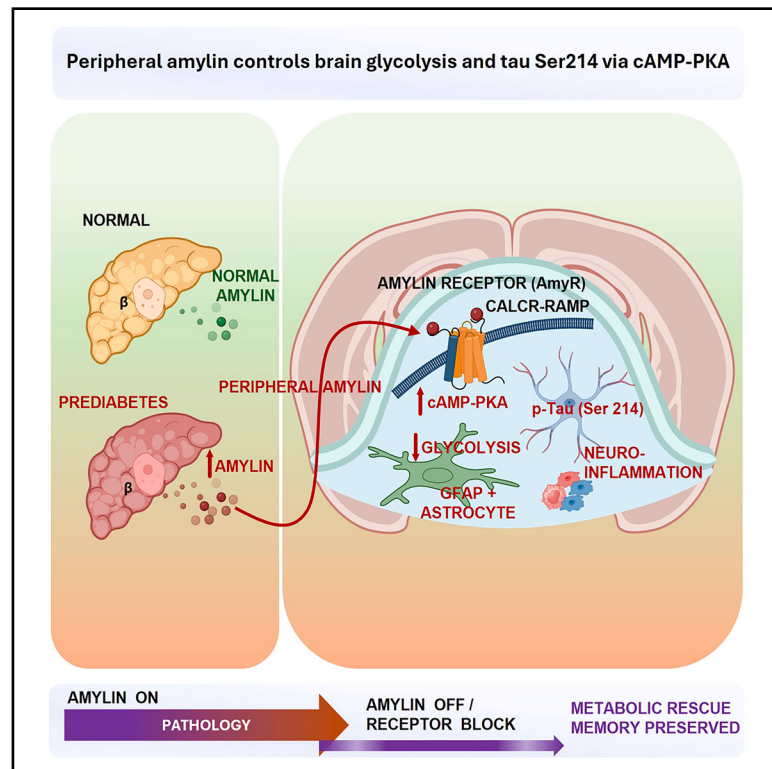


Peripheral amylin modulation rebalances brain glycolysis and Tau-Ser214 phosphorylation via cAMP-PKA signaling

Graphical abstract



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In brief

Neuroscience; behavioral neuroscience; endocrinology

Highlights

- Prediabetic hyperamylinemia overactivates cAMP-PKA signaling
- Amylin-cAMP-PKA signaling blocks glycolysis and promotes pTau and pTau-A β coupling
- Glycolytic deficits suppresses glucose transport driving maladaptive brain remodeling
- Inhibiting excess amylin protects against metabolic dysfunction and AD-like pathology



Article

Peripheral amylin modulation rebalances brain glycolysis and Tau-Ser214 phosphorylation via cAMP-PKA signaling

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SUMMARY

Brain glucose dysregulation is shared by Alzheimer's disease (AD) and diabetes, but whether it arises from central or peripheral mechanisms remains unclear. Amylin, a pancreatic hormone, normally supports CNS cAMP-PKA signaling, metabolism and memory; however, prediabetes-associated hypersecretion disrupts this balance. Using human amylin-inducible mice, we show that toggling amylin secretion during metabolic stress bidirectionally regulates brain glycolysis and function. Excess amylin overactivates cAMP-PKA signaling, suppressing glycolysis and inducing Tau-Ser214 phosphorylation, two core features of AD pathology. This state is accompanied by activation of the amino acid starvation response, Tau-T231 hyperphosphorylation, pTau-A β coupling, neuroinflammation and memory deficit. In contrast, reducing amylin in prediabetes preserves glycolysis, ATF4-dependent proteostasis and cognition. Astrocytes emerge as primary targets, as amylin receptor blockade prevents glycolytic deficits *ex vivo*, and amylin accumulates in GFAP-enriched regions *in vivo*. Together, these results define prediabetic hyperamylinemia as an upstream, modifiable driver of PKA-mediated tau pathology linking metabolic dysfunction to AD.

INTRODUCTION

Type-2 diabetes is a known risk factor for cognitive decline,¹ with earlier onset linked to an increased likelihood of developing dementia later in life.² In prodromal Alzheimer's disease (AD), marked reductions in neuronal glucose uptake and glycolytic enzyme activity are detectable prior to accumulation of hyperphosphorylated tau (pTau) and β -amyloid (A β).^{3–10} Impaired glycolytic flux contributes to endoplasmic reticulum stress, disrupted proteostasis and ultimately neurodegeneration.¹¹ As a result, restoring brain glucose homeostasis emerges as a promising therapeutic strategy for AD.

Prediabetes is associated with hypersecretion of the two β cell main hormones, insulin and amylin.^{12,13} Amylin normally signals through class B G protein-coupled receptors composed of calcitonin receptor (CalcR) and receptor activity-modifying proteins

(RAMP1–3) heterodimers.^{14–18} These receptors activate adenylyl cyclase, leading to cyclic AMP (cAMP) production.¹⁷ Transient cAMP elevation and subsequent PKA activation support a plethora of biological events, including metabolism, differentiation, proliferation, and memory.^{19–24} However, prediabetes-driven amylin hypersecretion may disrupt this balance. PKA phosphorylates glycolytic enzymes,^{25–29} reducing their activity, while also primes tau for hyperphosphorylation.³⁰ Thus, chronic activation of the amylin-cAMP-PKA pathway may simultaneously suppress glycolysis and promote tau phosphorylation, two key features of AD pathology.^{3,7}

Individuals with AD and comorbid diabetes or prediabetes commonly have elevated amylin levels in brain parenchyma, cerebrospinal fluid, and blood^{1,31–40}; yet, the functional consequences of excess brain amylin remain poorly understood.



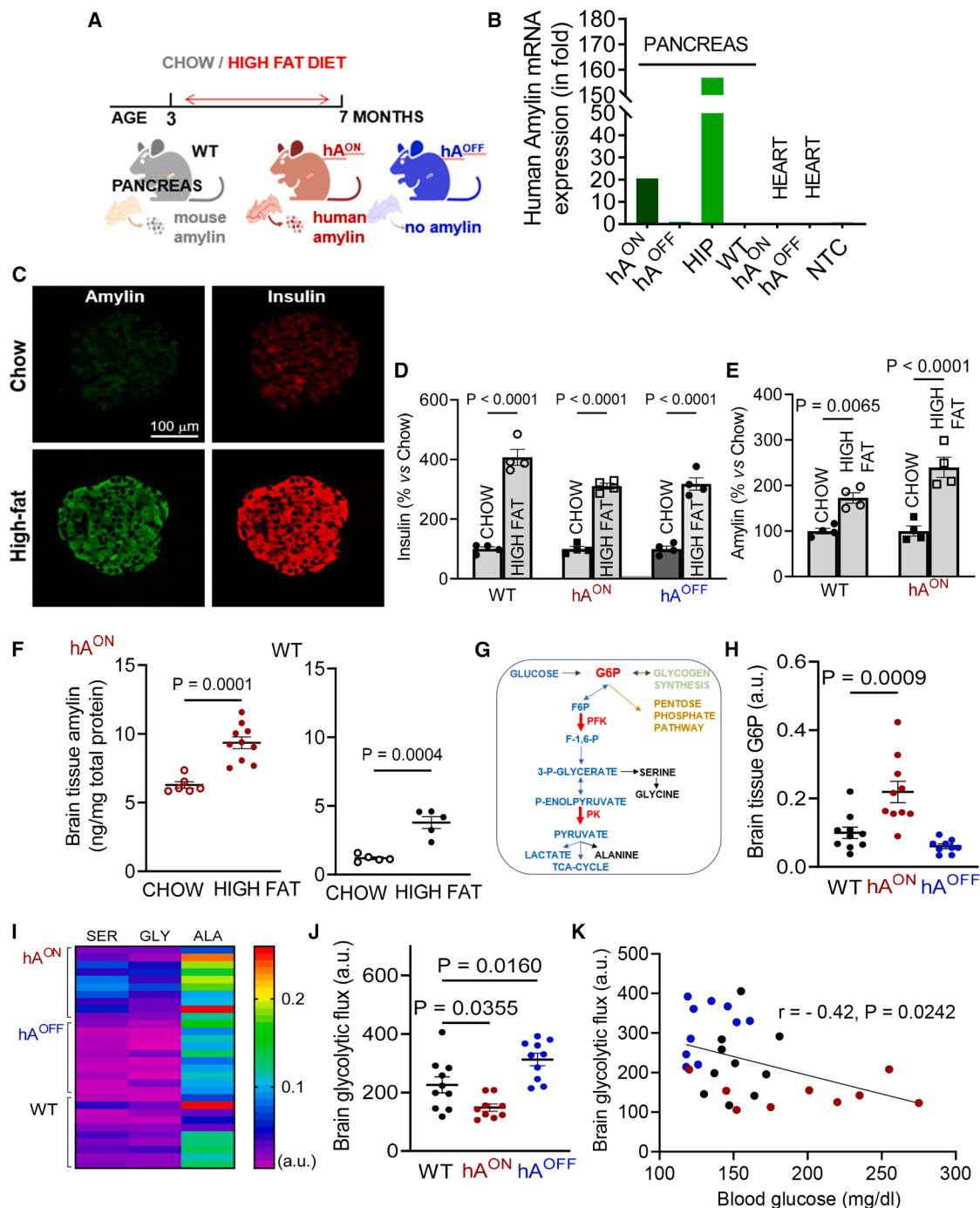


Figure 1. Impact of amylin deficiency and human vs. mouse amylin secretion on brain glucose regulation during prediabetes-like stress
(A) Timeline of diet-induced metabolic stress for comparative analyses in mice expressing mouse amylin (wild-type; WT mice), human amylin (hA^{ON} mice) and no amylin (hA^{OFF} mice). Mice were switched to a high fat diet at 3 months of age or maintained on regular chow and investigated at 7 months of age.
(B–E) Validation of pancreatic β -cell-specific expression of the human amylin transgene and confirmation of endogenous amylin gene deletion. (B) Amylin mRNA expression levels in pancreatic tissues from hA^{ON} , hA^{OFF} , and WT mice, heart tissue from hA^{ON} and hA^{OFF} mice and pancreatic tissue from HIP rats overexpressing human amylin (positive control). NTC stands for no template control. (C) Representative confocal microscopy images of immunostaining pancreatic islets for amylin and insulin in hA^{ON} mice on chow vs. high-fat diets. (D and E) Four months of high-fat feeding induces pancreatic hypersecretion of amylin and insulin as indicated by immunofluorescence signal intensities of insulin (D) and amylin (E) staining in islets from hA^{ON} , hA^{OFF} , and WT mice on chow vs. high-fat diets.
(F) Brain tissue amylin levels in high-fat-fed hA^{ON} and WT male mice vs. littermates on chow diet.

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Here, we used human amylin-inducible mice to mechanistically investigate how prediabetes-driven excess amylin influences peripheral-CNS signaling. The inducible system provides control over prediabetes related excess amylin, enabling delineation between signaling-driven and aggregation-driven effects, as human amylin is amyloidogenic.¹² The results of this study may help determine whether targeting excess circulating amylin during brain metabolic failure can mitigate neurodegeneration while reducing the risks associated with direct CNS intervention.

RESULTS

Prediabetes-driven excess amylin disrupts cerebral glycolytic flux and impairs memory

Using human amylin knock-in mice (hA^{ON}), amylin knockout (AKO) mice (hA^{OFF}) and wild-type (WT) mice expressing endogenous mouse amylin (controls), we investigated how the presence or absence of amylin, and the secretion of human versus mouse amylin affects cerebral glucose homeostasis under prediabetes-like metabolic stress. Schematic experimental protocol and RT-qPCR validation of amylin transgene specificity are shown in Figures 1A and 1B. Males and females were subjected to four months of high-fat diet to induce prediabetic hyperglycemia, while genotype-matched controls remained on regular chow. Data show that high-fat diet induced compensatory hypersecretion of amylin (along with insulin), compared to chow-fed littermates (Figures 1C–1E), and that amylin ON or OFF modulated energy homeostasis in a sex-dependent manner (Figure S1). Male hA^{ON} mice on a high-fat diet gained more weight, developed hyperglycemia and showed behavioral deficits compared with WT and hA^{OFF} controls (Figures S1A–S1K), whereas female mice exhibited delayed memory deficits and metabolic changes (Figures S1L–S1P), consistent with known sex-specific differences, as reported by Ly et al.³⁵

Prediabetes-driven excess amylin promoted brain amylin accumulation in both “humanized amylin” mice and in WT mice (Figure 1F). Using a targeted metabolomics approach, we quantified glucose-6-phosphate (G6P) (the first glycolysis intermediate) along with three glycolysis amino acids: serine, glycine, and alanine (schematic Figure 1G). Glycolytic flux was estimated by calculating the ratio of (serine + glycine + alanine) to G6P (Figures 1H–1J), as previously described by An et al.⁵ These amino acids are derived from glycolytic intermediates; therefore, their concentrations relative to intracellular glucose (i.e., G6P) serve as a proxy for the overall enzymatic activity driving glycolytic flux.^{25–29} This ratio was significantly reduced in WT mice compared to hA^{OFF} mice and further decreased in hA^{ON} mice. Cerebral glycolytic flux inversely correlated with blood glucose

(Figure 1K), linking prediabetes-driven excess amylin to cerebral glycolysis deficits.

