP conjugated (1:30,000; NA934VS; GE Healthcare) and anti-mouse IgG HRP conjugated (1:20,000; NXA931; GE Healthcare) were primary antibodies. Immunoprecipitated rat A β from brain homogenates and matched plasma (50 μ g of protein from tissue homogenate or immunoprecipitated rat A β elution) were loaded on 8% SDS-PAGE gel. Aggregated A β from brain homogenates were resolved in native-PAGE (non-reducing; non-denatured). Monomeric A β peptides were resolved in acidic Bis-Tris gel with 8M urea [35]. To enhance signal for monomeric A β , membranes were boiled for 3 minutes in PBS before the blocking step. LRP1 in cell and brain capillary lysates was resolved using 4-12% Bis-Tris gel under non-reducing condition. HRP-conjugated anti-rabbit or anti-mouse were secondary antibodies. Equal loading in Western blot experiments was verified by re-probing with a monoclonal anti- β actin antibody (raised in mouse, clone BA3R, Thermo Scientific; 1:2000). Protein levels were compared by densitometric analysis using ImageJ software.

Levels of amylin in the rat plasma and red blood cells were measured using amylin ELISA kits (EZHA-52K, Millipore), according to the manufacturer's protocol.

Lipid peroxidation and reactive oxygen species (ROS)

Lipid peroxidation and ROS were measured in cultured rat brain microvascular ECs using previously published protocols [26,39].

Statistical analysis

Parametric comparison of two groups was done using two-tailed unpaired t-test. Welch's correction was used with t-test to account for unequal variance from unequal sample sizes, if necessary. Parametric comparisons between three groups or more were performed using one-way or two-way ANOVA with Dunnett's *post hoc* or Tukey's *post hoc* tests. Data are presented as mean $\pm$ S.E.M. Difference between groups was considered significant when $P < 0.05$. All analyses were performed using GraphPad Prism 8.1 software.

Results

1. A β deposition in perivascular spaces co-occurs with amylin accumulation in vessel wall.

Co-staining of brain sections from the AD and CU individual groups with anti-amylin and anti-A β antibodies identified amylin and A β deposits in both groups, consistent with previous reports [16-21]. In AD brains, amylin immunoreactivity (brown) on the luminal side of small arterioles co-occurred frequently with patchy areas of A β immunoreactivity (green) within Virchow-Robin spaces (Fig. 1a-1c). Confocal microscopy analysis of AD brain sections confirmed amylin deposition (green) on the luminal side and A β deposits (red) in perivascular spaces (Fig. 1d). The analysis also indicated amylin-A β tangled within the vessel wall (Fig. 1e). To confirm amylin

accumulation within the vessel wall, we triple-stained AD brain sections for amylin, collagen and smooth muscle actin. Confocal microscopy analysis (Fig. 1f and 1g) shows a propensity for amylin deposition at the vascular luminal side.

The results of our exploratory study in human brains revealed histological evidence of interaction between amylin secreted from the pancreas and A β at the blood-brain interface, in AD. Tangled amylin-A β deposits across cerebral blood vessel walls were identified in AD brains from individuals with AD independent of comorbid type-2 diabetes, consistent with previous studies [16]. The presence of amylin deposition at the luminal side of small blood vessels and A β in perivascular spaces suggest that systemic amylin dyshomeostasis may contribute to impaired A β efflux from the brain into the bloodstream in individuals with AD.

2. Tangled amylin-A β across the BBB impairs the A β efflux from the brain, in rats.

To study *in vivo* how systemic pancreatic amylin dyshomeostasis impairs A β transcytosis across the BBB, we used HIP rats that express amyloid-forming human amylin in pancreatic β -cells [36] and accumulate amylin in brain capillaries [18]. The average circulating level of amylin in 16-month old HIP rats was ~ 2-fold higher compared to that in wild type (WT) littermates (Supplementary Fig. S1a). This was associated with amylin accumulation in small cerebral arterioles (Supplementary Fig. S1b) and presumable capillaries (Supplementary Fig. S1c; arrows), which was not detected in WT rat brains (Supplementary Fig. S1d).

Co-staining of brain slices from HIP and WT rat brains with anti-A β (green) and anti-amylin (brown) antibodies showed vascular amylin-A β interaction in HIP, but not WT rats (Fig. 2a-e). Pancreatic tissue from HIP (Supplementary Fig. S1e) and amylin knockout (AKO) (Supplementary Fig. S1f) rats served as positive and negative controls, respectively. Brain slices

from age-matched APP/PS1 rats were the positive control for A β deposits (Supplementary Fig. S2a). In HIP rat brains, amylin immunoreactivity was detected solely on the luminal side of the blood vessel, whereas those of A β were seen within perivascular, Virchow-Robin spaces (Fig. 2a) and within the blood vessel wall (Fig. 2b). In addition, brain slices from HIP rats had sporadic amylin-A β deposits (Fig. 2c; circle) that were seen in association with capillaries positive for luminal amylin accumulation and for A β deposition within the surrounding tissue (Fig. 2c; arrows), consistent with our findings in human AD brains (Fig. 1a-c). Scattered A β immunoreactivity was also detected in HIP rat brains (Supplementary Fig. S2b), but not in brains of WT littermates (Supplementary Fig. S2c).

