Adropin expression correlates with age-related neuropathologies in the human brain and improves neuroinflammation and cognitive function in aging mice

Subhashis Banerjee

Saint Louis University

Sarbani Ghoshal

Saint Louis University

Clemence Girardet

Saint Louis University

Kelly M. DeMars

McKnight Brain Institute, University of Florida

Changjun Yang

McKnight Brain Institute, University of Florida

Michael L. Niehoff

Saint Louis University

Andrew D. Nguyen

Saint Louis University

Prerana Jayanth

Saint Louis University

Brittany A. Mersman

Saint Louis University

Fenglian Xu

Saint Louis University

William A. Banks

Veterans Affairs Puget Sound Health Care System, 7University of Washington School of Medicine

Kim M. Hansen

Veterans Affairs Puget Sound Health Care System

Jinsong Zhang

Saint Louis University

Eduardo Candelario-Jalil

McKnight Brain Institute, University of Florida

Susan A. Farr

Saint Louis Veterans Affairs Medical Center and Saint Louis University

Andrew A. Butler (✉ Andrew.butler@health.slu.edu)

Saint Louis University

Research Article

Keywords: dementia, drug discovery, inflammation, metabolic disease, neural metabolism, neuroinflammation, neuroprotection, peptides

Posted Date: January 14th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-99159/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Abstract

Adropin is most abundant in neural tissues yet its neurological functions are unclear. Data from post-mortem human brain tissue samples indicates adropin expression occurs predominantly in astrocytes, peaks during critical post-natal periods of brain development, and then declines with aging. Previous experiments indicate adropin regulates mitochondrial metabolism. Gene clusters correlating with adropin are age- and dementia-specific, possibly indicating survivor bias. In people aged <40y adropin correlates positively with genes involved in mitochondrial metabolism, APOE and Clusterin. In the 'old-old' (>75y) with dementia, adropin expression correlates with genes linked to mitochondrial metabolism and neurodegenerative conditions. In the 'old-old' (>75y) without dementia, adropin correlates with genes involved in morphogenesis, growth of neuronal processes (dendrites, axons) and synapse function. Accordingly, adropin elicits neurotrophic responses in primary cultured neurons. Adropin expression also correlates positively with protein markers of tau-related neuropathologies and inflammation, particularly in people without dementia, indicating a link to cellular stressors. How variation in brain adropin expression affects neurological aging was investigated using C57BL/6J mice. In mice, adropin is more widely expressed in neurons, oligodendrocyte progenitor cells, oligodendrocytes, and microglia. Preventing the decline in expression observed with aging of mice using transgenesis improved cognitive function and resilience, while also reducing mRNA markers of inflammation in 18-month old mice. Treating 18-month old mice with adropin peptide also improved cognitive performance. These results link adropin expression to cellular energy metabolism and stress responses in the brain and indicates a possible relationship with aging-related cognitive decline.

Introduction

Aging associates with a decline in cognitive flexibility and capacity for tracking the “what/where/when” of daily activities (episodic memory) ¹⁻³. Cognitive decline that is perceived by individuals without loss of independence has been described as Mild Cognitive Impairment (MCI) ^{4,5}. Increased risk for dementia and dementia-related neuropathologies suggests MCI is an early stage in the spectrum of life-threatening dementias, of which Late-Onset Alzheimer's Disease (LOAD) is the most common ⁶⁻⁹. 'Risk magnifiers' for dementia include hypertension, hyperlipidemias, type 2 diabetes and sedentary lifestyle, all of which correlate with obesity ^{10,11}. These conditions are increasing common ¹² and will contribute to an increased prevalence of dementia during this century ¹³. Identifying new treatments that target the neuropathologies causing dementia is thus considered an important goal.

Proteogenomics identified >3000 small open reading frames (sORF) of <100 aa in human genomes that appear to modulate cellular stress responses and metabolic homeostasis ¹⁴⁻¹⁶. We identified a sORF (adropin¹⁻⁷⁶) encoded by the Energy Homeostasis Associated (*ENHO*) gene ¹⁷. A signal sequence (adropin¹⁻³³) is thought to target the secretory pathway ¹⁷⁻¹⁹. Adropin immunoreactivity has also been detected in plasma ^{17,20}, suggesting a mechanism for release into the circulation. Adropin³⁴⁻⁷⁶ is

sufficient for biological activity in cultured cells and rodent models ^{17,21-30}. However, the exact sequence and post-translational modifications of the mature peptide are not known.

Adropin regulates peripheral carbohydrate and lipid metabolism ^{17,20,21,23,25,26,30-37} and vascular function ^{24,38-40}. Expression of the *ENHO* transcript is however several orders of magnitude higher in the brain relative to non-neural tissues ^{17,34,41-44}. The two candidates for cell-surface adropin receptors are also highly expressed in the CNS, and each appears to or is known to regulate brain development. The orphan G protein coupled receptor GPR19 appears to be obligatory for adropin signaling ^{25,28,29}, and is highly expressed in the CNS relative to other tissues ^{45,46}. High GPR19 expression in the embryonic brain suggests a role in development ⁴⁷. Evidence for coupling of adropin with GPR19 is however controversial ⁴⁸. Another group reported an interaction between adropin retained in plasma membranes with NB-3/Contactin6 (CNTN6) using protein-protein yeast two hybrid screening ⁴¹. CNTN6 is a brain-specific non-canonical membrane-tethered Notch1 ligand that belongs to the immunoglobulin cell adhesion molecule superfamily (IgCAMs). Contactins function as cell-adhesion proteins in the developing nervous system where they regulate axonal guidance, development of neurites, synapse formation, synaptic plasticity and neural regeneration ⁴⁹.

Two recent studies suggest adropin expression is relevant to neurological aging. In Sprague Dawley rats, aging-related declines of plasma adropin concentrations and protein expression in the brain correlate with protein markers of oxidative stress ⁵⁰. While the origin of circulating adropin is unclear, plasma concentrations in humans also decline with aging, particularly in males ³³. Here we combined mouse and human transcriptomic data with experiments using mouse models and cultured cells to investigate the relationship between adropin expression and aging-related cognitive decline.

Results

In human post-mortem tissue samples, *ENHO* is predominantly expressed in brain structures (**Fig. 1A**). RNA-seq data from the Genotype-Tissue Expression (GTEx) project and Aging, Dementia and Traumatic Brain Injury (TBI) Study suggest sex differences, with higher levels in males (approx. 10%) (**Fig. S1, S2A**). Single-cell RNAseq identified mature astrocytes as a site of high *ENHO* expression in the human brain, with low expression in neurons, oligodendrocytes and endothelial cells (**Fig. 1B**) ⁵¹.

The Atlas of the Developing Human Brain (www.brainspan.org) maintains RNA-seq data from post-mortem samples collected from 4 months to 40 years of age. The GTEx project released data as ten-year bins in people aged 20 to 79 years (20-29, 30-39, and so on). *ENHO* expression appears to peak in early childhood in the Atlas of the Developing Human Brain dataset (**Fig. 1C**); GTEx data indicate expression is stable up to 79 years of age (data not shown). Analysis by sex and age-group is not possible in the GTEx data, however expression data shown in **Fig. S2A** suggests sex differences persist with advanced age.

Plasma adropin concentrations in humans are higher in males compared to females and decline with aging³³. Based on gene expression analysis, neural tissues appear to be a plausible site of origin for circulating adropin in humans.

Correlation with neural energy metabolism and LOAD risk genes

An unbiased screen for genes showing strong correlation coefficients was used to identify clusters of genes co-regulated with *ENHO*^{34,52}. In the Atlas of the Developing Human Brain, *ENHO* clusters with gene networks related to mitochondrial energy metabolism. Top ranked GO molecular functions for genes with correlation coefficients (*r*) of >0.7 included “oxidoreductase activity” (q-value FDR 1.02×10^{-18}), “oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor” (1.29×10^{-6}), “electron transfer activity” (4.30×10^{-6}), “NADH dehydrogenase (quinone) activity” (1.08×10^{-4}), and “NADH dehydrogenase (ubiquinone) activity” (1.08×10^{-4}). GO biological processes included “oxidation reduction process” (1.24×10^{-20}), “drug metabolic process” (4.21×10^{-15}), “small molecule catabolic process” (2.72×10^{-10}), “electron transport chain” (2.60×10^{-9}), and “generation of precursor metabolites and energy” (2.60×10^{-9}). GO cellular component also indicated mitochondrial functions: “mitochondrion” (2.36×10^{-26}), “mitochondrial inner membrane” (4.21×10^{-21}), “organelle inner membrane” (2.00×10^{-20}), “mitochondrial envelope” (5.03×10^{-20}), and “mitochondrial membrane” (8.10×10^{-20}).