To investigate the underlying mechanism, we generated two inducible mouse models expressing human amylin in pancreatic β cells via Cre-Lox recombination. In one inducible mouse model, amylin is expressed from birth and can be suppressed with tamoxifen (hA^{i-OFF}) (Figures 2A–2C), whereas in the other, amylin is absent at birth and can be induced with tamoxifen (hA^{i-ON}) (Figures 2D–2F). Three-month-old male mice were fed a high-fat diet for two months to induce metabolic stress comparable to prediabetic hyperglycemia. Then, mice received tamoxifen or vehicle to switch amylin expression ON or OFF, continuing the diet until seven months (schematic Figures 2C and 2F). Suppressing amylin (hA^{i-OFF}) reduced plasma and brain amylin levels and improved recognition memory (Figures 2G–2I), whereas inducing amylin (hA^{i-ON}) increased plasma and brain amylin levels and impaired memory (Figures 2J–2L). Knockdown of amylin expression does not enable complete amylin clearance from brain in hA^{i-OFF} mice, whereas brain amylin accumulation following activation of amylin expression in hA^{i-ON} mice is lower than in hA^{i-OFF} mice (Figures 2H and 2K).

During diet-induced metabolic stress, amylin knockdown (hA^{i-OFF}) prevented progression of metabolic disturbances, compared with hA^{ON} littermates (Figures 2M–2O). Mice lacking amylin (hA^{OFF}) resisted diet-induced obesity (Figures S1G and S1H), mimicking the PKA-deficient lean phenotype,^{22,23} while amylin induction (hA^{i-ON}) reversed this resistance, driving glucose dysregulation, weight gain, and variable hyperinsulinemia (Figures 2P–2R). High plasma amylin levels correlated with increased brain amylin concentrations, which in turn negatively correlated with the recognition index (Figures 2S and 2T), indicating functional consequences of brain amylin accumulation.

Excess amylin impairs glycolysis by overactivating cAMP-PKA signaling

Brain amylin levels and downstream signaling were assessed in tissue encompassing multiple regions with high expression of amylin receptors (CalcR-RAMPs complexes), including cortex, hippocampus, hypothalamus, and thalamus, as identified by single-cell profiling of receptor subunits (Figure S2). Single-cell RNA sequencing showed that receptor expression is highest in the hypothalamus (Figures S2F and S2G) but broadly detectable throughout the brain (Figure S2B), consistent with the comparative analysis by Christopoulos et al.⁴¹ in rat and monkey brains. Across inducible amylin mice, high brain amylin levels correlated with increased brain cAMP concentrations (Figure 3A) and robust PKA activation (Figure 3B). Mice were further stratified into normoglycemic (126.1 ± 3.15 mg/dL) and high blood glucose (154.4 ± 5.39 mg/dL) subgroups. Within

(G) Schematic describing the first intermediate of glucose metabolism (glucose-6-phosphate; G6P), metabolic pathways, glycolytic amino acids, and glycolytic kinases facilitating G6P use by cells.

(H–J) Comparative analyses of brain tissue G6P levels (H), glycolytic amino acids serine (Ser), glycine (Gly), and alanine (Ala) (I) and cerebral glycolytic flux (J) in hA^{ON} , hA^{OFF} , and WT male mice.

(K) Pairwise correlation between cerebral glycolytic flux and blood glucose level in all mice investigated. Data points in (D and E) represent the mean fluorescence intensity in islets from the same mouse, $n = 5–10$ islets/mouse. Data are shown as individual values and mean $\pm$ s.e.m. Statistical analyses were performed using two-tail t test (D–F) and one-way ANOVA followed by Tukey’s multiple-comparisons test (H and J). Schematic A was created using BioRender. See also Figure S1.

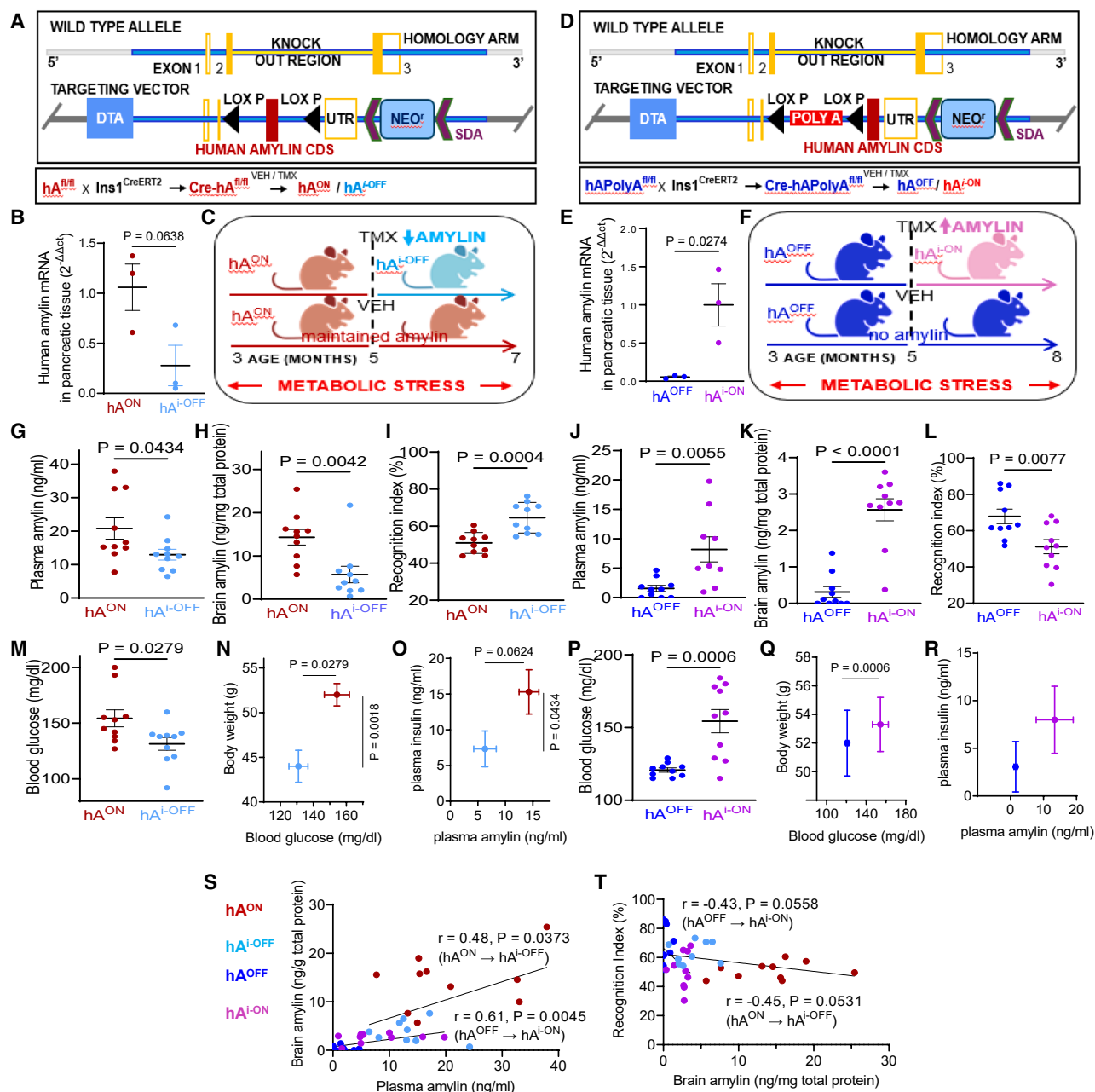


Figure 2. Human amylin-inducible mice to define the peripheral-CNS amylin axis in cerebral glucose regulation during prediabetes-like stress

(A) Construct for generating knock-in mice with human amylin flanked by *loxP* sites ($hA^{fl/fl}$). Crossing $hA^{fl/fl}$ with *Ins1*^{Cre-ERT2} mice produced *Cre-hA^{fl/fl}* (hA^{ON}) mice with conditional β -cell amylin expression. Tamoxifen suppressed amylin in hA^{ON} mice (hA^{i-OFF}).

(B) Pancreatic human amylin mRNA in hA^{ON} vs. hA^{i-OFF} .

(C) Timeline of tamoxifen injection in hA^{ON} mice ($\rightarrow hA^{i-OFF}$). VEH = vehicle.

(D) Construct for generating amylin knockout mice with inducible human amylin. Mouse amylin was replaced with a “*loxP*-PolyA-*loxP* human amylin CDS” cassette ($hAPolyA^{fl/fl}$). Crossing with *Ins1*^{Cre-ERT2} generated *Cre-hAPolyA^{fl/fl}* (hA^{OFF}) mice lacking both mouse and human amylin. Tamoxifen induced β cell amylin expression (hA^{i-ON}).

(E) Pancreatic human amylin mRNA in hA^{OFF} vs. hA^{i-ON} .

(F) Timeline of tamoxifen injection in hA^{OFF} mice ($\rightarrow hA^{i-ON}$).

(G–I) Endpoint plasma amylin, brain amylin, and recognition index in hA^{i-OFF} vs. hA^{ON} .

(J–L) Endpoint plasma amylin, brain amylin, index in hA^{i-ON} vs. hA^{OFF} .

(M–O) Endpoint blood glucose, body weight, and plasma insulin in hA^{i-OFF} vs. hA^{ON} .

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each, higher brain amylin levels were consistently associated with increased cAMP (Figure 3C) and PKA activation (Figure 3D). In cultured neonatal astrocytes, amylin exposure (15 μ M, 30 min) increased cAMP and PKA activity (Figures 3E and 3F). These effects were blunted by the amylin receptor antagonist AC187 (15 μ M) demonstrating that amylin activates astrocytic amylin receptors to trigger cAMP-PKA signaling (schematic, Figure 3G).

PKA phosphorylates glycolytic kinases (hexokinase, phosphofructokinase and pyruvate kinase),^{25–29} generally reducing their activity. Since amylin triggers cAMP-PKA signaling, we examined how the brain amylin level affects cerebral glycolytic flux under metabolic stress. Elevated brain amylin was consistently linked to higher G6P levels in both normoglycemic and hyperglycemic subgroups (Figure 4A), while glycolytic flux was inversely correlated with brain amylin levels (Figure 4B). To examine how amylin signaling affects glucose utilization, we cultured neonatal astrocytes, the primary brain cells that metabolize blood-derived glucose, with and without human amylin. Seahorse assays showed that amylin significantly reduced glycolysis and glycolytic capacity compared with controls (Figures 4C–4E). To further test receptor involvement, we silenced the CalcR subunit of the amylin receptor in astrocytes using siRNA (protocol in Figure 4F), with downregulation confirmed by western blot (Figure 4G). In CalcR-deficient astrocytes, amylin no longer affected ECAR, glycolysis or glycolytic capacity (Figures 4H–4J), demonstrating that amylin controls astrocytic glycolysis through receptor-mediated cAMP-PKA signaling.

To verify amylin receptor expression in astrocytes *in vivo*, we performed RNAscope *in situ* hybridization (ISH) on cortical sections from hA^{ON} mice. This analysis revealed specific signals for CalcR and RAMP1 within GFAP-positive astrocytes (Figure 5A), confirming receptor presence in these glial cells. These results align with recent single-cell RNA sequencing (scRNA-seq) atlases, which report similar expression patterns of CalcR and RAMP1 in astrocytes across various brain regions⁴² (Figure S2). To further contextualize receptor distribution, Figure 5B shows a uniform manifold approximation and projection (UMAP) plot of hypothalamic CalcR expression derived from adult mouse scRNA-seq data,⁴² highlighting the cell-type-specific localization of amylin receptors. Immunohistochemistry (IHC) analysis of cortical sections further revealed amylin-positive cells within GFAP-rich regions in hA^{ON} , hA^{i-OFF} and hA^{i-ON} mice, whereas hA^{OFF} brains (negative amylin controls) lacked amylin staining in these areas (Figure S3). Amylin knockdown in hA^{i-OFF} mice reduced amylin immunoreactivity signal intensity in GFAP-rich regions (Figure 5C), compared to hA^{ON} mice, in which amylin was continually expressed. Together, these findings (Figures 4 and 5) indicate that astrocytes are direct targets of amylin signaling that reduces glycolytic flux.