Western blot analysis of HIP rat brain homogenates shows accumulation of A β in the brain (Fig. 2f and Supplementary Fig. S2d). Rat A β ₄₀ peptide and brain homogenate from a 12-month old APP/PS1 rat were the positive controls for A β accumulation. To test whether both soluble and insoluble aggregated A β accumulated in HIP rat brains, we performed native-PAGE followed by Western blot (Fig. 2g). The levels of soluble and insoluble A β aggregates were higher in HIP rat brains compared to those in WT littermates.

AD model rats are genetically determined to develop brain A β pathology, whereas rats expressing human amylin in the pancreatic islets may accumulate A β in the brain due to changes associated with chronically elevated blood levels of human amylin. To test whether the A β efflux from the brain to bloodstream is altered in HIP vs. WT rats, we used immunoprecipitation to enrich A β in plasma samples and brain homogenates from age-matched rats in the two groups followed by Western blot analysis of A β (Fig. 3a and 3b). The ratio of plasma-to-brain A β levels was lower in HIP compared to WT rats (Fig. 3c), which suggests that A β efflux across the BBB is impaired in HIP rats.

Taken together, our results show that increased pancreatic secretion of amyloid-forming amylin is associated with: 1, amylin accumulation in brain capillaries (Figs. 2a-2c, Supplementary Fig. S1b and S1c); 2, tangled amylin-A β deposits across the blood vessel wall (Figs. 2a and 2b); 3, A β accumulation in the brain (Figs. 2f and 2g); and 4, impaired A β efflux across the BBB (Figs. 3a-3c).

3. High blood human amylin suppresses the A β efflux transporter expression.

Amylin deposition in the brain microvasculature may induce stress in ECs and decline of the A β efflux transporter LRP1 expression. To test this hypothesis, we analyzed LRP1 protein expression in brain capillary lysates from aged HIP rats vs. WT littermates and EC lysates from EC monolayers that were subjected to amylin-induced stress.

Vascular amylin-induced LRP1 downregulation in the brain endothelium. Brain capillaries were isolated from HIP and WT rats and tested for the presence of amylin deposition and LRP1 protein expression by immunofluorescence and Western blot. Confocal microscopy analysis of isolated brain capillaries (Fig. 3d) showed that amylin deposition (green) co-localized with caveolin-1 (red), a protein that is abundant in ECs and further confirmed amylin deposition in HIP brain capillaries (Fig. 3e). Staining for LRP1 revealed lower LRP1 immunoreactivity signal in brain capillaries from HIP rats compared to WT littermates (Fig. 3f-g). Consistent with this result, Western blot analysis showed reduced LRP1 protein levels in brain capillary lysates from HIP rats compared to those in WT littermates (Fig. 3h and 3i). In contrast to protein expression, LRP1 mRNA levels were increased in HIP vs. WT rat brain capillaries (Fig. 3j), suggesting that the decrease in LRP1 protein in HIP rat capillary ECs occurs at a post-transcriptional level.

Amylin-induced LRP1 downregulation in endothelial cells, in vitro. To further evaluate the relationship between vascular amylin deposition and LRP1 protein expression, we incubated rat brain microvascular ECs with various concentrations of human amylin for 24 hours followed by analysis of LRP1 protein expression by Western blot (Fig. 4a). LRP1 protein levels decreased with increasing concentrations of human amylin; LRP1 expression was reduced by more than 50% in ECs incubated with 10 μ M human amylin. This result was further confirmed by immunofluorescence measurements in ECs incubated for 24-hours with 10 μ M human amylin (Fig. 4b). In contrast, non-amyloidogenic rat amylin (10 μ M; 24-hour incubation time) had no effect on LRP1 protein levels (Fig. 4b-d), as indicated by analyses of immunoreactivity by confocal microscopy (Fig. 4b) and Western blot (Fig. 4c). Viability of the ECs was not affected by incubation with human amylin (Supplementary Fig. S3), indicating that decreased LRP1 protein expression is not due to cell death. Consistent with this result, the capacity of ECs to induce transcript expression of LRP1 was not affected by amylin stress; consistent with the findings in HIP rat cerebral capillaries, LRP1 mRNA levels were greatly elevated in ECs incubated with human amylin vs. control cells and ECs incubated with rat amylin (Fig. 4d).

Impaired endothelial A β transcytosis by amylin in a 3-dimensional BBB model. To determine whether the amylin stress-induced LRP1 downregulation affects A β transcytosis, we employed a well-established model of BBB [37] in which the EC monolayer was exposed to human amylin on the luminal side (as shown in Fig. 4e). The BBB model was tested for monolayer formation and tightness by measuring TEER (Fig. 4f). All experiments were done with a fully formed EC monolayer characterized by the maximum $TEER = 110 \pm 5 \Omega/cm^2$.

First, we tested the effect of human amylin on EC monolayer structural integrity. We then measured A β transcytosis across the BBB using FAM tagged A β_{42} (A β_{42} -FAM) and FITC-

dextran as a paracellular diffusion marker. *TEER*, cell morphology within the EC monolayer and permeability to FITC-dextran (4 kDa) were measured following the incubation of the ECs for 24-hours with 10 μ M human amylin or similar concentrations of rat amylin or vehicle (Supplementary Fig. S4a-S4e). Although *TEER* decreased following the treatment with human amylin (Supplementary Fig. S4b), neither the intercellular space, as assessed from FITC-dextran permeability (Supplementary Fig. S4c-d), nor EC monolayer morphology (Supplementary Fig. S4E) were significantly altered by the treatment with human amylin. To assess the impact of amyloid-forming amylin on A β transcytosis across the BBB, human amylin or vehicle were applied at the luminal (blood) side for 24-hours followed by washing of the EC monolayers with PBS and application of FITC-Dextran or A β ₄₂-FAM at abluminal (brain) side of the BBB for 1-hour. The amounts of A β ₄₂-FAM and FITC-Dextran that cross the monolayer were estimated from the fluorescence intensity in the medium samples collected from luminal side and used to calculate the A β transcytosis quotient [38]. Amylin-pretreatment reduced the A β transcytosis quotient by $20 \pm 5\%$ ($P < 0.05$) (Fig. 4g), which indicates that amylin blocks transcytosis of A β ₄₂ across the *in vitro* BBB.