APOE variants (e2, e3, e4) are the strongest genetic risk factors for LOAD, with the e4 allele increasing risk and e2 allele conferring protection^{53,54}. Clusterin (CLU, also known as ApoJ) is an extracellular chaperone linked to Ab clearance and toxicity⁵⁵. Strong positive correlations are observed between *ENHO* and both *APOE* and CLU expression (**Fig. 1D, E**). The insulin signaling pathway also has an important role in brain health. Tau phosphorylation by glycogen synthase kinase 3 (GSK3) contributes to LOAD neuropathology⁵⁶. There was a strong negative correlation with *GSK3B* (**Fig. 1F**) and *AKT3* (**Fig. 1G**), the predominant AKT isoform expressed in the brain upstream of GSK3B⁵⁷.

We next determined whether these correlations are also observed in laboratory animal models. Enrichment for proteins involved in mitochondrial energy metabolism is observed in gene clusters correlating with *ENHO* expression suggest in NHP (GSE98965, PRJNA236446) (^{43,58} and in the entorhinal cortex of mice (GSE125957)⁵⁹. Positive correlations between *ENHO* and APOE, CLU and GSK3 are also observed in NHP and mice.

These results indicate adropin expression correlates brain energy metabolism and LOAD-risk marker genes linked to lipid metabolism in the brain. Importantly, these associations have been conserved during evolution. Moreover, plasma adropin concentrations correlates with indices of cholesterol metabolism in humans³³ and NHP³⁴.

Correlations in the ‘old-old’ human brain is dependent on dementia status

Data from The Aging, Dementia and TBI Study (<http://aging.brain-map.org/>) were used to investigate relationships between aging-related dementia and *ENHO* expression. This study involved 107 people selected from the Adult Changes in Thought (ACT) cohort⁶⁰, a population-based, prospective analysis of neurological aging⁶¹⁻⁶⁴. Selected participants were mostly male (63 males, 44 females), varied in years of education and were in the 'old-old' age group (77–102y at time of death, median 90y); 52 were diagnosed with dementia (D) by the time of death (30 with AD; 12 with dementia of multiple etiologies; four with vascular dementia). Post-mortem samples were collected from brain regions known to exhibit neurodegeneration and pathology resulting from LOAD and Lewy body disease: frontal white matter (FWM), hippocampus (HIP), parietal cortex (PCx), and temporal cortex (TCx). *ENHO* expression was first compared between brain structures, between individuals based on dementia state and APOE allele.

ENHO expression is not affected by dementia status in humans (**Fig. S2B**). GSE125957 includes transgenic mouse models used to investigate the progressive neuropathologies of AD⁵⁹. *ENHO* expression in these models were also normal (data not shown). Interestingly, there was however a trend ($p < 0.1$) for higher *ENHO* expression in TCx and FWM samples from participants with >1 APOEε4 allele (**Fig. S2C**).

Correlations between brain structures in the expression of gene networks indicates regulation by systemic phenotypes⁶⁰. Subtle differences of are observed between structures, however *ENHO* expression is generally similar (**Fig. S2D**) and correlates between brain structures (**Fig. S2E-K**). The regulatory factors that affect *ENHO* transcript levels appear to act throughout the brain.

A “find correlates” approach to identify gene clusters that correlate with *ENHO* was applied to groups divided by dementia diagnosis (Dementia or No Dementia, referred to as D and ND hereafter). The rationale for separating into two groups is based on relationships between neurodegenerative disease state, RNA quality and agonal conditions that may result from ischemia that can complicate gene expression data analysis in post mortem brain samples^{60,65,66}.

Correlations between *ENHO* and other genes are noticeably weaker with aging. The reasons for this are unknown but could indicate deteriorating fidelity in the regulation of gene networks with advanced age. We therefore initially analyzed the top 1% of genes (502 out of ~50,200) ranked high to low by correlation coefficient (r) against *ENHO*.

Correlation coefficients between *ENHO* and other genes in the top 1% of genes are significantly higher in people with dementia compared to those not diagnosed with dementia (**Fig. 2A, B**). There was modest (25%) overlap in the genes correlating with *ENHO* in people with or without dementia (**Fig. 2C-F**). Functional enrichment analysis of the two datasets using ToppGene indicated a divergence in pathways correlating with *ENHO* expression (**Fig. 3**). The top 10 listed GO pathways for people with ND centered around morphogenesis and development of neuronal processes. The association between adropin expression and neural development was investigated using primary cultured neurons. Adropin³⁴⁻⁷⁶ had

potent trophic properties, promoting the development of neurites and increasing spontaneous firing activity (**Fig. 4A-F**)

Metabolic processes dominated the list of GO biological processes in study participants with dementia (**Fig. 3**). Adropin regulates angiogenesis and endothelial function^{24,39,40,50}. Clinical studies have identified correlations with aging-related endothelial dysfunction⁶⁷. Enrichment of genes involved in vascular development and function was specific for people with D (for example, GO processes “tube development”, “vascular process in circulatory system”, “vasculature development”, “epithelial cell proliferation”, and “response to epidermal growth factor”).

A larger unbiased screen using the entire dataset used multiple enrichment tools (Reactome, KEGG) (http://pharmacology.slu.edu/results/andrew/TBI_Dementia/). The outcomes from this analysis are consistent with a divergence between people with or without dementia. For this analysis, genome-wide gene set enrichment was performed to unbiasedly reveal biological pathways enriched or depleted in a manner correlated with *ENHO* expression. Four independent studies were performed to include: (i) all 5529 biological pathways, designated “All pathways”, or a subset of these pathways belonging to (ii) REACTOME, designated “REACTOME pathways”, (iii) KEGG, designated “KEGG pathways”, or (iv) the remaining pathways, designated “non-REACTOME-KEGG pathways”. Pathways positively correlated with *ENHO* expression were shown by positive NESs (normalized enrichment scores). Pathways negatively correlated with *ENHO* expression shown by negative NESs.

ENHO expression was positively associated with pathways related to catabolism and negatively associated with pathways related to anabolism. For example, “KEGG_OXIDATIVE_PHOSPHORYLATION”, “KEGG_FATTY_ACID_METABOLISM”, “REACTOME_RESPIRATORY_ELECTRON_TRANSPORT”. were strongly associated with *ENHO* expression. In contrast, “KEGG_STEROID_BIOSYNTHESIS”, “KEGG_RIBOSOME” and “KEGG_DNA_REPLICATION”, and “REACTOME_CHOLESTEROL_BIOSYNTHESIS” were depleted in *ENHO*-expressing samples. Interestingly, *ENHO* expression was also associated with low expression of some inflammatory pathways, such as “REACTOME_INFLAMMASOMES” and “REACTOME_THE_NLRP3_INFLAMMASOME”.

ENHO expression was also found to be associated with common neurodegenerative disorders, such as the Parkinson's disease, the Huntington's disease, or the Alzheimer's disease. This was shown by the significant positive or negative enrichment of “KEGG_PARKINSONS_DISEASE”, “KEGG_HUNTINGTONS_DISEASE” and “KEGG_ALZHEIMERS_DISEASE”, or the related pathways in *ENHO*-expressing or *ENHO*-depleted patient samples. Given that *ENHO* expression was also associated with increased mitochondrial activities indicated by the enrichment of pathways such as “KEGG_OXIDATIVE_PHOSPHORYLATION”, *ENHO* expression can be beneficial for these patients. Indeed, the enrichment of these neurodegenerative pathways were more significant in patients with Dementia than patients without Dementia. Finally, female patients show stronger enrichment of these pathways than male patients, suggesting that *ENHO* expression might be of more benefit to female relative to male patients with advanced dementia.

Correlations with aging-related neuropathologies

The source study also provides access to protein markers of dementia-related pathologies and inflammation by immunohistochemistry (IHC) on fresh frozen and formalin fixed paraffin embedded (FFPE) postmortem tissue samples⁶⁰. We first performed a simple bivariate correlation analysis using all samples, and in the ND and D subgroupings. Significant positive associations were observed between the levels of *ENHO* transcript with the expression of tau pathologies (AT8, ab plaques, AB40, pTau and pTau/Tau ratio) in the ND group (**Fig. 5A-D**), but less so in the D group (**Fig. 5E-H**). These relationships were further investigated by dividing participants into two groups ranked by *ENHO* expression (below or above the median) (**Fig. 5I-O**). More severe Tau-related pathologies appear to be evident in participants with high *ENHO* expression, particularly in participants with no dementia. Weaker relationships appear to be observed with inflammatory markers (for example with TNF α , MCP1 and IL1 β in **Fig. 6**, data not shown). There was no correlation between *ENHO* expression (averaged for all 4 structures) and CERAD, BRAAK, or NIA REAGAN scores (data not shown).

Adropin expression in the mouse brain

The observed results in humans suggest a complex relationship to known markers of dementia risk. Transcriptomic data suggest a positive correlation with pathways that could benefit brain function later in life (e.g., enhanced energy metabolism, morphogenesis). On the other hand, high *ENHO* expression also correlates with neuroinflammation. To determine whether increases in adropin function support the functions of neural networks in aging, we moved to the mouse model.