RNA sequencing (RNA-Seq) analysis of brain tissues from hA^{OFF} vs. hA^{ON} mice further revealed that the CalcR component of the amylin receptor is a top differentially expressed gene, with a ~ 7 -fold higher expression in hA^{OFF} brains (Figure 5D). A similarly large compensatory increase in CalcR expression was detected in hA^{i-OFF} vs. hA^{ON} brains (Figure 5E). RT-qPCR confirmed a marked increase of brain CalcR mRNA levels in hA^{OFF} compared with hA^{ON} mice (Figure 5F), with a similar trend ($p = 0.0875$) for RAMP1 (Figure 5G), while RAMP2 and RAMP3 expression remained unchanged across the inducible amylin mouse lines (Figures 5H and 5I). Even after tamoxifen-induced amylin knockdown, the expression of amylin receptors remained low in hA^{i-OFF} mouse brains (Figures 5F–5I), suggesting a persistent “molecular memory” of chronic amylin signaling. Similarly, inducing amylin does not significantly downregulate amylin receptor components CalcR or RAMPs (hA^{OFF} vs. hA^{i-ON} mice) during the amylin exposure timeline (Figures 5F–5J).

Restricting amylin in prediabetes rebalances cAMP-PKA signaling and Atf4-regulated proteostasis

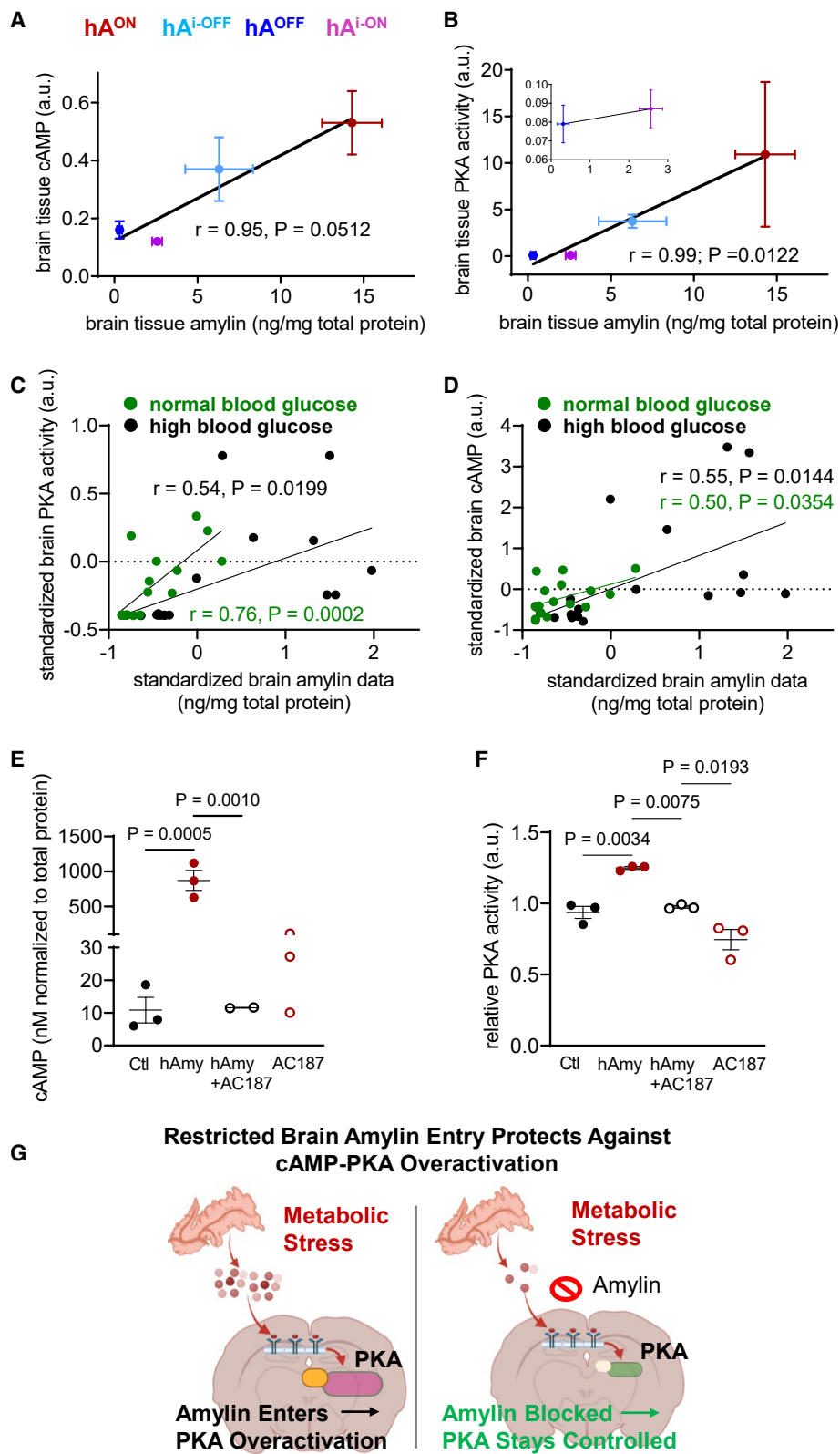
Differentially expressed brain gene transcripts ($p < 0.05$) in hA^{i-OFF} vs. hA^{ON} mice (Figure 5D) were analyzed using the Ingenuity Pathway Analysis (IPA) database, identified in canonical pathways (Figure 6A) and further categorized based on gene ontology into biological processes (Figure 6B). Differentially expressed genes include genes involved in mTOR signaling, metabolism, redox processes and the amino acid starvation response. A central node shared by these pathways is the cAMP-dependent transcription factor 4 (Atf4), which is necessary for both canonical regulation of nutrient-sensing response and protein synthesis (proteostasis).⁴³ Brain Atf4 levels were increased in metabolically stressed mice with low brain amylin levels (Figure 6C). The aspartate-to-asparagine ratio, which reflects the activity of asparagine synthetase (Asns), was increased in brain tissues with elevated Atf4 levels (Figure 6D), consistent with the Atf4-regulated amino acid starvation response.⁴³ Moreover, the Atf4 level was negatively associated with the brain tissue amylin levels in mice with matched blood glucose levels (Figure 6E).

Because insulin secretion parallels that of amylin,¹³ we evaluated whether plasma insulin changes contributed to these effects. Statistical modeling showed that molecular outcomes (cAMP and ATF4) correlated more strongly with amylin than with insulin levels (Figures 6F–6I).

In mice with similar blood glucose levels (i.e., normal vs. high glucose), GLUT1 and GLUT3 glucose transporters were significantly higher in brain tissues from hA^{OFF} and hA^{i-ON} mice, compared to hA^{ON} and hA^{i-OFF} mice (Figures 6J and 6K), indicating an inverse relationship with brain amylin levels. Thus, lowering amylin levels under metabolic stress may positively influence cerebral glucose utilization.

(P–R) Endpoint blood glucose, body weight, and plasma insulin in hA^{i-ON} vs. hA^{OFF} .

(S and T) In the same mice, correlation analyses in plasma amylin vs. brain amylin (S) and brain amylin vs. recognition index. The diagrams (C and F) were created using BioRender. An outlier was excluded from data shown in (H). Results are shown as individual data points and mean $\pm$ s.e.m (B, E, and G–R). Statistical analyses were performed using two-tail *t* test. See also Figure S2.



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Peripheral hyperamylinemia drives tau pathology

Phosphorylation of tau at threonine 231 (pT231-tau) shows a strong association with brain accumulation of β amyloid ($A\beta$) and early neurodegeneration, as reported by Paspalas et al.⁴⁴ Using brain tissues from the same mice described in above, we measured pT231-tau, total tau, $A\beta_{40}$ and $A\beta_{42}$ species by meso scale discovery immunoassay platform. Turning circulating amylin ON or OFF influences brain tissue protein levels of pT231-tau, total tau, $A\beta_{40}$ and $A\beta_{42}$ (Figures 7A–7D). Amylin knockdown (hA^{i-OFF}) mice were resistant to metabolically induced AD-like pathology, compared to hA^{ON} mice with continued to secrete amylin (Figures 7A and 7C). Conversely, inducing amylin expression (hA^{i-ON}) in hA^{OFF} mice reversed this resistance, triggering tau phosphorylation and $A\beta$ generation (Figures 7B and 7D).

Incubation with human amylin (1 μ M for 4 h) elevated both the $A\beta$ immunofluorescence signal (Figure 7E) and the level of secreted $A\beta_{42}$ in the culture media (Figure 7F). The pTau/total Tau ratio increased in neuronal lysates (Figure 7G). In cultured neonatal astrocytes, amylin exposure (15 μ M, 30 min) increased tau phosphorylation (Figure 7H), while this effect was blunted by the amylin receptor antagonist AC187 (10 μ M) or the PKA inhibitor H-89 (20 μ M).

IHC analysis of cortical sections from hA^{ON} mice revealed sparse amylin deposits and no $A\beta$ deposits (Figures 7I and S4). These findings suggest that more prolonged brain exposure to pancreatic human amylin may be required to induce cerebral $A\beta$ plaque formation, similar to what has been observed in the HIP rat model of pancreatic human amylin overexpression.⁴⁵ The HIP rats secrete pancreatic human amylin in a similar range as in persons with dementia, as reported by Verma et al.³⁷ without any dietary manipulation. Next, we used the HIP rat model to test whether chronically elevated blood amylin levels independently trigger cerebral glycolysis deficits and tau pathology. HIP rats, WT littermates and AKO rats were maintained on standard chow and analyzed at 16 months of age (Figure 8A). Amylin hypersecretion in HIP rats induced hyperglycemia and brain amylin accumulation (Figures 8B and 8C), accompanied by impaired cerebral glycolysis and increased AD biomarkers (Figures 8D–8J). Elevated pTau further led to tau deposition and intracellular tangle formation (Figures 8K and 8L). Thus, chronically elevated human amylin alone can induce brain glycolytic deficits and AD-like pathology without dietary intervention, indicating amylin stress as a contributing factor to mixed amylin-AD pathology observed in humans.^{1,31–40}

Restricting amylin in prediabetes rebalances PKA-dependent tau Ser214 phosphorylation

PKA phosphorylates tau at serine 214 (pS214-tau) and is required for increasing pT231-tau, as shown by Liu et al.⁴⁶ Inhibiting PKA prevents this cascade from occurring (Figure 7F). The regression analysis of brain pT231-tau versus amylin levels, adjusted for

PKA activity, reads $Y = 6.4 + 1.37 (\text{amylin}) + 86 (\text{PKA activity})$ and predicts that for a given brain PKA activity level, each ng/mg decrease of brain amylin level is associated with an average decrease in brain tissue pT231-tau of 1.37 a.u./mg total protein. We examined this relationship by comparing pS214-tau and amylin levels in brain tissues from hA^{ON} mice (high PKA activity) and hA^{OFF} mice (low PKA activity). Confocal microscopy of cortical sections from hA^{ON} mice revealed that MAP2-positive neurons exhibiting amylin staining also contained phosphorylated tau (Figure 9A). The absence of a pTau response in hA^{OFF} mice (Figure 9B), which lack amylin, further provides direct evidence of an amylin-induced pTau response.

Next, we quantified pS214-tau by ELISA in brain tissues from the same mice described in above. The data show that circulating amylin ON vs. OFF status alters the relationship between brain tissue pS214-tau and amylin levels (Figure 9C). Moreover, the association between brain tissue pS214-tau and PKA activity (Figure 9D) closely reflects the amylin-cAMP-PKA pattern (Figures 3A and 3B). Switching OFF or ON amylin expression influences the relationship between pT231-tau and $A\beta_{42}$ (Figures 9E and 9F).