From these results (Fig. 4), we infer that amylin accumulation at the luminal side of the BBB impairs A β transcytosis across the ECs via LRP1 downregulation. As the LRP1 transcript was upregulated in response to amylin effects on ECs both *in vivo* (Fig. 3j) and *ex vivo* (Fig. 4d), this suggests that LRP1 protein downregulation may involve altered protein translation.

4. A β efflux transporter expression is suppressed by amylin-induced endothelial cell stress.

Paralog miRNAs miR-103 and miR-107 are upregulated by oxidative stress [42] and repress LRP1 translation in several cell lines [32]. Thus, we investigated if these miRNAs are involved in amylin-induced LRP1 downregulation in the BBB.

Amylin accumulation in brain capillaries induced oxidative stress in ECs by forming deposits with biochemical properties of amyloid (Fig. 5a), which was shown to alter structural stability of the cellular membranes [25,26,39]. This is evidenced by accumulation of the lipid peroxidation marker 4-hydroxynonenal (4-HNE) (Fig. 5b). Oxidative stress also occurred in rat brain microvascular ECs incubated with human amylin (10 μ M amylin; 24-hour incubation time), as indicated by lipid peroxidation of the EC membranes (Fig. 5c-e) and increased generation of ROS (Fig. 5f and 5g). Pancreas from a HIP rat was the positive control (Supplementary Fig. S5a) and pancreas tissue from an AKO rat was the negative control (Supplementary Fig. S5b) for amylin amyloid. The amylin stress on ECs was associated with elevated levels of miR-103 and miR-107 (Fig. 5h). Brain capillary lysates from HIP rats also had elevated miR-103 and miR-107 levels compared to those in WT littermates (Fig. 5i).

Next, we pre-treated ECs with poloxamer 188, a surfactant that decreases lipid peroxidation in cellular membranes [26,39]. Surfactant molecules blocked lipid peroxidation and consequent ROS production (Fig. 5c-g; magenta bars); however, the surfactant neither normalized miR-103/107 levels (Fig. 5h) nor rescued LRP1 expression (Fig. 5j) upon amylin-induced EC damage.

The results support the hypothesis that miRNA is upregulated by amylin-mediated endothelial stress and suggest that additional pathways may compromise the capability of ECs to express LRP1, which were not linked to peroxidative membrane injury.

5. Antisense microRNAs rescue amylin-induced suppression of A β efflux transporter.

TargetScan predicts that miR-103 and miR-107 bind directly to *LRP1*, with the binding site located at the 3'UTR region of rat *LRP1* (Fig 6a). To further determine whether high miR-103

and miR-107 levels suppress LRP1 translation, we co-transfected miR-103 and miR-107 mimics (100 nM) into rat brain microvascular ECs. Cell lysates were tested after 24-hours for LRP1 protein expression by Western blot. The average LRP1 expression level was lower in ECs co-transfected with miR-103 and miR-107 mimics compared to miR-control (Fig. 6b).

Antisense microRNAs are used to target aberrant miRNA [43]. We used antagomir (amiR) 103 and amiR-107 to test the hypothesis that silencing amylin-induced upregulation of miR-103 and miR-107 rescues LRP1 expression. Our results show that amiR-103/107 rescued LRP1 expression in ECs following amylin-induced cell stress (Fig. 6c).

These results indicate that: 1, endothelial LRP1 downregulation associated with amylin stress is a miRNA-based translational repression mechanism; and 2, LRP1 downregulation by amylin-induced stress on ECs can be reversed by modulating miR-103 and miR-107.

Discussion

By investigating brain tissues from humans with AD and from non-AD rats with chronically elevated blood levels of human amylin, we found that A β clustering in the cerebral capillary bed consistently co-localized with luminal accumulation of amylin amyloid secreted by pancreatic β -cells. We then used aged rats with pancreatic expression of amyloid-forming human amylin vs. littermates expressing non-amyloidogenic rat amylin and found that chronically elevated blood levels of amyloid-forming human amylin results in tangled amylin-A β across the BBB and endothelial transcytosis dysfunction (see Fig. 6d for the proposed mechanism). These results suggest that LRP1-mediated A β efflux could be affected (or restored) through cellular pathways modulated from the luminal BBB side.

Although APOE/LRP1-regulated metabolic pathways have a well-established role in A β clearance [44], a large fraction of A β fragments is eliminated from the central nervous system through transcytosis across the BBB, as shown by kinetic studies in humans [45] and in mice [33,35]. This pathway of A β clearance from the brain is reduced early in the onset of AD causing A β deposition in capillary beds [46]. In contrast to rats expressing endogenous non-amyloidogenic rat amylin that had unimpaired A β efflux across the BBB, human amylin-expressing rats showed amylin amyloid deposition in the brain microvasculature and lower A β efflux across the BBB. This was caused by miRNA-based translational repression of LRP1 and was reversed by antisense microRNA. Our results suggest that miRNA-mediated suppression of endothelial LRP1 may be a mechanism by which amylin accumulation in the brain microvasculature reprogram ECs toward an AD phenotype.