Open access data bases reporting single cell RNA-seq indicate less specificity in the cell types expressing the *ENHO* transcript expression in the mouse brain (**Fig. 4G**). High expression is observed in astrocytes, neurons and oligodendrocyte precursor cells; newly formed and myelinating oligodendrocytes and microglial/macrophages also express significant levels, while low expression is observed in endothelial cells⁶⁸. *ENHO* expression appears to peak between the 1st and 5th week of life in the cortex, hippocampus and striatum, and declines thereafter (**Fig. 4H-J**)⁶⁹. We also observed a decline in adropin protein levels in brain samples from 18-month old B6 mice compared to 4-month old animals (**Fig. 4K**). This pattern of adropin protein expression is similar to Sprague Dawley rats in which adropin protein levels in plasma and brain extracts are lower in 18-month old relative to 4-month old animals⁵⁰.

To investigate cell-specific patterns of *ENHO* expression, we generated a knockin-mouse model by inserting Cre into the *ENHO* locus (**Fig. 7**); mating these mice with CAG-lox-STOP-lox-tdTomato mice allowed us to assess cell-specific *ENHO* expression in the nervous system (**Fig. 7A-K**). Expression was observed throughout the brain. Of interest, expression was evident in hippocampal neurons, but surprisingly not in astrocytes (**Fig. 7A-F**). Expression was also observed in cortical neurons and endothelial (CD31⁺) cells (**Fig. 7G-K**). The latter observation is consistent with previous experiments using mouse and human endothelial cell lines²⁴. It is also consistent with our data showing an

association between *ENHO* expression GO processes involved in vascular function in the aged human brain.

Adropin treatment improves inflammatory markers in 18-month old B6 mice.

Male B6 mice exhibit evidence of cognitive impairment at around 18 months of age, corresponding to a human age of 56-69y⁷⁰. To determine whether increasing adropin expression improves neuroinflammation, we used transgenic mice over expressing adropin¹⁻⁷⁶ (AdrTG) under the control of a human b-actin promoter^{17,21,33,52}.

Increased adropin protein levels are observed in brain lysates from AdrTG mice compared to WT controls, irrespective of age (**Fig. 4K, L**). AdrTG aged up to 6 months exhibit modest but significant reductions of adiposity and protection from diet-induced obesity^{17,34}. Body weight and composition by NMR suggested a modest nutrient partitioning phenotype is still evident at 18 months of age (**Fig. S3A-F**). However, glucose tolerance was not significantly different (**Fig. S3G**).

Hippocampal expression of candidate genes representing pathways involved in inflammation, mitochondrial function, cellular stress responses and morphogenesis were compared in 18-month old male AdrTG and WT littermate controls. Reduced expression of genes involved in inflammation were observed (CCL2, IL13, IL17), however the other genes selected exhibit normal levels of expression (**Fig. 7A**). Similar differences in the expression of genes involved in inflammation were observed in samples from the cortex (CCL2, TNFA, IL17) (**Fig. 7B**). Measurement of serum cytokines identified a significant difference ($p < 0.05$) in IL-6 concentrations, with 60% lower levels in AdrTG mice (**Fig. 7C**). However, levels of TNFA (**Fig. 7C**) and other cytokines were not significantly different (**Fig. S4**). We also assessed for tau phosphorylation and markers of oxidative stress. However, levels of both were highly variable and mostly below the limits of detection (data not shown).

Improved cognitive performance and body weight homeostasis in 18-month old AdrTG mice

Mice aged 16-18 months were subjected to a series of tests investigating cognitive function and energy balance. Male AdrTG mice performed significantly better in tests of spatial learning and memory, and of recognition memory compared to age-matched WT controls (**Fig. 7D-E**). However, there was no difference in females.

One caveat to the transgenic mice approach is the phenotype is secondary to altered metabolic control and developmental processes earlier in life. To determine if adropin can reverse age-related cognitive decline, 18-month old male B6 mice were treated with synthetic peptide (adropin³⁴⁻⁷⁶). Mice were split into two weight matched groups (**Fig. S5A**), acclimated to handling and then treated with adropin³⁴⁻⁷⁶ (90 nmol/kg/d) suspended in 0.9% saline plus 0.1% BSA over 4 weeks. Controls were treated with diluent; injections were given daily at 0900h. The dose selected is compatible with previous experiments using male B6 mice in which significant biological responses were observed^{17,22,23}. After 2 weeks of treatment, trials examining spatial learning and memory indicated improvements in performance that were

compatible to those observed in AdrTG (**Fig. 7D-E**). Fasting glucose and insulin data were recorded at the completion of the study; no significant treatment effects were observed for either parameter (**Fig. S5B**)

We also compared the effects of aging on whole-body energy balance and ambulatory movement. This experiment used mice that were either 'middle aged' (M, 8-10 mo) or 'old' (O, 16-20 mo.).

The respiratory exchange ratio (RER, VCO_2/VO_2) provides an estimate of whole body substrate selection⁷¹. The RER increases during the dark period as chow-fed mice move to oxidizing ingested carbohydrates and relying less on mobilized fatty acid (**Fig. S6A, B**). WT-O mice exhibited low RER, indicating increased dependency on fat reserves (**Fig. S6A**). In females, the RER transition between light and dark periods was more pronounced in AdrTG, irrespective of age (**Fig. S6B**). Oxygen consumption exhibited the diurnal rhythm predicted from increased ambulatory activity and food intake during the dark period (**Fig. S6C, D**). This feature of energy metabolism was not markedly affected by genotype.

Food intake and changes in body weight during the recordings were consistent with RER data, indicating improved maintenance of body weight in M and O mice. Male WT-O mice lost weight during the recording (mean $\pm$ SD and n for weight gain/loss in g, young-WT -0.1 ± 0.2 , n=4; old-WT -5.8 ± 3.4 , n=3; young-AdrTG -0.4 ± 1.0 , n=4; old-AdrTG -1.7 ± 0.5 , n=3). Female WT mice also tended to lose more weight (mean $\pm$ SD for weight gain/loss in g, young-WT -3.0 ± 2.5 ; old-WT -1.6 ± 2.9 ; young-AdrTG -0.1 ± 0.5 ; old-AdrTG -0.6 ± 0.5 , n=4 all groups). Weight loss correlated with lower food intake during the recording period (**Fig. S6E**). Pooling data from male and female mice indicated WT mice were more prone to weight loss during recordings (-2.4 ± 3.0 vs. -0.6 ± 0.8 , $p<0.05$). Advanced age may exacerbate the phenotype (**Fig. S6F**).

Ambulatory movement exhibited the predicted diurnal profile, irrespective of age or sex or genotype (**Fig. S6G, H**). Aging of WT mice, but not in AdrTG mice, appeared to associate with reduced movement (**Fig. S6G-I**). A negative energy balance indicated by low food intake and weight loss might be predicted to account for this phenotype. Food intake is a strong predictor of weight gain/loss (**Fig. S7A**). However, it is not a strong predictor of ambulatory movement (**Fig. S7B**). Weight gain/loss is also only a weak predictor of ambulatory movement (**Fig. S7C**).

Overall, these results indicate a genotype effect on feeding behavior, substrate selection (glucose vs. fat oxidation) and ambulatory movement. As prolonged anorexia is not compatible with survival, a plausible explanation for the phenotype is superior adaptation of AdrTG after the transition from the home cage to metabolic caging.

Discussion

Gene and protein expression data from mammalian species clearly indicate adropin functions as a neuropeptide. Indeed, adropin protein is sufficiently abundant to allow measurement in the mouse brain using simple western blot techniques^{41,50,52}. The current results indicate that the functions of this abundant neuropeptide support brain health during aging. In human, NHP and mice transcriptomes expression of the *ENHO* transcript encoding adropin correlates positively with gene networks that support

mitochondrial energy metabolism in the brain. Glucose is the primary fuel sources of neurons. However, there is also evidence of an association with genes implicated in cholesterol metabolism.

Adropin regulates mitochondrial fuel selection to favor glucose oxidation in cardiac and skeletal muscle^{21,22,25,30}. The correlations with mitochondrial energy metabolism in children and mature adults suggest that this role extends to the nervous system. The brain accounts for 20% of the body's energy consumption, with glucose being the primary fuel source⁷². It is therefore tempting to speculate that there will be positive correlation between adropin expression and glucose utilization by the brain. However, the inverse associations with AKT3 and GSK3B indicate this interpretation is too simplistic. AKT3 belongs to a family of serine/threonine kinases (AKT1-3), and regulates brain development and cognitive function⁷³. The phosphorylation of GSK3 by AKT3 may also affect behavior⁷³. Given the roles of GSK3 in mediating tau hyperphosphorylation⁵⁶ and affecting behavior, the inverse association with *GSK3B* is also potentially of interest. Further experiments investigating the regulation of glucose metabolism in astrocytes and neurons, and of the relationships between adropin and GSK3 activity in the brain, are clearly needed.

The peaks observed in adropin expression early in life suggests a role for adropin in developmental processes. Adropin knockout mice display evidence of deficits in synapse formation, coupled with decreased locomotor activity and impaired motor coordination⁴¹. Growth and development of the human brain requires an abundance of calorie-dense diets that include essential fatty acids to provide energy and substrates for cell division, cellular morphogenesis and synaptic functions⁷⁴. With this in mind, it is interesting to note the positive correlations between circulating adropin levels and selection of calorie-dense diets with high fat content^{75,76}. Nutrient-sensing mechanisms have been identified that drive macronutrient preferences to match macronutrients selection with demand^{77,78}. It is tempting to speculate that the preference for high fat diets in people with high adropin levels correlates with increased demand in the nervous system. Future studies examining the relationship between adropin expression, energy requirements of the brain and nutrient selection are clearly important.