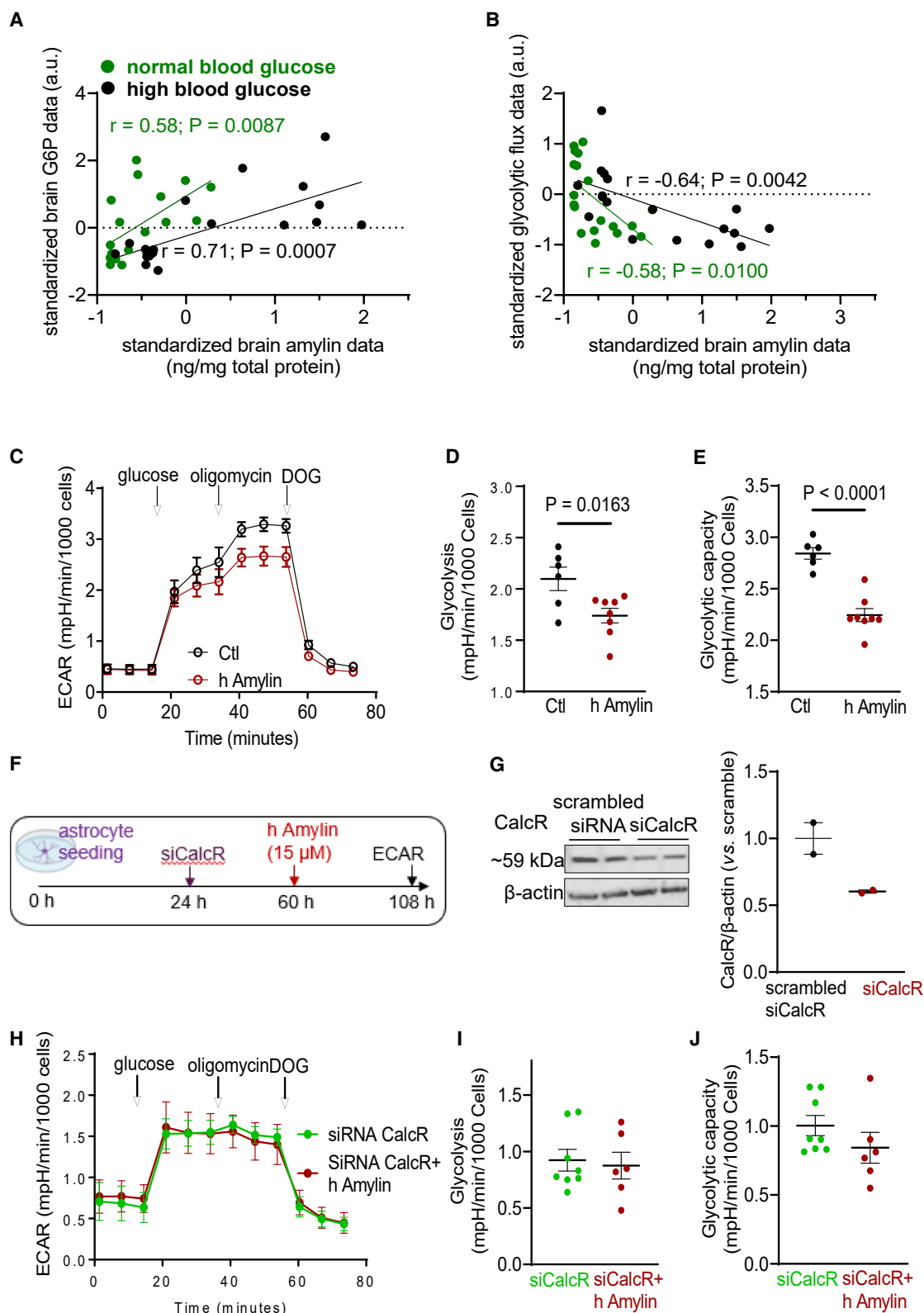
Furthermore, restricting brain amylin accumulation during diet-induced metabolic stress resulted in lower brain tissue levels of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α , IFN- γ , and CXCL1) in hA^{i-OFF} mice compared to hA^{ON} mice with continued amylin expression (Figure 9G). In the reciprocal experiment, turning circulating amylin ON during metabolic stress induced neuroinflammatory signaling in hA^{i-ON} mice versus hA^{OFF} mice (Figure 9H).

Principal component (PC) analysis of composite variables, including amylin; metabolic covariates (blood glucose, G6P, cAMP, and ATF4); AD covariates (tau, pTau, $A\beta_{40}$, and $A\beta_{42}$); and neuroinflammatory markers (IL-1 β , IL-6, TNF- α , IFN- γ , and CXCL1), revealed strong clustering based on the OFF/ON status of pancreatic amylin secretion (Figure 9I). The loading plot (Figure 9J) indicates that this clustering is primarily driven by contributions of amylin to the first principal component (PC1).

DISCUSSION

Together, our findings indicate that modulation of amylin secretion during metabolic stress is sufficient to influence brain metabolic signaling and cognitive outcomes. As summarized in Figure 9K, prediabetes-associated amylin hypersecretion enhances brain amylin-cAMP-PKA signaling, disrupts cerebral glycolysis and accelerates tau pathology, tau- $A\beta$ coupling and neuroinflammation, culminating in memory impairment. Conversely, limiting brain amylin accumulation preserves metabolic homeostasis, restrains AD-related pathogenic pathways, and sustains cognitive performance despite continued metabolic stress. Notably, transgenic

Figure 3. Amylin-dependent cAMP-PKA regulation *in vivo* under prediabetic stress and in neonatal astrocytes following receptor blockade (A–D) In the same mice as in Figure 2, average brain amylin level vs. average cAMP (A) and PKA activity (B), with correlation analyses in glucose-matched subgroups (normal, 126.1 ± 3.15 mg/dL; high, 154.4 ± 5.39 mg/dL) (C and D). (E, F) Effect of amylin on cAMP (E) and PKA activity (F) in cultured murine astrocytes. Cells were incubated for 30 min under control conditions or in the presence of human amylin (15 μ M) with and without the amylin receptor antagonist AC187 (10 μ M). (G) Schematic illustrates that elevated brain amylin entry drives PKA overactivation under metabolic stress, while restricted entry maintains controlled signaling. The diagram (G) was created using BioRender.



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rats with chronically elevated human amylin develop brain glycolytic deficits and AD-like pathology even in the absence of dietary challenge, supporting a causal role for excess amylin independent of diet. Collectively, these observations indicate amylin dysregulation as a key upstream contributor to brain glucose impairment and AD-related metabolic pathology.

Multiple reports from cancer and muscle cells demonstrated that amylin increases G6P, reduces insulin-stimulated glucose uptake and inhibits glycolysis in a dose-dependent manner.^{47–52} Here, we showed that blocking or silencing amylin receptors in cultured astrocytes prevents the glycolytic impairment triggered by amylin exposure. Consistent with this *in vitro* effect, IHC analyses revealed amylin-positive cells within GFAP-rich regions in amylin-inducible mice, whereas amylin-knockout controls showed no such staining. These convergent findings indicate that astrocytes are direct targets of amylin signaling and support a model in which excess amylin suppresses astrocytic glycolytic flux. This mechanism indicates the peripheral-CNS amylin axis as a potential therapeutic target for restoring brain energy metabolism. In addition, amylin receptors, similar to other GPCRs, have the capacity to modulate the A β cascade through effects on α -, β -, and γ -secretases (reviewed in Thathiah and De Strooper⁵³), suggesting broader implications for AD-related pathological pathways. Yet, despite single-cell RNA-seq evidence for cell type-specific expression of amylin receptor components (CalcR and RAMP1-3), the region-specific consequences of amylin signaling for brain glucose metabolism and AD pathology remain to be defined.

PKA and pS214-tau are mechanistically linked and promote downstream tau hyperphosphorylation.^{30,46,54} In the brains of human amylin-expressing rats and in dementia patients with type-2 diabetes, we previously observed chronic CaMKII activation,⁵⁵ another contributor to tau pathology. Here we showed that regulating pancreatic amylin secretion inhibits amylin-cAMP-PKA signaling, preventing tau Ser214 phosphorylation and tau-A β coupling. These findings have important implications for anti-tau therapeutics. Most tau-directed approaches aim to block aggregation, inhibit spread, or target specific kinases,⁵⁴ yet they do not address the upstream factors that initiate and sustain tau phosphorylation. A key challenge in AD is that tau pathology is sustained by multiple signaling inputs (PKA, CaMKII, GSK3 β , and stress kinases). Our findings show that peripheral amylin is a major upstream node feeding into this network. Such a peripheral-to-CNS drive may counteract the benefits of anti-tau agents, particularly in individuals with prediabetes or type 2 diabetes. By attenuating this amylin-dependent signaling, interventions targeting the peripheral-CNS amylin axis may reduce the phosphorylation burden on tau and

potentially enhance the efficacy of emerging tau-directed therapies.

Under physiological conditions, amylin secretion rises several-fold after meals and then rapidly returns to baseline (within ~ 45 min⁵⁶). This transient activation of the peripheral-CNS amylin axis, acting through circumventricular organs such as the area postrema, is thought to contribute to short-term regulation of feeding behavior.^{15–17,19} In contrast, our findings show that prediabetes-driven amylin hypersecretion leads to chronic overactivation of the amylin-cAMP-PKA pathway and sustained suppression of cerebral glycolysis. Such inhibition is likely to impair the astrocyte-neuron lactate shuttle, a key metabolic support system, thereby promoting neurodegeneration.¹¹ Notably, CNS amylin receptors and glucose transporters (GLUT1 and GLUT3) remained downregulated in mice with elevated brain amylin even after normoglycemia was restored, indicating that prolonged amylin signaling leaves a persistent “molecular memory” reflected in long-lasting alterations in gene expression. In human amylin-inducible mice, this effect is mediated by the cAMP-dependent Atf4 regulation of nutrient-sensing response and proteostasis. Thus, lowering amylin levels under metabolic stress may help restore proteostatic and metabolic homeostasis.

Mice lacking amylin (hA^{OFF}) on chow diet maintained normal body weight and glucose levels, consistent with previously reported data by Gebre-Medhin et al.⁵⁷ Similarly, AKO rats displayed normal brain glucose homeostasis as well as normal tau and A β processing. Together, these results indicate that amylin does not impair metabolic or AD-related pathways under basal conditions, and that its detrimental effects emerge primarily when present in excess, as occurs during prediabetic metabolic stress. Under diet-induced metabolic stress, however, hA^{OFF} mice had lower body weight and reduced blood glucose, mimicking the PKA-deficient lean phenotype.^{22,23} Notably, hA^{OFF} mice showed enhanced cerebral glucose utilization and resistance to AD-like pathology, compared with amylin-expressing mice. Amylin induction (hA^{i-ON}) in hA^{OFF} mice reversed this resistance, driving glucose dysregulation, weight gain and AD-like pathology. These observations suggest that amylin is a key mediator linking metabolic stress to AD-related pathways. Further studies are warranted to clarify how altered energy expenditure contributes to the vulnerability of these pathways under metabolic stress.

Previous studies in humans showed that brain amylin accumulation contributes to AD pathology.^{1,31–40} Consistently, pancreatic specific overexpression of amyloid-forming human amylin transgene in mice and rats induces diabetes-like pathology,^{45,58,59} accelerates AD-like pathology^{35–37} and memory impairment.^{35,36,60,61} Conversely, AKO³⁵ or amylin receptor silencing⁶²

Figure 4. Glycolytic outcomes in neonatal astrocytes following amylin receptor downregulation

(A and B) Correlations between brain amylin and G6P (A) or glycolytic flux (B) in the same glucose-matched subgroups as in Figure 2. (C–E) Extracellular acidification rate (ECAR) (C), glycolysis rate (D), and glycolytic capacity (E) measured by Seahorse analyzer in rat astrocytes cultured for 48 h under control conditions or in the presence of human amylin (15 μ M). Individual data points and mean $\pm$ s.e.m for 6–8 wells/group. (F) Timeline of siRNA-mediated suppression of CalcR expression in cultured murine astrocytes and subsequent treatment with human amylin. (G) Western blot analysis to verify the siRNA-induced silencing of CalcR in murine astrocytes. (H–J) ECAR (H), glycolysis rate (I), and glycolytic capacity (J) in CalcR siRNA-transfected murine astrocytes incubated with or without 15 μ M recombinant human amylin. Data are shown as individual points and mean $\pm$ s.e.m. Statistical analysis was performed using two-tail *t* test in (D, E, G, I, and J).

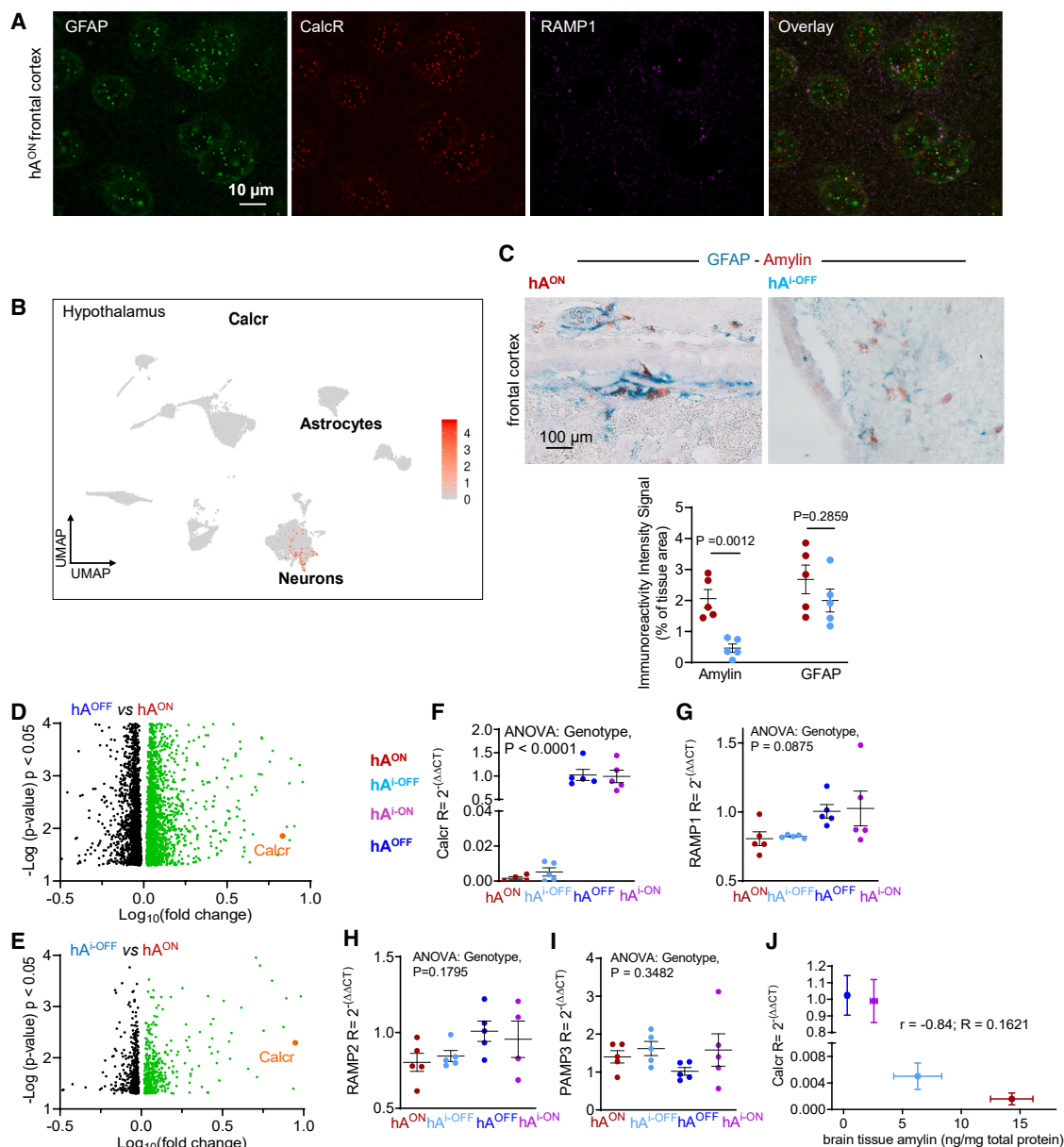


Figure 5. Transcriptomic and single-cell RNA-seq evidence that amylin directly targets astrocytes to suppress glycolytic flux