Because amylin is co-secreted with insulin by pancreatic β -cells [22], conditions associated with hyperinsulinemia and prediabetic insulin resistance always coincide with hypersecretion of amylin. Patients with type-2 diabetes accumulate amylin amyloid in pancreatic islets that causes oxidative stress and inflammation leading to cell apoptosis and depletion of β -cell mass [22]. We reported the presence of aggregated amylin with similar molecular weights in matched plasma and brain homogenate from humans with AD [16], and suggested the hypothesis that toxic aggregated amylin circulates in the blood in humans with AD. Soluble aggregated amylin is endocytosed into the cells through macropinocytosis or caveolae-mediated pathways and degraded in the acidic compartment of lysosomes [47]. In human amylin-expressing rats, we found amylin deposition in the brain microvasculature, consistent with amylin deposition in brains of humans with AD [16,17,18,19, 20]. From these results, we infer that chronically

elevated blood amylin overwhelms the EC capacity to clear aggregated amylin through endocytosis letting the BBB vulnerable to aggregated amylin toxicity.

MiR-103 and miR-107 are upregulated by oxidative stress [42] and repress LRP1 translation *in vitro* [32]. Several human diseases [30,31,48,49], including AD [31] and type-2 diabetes [49] are associated with altered miR-103/107. Our results show that, although amylin deposition in the brain microvasculature in HIP rats causes stress on the brain endothelium, the brain capillary ECs can induce transcript expression of LRP1; however, a miRNA feedback loop prevents the translation of LRP1 transcripts. In support to this proposed mechanism (Fig. 6d), we found evidence of dose-dependent regulatory mechanism of LRP1 expression that occurred at the post-transcriptional level in ECs incubated with amyloid-forming human amylin (but not non-amyloidogenic rat amylin) and by miR-103 and 107 transfections in ECs. In future studies, it would be interesting to determine whether miRNA-mediated suppression of LRP1 show an inverse expression pattern with lowering blood amylin concentration.

Finding A β accumulation in the brains of non-AD rats that express human amylin in pancreatic islets was unanticipated. AD model rats are genetically determined to develop brain A β pathology, whereas rats expressing human amylin in the pancreas accumulate A β in the brain in association with amylin deposition in the brain microvasculature and impairment of LRP1-mediated A β efflux across the BBB. Our results suggest that rats “humanized” for amylin expression may serve as a model for the amylin contribution to late-onset AD pathology.

LRP1 downregulation in brain capillaries is common in aging [50] and further accelerated in AD [33,34,35]. We showed that changing blood components in rats by pancreatic expression of human amylin provoked miRNA-based LRP1 downregulation in brain capillary endothelium, which suggests that high circulating human amylin is a specific stressor on the

luminal side of the BBB. Thus, lowering blood amylin levels or/and inhibiting miRNA-mediated LRP1 downregulation caused by amylin stress on the BBB could improve A β clearance from the brain and reduce AD pathology. Finding that endothelial LRP1 expression/ function can be restored via cellular pathways that are modulated from the luminal side of the BBB has the potential to change the current paradigms in the treatment of AD.

Conclusions

While the association between dysregulation of circulating amylin and AD pathology is not yet as widely studied as other aspects of AD biology, accumulating evidence from multiple research teams describe amylin-A β cross-seeding in brains of individuals with sporadic AD [16-21]. Here, we show that high blood human amylin levels cause cerebral microvascular dysfunction and interfere with A β efflux across the BBB through miRNA-mediated LRP1 downregulation. This study constitutes a basis for additional hypothesis driven research and evidence-based validation studies to help understanding what triggers amylin deposition in the cerebral microvasculature and whether lowering the blood amylin level in early AD could improve A β clearance from the brain and may reduce the progression of AD pathology.

List of abbreviations

AD	Alzheimer's disease
CU	Cognitively unaffected
A β	Amylin- β -amyloid
IAPP	Islet amyloid polypeptide
HIP rats	Rats express human amylin gene in the pancreas
PSEN1 or PS1	Presenilin 1
APP	Amyloid precursor protein

WT rats	Wild-type rats
AKO rats	amylin knockout rats
LRP1	low-density lipoprotein receptor-related protein 1
miRNAs	microRNAs
APOE	apolipoprotein E
EC	endothelial cell
BBB	blood-brain barrier
ROS	reactive oxygen species
4-HNE	4-hydroxynonenal

Declarations

Ethics approval and consent to participate

The protocol concerning the use of autopsy tissues from patients was approved by the University of Kentucky Institutional Review Board (IRB) and informed consent was obtained prospectively.

Experimental studies involving animals conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Consent for publication

Not applicable

Competing interests

The authors declare that no conflict of interest exists.

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Author's Contributions

Conceptualization, F.D.; methodology, G.V.V, H.L., N.V., S.S., S.D., P.T.N., L.B.G., and F.D.; formal analysis, G.V.V., H.L., N.V., S.S., S.D., and F.D.; resources, P.T.N. and F.D.; writing, F.D. wrote the first draft of the manuscript, all other authors contributed to the final form of the manuscript.