The correlation between *ENHO*, *APOE* and *CLU/ApoJ* expression in the group aged from birth to 40y is also of interest to aging. *APOE* has a critical role in mediating cholesterol transport in the brain⁵³. *CLU* has also been implicated in lipid metabolism, and also functions as extracellular chaperone linked to Ab clearance and toxicity⁵⁵. Our recent analysis of human clinical samples and nonhuman primates suggests a strong inverse correlation between plasma adropin concentrations and plasma markers of cholesterol metabolism^{33,34}. The relationship observed here suggests that high adropin expression in humans may correlate with significant differences in brain cholesterol metabolism.

The correlations between adropin and metabolic pathways appear to be conserved in mammals. However, in humans advanced age associates with changes in the pathways correlating with *ENHO* expression. The correlations observed in those with advanced age are also dependent on dementia status. In the 'old-old' without dementia, correlations develop with gene networks involved in

morphogenesis, including the growth of axonal and synaptic processes. Adropin has been implicated in brain development in mice ⁴¹, and here we observed potent effects of synthetic adropin on development of neuronal processes in cultured neurons, indicating a direct effect (see Fig. 4A). This could suggest that adropin plays a role in maintaining neuronal processes critically involved in cognitive function during aging.

In 'old-old' people diagnosed with dementia, the *ENHO* transcript clustered with gene networks involved in mitochondrial energy metabolism and angiogenesis. Synthetic adropin has been shown to regulate differentiation of human umbilical vein endothelial cells (HUVEC) into capillary-like structures, while transgenic over expression of adropin stimulated angiogenesis and improved blood flow in a mouse model of ischemia ²⁴. Clinical studies have observed positive correlations between circulating adropin levels and endothelial function ³⁸. These actions may have relevance to dementia, as reduced cerebrovascular blood flow has been linked to aging-related cognitive impairment and dementia ⁷⁹.

The results from studies using aged mice indicate higher adropin expression is beneficial in terms of cognitive performance. Furthermore, providing synthetic adropin as a supplement to aged mice appears to have therapeutic benefits in a situation where native expression in the brain is low. On the other hand, the positive correlations between *ENHO* expression and protein markers of neuroinflammation and tau pathology contradict this conclusion. There is considerable variation in signatures of brain inflammation; these markers do not always correlate with dementia status in the 'old-old' cohort used for the current study ⁶⁰.

It is unclear how to interpret the differences observed between the correlative data observed in post-mortem samples from people aged from birth to 40 years (mature adults) and the 'old-old'. Reduced fidelity in transcriptional and post-transcriptional mechanisms affecting transcript abundance is a plausible explanation. However, survivorship bias should also be considered. The transition to correlating with pathways involved in neural morphogenesis could indicate a survival advantage in the 'old-old' without dementia. A similar reasoning could be applied to the development of associations with gene networks involved in angiogenesis in the 'old-old' with dementia. This does not necessarily imply that high levels of adropin expression *per se* confers a survival benefit. Rather, the retention of gene networks including the *ENHO* gene may act in concert to confer a survival advantage in the old-old.

Another plausible theory is that aging-related transitions in gene networks that correlate with *ENHO* are a response to changes in the cellular environment. Cellular stress responses driving age-related inflammation could dominate, thereby altering the regulatory framework driving the transcriptional and post-transcriptional processes affecting the levels of *ENHO* and other transcripts. The observation that the gene networks correlating with *ENHO* differ between the 'old-old' diagnosed with dementia (mostly LOAD) and those who died with normal cognitive function is consistent with this theory.

Similarly, the trend for higher *ENHO* expression in the 'old-old' carriers of the APOE e4 allele could indicate survivor bias. GWAS have linked APOE gene variants to longevity ⁸⁰. Inheritance of the e4 allele increases

risk of early mortality from cardiovascular disease and Alzheimer's Disease. That *ENHO* expression tends to be higher in people aged over 75y with >0 APOE e4 alleles could indicate a selection advantage. On the other hand, the APOE e4 allele could associate with cellular phenotypes in the nervous system that increase *ENHO* expression.

It is important to note limitations of the human study. For example, differences in nutrition and environment during childhood between participants of the GTEx and the Aging, Dementia and TBI Study could be important. The participants in the latter study were born in the early 20th Century when obesity and type 2 diabetes were less prevalent. On the other hand, post-mortem samples in the GTEx portal come from people living in an obesogenic environment.

Mouse models were used to investigate whether higher adropin expression confers an advantage in aging. The AdrTG mice used for these studies exhibit increased adropin protein expression in the brain. However, the use of a ubiquitous promoter will increase expression in all tissues^{17,21,33,52}. The results from experiments using 18-month old male mice suggests that increased expression of adropin correlates with improved cognitive function, as assessed by tests of spatial learning and memory and novel object recognition. The use of mice over expressing adropin throughout the lifespan complicates the interpretation of these results. However, injections of adropin peptide improved cognitive performance in 18-month old B6 mice. The effects of adropin to improve cognitive performance can thus be dissociated from metabolic phenotypes earlier in life. Moreover, the results from this acute treatment experiment suggest that treatment with synthetic adropin can reverse aging-related declines of cognitive function.

The results from assessing whole body energy balance could be interpreted as indicating increased resilience in response to a novel environment. Old mice transferred to metabolic cages exhibit anorexia, reduced activity, weight loss and mobilization of fatty acids. This response was not evident in age matched AdrTG. Future studies examining how adropin over expression affect behavioral flexibility and stress responses in aging are clearly warranted. While technically challenging, behavioral studies using 'old-old' mice aged >2 years could also be informative.

A weakness with the mouse studies is the lack of apparent clear mechanism explaining the phenotype. Gene expression analysis suggests reduced inflammation as a potential factor. However, other mechanisms based on the known functions of adropin are possible that include effects on blood flow²⁴ or the blood brain barrier⁴⁰. Direct neurotrophic actions on neurons were also observed. However, it is not known whether adropin³⁴⁻⁷⁶ administered ip. penetrates the blood brain barrier.

The current results nevertheless provide an indication that high expression during the later stages of life, or administration of the synthetic peptide or derivative thereof, confer a benefit. Further experiments exploring mechanism are clearly indicated. Whether adropin expression correlates with lifespan, and whether over expression is effective in mouse models of severe LOAD, also needs to be explored.

In summary, current demographic trends indicate that the burden of dementia will increase this century as the population ages. The current study has applied a translational approach to identify a novel candidate for developing therapies to improve cognitive function in aging. Further investigation of the adropin neuropeptide with respect to cognitive function and brain health are clearly warranted.

Methods

Transcriptomic analysis.

Dataset pre-processing – The Aging, Dementia and TBI Study Patient datasets were downloaded from <https://aging.brain-map.org/download/index>. The data included: i) de-identified clinical information for 107 patient donors, ii) normalized gene-level FPKM (Fragments Per Kilobase of transcript per Million mapped reads) expression for 377 unique samples obtained from these donors, iii) A sample metadata table and iv) a gene ID and gene symbol table for 50281 unique genes. To determine whether the samples were normalized, we plotted the density of histograms for all samples. To perform the correlation analysis, we pre-processed the expression matrix using “ $\log_2(\text{FPKM}+1)$ ”. All analyses were performed using Bioconductor^{81,82} in R.

GSEA (Genome-wide gene set enrichment analysis) used a scaled correlation matrix to reveal *ENHO*-coregulated gene pathways⁸³. GSEA is used to evaluate the enrichment or depletion of a given gene set/pathway relative to one of the two functional states, which are distantly connected by a gradient of genes pre-ranked based on a measurable activity. Typically, these are differentially expressed genes. In our analysis, we defined the two states as, (i) a gene state positively associated with *ENHO* expression, and (ii) a gene state antagonistic or incompatible with *ENHO* expression.

To perform GSEA 5529 experimentally-verified biological pathways were downloaded from the Molecular Signature Database⁸⁴ (V7.1 C2) (<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>). Pearson Correlation coefficients were calculated between *ENHO* and each of the 50281 genes. This was done using the entire dataset containing all 377 samples or only a portion of them with specific features (Dementia/No Dementia, Male/Female or both). The correlation coefficients were then ranked, centered and scaled. The resulting pre-ranked correlation coefficients were used to search for enriched or depleted pathways from the 5529 experimentally verified biological pathways using the “fgsea” package in R. Genes not included in the MSigDB pathways were removed from the list of pre-ranked genes. Significant pathways with an adjusted p-value ≤ 0.05 were selected for further analyses. Top 20 enriched or depleted pathways were plotted. These significant pathways were saved into excel files.

For the other datasets referenced in the Result sections, data were downloaded and log transformed if needed. Genes were ranked by the correlation coefficient determined using Microsoft Excel. Functional enrichment analysis used the ToppFun tool (<https://toppgene.cchmc.org>).