(A) Representative confocal microscopy images of RNAscope *in situ* hybridization (ISH) showing fluorescent signals (puncta) that correspond to GFAP, CalcR and RAMP1 RNA molecules within cortical sections from *hA^{ON}* mice ($n = 5$ slices/mouse from $n = 3$ mice).

(B) UMAP plot of hypothalamic CalcR expression from adult mouse single-cell RNAseq data.⁴² (C) Representative images of IHC analyses of cortical slices from *hA^{ON}* and *hA^{I-OFF}* mice co-stained for GFAP and amylin ($n = 5$ slices/mouse from $n = 5$ mice/group).

(D and E) The Log_{10} (p -value) versus Log_{10} (fold change) of the differentially expressed (DE) genes ($p < 0.05$) in brain tissues from *hA^{OFF}* vs. *hA^{ON}* (D) and *hA^{I-OFF}* vs. *hA^{ON}* mice (E). Upregulated genes are green, downregulated black, with CalcR highlighted in orange.

(F–I) (B) Cortical CalcR and RAMP1–3 mRNA in *hA^{ON}*, *hA^{I-OFF}*, *hA^{I-ON}*, and *hA^{OFF}* mice ($n = 5$ mice/group).

(J) In same mice, average cortical CalcR mRNA level vs. average cortical amylin level in same mice. Measurements were performed in duplicate. Results are shown as individual data points and/or mean $\pm$ s.e.m. and statistical analyses were performed using two-tail t test in (C) and one-way ANOVA followed by Tukey's multiple-comparisons test (F–I). See also Figures S2 and S3.

reduces AD-like pathology in these models. Using human amylin-inducible mice, we extended these observations by identifying an amylin receptor-initiated signaling mechanism that becomes

active before overt amylin amyloid deposition. Thus, targeting amylin secretion or signaling, already of interest for weight-loss therapeutics,⁶³ offers a dual-action approach: restoring brain

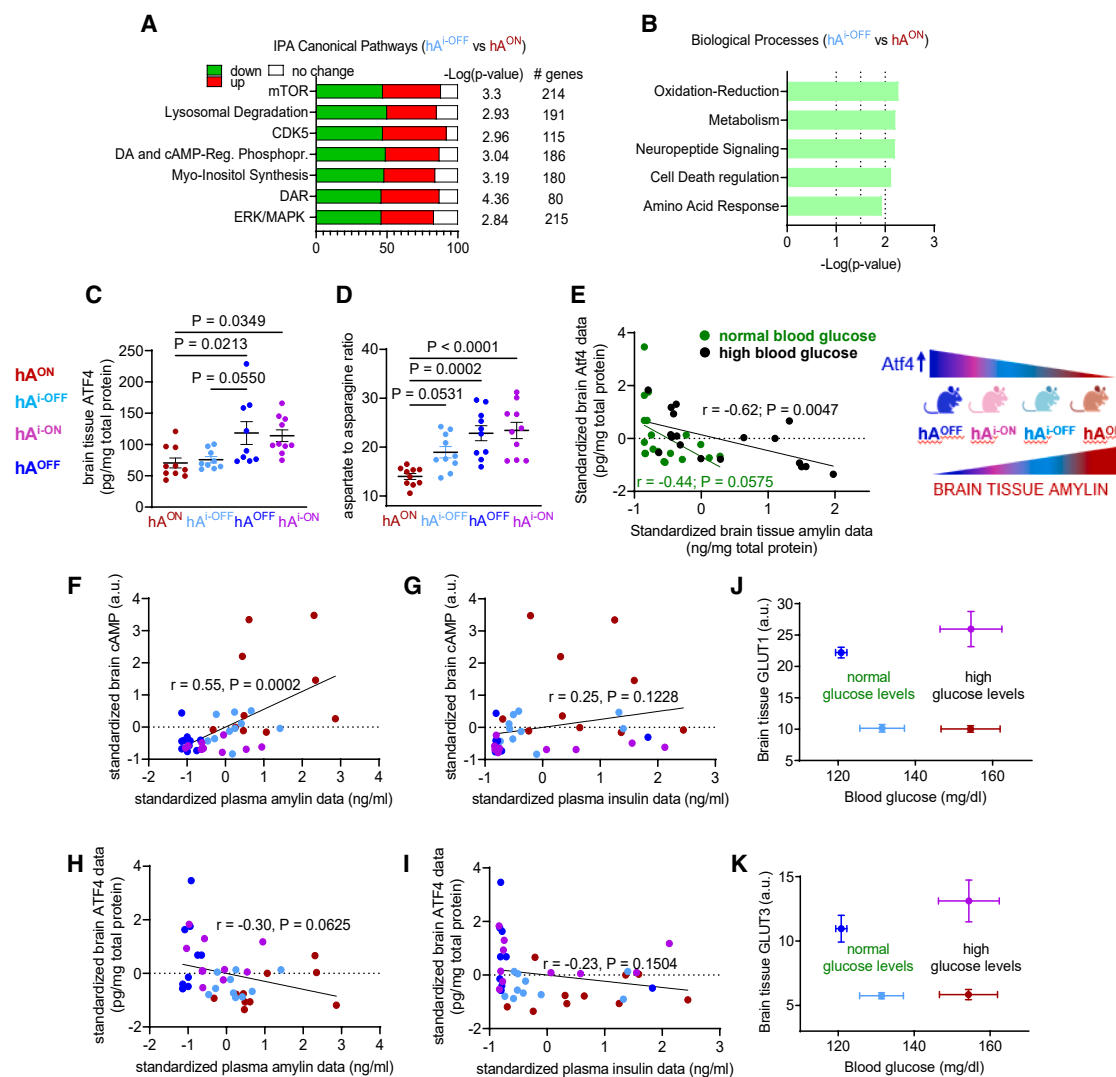


Figure 6. ATF4 signaling, glucose transporters and amino acid responses in low vs. high brain amylin under metabolic stress

(A and B) Top seven canonical pathways from ingenuity pathways analysis (A) and (B) top five GO biological processes enriched in hA^{i-OFF} vs. hA^{ON} brains.

(C) Brain ATF4 levels in hA^{ON} , hA^{i-OFF} , hA^{OFF} , and hA^{i-ON} mice. Main effect of animal model, $p = 0.0049$.

(D) Aspartate to asparagine ratio in brain tissues from hA^{ON} , hA^{i-OFF} , hA^{OFF} , and hA^{i-ON} mice. Main effect of animal model, $p < 0.0001$.

(E) Correlation between brain amylin and ATF4, with mice stratified by normal (126.1 ± 3.15 mg/dL) or high (154.4 ± 5.39 mg/dL) glucose.

(F–I) Statistical modelling comparing molecular outcomes (cAMP and ATF4) of changes in plasma amylin vs. insulin levels in same mice.

(J and K) GLUT1 and GLUT3 levels in brain tissues from hA^{ON} , hA^{i-OFF} , hA^{OFF} , and hA^{i-ON} mice as a function of blood glucose levels. Measurements were performed in duplicate. Results are shown as individual data points and/or mean $\pm$ s.e.m. and statistical analyses were performed using One-way ANOVA followed by Tukey's multiple-comparisons test (C and D).

energy metabolism while blocking pathological protein accumulation and neuroinflammation. Such strategies may succeed by addressing both the metabolic origins and neurodegenerative consequences of disease.

Limitations of the study

The extent to which human amylin activates mouse amylin receptors *in vivo* remains uncertain. *In vitro* data⁶⁴ show that human and rat amylin elicit similar concentration-response curves for cAMP production in Cos-7 cells expressing either rat or human amylin receptors. Likewise, present pharmacological and functional

data (Figures 3E, 3F, 4G–4J, and 7E–7H) support cross-species recognition of amylin receptors. Nonetheless, these findings should be validated in mice humanized for amylin receptors.

Brain metabolism is highly region-specific; yet, we measured amylin levels and downstream metabolic signatures in tissue spanning multiple regions with high receptor expression, including cortex, hippocampus, hypothalamus, and thalamus, identified via single-cell profiling. This broad approach was intended to capture global effects of systemic amylin elevation rather than region-specific changes. While we focused on the amylin-cAMP-PKA pathway, other receptor-mediated cascades and compensatory

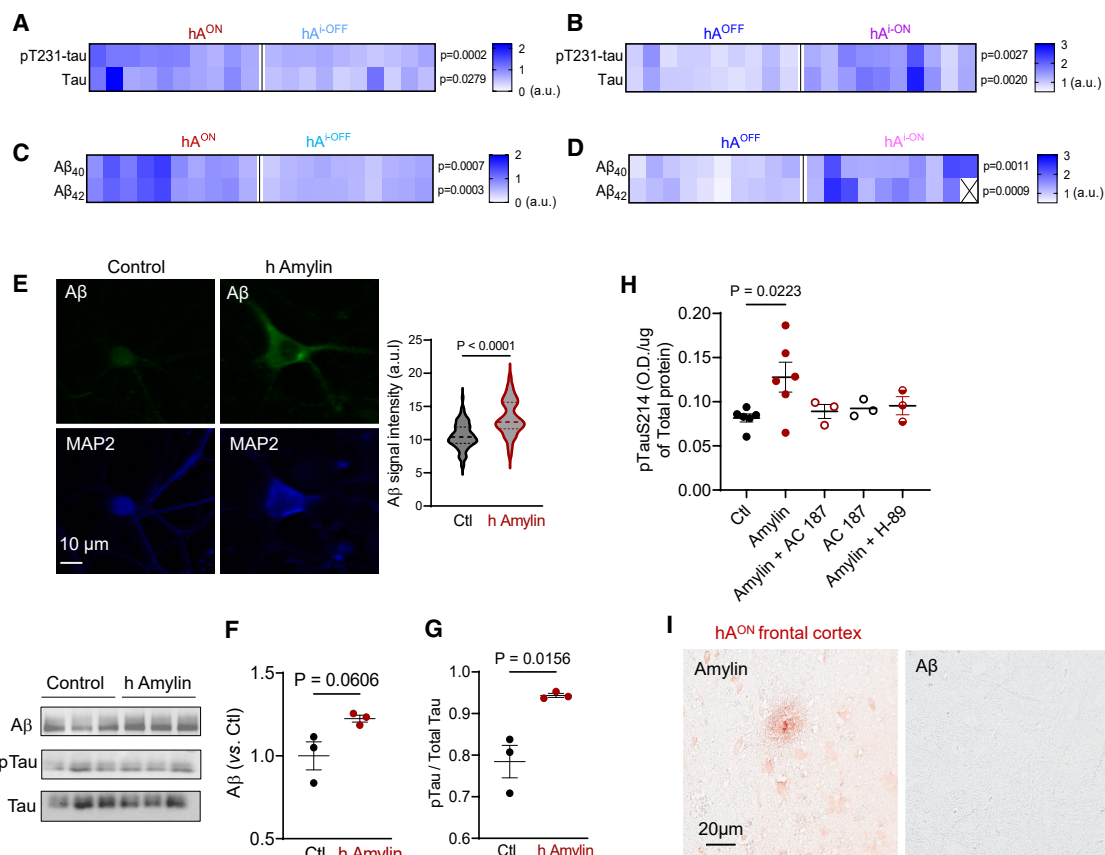


Figure 7. Effects of circulating amylin modulation on AD-like pathology *in vivo* and in human amylin-treated neonatal neurons

(A–D) Heat maps comparing the brain tissue levels of pT231-tau, tau, Aβ₄₀, and Aβ₄₂ in the same mice as in Figures 2, 3, 4, 5, and 6 (i.e., hA^{ON} vs. hA^{1-OFF} mice and hA^{OFF} vs. hA^{1-ON} mice).