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Figure Legends

Fig. 1: Amylin-A β interaction at the blood-brain interface in human AD brains. a, b

Representative immunohistochemical (IHC) micrographs of brain sections from patients with sporadic AD (**a**) and cognitively unimpaired (CU) (**b**) individuals that were co-stained with anti-amylin (brown) and anti-A β (green) antibodies. **c** Analysis of the number of blood vessels with vascular mixed amylin-A β deposition (per mm²) in brains from AD ($n = 6$) and CU ($n = 3$) (2 slides/brain) groups assessed from IHC. **d, e** Representative images of confocal microscopy analysis of brain sections from the same AD individuals ($n = 6$; 2 slides/brain) as in above showing amylin (green) and A β (red) deposits in small-type vessels in which amylin is present on the luminal side (**d**) and within the vessel wall (**e**). **e-g** Representative immunofluorescence images of AD brain sections triple stained with anti-amylin (green), anti-collagen IV (red) and anti-smooth muscle actin (blue) ($n = 3$; 2 slides/brain). Scale bars, 50 μ m. Data are mean $\pm$ SEM. $P \leq 0.05$ *; by two-tailed, unpaired t test (**c**).

Fig. 2: Pancreatic amylin accumulates in the brain microvasculature and impairs A β efflux from the brain. a-d

Representative IHC micrographs of brain sections from HIP (**a-c**) and WT (**d**) rats co-stained with anti-amylin (brown) and anti-A β (green) antibodies. **e** Analysis of the number of blood vessels with vascular amylin-A β deposition (per mm²) in brains of WT and HIP rats ($n = 5$ /group) (3 slides/brain), assessed from IHC. **f** Representative Western blot and densitometry quantification of A β in brain homogenates from HIP rats and WT littermates using acidic urea gel ($n = 3$ /group) to resolve monomers. Rat A β ₄₀ peptide and APP/PS1 rat brain homogenate were used as positive controls. A β densitometry was normalized to loading control actin. A β densitometry quantification was calculated from two experiments ($n = 7-8$ /group). **g** Representative Western blot and densitometry analyses of aggregated A β in brain homogenates

from HIP rats and WT littermates ($n = 7/\text{group}$) using native-PAGE. Scale bars, 50 μm (**a-d**).

Data are mean $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.01$ **; by two-tailed, unpaired t test (**e-g**)

Fig. 3: High blood amylin levels downregulate the A β efflux transporter at the BBB. a-b

Immunoprecipitation and western blot analyses of A β in the plasma (**a**) and brain homogenates (**b**) from WT and HIP rats ($n = 5/\text{group}$). Age-matched APP/PS1 rat brain homogenate were used as a positive control. **c** Ratio of plasma A β -to-brain A β levels in HIP and WT rats assessed from Western blot analysis of A β enriched by immunoprecipitation from plasma (**a**) and brain (**b**) homogenates. **d** Confocal fluorescent micrographs of amylin (green) and endothelial cell marker caveolin-1 (red) in brain capillaries isolated from HIP rats and WT littermates. **e** Levels of amylin fluorescence intensity signals quantified from $n = 21$ and $n = 47$ isolated capillaries from WT rats and HIP littermates ($n = 3$ rats/group), respectively, and averaged for each rat. **f** Confocal immunofluorescent micrographs of LRP1 (green) and nuclei (blue) in isolated capillaries from WT rats and HIP littermates. **g** Levels of LRP1 fluorescence intensity signals quantified from WT rats ($n = 4$) and HIP littermates ($n = 4$) and averaged for each rat. **h** Representative western blot and densitometry quantification (**i**) of LRP1 in brain capillary lysates isolated from HIP rats and WT littermates ($n = 3$ rats/group). **j** LRP1 mRNA levels (fold difference using $2^{-\Delta\Delta C_t}$ method) in brain capillary lysates isolated from the same HIP and WT rats as in (**h**) ($n = 3$ rats/group). Scale bars, 10 μm (**d, e**). Data are mean $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.01$ ** by two-tailed, unpaired t test (**c, e, g, i, j**).

Fig. 4: *In vitro* test of amylin-induced impairment of A β efflux across the BBB. a

Representative Western blot and densitometry quantification of LRP1 in lysates from primary rat brain microvascular vascular endothelial cells (ECs) treated with vehicle or various concentrations of human amylin (500 nM, 1 μM , 5 μM , and 10 μM) for 24 hours ($n = 3$

preparations/test). **b** Immunofluorescent micrographs (LRP1-red; nuclei - blue) and quantification of LRP1 in ECs treated with vehicle, human amylin (10 μ M) or rat amylin (10 μ M) ($n = 30$ cells/group from 3 replicates per group). **c** Western blot and densitometry quantification of LRP1 in lysates from ECs treated with vehicle, human amylin or rat amylin as in **(b)** ($n = 3$ preparations/test). **d** LRP1 mRNA levels (fold difference using $2^{-\Delta\Delta C_t}$ method) measured with qRT-PCR in lysates from ECs treated with vehicle, human amylin or rat amylin (same 3 preparations/test used in panels **b** and **c**). **e** Cartoon representation of the *in vitro* BBB model (ECs monolayer - luminal chamber; astrocytes - abluminal chamber) used in A β transcytosis experiments. **f** Transendothelial electrical resistance (*TEER*) in EC monolayers ($n = 20$ preparations) as a function of days in culture. **g** The A β_{42} transcytosis quotient (TQ) across the *in vitro* BBB, in vehicle- and human amylin-treated EC monolayers. Scale bars, 10 μ m (**b**). Data are mean $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.0001$ ****; by one-way ANOVA with Dunnett's *post-hoc* (**a**) or Tukey's *post-hoc* (**b-d**); two-tailed, unpaired *t* test (**g**).