Data availability – results from the analysis are available online (http://pharmacology.slu.edu/results/andrew/TBI_Dementia/); an interactive shiny serve is also available

at http://pharmacology.slu.edu/shiny/tbi_shiny/.

Experiments using B6 mice

Studies involving mice were conducted with the approval of the Animal Care and Use Committee at the Saint Louis University School of Medicine; St. Louis, MO. Sentinels from the facility were tested regularly to ensure our facility is virus- and pathogen- free. Food and water were available on an *ad libitum* basis and the rooms had a 12 hour light-dark cycle with lights on at 0600 hours. Behavioral experiments were conducted between 0730 and 1400 hours.

C57BL mice were purchased at 18 months of age from Jackson Laboratories (Bar Harbor, ME). Mice were acclimated to local conditions for 2 wk and then divided into two weight matched groups (n=15/group). The mice were treated with 90 nmol/kg/d adropin³⁴⁻⁷⁶ (ABClonal Science, 98.66% purity) suspended in 0.9% sterile saline with 0.1% BSA. The peptide was resuspended daily prior to injection and administered as a single ip. injection (0.1ml volume) at 0900h. Controls received saline diluent only. Mice were weighed daily; after 2 weeks of injections, mice were subjected to behavioral testing. Treatment continued during behavioral testing. At completion, mice were fasted for 6h. Blood glucose (tail snip) levels were recorded using a glucometer; blood samples were collected for analysis of measurement of insulin by ELISA (Ultra Sensitive Mouse Insulin ELISA kit, Crystal Chem USA, IL, USA). Brain tissue samples were collected and snap frozen using liquid nitrogen.

AdrTG were generated and maintained as previously described¹⁷. For aging studies, male mice were maintained in group housing (3-4/cage) on standard rodent chow. Fasting glucose and glucose clearance were assessed in mice fasted for 6h. Glucose tolerance tests were performed using 1 mg/kg dextrose administered intraperitoneally (ip.), as previously described⁵².

For visualization of cells expressing adropin, an IRES-Cre was inserted into the 3'untranslated region (UTR) of the adropin open reading frame in exon 2. The targeting vector was constructed using recombineering system. Isogenic DNA containing the Enho locus was retrieved from genomic colony RP23-100C7 of C57Bl/6 BAC genomic library via gap repair. An IRES-Cre-Frt-neo-Frt was inserted into 3' 53 bp downstream of the translational stop codon in exon 2. For gene targeting, 50µg of linearized targeting vector consisting of 3.5kb 5'arm and 7.2 kb 3' arm was electroporated into Bruce4 B6 embryonic stem (ES) cells. Homologous recombination in targeted clones was confirmed with Fidelity PCR at the 5' and 3' ends. The fragments produced from Fidelity PCR with these primers were sequenced to verify the correctness of recombination.

Correctly targeted ES cells were injected into Albino B6 blastocysts; germline transmitting chimeric mice were obtained and mated with Albino B6 mice to generate heterozygous carriers of the EnhoIRES-Cre-Frt-neo-Frt on the B6 background. The Frt-neo-Frt sequence was removed using B6;SJL-Tg(ACTFLPe)9205Dym/J transgenic mice purchased from the Jackson laboratory. EnhoIRES-Cre mice were then crossed onto the B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J strain in which a loxP-

flanked stop cassette prevents transcription of a red fluorescent protein variant (tdTomato) driven by a CAG promoter (5).

T-Maze training and testing procedures, Novel Object Recognition

The T-maze is both a learning task based on working-memory and a reference-memory task. The T-maze consisted of a black plastic alley with a start box at one end and two goal boxes at the other. The start box was separated from the alley by a plastic guillotine door that prevented movement down the alley until raised at the onset of training. An electrifiable floor of stainless-steel rods run throughout the maze to deliver a mild scrambled foot-shock.

Mice were not permitted to explore the maze prior to training. A block of training trials began when a mouse was placed into the start box. The guillotine door was raised and a cue buzzer sounded simultaneously; five seconds later, foot-shock was applied. The arm of the maze entered on the first trial was designated “incorrect” and the mild foot-shock was continued until the mouse entered the other goal box, which in all subsequent trials was designated as “correct” for the particular mouse. At the end of each trial, the mouse was returned to its home cage until the next trial.

Mice were trained until they made one avoidance. Training used an inter-trial interval of 60 seconds, the buzzer was of the door-bell type sounded at 55 dB, and shock was set at 0.35 mA (Coulbourn Instruments scrambled grid floor shocker model E13-08). Retention was tested one week later by continuing training until mice reached the criterion of five avoidances in six consecutive trials. The results were reported as the number of trials to criterion for the retention test.

Novel object recognition (NOR) was tested the five days following T-maze retention testing. NOR is a declarative memory task that involves the hippocampus when, as performed here, the retention interval is 24 hours after initial exposure to the objects⁸⁵. Mice were habituated to an empty apparatus for 5 minutes a day for 3 days prior to entry of the objects. During the training session, the mouse was exposed to two identical objects which it was allowed to examine for 5 minutes. The apparatus and the objects were cleaned between each mouse. 24 hours later, the mouse was exposed to one of the original objects and a new novel object in a new location and the amount of time spent examining each object was recorded. The novel object was made out of the same material as the original object and of the same size, but a different shape. This eliminated the possibility of smell associated with a particular object being a factor. The underlying concept of the task is based on the tendency of mice to spend more time exploring new, novel objects than familiar objects. The greater the retention/memory at 24 hours, the greater the discrimination index (DI). The time with new object (t_n) and time spent with the old object (t_o) was used to calculate the DI [$DI = (t_n - t_o) / (t_n + t_o)$]⁸⁶.

Measurements of gene and protein expression

Expression of candidate genes was assessed using qRT-PCR⁵². The extraction of the total RNA from was performed by using kits from Applied Biosystems. cDNA was synthesized with cDNA reverse transcription

kit from Quanta. PCR was conducted using a QuantStudio Realtime PCR machine, Applied Systems using primers from Integrated DNA Technology. Data were normalized using three reference genes (HPRT1, 36B4, PPIB; a list of primers and their sequence is provided in **Table S1**). Quantitative PCR was performed in 96-well plates using Taqman gene expression and QuantStudio 7 Detection Systems (Applied Biosystems, Life Technologies).

Adropin peptide levels in brain tissues lysate were assessed by Western blot as previously described using a mouse monoclonal antibody (Cayman Chemical cat. no. 14117, Ann Arbor, MI) (6). An HSP90 rabbit polyclonal antibody (Cell Signaling Technology cat. no. 4874, Danvers, MA) was used as a reference for loading.

Neuronal Cell Culture, Phase Contrast Imaging

Hippocampal neurons were dissected from B6 mice and cultured from newborn pups (postnatal day 2, P2) as described previously^{87,88}. Briefly, hippocampal tissue was isolated and single cells were dissociated with papain (50 µg/mL). Single-cell suspensions were created via trituration with pipettes of decreasing size. The cells were diluted in culture media (neurobasal medium, 2% B27, L-Glutamine [200mM], 4% FBS, and penicillin-streptomycin [Invitrogen]) and plated on dishes coated with poly-D-lysine (100 µg/mL) and laminin (2 µg/mL). For phase-contrast imaging, cells were cultured on glass-bottom petri dishes. For neuronal activity recording, primary cultured neurons were cultured on Multi-electrode array (MEA) Neurochip (Multi Channel Systems) was coated with poly-D-lysine and laminin, and cells were plated directly on the Neurochip. Hippocampal neurons were kept at 37°C in an airtight modular incubator chamber (Thermo-Forma) circulated with 5% carbon dioxide and medical air. Every 3-4 days, half of the culture media was replaced with fresh media.

To examine the effect of adropin on the formation of early networks, culture media supplemented with 1nM, 10nM, or 100nM adropin was added to culture dishes on the same day as plating (day 0). Hippocampal neurons grew for two days (day 2), and phase contrast images were taken with an inverted microscope (Olympus CKX53). To examine adropin effects on developed neuronal networks, primary hippocampal neurons were grown for nine days in normal neurobasal culture media. On day 9, 1nM, 10nM, or 100nM adropin was added to the culture media, and phase contrast images were taken five days later (day 14). In all phase contrast experiments, control dishes received no adropin, and microscope parameters remained the same between control and treatment dishes.

Recording of Neuronal Activity

The MEA2100-System Neurochip consists of 60 electrodes organized in an 8*8 grid, designed to capture neuronal electrical activity (Multi Channel Systems). Hippocampal neurons were cultured on the Neurochip as described above and grown for nine days (day 9) to allow for synapse formation. Neuronal activity was measured with the MEA2100-Lite headstage connected to the MCS-IFB interface board. The baseline of neuronal activity was first recorded for four minutes or until the neuronal activity among majority of electrodes was stabilized to act as a pre-treatment baseline. After four minutes, 100nM

adropin was added to the medium, and electrical activity recordings were taken for eight more minutes by the Multichannel Experimenter software.