(E) Aβ immunofluorescence signal for Aβ (green) of cultured primary neurons incubated with human amylin (hAmylin; 1 μM for 4 h) or under control conditions (control). MAP2 (blue) was used to identify the neurons. n = 86 neurons from 3 primary neonatal rat neuron cultures.

(F) Total Aβ level secreted in the culture media by primary neurons incubated with human amylin (hAmylin; 1 μM for 2 h) or under control conditions (control). Aβ was enriched by immunoprecipitation and measured by Western blot.

(G) pTau and total Tau lysates from primary neurons incubated with human amylin (hAmylin; 1 μM for 2 h) or under control conditions (control). pTau and Tau were enriched by immunoprecipitation and measured by western blot. Individual data points and mean ± s.e.m for three neuronal cultures.

(H) Effect of amylin on pS214-tau in cultured murine astrocytes. Cells were incubated for 30 min under control conditions or in the presence of human amylin (15 μM) with and without the amylin receptor antagonist AC187 (10 μM) or PKA inhibitor H-89 (10 μM). pS214-tau was measured by ELISA. Individual data points and mean ± s.e.m for three cell cultures.

(I) Representative images of IHC analyses of cortical slices from hA^{ON} mice stained for amylin (top) and for total Aβ (bottom) (n = 5 slices/mouse from n = 3 mice). Statistical analyses were performed using one-way ANOVA followed by Dunnett's multiple-comparisons test (H) and two-tail t test (E–G). See also Figures S4–S6.

receptor upregulation may also contribute. *In vivo* rescue experiments targeting PKA or tau were beyond the scope of this study.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Florin Despa (f.despa@uky.edu).

Materials availability

Mouse lines generated in this study will be made available upon request to the lead contact. However, distribution may require a completed material transfer agreement and/or payment if the request involves potential commercial applications.

Data and code availability

- Standardized data have been deposited at Dataverse as (<https://dataverse.harvard.edu/previewurl.xhtml?token=1fcc7c88-84f4-477f-a8a0-56c4111d2e97>) and are publicly available as of the date of publication. The original, unprocessed data are available in Mendeley Data <https://data.mendeley.com/preview/666zbc6j59?a=03459c89-68f0-4010-b823-7c293c7231ab>
- This study analyzes existing, publicly available single-cell RNA sequencing data accessible at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (GSE132355, GSE116470, and GSE129788 accession numbers).
- RNA-seq data have been deposited at GEO (GSE309843 accession number).
- Any additional information required to reanalyze the data reported in this study is available from the lead contact upon request.

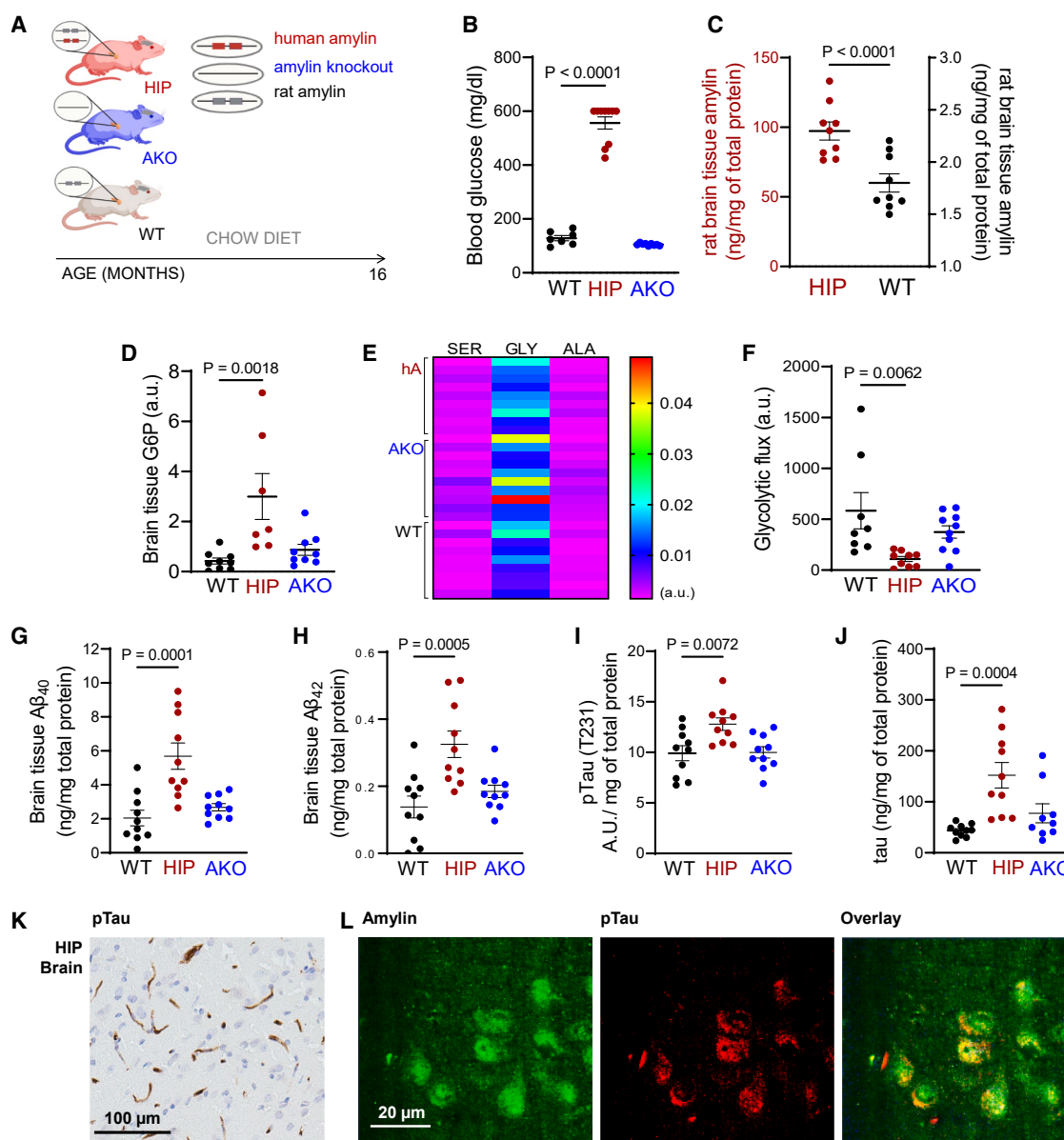


Figure 8. Cerebral glycolysis impairment and AD-like pathology in rats with genetically elevated pancreatic human amylin secretion

(A) Schematic of the experimental approach for assessing cerebral glycolytic flux, $A\beta_{40}$, $A\beta_{42}$, pTau, and total tau levels in rats expressing WT rat amylin vs. pancreatic human amylin (HIP rats) vs. amylin knockout (AKO) rats. All rats were maintained on chow diet through the endpoint (16 months of age).

(B) Endpoint blood glucose concentrations in HIP, WT, and AKO rats.

(C) Brain tissue amylin levels in HIP and WT rats measured at the endpoint.

(D–F) Comparative analyses of brain tissue G6P levels (D), glycolytic amino acids (Ser, glycine (Gly), and alanine (Ala) (E) and cerebral glycolytic flux (F) in the same rats as in (B).

(G–J) Brain tissue levels of $A\beta_{40}$, $A\beta_{42}$, pTau, and total Tau in the same rats as in (B).

(K and L) Representative images of immunohistochemistry analysis of pTau in HIP brain tissue (K) and confocal microscopy analysis of brain sections from the same rats stained with a combination of anti-amylin and anti-pTau antibodies (L). Three sections/brain. The diagram in (A) was created using BioRender. Data are mean $\pm$ s.e.m from 7 to 10 male mice/group. Statistical analyses were performed using One-way ANOVA followed by Dunnett's multiple-comparisons test (B and D–J) and two-tail t test (C).

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(I) In same mice, principal component (PC) analysis of amylin, AD markers ($A\beta_{40}$, $A\beta_{42}$, pTau, and total tau), neuroinflammation (IL-1 β , IL-6, TNF- α , IFN- γ , and CXCL1), and metabolic covariates (glucose, G6P, cAMP, and ATF4).

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AUTHOR CONTRIBUTIONS

Conceptualization, F.D.; methodology, F.D., S.D., P.K., P.K.S., M.S.G., D.R., V.C., and K.C.C.; investigation, R.S.D., N.V., N.L., D.K., G.V.V., K.C.C., P.K.S., H.L., D.R., H.C., S.D., and F.D.; funding acquisition, F.D.; project administration, N.V.; supervision, F.D., S.D., V.C., P.K., and M.S.G.; writing – original draft, F.D.; writing – review and editing, F.D., S.D., D.R., R.S.D., N.V., D.K., N.L., H.C., P.K., P.K.S., and M.S.G.

DECLARATION OF INTERESTS

Authors declare that they have no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the corresponding author used ChatGPT3.5 to correct grammar errors and improve readability. After using this tool or service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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(J) PC loading plot suggesting that clustering is largely driven by contributions of amylin, cAMP and Aβ covariates to the main principal component (PC1), whereas G6P and tau pathology drive the variation on PC2.

(K) Schematic diagram (using BioRender): toggling amylin secretion under pre-diabetic stress bidirectionally regulates brain glucose metabolism and memory. Excess amylin amplifies amylin-cAMP-PKA signaling, suppresses astrocytic glycolysis and promotes tau phosphorylation, tau-Aβ coupling and neuroinflammation, whereas restricting brain amylin preserves metabolic function and memory.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Calcitonin Receptor Polyclonal Antibody	ThermoFisher	RRID: AB_2633247
anti-A β antibody	Cell Signaling Technology	RRID: AB_2056585
beta Amyloid Recombinant Mouse Monoclonal Antibody (6E10)	Mouse/IgG	RRID: AB_3250486
MAP2 Antibodies	Novus Biologicals	RRID: AB_2138178
β -actin	Invitrogen	RRID: AB_2537661
goat anti-mouse Alexa Fluor 488	Thermo Fisher Scientific	RRID: AB_2534088
goat anti-chicken IgY Alexa Fluor 405	Abcam	RRID: AB_2810980
anti-rabbit IgG-HRP-conjugated secondary antibody	Millipore-Sigma	RRID: AB_772206
GFAP	Cell Signaling Technology	RRID: AB_561049
anti-pTau (Ser 202, Thr205) monoclonal antibody	Thermofisher	RRID: AB_223647
anti-amylin	Santa Cruz	RRID: AB_10989354
Chemicals, peptides, and recombinant proteins		
amidated human amylin peptide	Anaspec	AS-60254-1
Amylin receptor antagonist AC187	Tocris	3419
PKA inhibitor H-89	Abcam	ab120341
Critical commercial assays		
Amylin ELISA	RayBiotech	EIA-AMY-1
Insulin ELISA	Crystal Chem	62100
GLUT1 ELISA	MyBiosource	MBS2503235
GLUT3 ELISA	MyBiosource	MBS2501633
ATF4	Proteintech	KE00147
pS214-tau	Cell Signaling	44177
V-Plex ELISA A β_{42} and A β_{40}	Meso Scale Discovery	K15199G
V-Plex ELISA Tau and pT231-tau	Meso Scale Discovery	K15121D
V-Plex ELISA proinflammatory cytokines interferon-gamma (IFN- γ), interleukin (IL)-1 β , IL-6, keratinocyte chemoattractant/human growth-regulated oncogene (KC-GRO), and tumor necrosis factor-alpha (TNF- α)	Meso Scale Discovery	K15048D-1
ViewRNA™ Tissue Fluorescence Assay for GFAP, CalR and RAMP in brain sections	ThermoFisher	VB4-3111883-VCP, VB1-3034007-VC, and VB6-3197831-VC
RNeasy Mini Kit	Qiagen	74104
Deposited data		
Bulk RNASeq data	This paper	GSE309843
Standardized data	This paper	Dataverse token = 1fcc7c88-84f4-477f-a8a0-56c4111d2e97
Experimental models: Cell lines		
Rat astrocytes	Sigma	R882A-05N
Experimental models: Organisms/strains		
Inducible-human amylin mice	This paper	hA ^{ON} , hA ^{1-OFF} , hA ^{OFF} , and hA ^{1-ON} mice
transgenic rats expressing pancreatic human amylin	Charles Rivers Laboratories	HIP rats, SD-Tg (Ins2-IAPP)

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
amylin knockout rats	Despa laboratory, University of Kentucky	AKO rats
Software and algorithms		
STATA	STATA	BE-17
PartekFlow	Partek	Illumina HiSeq 2500
Prism 9	GraphPad	

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Generation of hA^{ON} , hA^{i-OFF} , hA^{OFF} , and hA^{i-ON} mice