Fig. 5: MiRNA upregulation and LRP1 downregulation by amylin amyloid-mediated stress in vascular endothelial cells. **a** Representative fluorescent images of Thioflavin S (Thio S, green) and amylin (red) staining in brain tissue sections from HIP rats ($n = 3$ rats) showing the presence of amylin amyloid in a brain capillary. **b** Staining for the lipid peroxidation marker 4-HNE (red) and amylin (green) same as in **(a)**. **c, d** Representative fluorescent images and analysis (**e**) of lipid peroxidation levels in ECs incubated with human amylin ($n = 53$ cells) or with poloxamer 188 (P188) prior to incubation with human amylin ($n = 161$ cells), compared to controls ($n = 141$ cells). **f** Representative fluorescent images and analysis (**g**) of ROS levels in ECs incubated with human amylin ($n = 103$ cells) or with P188 prior to incubation with human amylin ($n = 85$ cells), compared to controls ($n = 92$ cells). **h** Expression of miR-103 and miR-107

measured by qRT-PCR (fold difference using $2^{-\Delta\Delta C_t}$ method) in ECs incubated with human amylin or with P188 prior to incubation with human amylin ($n=3$ preparations/treatment). **i** Expression levels of miRNA (miR)-103 and miR-107 measured by qRT-PCR (fold difference using $2^{-\Delta\Delta C_t}$ method) in lysates from HIP rat brain capillaries compared to those in WT rats ($n = 4$ rats/group). miR-U6 was used as internal control (**h, i**). **j** Representative Western blot and densitometry quantification of LRP1 from rat brain microvascular ECs treated with human amylin or with poloxamer 188 (P188) prior to incubation with human amylin, compared to control vehicle ($n = 3$ preparations/treatment). Scale bars, 20 μm (**a-d, f**). Data are mean $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***, $P \leq 0.0001$ ****; by one-way ANOVA with Tukey's *post-hoc* (**e, g, h, j**); two-tailed, unpaired *t* test (**i**).

Fig. 6: Amylin-induced suppression of A β transporter is rescued by antisense microRNAs.

a TargetScan schematic showing consensus regions for miR-205, miR200bc-3p/429, and miR-103 and miR-107. **b** Western blot and densitometry quantification of LRP1 from miRNA (miR) 103 and miR-107 treated ECs compared to miR-control ($n = 3$ preparations/group). **c** Western blot and densitometry quantification of LRP1 from antagomir (amiR) 103 and amiR-107 treated ECs compared to amiR-control treated cells ($n = 3$ preparations/group). **d** Schematic summary of the effect of amyloid-forming human amylin on the A β efflux across the BBB and the rescue mechanism. Rats expressing endogenous non-amyloidogenic rat amylin that have unimpaired A β efflux across the BBB (left panel). Human amylin-expressing rats have amylin amyloid deposition in the brain microvasculature and impaired A β efflux across the BBB (right panel). This was caused by miRNA-based translational repression of LRP1 (red pathway) and was reversed by antisense microRNA (green pathway). Data are mean $\pm$ SEM. $P \leq 0.05$ *; by two-tailed, unpaired *t* test (**b, c**).

Amylin - A β

Sporadic AD

Figure 1

Amylin - A β

Fig. 3

Fig. 5

Fig. 6

Supplemental figure legends

Fig S1. Immunochemical analyses of amylin in blood, brain and pancreatic tissues from transgenic and wild-type rats. **a** Levels of amylin by ELISA in plasma and lysates of red blood cells (RBCs) from HIP and WT rats ($n = 7/\text{group}$). Levels of amylin in the RBC lysates are normalized to the total protein input. **b-d** IHC analysis with anti-amylin antibody (brown) on brain sections from HIP (**b, c**) and WT (**d**) rats ($n = 5/\text{group}$). **e, f** Images of IHC staining of amylin in HIP rat pancreas (positive control for amylin deposition; **b**) and AKO rat pancreas (negative control for amylin immunoreactivity signal; **c**). Scale bars, 50 μm (**b-f**). Data are means $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.01$ **; by two-tailed, unpaired t test.

Fig S2. Immunochemical analyses of A β in brain tissues from transgenic and wild-type rats **a-c** Images of IHC staining with anti-A β antibody (brown) on brain sections from AD (**a**), HIP (**b**) and WT (**c**) rats ($n = 5$ rats/group). Scale bars, 50 μm (**a-c**). **d** Western blot analysis of A β in brain homogenates from HIP rats and WT littermates, using acidic 8M urea gel ($n = 4-5/\text{group}$).

Fig S3. Viability of endothelial cells under amylin-induced stress. Percent cell viability from the MTS assay in ECs treated with various concentrations of human amylin (500 nM, 1 μM , 5 μM , and 10 μM) or vehicle, for 24 hours. Data are mean $\pm$ SEM. $P > 0.05$; not significant by one-way ANOVA with Dunnett's *post-hoc*.