Statistics

Mouse data were analyzed using Microsoft Excel or Graph Pad Prism software; differences between control and treatment groups were assessed by Student's *t*-test.

Declarations

Acknowledgements.

Andrew A. Butler acknowledges the financial support provided by Saint Louis University and NIH grant R21NS108138. Fenglian Xu acknowledges the financial support of Saint Louis University. Eduardo Candelario-Jalil acknowledges the support provided by NIH grants NS108138 and NS103094. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. Data used for the analyses described in this manuscript were obtained from the GTEx Portal on 09/14/20 .

The authors thank Jingying Zhang and Randall L. Mynatt at the Pennington Biomedical Research Center Transgenic Core for construction of the ENHO-Cre mice. The Transgenic Core is supported by the Clinical Nutrition Research Unit Center Grant 1P30 DK072476 entitled "Nutritional Programming: Environmental and Molecular Interactions" sponsored by the National Institutes of Health through the NIDDK (Eric Ravussin, principal investigator).

References

- 1 Korkki, S. M., Richter, F. R., Jeyarathnarajah, P. & Simons, J. S. Healthy ageing reduces the precision of episodic memory retrieval. *Psychol Aging* **35**, 124-142, doi:10.1037/pag0000432 (2020).
- 2 Langnes, E. *et al.* Anterior and posterior hippocampus macro- and microstructure across the lifespan in relation to memory-A longitudinal study. *Hippocampus* **30**, 678-692, doi:10.1002/hipo.23189 (2020).
- 3 Tromp, D., Dufour, A., Lithfous, S., Pebayle, T. & Despres, O. Episodic memory in normal aging and Alzheimer disease: Insights from imaging and behavioral studies. *Ageing Res Rev* **24**, 232-262, doi:10.1016/j.arr.2015.08.006 (2015).
- 4 Petersen, R. C. Mild cognitive impairment as a diagnostic entity. *J Intern Med* **256**, 183-194, doi:10.1111/j.1365-2796.2004.01388.x (2004).
- 5 Bennett, D. A. *et al.* Natural history of mild cognitive impairment in older persons. *Neurology* **59**, 198-205, doi:10.1212/wnl.59.2.198 (2002).

- 6 Morley, J. E. Mild cognitive impairment-a treatable condition. *J Am Med Dir Assoc* **15**, 1-5, doi:10.1016/j.jamda.2013.11.001 (2014).
- 7 Stefaniak, J. & O'Brien, J. Imaging of neuroinflammation in dementia: a review. *J Neurol Neurosurg Psychiatry* **87**, 21-28, doi:10.1136/jnnp-2015-311336 (2016).
- 8 Chandra, A. *et al.* Applications of amyloid, tau, and neuroinflammation PET imaging to Alzheimer's disease and mild cognitive impairment. *Hum Brain Mapp* **40**, 5424-5442, doi:10.1002/hbm.24782 (2019).
- 9 Bradburn, S., Murgatroyd, C. & Ray, N. Neuroinflammation in mild cognitive impairment and Alzheimer's disease: A meta-analysis. *Ageing Res Rev* **50**, 1-8, doi:10.1016/j.arr.2019.01.002 (2019).
- 10 Sindi, S. *et al.* Healthy Dietary Changes in Midlife Are Associated with Reduced Dementia Risk Later in Life. *Nutrients* **10**, doi:10.3390/nu10111649 (2018).
- 11 Kivipelto, M., Mangialasche, F. & Ngandu, T. Lifestyle interventions to prevent cognitive impairment, dementia and Alzheimer disease. *Nat Rev Neurol* **14**, 653-666, doi:10.1038/s41582-018-0070-3 (2018).
- 12 Jastreboff, A. M., Kotz, C. M., Kahan, S., Kelly, A. S. & Heymsfield, S. B. Obesity as a Disease: The Obesity Society 2018 Position Statement. *Obesity (Silver Spring)* **27**, 7-9, doi:10.1002/oby.22378 (2019).
- 13 in *Future Directions for the Demography of Aging: Proceedings of a Workshop* (eds M. K. Majmundar & M. D. Hayward) (2018).
- 14 Makarewich, C. A. & Olson, E. N. Mining for Micropeptides. *Trends Cell Biol* **27**, 685-696, doi:10.1016/j.tcb.2017.04.006 (2017).
- 15 Andrews, S. J. & Rothnagel, J. A. Emerging evidence for functional peptides encoded by short open reading frames. *Nat Rev Genet* **15**, 193-204, doi:10.1038/nrg3520 (2014).
- 16 Saghatelian, A. & Couso, J. P. Discovery and characterization of smORF-encoded bioactive polypeptides. *Nat Chem Biol* **11**, 909-916, doi:10.1038/nchembio.1964 (2015).
- 17 Kumar, K. G. *et al.* Identification of adropin as a secreted factor linking dietary macronutrient intake with energy homeostasis and lipid metabolism. *Cell metabolism* **8**, 468-481, doi:10.1016/j.cmet.2008.10.011. PMID: 2746325 (2008).
- 18 Uhlen, M. *et al.* Proteomics. Tissue-based map of the human proteome. *Science* **347**, 1260419, doi:10.1126/science.1260419 (2015).
- 19 Wang, P. *et al.* Mapping spatial transcriptome with light-activated proximity-dependent RNA labeling. *Nat Chem Biol* **15**, 1110-1119, doi:10.1038/s41589-019-0368-5 (2019).

- 20 Butler, A. A. *et al.* Low circulating adropin concentrations with obesity and aging correlate with risk factors for metabolic disease and increase after gastric bypass surgery in humans. *The Journal of clinical endocrinology and metabolism* **97**, 3783-3791, doi:10.1210/jc.2012-2194. PMCID: 3462944 (2012).
- 21 Gao, S. *et al.* Regulation of substrate oxidation preferences in muscle by the peptide hormone adropin. *Diabetes* **63**, 3242-3252, doi:10.2337/db14-0388. PMCID: 4171656 (2014).
- 22 Gao, S. *et al.* Therapeutic effects of adropin on glucose tolerance and substrate utilization in diet-induced obese mice with insulin resistance. *Mol Metab* **4**, 310-324, doi:10.1016/j.molmet.2015.01.005. PMCID:PMC4354928 (2015).
- 23 Gao, S. *et al.* The peptide hormone adropin regulates signal transduction pathways controlling hepatic glucose metabolism in a mouse model of diet-induced obesity. *J Biol Chem* **294**, 13366-13377, doi:10.1074/jbc.RA119.008967 (2019).
- 24 Lovren, F. *et al.* Adropin is a novel regulator of endothelial function. *Circulation* **122**, S185-192, doi:10.1161/CIRCULATIONAHA.109.931782 (2010).
- 25 Thapa, D. *et al.* Adropin regulates pyruvate dehydrogenase in cardiac cells via a novel GPCR-MAPK-PDK4 signaling pathway. *Redox Biol* **18**, 25-32, doi:10.1016/j.redox.2018.06.003 (2018).
- 26 Thapa, D. *et al.* Adropin reduces blood glucose levels in mice by limiting hepatic glucose production. *Physiol Rep* **7**, e14043, doi:10.14814/phy2.14043 (2019).
- 27 Thapa, D. *et al.* Adropin treatment restores cardiac glucose oxidation in pre-diabetic obese mice. *J Mol Cell Cardiol*, doi:10.1016/j.yjmcc.2019.02.012 (2019).
- 28 Stein, L. M., Yosten, G. L. & Samson, W. K. Adropin acts in brain to inhibit water drinking: potential interaction with the orphan G protein-coupled receptor, GPR19. *Am J Physiol Regul Integr Comp Physiol* **310**, R476-480, doi:10.1152/ajpregu.00511.2015 (2016).
- 29 Rao, A. & Herr, D. R. G protein-coupled receptor GPR19 regulates E-cadherin expression and invasion of breast cancer cells. *Biochim Biophys Acta Mol Cell Res* **1864**, 1318-1327, doi:10.1016/j.bbamcr.2017.05.001 (2017).
- 30 Altamimi, T. R. *et al.* Adropin regulates cardiac energy metabolism and improves cardiac function and efficiency. *Metabolism* **98**, 37-48, doi:10.1016/j.metabol.2019.06.005 (2019).
- 31 Ganesh Kumar, K. *et al.* Adropin deficiency is associated with increased adiposity and insulin resistance. *Obesity (Silver Spring)* **20**, 1394-1402, doi:10.1038/oby.2012.31 (2012).
- 32 Lian, A., Wu, K., Liu, T., Jiang, N. & Jiang, Q. Adropin induction of lipoprotein lipase expression in tilapia hepatocytes. *J Mol Endocrinol* **56**, 11-22, doi:10.1530/JME-15-0207 (2016).