Human amylin is amyloidogenic, while rodent amylin is not,⁶⁵ enabling the modeling of amylin dyshomeostasis and its pathological effects under metabolic stress. The human amylin sequence differs from the rodent form by six amino acids, which confer the propensity to aggregate and to activate inflammatory and stress signaling.⁶⁵ To investigate *in vivo* peripheral-CNS amylin axis (Figure 1A), we generated two inducible mouse models (hA^{ON} and hA^{OFF}) expressing human amylin in pancreatic β -cells via Cre-Lox recombination. hA^{ON} and hA^{OFF} mice were generated in collaboration with Cyagen Biosciences, California. Human amylin gene (NCBI Reference Sequence: NM_000415.2), located on chromosome 12, was used to replace the mouse amylin gene (NCBI Reference Sequence: NM_010491.2) located on chromosome 6. Both human and mouse amylin genes are identified with three exons with ATG start codon in exon 2 and stop codon in exon 3. To generate the hA^{ON} mice, the coding region of mouse amylin (ATG start codon to TAA stop codon) was replaced by a “loxP-human amylin CDS-loxP” cassette in embryonic stem (ES) cells from C57BL/6 mice using CRISPR/Cas9 technology (Figure 2A). Homology arms were generated by PCR using BAC clone RP24-81G21 and RP23-356E23 from the C57BL/6 library as template. In the targeting vector, the *Neo* cassette is flanked by self-deletion anchor (SDA) sites for negative selection. Positive targeted ES cell clones were injected into C57BL/6 albino embryos, which were then re-implanted into CD-1 pseudo-pregnant females. Founder animals were identified by their coat color. Germline transmission was confirmed by breeding with C57BL/6 females and subsequent genotyping of the offspring. The *Neo* cassette is self-deleted in germ cells so the offspring were *Neo* cassette-free. Heterozygous founders were shipped to University of Kentucky from Cyagen Biosciences, where they were bred to obtain homozygous mice for the transgene ($hA^{fl/fl}$ mice). $hA^{fl/fl}$ mice were crossed with *Ins1Cre-ERT2* mice (Jackson Laboratories, stock number 026802) to generate *Cre-hA^{fl/fl}* mice (called hA^{ON} mice throughout the study) that have conditional expression of human amylin in the pancreatic β -cells. Injection of tamoxifen (75 mg/kg BW; IP, daily for 1 week) knocks down β -cell human amylin expression in hA^{ON} mice (Figures 1B and 1C). hA^{ON} mice injected with tamoxifen are referred to as hA^{i-OFF} mice throughout the study. A similar strategy was used to generate the hA^{OFF} mice, except that the coding region of mouse amylin was replaced by a “loxP-PolyA-loxP human amylin CDS” cassette (Figure 1D). Homozygous mice for the transgene ($hAPolyA^{fl/fl}$ mice) were crossed with *Ins1Cre-ERT2* mice to generate *Cre-hAPolyA^{fl/fl}* mice (called hA^{OFF} mice throughout the study). The hA^{OFF} mice do not express either mouse or human amylin. Injection of tamoxifen in hA^{OFF} mice induces human amylin expression in β -cells (Figures 1E and 1F). hA^{OFF} mice injected with tamoxifen are called hA^{i-ON} throughout the study. Tamoxifen off-target effects are known to be dose- and duration-dependent. The limited tamoxifen regimen used here, followed by analyses more than 7 weeks later, is consistent with established Cre-lox practices designed to achieve efficient recombination while minimizing off-target effects.

HIP rats

Breeding pairs of heterozygous rats with pancreatic β -cell specific expression of human amylin (HIP rats; SD-Tg (*Ins2-IAPP*)⁴⁵ were purchased from Charles Rivers Laboratories, and rats were maintained in our colony.

AKO rats

Amylin-knockout rats (AKO rats) were generated and characterized as previously described by Ly et al.³⁵

APP/PS1 rats

TgF344-19 rats from the Rat Resource and Research Center, Univ. of Missouri, Columbia, MO (APP/PS1 rats) are Fischer rats that express human A β (A4) precursor protein (hAPP) gene with the Swedish mutation (K595N/M596L), and *presenilin 1* (*PSEN1*) gene with a deletion of exon 9, driven by mouse prion promoter (*Prp*). Brain tissues from APP/PS1 rats served as positive control for A β deposits.

METHOD DETAILS

All animal experiments comply with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments), were conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky under the code of the ethical approval 2019-3235. Sex-based analyses were included in this study. We used $N = 112$ mice (86 males and 26 females) and $N = 31$ rats (all males). Throughout the study, animals were socially housed in individually ventilated cages on a 12 h light cycle and had *ad libitum* access to food and water. For some studies, mice were randomly assigned to chow or high fat diet (60% fat; D12492 Research Diets) at 3 months of age to induce metabolic stress. Sustained nutrient overload induced by high-fat feeding alters systemic and cellular energy homeostasis. All rats were fed a standard chow diet.

Blinding

Investigators conducting experiments and analyzing data were blinded to group allocation.

Novel object recognition test

Mice were acclimatized to an empty open field arena for 1 h. Twenty-four hours later, mice were returned to the same arena, now containing two identical objects, and allowed to explore freely for 10 min. Four hours later, mice were reintroduced to the arena for 10 min, but one of the familiar objects was replaced with a novel object (the trial phase). The time spent exploring each object was recorded. All mice met the minimum exploration time of 10 s. The recognition index was calculated as the ratio between the amount of time spent to explore the novel object and the total time spent exploring the two objects (expressed as the percentage of time spent exploring the novel object).

Open field test

The open field test was conducted to assess exploratory and anxiety-related behaviors. The apparatus consisted of a square Plexiglas box measuring 40 cm $\times$ 40 cm $\times$ 40 cm. Each animal was placed in the center of the box and allowed to freely explore for 10 min. Movements were recorded using video-tracking software (Ethovision). Parameters such as movement velocity and frequency of entries into the center zone were analyzed using the software.

Blood glucose measurements

Blood glucose levels were measured using a OneTouch Ultra glucometer.

qRT-PCR

Total RNA was extracted from pancreas samples and converted to cDNA by reverse transcription. SYBR Green (1725121; Bio-Rad) based qPCR was then performed to quantify the human amylin mRNA (forward primer: 5'-AGCTGCAAGTATTTCTCATTGTG-3', reverse primer 5'-TCCGCTTTTCCACCTGATG-3') and the CalcR mRNA (forward primer: 5'-ACCTTAGGTGGACGCAGGAA-3', reverse primer 5'-TCCGCTTTTCCACCTGATG-3'); RAMP1 mRNA (); RAMP2 mRNA (); and RAMP3 mRNA (). TaqMan based RT-PCR using predesign primers and probes were performed for RAMP1 mRNA (ThermoFisher; Mm00489796_m1, cat# 4331182), RAMP2 mRNA (ThermoFisher; Mm00490256_g1, cat# 4331182) and RAMP3 mRNA (ThermoFisher; Mm07297570_m1, cat#4351372). Actin was used as the internal control.

ELISA assays

ELISA kits were used to measure the levels of amylin (EIA-AMY-1, RayBiotech), insulin (62100, Crystal Chem), Glut-1 (MBS2503235, MyBiosource), Glut-3 (MBS2501633, MyBiosource), Glut-5 (MBS9302430, MyBiosource) and ATF4 (KE00147, Proteintech). V-Plex ELISA kits were used to measure the levels of $A\beta_{42}$ and $A\beta_{40}$ (K15199G, Meso Scale Discovery), Tau and pT231-tau (K15121D, Meso Scale Discovery), pS214-tau (Cell Signaling cat# 44177) and proinflammatory cytokines (K15048D-1, Meso Scale Discovery). The cytokines assessed include interferon-gamma (IFN- γ), interleukin (IL)-1 β , IL-6, keratinocyte chemoattractant/human growth-regulated oncogene (KC-GRO), and tumor necrosis factor-alpha (TNF- α). Experiments were carried out according to manufacturer's protocols. All target protein concentrations were normalized to total protein input, as determined by the bicinchoninic acid (BCA) assay.

Metabolomics determination of amino acids, glucose-6 phosphate and cAMP in mouse brain homogenates by UHPLC-MS

UHPLC-MS was used to measure amino acids, glucose-6 phosphate and cAMP in mouse brain homogenates. Brain tissue was flash frozen in liquid nitrogen, weighed (25–50 mg wet weight), placed in buffer (50% acetyl-nitrile, 50% water, 0.3% formic acid) at a standard concentration of 50 mg wet weight per mL buffer, then fully homogenized on ice for 10–25 s and placed on dry ice/stored at -80°C . Homogenates were analyzed using a 1290 Infinity UHPLC (Agilent Technologies) and 6546 LC/Q-TOF mass spectrometer (Agilent Technologies), as previously described (58). Briefly, samples were run on a 150 $\times$ 4.6 mm Luna Omega 3 μM polar column using water containing 0.1% formic acid (FA) as mobile phase A and acetonitrile (ACN) containing 0.1% FA as

mobile phase B. The following gradient was used for separation with a flow rate of 0.75 mL/min: 0–5.5 min (10% B), 5.5–11.50 min (10–100% B), and 11.60–16 min (100–10% B). The MS was run in negative and positive electrospray ionization mode with the following parameters: gas temperature, 300°C; drying gas flow rate, 12 L/min; nebulizer, 35 psi; sheath gas temperature, 275°C; sheath gas flow 12 L/min; fragmenter voltage, 120 V; skimmer voltage, 65 V; nozzle voltage, 750 V; capillary voltage, 3500 V. Data were analyzed using MassHunter-Qualitative and -quantitative software.

Measurement of amino acids and glucose-6 phosphate in rat brain tissue using gas chromatography-mass spectrometry (GCMS)

Approximately 20 mg of pulverized rat brain tissue was extracted using 1 mL of ice-cold 50% methanol supplemented with 200 μ M L-norvaline. After vortexing, the samples underwent 5 min of mechanical cell disruption and were subsequently incubated on ice for 30 min. Following the incubation, samples were centrifuged at 15,000 rpm for 10 min at 4°C to separate the polar metabolites (in the supernatant) from proteins and lipids (in the pellet). The supernatant containing the polar phase was rapidly snap-frozen in liquid nitrogen and stored at –80°C. The protein-rich pellet was hydrolyzed in 100 μ L of 6N hydrochloric acid by heating at 95°C for 2 h. To neutralize the reaction, 200 μ L of 100% methanol containing 200 μ M L-norvaline was added. Samples were thoroughly vortexed and then centrifuged again at 15,000 rpm for 10 min at 4°C. The resulting supernatant, containing digested proteins along with glycogen and glycans, was transferred into a new tube. Both the polar and protein-derived fractions were dried using a speed vacuum concentrator for 2 to 4 h. Dry extracts were derivatized by sequentially adding 20 mg/mL methoxyamine hydrochloride in pyridine, followed by the silylating reagent N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), with thorough mixing at 37°C. Samples were subsequently analyzed via gas chromatography-mass spectrometry (GC-MS) in full scan mode using an Agilent 7800B GC system coupled to a 5977B single quadrupole mass spectrometer. The analytical method was based on previously published protocols,⁶⁶ but employed a modified GC oven temperature program: starting at 130°C for 4 min, ramping at 6 °C/min to 243°C, then at 60 °C/min to 280°C, where it was held for 2 min. Batch data were processed with the DExSI software suite, which is tailored for stable isotope-labelled metabolite analysis. Metabolites were identified by spectral deconvolution using the AMDIS software tool.⁶⁷ To correct for sample loss, metabolite levels in both fractions were first normalized to L-norvaline added to each, and then adjusted based on the total protein input estimated from amino acid content in the pellet.

RNAseq analysis

For canonical pathway analysis, we compared the hA^{i-OFF} and hA^{ON} groups (amylin knockdown vs. continued amylin expression) and the hA^{OFF} and hA^{ON} groups (absence vs. presence of human amylin). The first comparison identifies pathways associated with partial amylin suppression, whereas the second captures the most distinct amylin activity states, absence versus presence of human amylin under comparable metabolic load. Together, these analyses enable robust identification of amylin-dependent signaling pathways. RNA was isolated from the cerebral cortex using the RNeasy Mini Kit (Qiagen, #74104). RNAseq library preparation and sequencing was conducted by Omega Bioservices using the Illumina HiSeq 2500; data were analyzed with PartekFlow software (Partek, MO).

Mouse reference genome *Mus musculus*-mm10 was used for the alignment of Fastq files, and gene level quantification was conducted using Ensembl105 annotations. RNAseq read counts were normalized and analyzed for differential expressing between hA^{i-OFF} vs. hA^{ON} and hA^{OFF} vs. hA^{ON} groups. We have processed the RNA-seq data with DESeq2 algorithm, applying Benjamini-Hochberg correction (adjusted $p < 0.05$). RNA transcripts with p -values ≤ 0.05 between the groups were queried for enrichment of canonical pathways using Ingenuity Pathway Analyses software (IPA, Qiagen) and for enrichment in Gene Ontology (GO) biological processes of these differentially expressed (DE) genes using DAVID (NIH).