Fig. S4. Structural integrity test of the EC monolayer following amylin-induced stress. **a** Diagrammatic representation of the *in vitro* BBB model (ECs monolayer - luminal chamber;

astrocytes - abluminal chamber) used in testing the structural integrity of the EC monolayer. **b** At maximum *TEER*, EC monolayers were treated for 24 hours with vehicle, human amylin (10 μ M) or rat amylin (10 μ M) ($n = 5$ preparations/group); *TEER* was measured at different time points (3, 6, 12 and 24 hours). **c, d** Measurement of the paracellular transport of FITC-Dextran (4 kDa) (**c**) after the treatments described in (**b**) and permeability coefficients for FITC-Dextran (**d**) ($n = 5$ preparations/group). **e** Bright field micrographs of ECs after vehicle, human amylin or rat amylin treatments. Scale bars, 100 μ m (**e**). Data are mean $\pm$ SEM. $P \leq 0.0001$ ****; $P > 0.05$, not significant by one-way ANOVA (**d**) or two-way ANNOVA (**b, c**) with Tukey's *post-hoc*.

Fig. S5. Test of biochemical properties of amyloid in pancreatic tissue from transgenic rats

a Representative images of immunofluorescence staining of human amylin (red) and ThioS (green) in HIP rat pancreas (positive control for amylin amyloid); **b** Same as in (**a**) for AKO rats pancreas (negative control for amylin immunoreactivity signal) Scale bars, 50 μ m.

Fig. S1

Brain tissues

A β

Fig. S2

Fig. S3

Fig. S4

Fig. S5

	Sex	APOE	Age	Diabetes	Braak stage	CAA severity	AD	CERAD
1	M	4/4	93	Yes	5	2	Yes	3
2	M	3/4	91	Yes	5	0	Yes	3
3	F		92	Yes	5	3	Yes	3
4	M	3/3	95	No	5	1	Yes	2
5	F	3/4	82	No	5	0	Yes	3
6	F	4/4	86	No	5	2	Yes	3
7	F	3/3	91	No	2	3	No	0
8	F	3/3	83	No	2	0	No	0
9	M	3/3	80	No	1	0	No	0

Supplementary Table 1: Neuropathological information, including neuritic amyloid plaques (Consortium to Establish a Registry for Alzheimer’s Disease; CERAD), Braak NFT stage and CAA severity, along with *APOE* genotype, absence/presence of diabetes, age and sex of each individual included in the present study.

Figures

Figure 1

Figure 1

Amylin-A β interaction at the blood-brain interface in human AD brains. a, b Representative immunohistochemical (IHC) micrographs of brain sections from patients with sporadic AD (a) and cognitively unimpaired (CU) (b) individuals that were co-stained with anti-amylin (brown) and anti-A β (green) antibodies. c Analysis of the number of blood vessels with vascular mixed amylin-A β deposition (per mm²) in brains from AD (n = 6) and CU (n = 3) (2 slides/brain) groups assessed from IHC. d, e Representative images of confocal microscopy analysis of brain sections from the same AD individuals (n = 6; 2 slides/brain) as in above showing amylin (green) and A β (red) deposits in small-type vessels in which amylin is present on the luminal side (d) and within the vessel wall (e). e-g Representative immunofluorescence images of AD brain sections triple stained with anti-amylin (green), anti-collagen IV (red) and anti-smooth muscle actin (blue) (n = 3; 2 slides/brain). Scale bars, 50 μ m. Data are mean $\pm$ SEM. $P \leq 0.05$ *; by two-tailed, unpaired t test (c).

Figure 2

Figure 2

Pancreatic amylin accumulates in the brain microvasculature and impairs A β efflux from the brain. a-d Representative IHC micrographs of brain sections from HIP (a-c) and WT (d) rats co-stained with anti-amylin (brown) and anti-A β (green) antibodies. e Analysis of the number of blood vessels with vascular amylin-A β deposition (per mm²) in brains of WT and HIP rats (n = 5/group) (3 slides/brain), assessed from IHC. f Representative Western blot and densitometry quantification of A β in brain homogenates from HIP rats and WT littermates using acidic urea gel (n = 3/group) to resolve monomers. Rat A β 40 peptide and APP/PS1 rat brain homogenate were used as positive controls. A β densitometry was normalized to loading control actin. A β densitometry quantification was calculated from two experiments (n = 7-8/group). g Representative Western blot and densitometry analyses of aggregated A β in brain homogenates from HIP rats and WT littermates (n = 7/group) using native-PAGE. Scale bars, 50 μ m (a-d). Data are mean $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.01$ **; by two-tailed, unpaired t test (e-g)

Fig. 3

Figure 3

High blood amylin levels downregulate the A β efflux transporter at the BBB. a-b Immunoprecipitation and western blot analyses of A β in the plasma (a) and brain homogenates (b) from WT and HIP rats ($n = 5/\text{group}$). Age-matched APP/PS1 rat brain homogenate were used as a positive control. c Ratio of plasma A β -to-brain A β levels in HIP and WT rats assessed from Western blot analysis of A β enriched by immunoprecipitation from plasma (a) and brain (b) homogenates. d Confocal fluorescent micrographs of amylin (green) and endothelial cell marker caveolin-1 (red) in brain capillaries isolated from HIP rats and WT littermates. e Levels of amylin fluorescence intensity signals quantified from $n = 21$ and $n = 47$ isolated capillaries from WT rats and HIP littermates ($n = 3$ rats/group), respectively, and averaged for each rat. f Confocal immunofluorescent micrographs of LRP1 (green) and nuclei (blue) in isolated capillaries from WT rats and HIP littermates. g Levels of LRP1 fluorescence intensity signals quantified from WT rats ($n = 4$) and HIP littermates ($n = 4$) and averaged for each rat. h Representative western blot and densitometry quantification (i) of LRP1 in brain capillary lysates isolated from HIP rats and WT littermates ($n = 3$ rats/group). j LRP1 mRNA levels (fold difference using $2^{-\Delta\Delta\text{Ct}}$ method) in brain capillary lysates isolated from the same HIP and WT rats as in (h) ($n = 3$ rats/group). Scale bars, 10 μm (d, e). Data are mean $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.01$ ** by two-tailed, unpaired t test (c, e, g, i, j).