- 33 Ghoshal, S. *et al.* Adropin: An endocrine link between the biological clock and cholesterol homeostasis. *Mol Metab* **8**, 51-64, doi:10.1016/j.molmet.2017.12.002 (2018).
- 34 Butler, A. A. *et al.* Low plasma adropin concentrations increase risks of weight gain and metabolic dysregulation in response to a high-sugar diet in male nonhuman primates. *J Biol Chem* **294**, 9706-9719, doi:10.1074/jbc.RA119.007528 (2019).
- 35 Chen, X. *et al.* Adropin protects against liver injury in nonalcoholic steatohepatitis via the Nrf2 mediated antioxidant capacity. *Redox Biol* **21**, 101068, doi:10.1016/j.redox.2018.101068 (2019).
- 36 Chen, X. *et al.* Adropin regulates hepatic glucose production via PP2A/AMPK pathway in insulin-resistant hepatocytes. *FASEB J*, doi:10.1096/fj.202000115RR (2020).
- 37 Zhang, C., Zhang, Q., Huang, Z. & Jiang, Q. Adropin inhibited tilapia hepatic glucose output and triglyceride accumulation via AMPK activation. *J Endocrinol* **246**, 109-122, doi:10.1530/JOE-20-0077 (2020).
- 38 Kwon, O. S., Andtbacka, R. H. I., Hyngstrom, J. R. & Richardson, R. S. Vasodilatory function in human skeletal muscle feed arteries with advancing age: the role of adropin. *J Physiol*, doi:10.1113/JP277410 (2019).
- 39 Wu, L., Fang, J., Yuan, X., Xiong, C. & Chen, L. Adropin reduces hypoxia/reoxygenation-induced myocardial injury via the reperfusion injury salvage kinase pathway. *Exp Ther Med* **18**, 3307-3314, doi:10.3892/etm.2019.7937 (2019).
- 40 Yang, C., DeMars, K. M., Hawkins, K. E. & Candelario-Jalil, E. Adropin reduces paracellular permeability of rat brain endothelial cells exposed to ischemia-like conditions. *Peptides*, doi:10.1016/j.peptides.2016.03.009 (2016).
- 41 Wong, C. M. *et al.* Adropin is a brain membrane-bound protein regulating physical activity via the NB-3/Notch signaling pathway in mice. *J Biol Chem* **289**, 25976-25986, doi:10.1074/jbc.M114.576058 (2014).
- 42 Fagerberg, L. *et al.* Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics* **13**, 397-406, doi:10.1074/mcp.M113.035600 (2014).
- 43 Mure, L. S. *et al.* Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. *Science* **359**, doi:10.1126/science.aao0318 (2018).
- 44 Consortium, G. T. The Genotype-Tissue Expression (GTEx) project. *Nat Genet* **45**, 580-585, doi:10.1038/ng.2653 (2013).

- 45 O'Dowd, B. F. *et al.* A novel gene codes for a putative G protein-coupled receptor with an abundant expression in brain. *FEBS Lett* **394**, 325-329, doi:10.1016/0014-5793(96)00901-5 (1996).
- 46 Hoffmeister-Ullrich, S. A., Susens, U. & Schaller, H. C. The orphan G-protein-coupled receptor GPR19 is expressed predominantly in neuronal cells during mouse embryogenesis. *Cell Tissue Res* **318**, 459-463, doi:10.1007/s00441-004-0948-9 (2004).
- 47 Alexander, S. P. *et al.* Class A Orphans (version 2019.5) in the IUPHAR/BPS Guide to Pharmacology Database. *IUPHAR/BPS Guide to Pharmacology CITE* (**5**), doi:doi.org/10.2218/gtopdb/F16/2019.5. (2019).
- 48 Foster, S. R. *et al.* Discovery of Human Signaling Systems: Pairing Peptides to G Protein-Coupled Receptors. *Cell* **179**, 895-908 e821, doi:10.1016/j.cell.2019.10.010 (2019).
- 49 Chatterjee, M., Schild, D. & Teunissen, C. E. Contactins in the central nervous system: role in health and disease. *Neural Regen Res* **14**, 206-216, doi:10.4103/1673-5374.244776 (2019).
- 50 Yang, C., DeMars, K. M. & Candelario-Jalil, E. Age-Dependent Decrease in Adropin is Associated with Reduced Levels of Endothelial Nitric Oxide Synthase and Increased Oxidative Stress in the Rat Brain. *Aging Dis* **9**, 322-330, doi:10.14336/AD.2017.0523 (2018).
- 51 Zhang, Y. *et al.* Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron* **89**, 37-53, doi:10.1016/j.neuron.2015.11.013 (2016).
- 52 Banerjee, S. *et al.* Hepatocyte expression of the micropeptide adropin regulates the liver fasting response and is enhanced by caloric restriction. *J Biol Chem*, doi:10.1074/jbc.RA120.014381 (2020).
- 53 Lanfranco, M. F., Ng, C. A. & Rebeck, G. W. ApoE Lipidation as a Therapeutic Target in Alzheimer's Disease. *Int J Mol Sci* **21**, doi:10.3390/ijms21176336 (2020).
- 54 Yamazaki, Y., Zhao, N., Caulfield, T. R., Liu, C. C. & Bu, G. Apolipoprotein E and Alzheimer disease: pathobiology and targeting strategies. *Nat Rev Neurol* **15**, 501-518, doi:10.1038/s41582-019-0228-7 (2019).
- 55 Foster, E. M., Dangla-Valls, A., Lovestone, S., Ribe, E. M. & Buckley, N. J. Clusterin in Alzheimer's Disease: Mechanisms, Genetics, and Lessons From Other Pathologies. *Front Neurosci* **13**, 164, doi:10.3389/fnins.2019.00164 (2019).
- 56 Hernandez, F., Lucas, J. J. & Avila, J. GSK3 and tau: two convergence points in Alzheimer's disease. *J Alzheimers Dis* **33 Suppl 1**, S141-144, doi:10.3233/JAD-2012-129025 (2013).
- 57 Miao, L. *et al.* mTORC1 is necessary but mTORC2 and GSK3beta are inhibitory for AKT3-induced axon regeneration in the central nervous system. *Elife* **5**, e14908, doi:10.7554/eLife.14908 (2016).

- 58 Sousa, A. M. M. *et al.* Molecular and cellular reorganization of neural circuits in the human lineage. *Science* **358**, 1027-1032, doi:10.1126/science.aan3456 (2017).
- 59 Castanho, I. *et al.* Transcriptional Signatures of Tau and Amyloid Neuropathology. *Cell Rep* **30**, 2040-2054 e2045, doi:10.1016/j.celrep.2020.01.063 (2020).
- 60 Miller, J. A. *et al.* Neuropathological and transcriptomic characteristics of the aged brain. *Elife* **6**, doi:10.7554/eLife.31126 (2017).
- 61 Kukull, W. A. *et al.* Dementia and Alzheimer disease incidence: a prospective cohort study. *Arch Neurol* **59**, 1737-1746, doi:10.1001/archneur.59.11.1737 (2002).
- 62 Larson, E. B. *et al.* Exercise is associated with reduced risk for incident dementia among persons 65 years of age and older. *Ann Intern Med* **144**, 73-81, doi:10.7326/0003-4819-144-2-200601170-00004 (2006).
- 63 Crane, P. K. *et al.* Glucose levels and risk of dementia. *N Engl J Med* **369**, 540-548, doi:10.1056/NEJMoa1215740 (2013).
- 64 Sonnen, J. A. *et al.* Neuropathology in the adult changes in thought study: a review. *J Alzheimers Dis* **18**, 703-711, doi:10.3233/JAD-2009-1180 (2009).
- 65 Monoranu, C. M. *et al.* pH measurement as quality control on human post mortem brain tissue: a study of the BrainNet Europe consortium. *Neuropathol Appl Neurobiol* **35**, 329-337, doi:10.1111/j.1365-2990.2008.01003a.x (2009).
- 66 Mills, J. D. *et al.* The alternative splicing of the apolipoprotein E gene is unperturbed in the brains of Alzheimer's disease patients. *Mol Biol Rep* **41**, 6365-6376, doi:10.1007/s11033-014-3516-8 (2014).
- 67 Kwon, O. S., Andtbacka, R. H. I., Hyngstrom, J. R. & Richardson, R. S. Vasodilatory function in human skeletal muscle feed arteries with advancing age: the role of adropin. *J Physiol* **597**, 1791-1804, doi:10.1113/JP277410 (2019).
- 68 Zhang, Y. *et al.* An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci* **34**, 11929-11947, doi:10.1523/JNEUROSCI.1860-14.2014 (2014).
- 69 Clarke, L. E. *et al.* Normal aging induces A1-like astrocyte reactivity. *Proc Natl Acad Sci U S A* **115**, E1896-E1905, doi:10.1073/pnas.1800165115 (2018).
- 70 Flurkey, K., Curren, J. M. & Harrison, D. E. in *The Mouse in Biomedical Research 2nd Edition* (eds J.G. Fox *et al.*) (Elsevier, 2007).