Single-cell RNA sequencing analysis

Single-cell RNA sequencing data were downloaded from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO). Seurat package (v5.1)⁶⁸ in R (v4.4.1) was used to analyze all data. All datasets analyzed were derived from adult C57BL6J male mice. Hypothalamus count matrices from the two available adult replicates were obtained from (GSE132355) (62). Genes expressed in fewer than 3 cells were excluded. As done in the original publication, cells with less than 200 genes were removed from the analysis.⁶⁹ Cells with more than 25% mitochondrial reads were excluded. All data were log-normalized and scaled using Seurat's standard workflow, normalizing to 10,000 transcripts per cell. The top 2000 variable features were identified using the "vst" method and the expression of these genes was scaled prior to PCA. Cells were clustered using Louvain clustering. UMAP embeddings (resolution = 0.4) were constructed using the first 100 principal components. DoubletFinder (v2.0.3) was applied to hypothalamus datasets to identify doublets and multiplets using recommended statistical parameters, with computed pK values of 0.02 and 0.01 for replicates 1 and 2, respectively. Expected doublet rates (nExp) were set at 5% and 5.6% of total cells before doublet removal based on recommended percentages provided by 10X Genomics. After doublet removal, both replicates were merged, and dimensionality reduction was performed as previously described. Harmony (v1.2.3) was then used to integrate both datasets. Cell types were assigned based on established brain cell markers, including *Rbfox3*, *Slc17a6*, and *Slc32a1* for neurons, and *Aqp4* and *Agt* for astrocytes. One cluster with non-specific marker genes characterized by high ribosomal and mitochondrial reads along with mixed glial and neuronal marker expression was excluded. Additionally, an erythroid cluster expressing

hemoglobin genes was also excluded. The final hypothalamus dataset contained 13,194 cells after Harmony integration. Whole-brain, data matrices were obtained from (GSE129788), consisting of 37,069 previously processed cells that had already undergone quality control filtering in the original study.⁴² For posterior cortex, hippocampus, and thalamus, raw digital expression matrices were obtained from (GSE116470) (63). Expression matrices were separated into individual replicates and processed individually for quality control filtering. As done in the original publication, cells with less than 400 expressed genes were excluded.⁴² Twenty principal components were used for graph-based clustering (resolution = 0.1). Doublets were removed with DoubletFinder and Harmony was used to integrate replicates. Final cell numbers were 97,263 for posterior cortex, 105,818 for hippocampus, and 89,001 for thalamus.

ViewRNA tissue fluorescence assay for GFAP, CalR and RAMP in brain sections

ViewRNA Tissue Assay Fluorescence for GFAP, CalR and RAMP1 was performed in mouse brain sections as per kit instructions. Tissue sections were deparaffinized in xylene and rehydrated in a dilution series of ethanol. Sections were then heated in 1x pretreatment buffer for 30 min at 90°C and then cooled down to RT. Sections were then treated with 1x protease solution for 10 min at 40°C and then washed with 1x PBS four times. After that, the sections were incubated in a 1:40 dilution of predesigned targeted probes (ThermoFisher; cat#VB4-3111883-VCP, VB1-3034007-VC and VB6-3197831-VC) at 40°C for 2 h and washed in wash buffer three times for 2 min each. Then sections were incubated in PreAmplifier Mix solution for 30 min at 40°C, followed by three washes in wash buffer. Sections were then incubated in fluorescence-tagged label probes for 30 min at 40°C and washed in wash buffer. To remove autofluorescence, sections were incubated in 0.2% Sudan black for 1 min and washed in 1xPBS three times and mounted with ProLong Glass Antifade Mountant, then allowed to cure overnight. Images were captured with a Nikon confocal A1R microscope.

PKA activity measurements

PKA activity was measured in brain homogenates and astrocyte lysates using a PKA kinase activity assay kit (Abcam, cat# ab139435) following the manufacturer's instructions. Briefly, microtiter plate wells were soaked with 50 μ L of Kinase Assay Dilution Buffer at room temperature for 10 min and then aspirated. Samples (30 μ L) and serial dilutions of positive controls were added to the wells, and the reaction was initiated with 10 μ L of reconstituted ATP. The plate was incubated at 30°C for 90 min, then the reaction was stopped by emptying the wells. Next, PKA phosphospecific substrate antibody (40 μ L) was added to each well except the blank and the plate was incubated at room temperature for 60 min, followed by washing. The plate was then incubated with 40 μ L anti-rabbit IgG-HRP conjugate (room temperature, 30 min). After washing, the plate was incubated at room temperature with 3,3',5,5'-tetramethylbenzidine (TMB) substrate for ~35 min. The reaction was then stopped by adding 20 μ L stop solution and OD 450 nm was measured with a microplate spectrophotometer (Bio-Rad, model xMark). Measurements were performed in duplicate.

Culture of astrocytes and neurons

Rat astrocytes were purchased from Sigma (R882A-05N) and cultured in rat astrocyte growth medium (R821-500, Sigma) supplemented with 10% FBS. Astrocytes were seeded into 6-well plates at a density of 1×10^6 cells per well. For some experiments, astrocytes were transfected with 30 nM calcitonin receptor siRNA (AM16708, Ambion) or 30 nM scrambled siRNA (AM4611, Ambion) using Lipofectamine 2000 (11668-027; Invitrogen). In other experiments, cells were pre-incubated for 30 min with AC 187 (10 μ M) followed by amylin treatment (15 μ M for 30 min). In other experiments, the cells were incubated in basic RPMI medium for 30 min, followed by a 30-min pre-incubation with either basic RPMI alone, RPMI containing the calcitonin receptor blocker AC-187 (10 μ M); Tocris, #3419, or RPMI containing the PKA inhibitor H-89 (20 μ M, Abcam, #ab120341). Cells were then treated with vehicle (DMSO) or 15 μ M amylin, either alone or in combination with AC-187 or H-89. After 30 min, the medium was removed and the cells were lysed in lysis buffer.

Neurons were isolated from hippocampi of one-day-old rat pups by digestion with 0.25% Trypsin-EDTA (11 min at 37°C) followed by repeated trituration as previously described.^{33,36} Neurons were then plated on poly-L-lysine coated coverslips and cultured in N21 Neurobasal medium (AR008; R&D systems; MN) and used in experiments after 10 days in culture. Primary neuronal cells were detected with mouse anti-MAP2 antibody (NB300-213; Novus Biologicals; CO). For some experiments, neurons were pre-incubated for 120 min with human amylin treatment (15 μ M) (amidated human amylin peptide, Anaspec, AS-60254-1). The 15 μ M human amylin concentration approximates transient local levels near amylin deposits in perivascular spaces under hyperamylinemic conditions (based on estimates from Ly et al.³⁵ and Verma et al.³⁷), and is thus physiologically relevant for modeling stress signaling rather than basal conditions.

Immunofluorescence of primary neonatal rat neurons

Primary neonatal rat neurons were cultured as described above, fixed with paraformaldehyde, permeabilized with 0.25% Triton X-100 and labeled with antibodies against A β (MA5-51794; Thermo Fisher Scientific, 1:300 dilution) and MAP2 (NB300-213; Novus Biologicals, 1:10000 dilution). After washing, cells were incubated with goat anti-mouse Alexa Fluor 488 (A11029; Thermo Fisher Scientific) and goat anti-chicken IgY Alexa Fluor 405 (ab175675; Abcam) secondary antibodies at 1:500 dilution. Images were collected with a confocal microscope (LSM 5 LIVE, Zeiss).

cAMP measurements in cultured astrocytes

cAMP levels were measured using the THUNDER cAMP TR-FRET assay kit (Bioauxilium, KIT-CAMP-1000). Astrocytes were harvested using non-enzymatic Versene solution (Thermo Fisher Scientific, 15040066), washed with Hank's buffered saline solution, and resuspended in stimulation buffer at a density of 5000 cells per 5 μ L. Assays were performed in 384-well microplates (20 μ L total volume). Standards and astrocyte suspensions (5 μ L each) were added to wells in triplicate. Standards received stimulation buffer, while astrocytes were incubated with 15 μ M recombinant human amylin (AS-60254-1, AnaSpec) or vehicle for 15 min at room temperature. Next, 5 μ L of FR-*anti*-cAMP antibody solution and 5 μ L of Eu-SA/biotin-cAMP Mix were added. Plates were sealed, incubated for 1 h at room temperature, and read using a TR-FRET reader (Agilent BioTek Cytation 5). Final cAMP concentration was calculated from the 615/665 emission ratio following manufacturer's instructions. Measurements were performed in triplicate.

Extracellular acidification rate (ECAR)

ECAR was measured in cultured rat astrocytes using a Seahorse XF Pro analyzer (Agilent) to assess glycolytic function. Astrocytes were plated in a 96-well Seahorse microplate and cultured under various experimental conditions. On the day of the experiment, cells were washed and equilibrated in Seahorse XF Base Medium for 1 h. Measurements were done using the standard Glycolysis Stress Test protocol, with sequential injections of glucose (10 mM), oligomycin (1 μ M), and 2-deoxy-D-glucose (50 mM) to assess basal glycolysis, glycolytic capacity, and glycolytic reserve.

Brain section immunofluorescence and immunohistochemistry

We used brain tissues from hA^{ON} , hA^{I-OFF} , hA^{OFF} and hA^{I-ON} mice and APP/PS1 and HIP rats, and pancreatic tissue from HIP rats. Tissues were processed as previously described.^{33,35–37} The specificity of the amylin antibody in brain tissues was established in previous studies.^{33,35–37} Pancreatic tissue from AKO rats was the negative control for amylin. Paraffin-embedded brain sections were deparaffinized, rehydrated, and washed in 0.01 M PBS 1X plus Triton X-100 0.01% three times for 5 min each. After blocking in normal goat serum for 1 h, sections were incubated in primary antibodies mixture for 24 h at 4°C. The primary antibodies mixture included anti-pTau (Ser 202, Thr205) monoclonal antibody (AT8) (1:200) (ThermoFisher), anti-amylin (clone E5; SC-377530) (1:200) (Santa Cruz), GFAP (1:400; 3670S, Cell Signaling Technology) and A β (1:400; clone 6E10, 803002, Biolegend) and MAP2 (1:400; NB300-213; Novus Biologicals; CO). The next day, the sections were washed in PBS 1 $\times$ 3 times for 10 min each and then incubated with goat anti-mouse Alexa Fluor 488 (1:700, Invitrogen) and goat anti-rabbit Alexa Fluor 568 (1:700, Invitrogen) for 1 h. Sections were washed and mounted in Fluoromount (Sigma, F4680) mounting medium with DAPI (Vector Laboratories). Images were collected with a confocal microscope (LSM 5 LIVE, Zeiss). For immunohistochemistry the anti-amylin (1:200, T-4157) (Bachem-Peninsula Laboratories) was used.

Western blot

Cultured astrocytes were lysed in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 2 mM EGTA, 1% NP-40, 50 mM NaF and protease and phosphatase inhibitor cocktail sets) and subjected to SDS-PAGE. Proteins were then transferred to PVDF membranes, blocked with 5% milk and incubated overnight at 4 °C with a primary antibody against CalcR (720296, ThermoFisher; 1:500 dilution). Membranes were then washed and incubated with an anti-rabbit IgG-HRP-conjugated secondary antibody (NA934; Millipore-Sigma, 1:20,000 dilution) for 1 h at 37 °C. β -actin (MA5-15739; Invitrogen) was used for loading control. Blots were visualized through chemiluminescent signals using the enhanced chemiluminescence method (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific, USA) and captured with a G:BOX gel imaging system (SynGene, Cambridge, United Kingdom).

QUANTIFICATION AND STATISTICAL ANALYSIS

The number of samples or animals in each analysis, the statistical analysis performed, and *p* values are reported in figures and figure legends. D'Agostino-Pearson and Kolmogorov-Smirnov test was used to test the normality distribution of continuous variables. Parametric comparisons of continuous variables with normal distributions were performed 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 of three groups or more group means were performed using one-way or two-way ANOVA with the Bonferroni post-test. Relationships between two continuous variables were analyzed by correlation analysis. Part of analyses were performed using GraphPad Prism 9 software. Principal Component (PC) analysis (GraphPad Prism 9 software) was performed to identify underlying patterns in the whole dataset owing to the OFF/ON pancreatic amylin secretion status in mice. The dataset includes amylin, AD covariates (tau, pTau, A β_{40} and A β_{42}), neuroinflammation covariates (IL-1 β , IL-6, TNF- α , IFN- γ and CXCL1) and metabolic covariates (blood glucose, G6P, cAMP and ATF4). Prior to applying PC analysis the data was standardized to have a mean of 0 and standard deviation of 1 for each variable. The first two principal components accounted for 41.7% and 28.7%, respectively, of the total variation. STATA BE-17 was used for conducting regression analyses of the relationships between brain tissue pTau and amylin levels adjusted for PKA activity.