Fig. 4

Figure 4

In vitro test of amylin-induced impairment of Aβ efflux across the BBB. **a** Representative Western blot and densitometry quantification of LRP1 in lysates from primary rat brain microvascular endothelial cells (ECs) treated with vehicle or various concentrations of human amylin (500 nM, 1 μM, 5 μM, and 10 μM) for 24 hours (n = 3 preparations/test). **b** Immunofluorescent micrographs (LRP1-red; nuclei - blue) and quantification of LRP1 in ECs treated with vehicle, human amylin (10 μM) or rat amylin (10 μM) (n = 30 cells/group from 3 replicates per group). **c** Western blot and densitometry quantification of LRP1 in lysates from ECs treated with vehicle, human amylin or rat amylin as in (b) (n = 3 preparations/test). **d** LRP1 mRNA levels (fold difference using 2-ΔΔCt method) measured with qRT-PCR in lysates from ECs treated with vehicle, human amylin or rat amylin (same 3 preparations/test used in panels b and c). **e** Cartoon representation of the in vitro BBB model (ECs monolayer - luminal chamber; astrocytes - abluminal chamber) used in Aβ transcytosis experiments. **f** Transendothelial electrical resistance (TEER) in EC monolayers (n = 20 preparations) as a function of days in culture. **g** The Aβ42 transcytosis quotient (TQ) across the in vitro BBB, in vehicle- and human amylin-treated EC monolayers. Scale bars, 10 μm (b). Data are mean ± SEM. P ≤ 0.05 *, P ≤ 0.0001 ****; by one-way ANOVA with Dunnett's posthoc (a) or Tukey's post-hoc (b-d); two-tailed, unpaired t test (g).

Fig. 5

Figure 5

MiRNA upregulation and LRP1 downregulation by amylin amyloid-mediated stress in vascular endothelial cells. a Representative fluorescent images of Thioflavin S (Thio S, green) and amylin (red) staining in brain tissue sections from HIP rats (n = 3 rats) showing the presence of amylin amyloid in a brain capillary. b Staining for the lipid peroxidation marker 4-HNE (red) and amylin (green) same as in (a). c, d Representative fluorescent images and analysis (e) of lipid peroxidation levels in ECs incubated with human amylin (n = 53 cells) or with poloxamer 188 (P188) prior to incubation with human amylin (n = 161 cells), compared to controls (n = 141 cells). f Representative fluorescent images and analysis (g) of ROS levels in ECs incubated with human amylin (n = 103 cells) or with P188 prior to incubation with human amylin (n = 85 cells), compared to controls (n = 92 cells). h Expression of miR-103 and miR-107 measured by qRT-PCR (fold difference using $2^{-\Delta\Delta Ct}$ method) in ECs incubated with human amylin or with P188 prior to incubation with human amylin (n=3 preparations/treatment). i Expression levels of miRNA (miR)-103 and miR-107 measured by qRT-PCR (fold difference using $2^{-\Delta\Delta Ct}$ method) in lysates from HIP rat brain capillaries compared to those in WT rats (n = 4 rats/group). miR-U6 was used as internal control (h, i). j Representative Western blot and densitometry quantification of LRP1 from rat brain microvascular ECs treated with human amylin or with poloxamer 188 (P188) prior to incubation with human amylin, compared to control vehicle (n = 3 preparations/treatment). Scale bars, 20 μ m (a-d, f). Data are mean $\pm$ SEM. $P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***, $P \leq 0.0001$ ****; by one-way ANOVA with Tukey's post-hoc (e, g, h, j); two-tailed, unpaired t test (i).

Fig. 6

Figure 6

Amylin-induced suppression of A β transporter is rescued by antisense microRNAs. a TargetScan schematic showing consensus regions for miR-205, miR200bc-3p/429, and miR- 103 and miR-107. b Western blot and densitometry quantification of LRP1 from miRNA (miR) 103 and miR-107 treated ECs compared to miR-control (n = 3 preparations/group). c Western blot and densitometry quantification of LRP1 from antagomir (amiR) 103 and amiR-107 treated ECs compared to amiR-control treated cells (n = 3 preparations/group). d Schematic summary of the effect of amyloid-forming human amylin on the A β efflux across the BBB and the rescue mechanism. Rats expressing endogenous non-amyloidogenic rat amylin that have unimpaired A β efflux across the BBB (left panel). Human amylin-expressing rats have amylin amyloid deposition in the brain microvasculature and impaired A β efflux across the BBB (right panel). This was caused by miRNA-based translational repression of LRP1 (red pathway) and was reversed by antisense microRNA (green pathway). Data are mean $\pm$ SEM. $P \leq 0.05$ *, by twotailed, unpaired t test (b, c).