- 71 Arch, J. R., Hislop, D., Wang, S. J. & Speakman, J. R. Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int J Obes (Lond)* **30**, 1322-1331, doi:10.1038/sj.ijo.0803280 (2006).
- 72 Magistretti, P. J. & Allaman, I. A cellular perspective on brain energy metabolism and functional imaging. *Neuron* **86**, 883-901, doi:10.1016/j.neuron.2015.03.035 (2015).
- 73 Ouimet, B. *et al.* Motor learning deficits and striatal GSK-3 hyperactivity in Akt3 knockout mice. *Behav Neurosci* **133**, 135-143, doi:10.1037/bne0000292 (2019).
- 74 Gomez-Pinilla, F. Brain foods: the effects of nutrients on brain function. *Nat Rev Neurosci* **9**, 568-578, doi:10.1038/nrn2421 (2008).
- 75 St-Onge, M. P. *et al.* Fasting plasma adropin concentrations correlate with fat consumption in human females. *Obesity (Silver Spring)* **22**, 1056-1063, doi:10.1002/oby.20631. PMCID: 3968187 (2014).
- 76 Stevens, J. R. *et al.* Inverse association between carbohydrate consumption and plasma adropin concentrations in humans. *Obesity (Silver Spring)* **24**, 1731-1740, doi:10.1002/oby.21557. PMCID:PMC5184848 (2016).
- 77 van der Klaauw, A. A. *et al.* Divergent effects of central melanocortin signalling on fat and sucrose preference in humans. *Nat Commun* **7**, 13055, doi:10.1038/ncomms13055 (2016).
- 78 Hill, C. M. *et al.* FGF21 and the Physiological Regulation of Macronutrient Preference. *Endocrinology* **161**, doi:10.1210/endocr/bqaa019 (2020).
- 79 Fouda, A. Y., Fagan, S. C. & Ergul, A. Brain Vasculature and Cognition. *Arterioscler Thromb Vasc Biol* **39**, 593-602, doi:10.1161/ATVBAHA.118.311906 (2019).
- 80 Abondio, P. *et al.* The Genetic Variability of APOE in Different Human Populations and Its Implications for Longevity. *Genes (Basel)* **10**, doi:10.3390/genes10030222 (2019).
- 81 Mei, H. *et al.* Expression array annotation using the BioMediator biological data integration system and the BioConductor analytic platform. *AMIA Annu Symp Proc*, 445-449 (2003).
- 82 Gentleman, R. C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**, R80, doi:10.1186/gb-2004-5-10-r80 (2004).
- 83 Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550, doi:0506580102 [pii] 10.1073/pnas.0506580102 (2005).
- 84 Subramanian, A., Kuehn, H., Gould, J., Tamayo, P. & Mesirov, J. P. GSEA-P: a desktop application for Gene Set Enrichment Analysis. *Bioinformatics* **23**, 3251-3253, doi:btm369 [pii]

10.1093/bioinformatics/btm369 (2007).

85 Hammond, R. S., Tull, L. E. & Stackman, R. W. On the delay-dependent involvement of the hippocampus in object recognition memory. *Neurobiol Learn Mem* **82**, 26-34 (2004).

86 Pedros, I. *et al.* Early alterations in energy metabolism in the hippocampus of APPswe/PS1dE9 mouse model of Alzheimer's disease. *Biochim Biophys Acta* **1842**, 1556-1566, doi:10.1016/j.bbadis.2014.05.025 (2014).

87 Getz, A. M., Xu, F., Visser, F., Persson, R. & Syed, N. I. Tumor suppressor menin is required for subunit-specific nAChR alpha5 transcription and nAChR-dependent presynaptic facilitation in cultured mouse hippocampal neurons. *Sci Rep* **7**, 1768, doi:10.1038/s41598-017-01825-x (2017).

88 Mersman, B., Zaidi, W., Syed, N. I. & Xu, F. Taurine Promotes Neurite Outgrowth and Synapse Development of Both Vertebrate and Invertebrate Central Neurons. *Front Synaptic Neurosci* **12**, 29, doi:10.3389/fnsyn.2020.00029 (2020).

Figures

Figure 1

ENHO expression in the 'old-old' human brain. (A) Comparison of ENHO transcript expression (in TPM) in human tissues. The expression data have been ranked high to low when reading left to right. (B) Single-cell RNA-seq data showing expression in cell populations in the human brain. (C) ENHO expression by age group. Data were pooled into 10-year bins (birth-9y, 10-19y, 20-29y, and 30-39y). The top panel shows transcript expression, the bottom panel shows age. * $p < 0.05$ vs. all other groups combined (D-E) Correlations between expression levels of the ENHO and APOE (D), Clusterin/ApoJ (E), GSK3B (F), and AKT3 (G) transcripts.

Figure 2

Clustering of ENHO with gene networks in the brain of the old-old depends on dementia stats. (A, B) Correlation coefficients (r) for the top 1% of genes ranked high to low by r. Data are shown as a scatterplot (A) or box-and-whisker plot (B) showing tighter correlations in people with dementia (D) compared to those with no dementia (ND). (C) Overlap in gene clusters shown in panel A. (D) Heatmaps for the most significant GO:Biological Process correlating with ENHO in people without dementia (ND) or with dementia (D). (E, F) Scatterplots showing correlations between relative ENHO expression (x-axis) and relative expression of FGFR3 and TN1K in ND participants (C), or with MAOA and LDHD in D participants. The two genes selected for each group had the highest correlation coefficients.

Figure 3

GO biological processes in gene groups meeting the selection criteria from participants with dementia (D), with no dementia (ND).

Figure 4

Adropin effects on neurogenesis and expression profile in the mouse brain. (A-F) Adropin promotes mouse hippocampal neural growth and neuronal firing activities of primary cultured hippocampal neurons. (A) Primary cultured hippocampal neurons were cultured in the absence (control) and presence of 1,10 and 100 nM adropin. Phase contrast images were taken at day 2 (early network) and day 14 (mature network). The preliminary data show that neurons cultured in the presence of adropin at all concentrations exhibited longer neurites at day 2. They already formed contact with neurons nearby and also farther away. Control neurons showed much shorter neurites and they form contact with neurons nearby only. At day 14, adropin treated neurons also showed more branches and interconnections, and their neurites are longer.(B) Hippocampal neurons cultured on a MEA chip. (C) Higher magnification image showing electrode and neurons. (D-F). Sample traces of neuronal spiking activity before and after exposure to adropin (100 nM) using the multichannel recording system. The preliminary data show that

Adropin increased the spontaneous spiking firing activity in neurons either previously quiescent (D, E) or active (F). (G-L) ENHO expression declines with in aging mouse brain. (G) Single-cell RNAseq profiling of ENHO expression in the mouse brain. (H-J) Expression profile in mouse brain structures by age showing a peak at postnatal day 32 (P32) following by a decline up to 2y of age. (K, L) Increased levels of adropin immunoreactivity in brain lysates of AdrTG mice compared to WT littermates. (K) Western blot with HSP90 used as a loading control. (F) Adropin protein expressed as a ratio to HSP90 (right). N=3/group.

Figure 5

Scatterplots showing relationships between relative ENHO expression (x-axis) and protein markers of aging-related neuropathology. Data are shown for study participants without no dementia (A-D) or with dementia (E-H). The markers shown reading top to bottom are AT8 (tau phosphorylation of Ser202 and Thr205), TAU (total phosphorylated tau, and ratio of phosphorylated to non-phosphorylated tau), A β , AB40 (A β 40), and p. Correlation coefficients are shown where associations are significant ($p<0.05$). For

AT8 and A β staining, data are shown from fresh frozen (ffpe) or paraffin-embedded sections. The data were subdivided into two groups (below or above the median) after being ranked by ENHO expression. The median is shown as the red dashed line in the scatterplots shown in panels A-H. Ranking occurred for all participants (All), or for subgroups separated by dementia status (ND, no dementia; D, dementia). Significantly different from low dementia status, ** $p < 0.01$; * $p < 0.05$.

Figure 6

Relationships between ENHO expression and protein markers of neuroinflammation in study participants. The top three panels show the data as scatterplots; TNF α , MCP1 or IL1 β are plotted against relative ENHO expression. The bottom three panels show TNF α , MCP1 or IL1 β in participants divided into two groups (below or above the median, labelled “low” or “high”) after being ranked by ENHO expression.

Figure 7

Identification of Enho+ve cells types in the mouse brain using an ENHO-Cre reporter mouse. The top panel is a schematic of the approach used to generate an ENHO-Cre reporter mouse. The lower panels (A-K) show photomicrographs from hippocampal sections (A-F) and high magnification photomicrographs of vessels in the cortex (G-K).

Figure 8

Reduced expression of genes involved in inflammation in the hippocampus and cortex of 18-month old AdrTG mice compared to age matched controls. Expression data (mean, SD) for candidate genes in the hippocampus (A) and cortex (B) are shown. (C) Serum levels of IL-6 but not TNFA are lower in AdrTG compared to controls. (D-E) Improved performance in tests of spatial learning and memory (aversive T-maze) or novel object recognition of 18-month old AdrTG or B6 mice treated with adropin34-76 for 2 weeks compared to the respective age-matched control group (WT littermates for AdrTG; mice treated with diluent with adropin34-76). Significance is indicated by * (p<0.05), ** (p<0.01), or *** (p<0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [BanerjeeGhoshalGirardetADROPINANDAGINGSupplInf.TableS1FigureS1S8.pdf](#)