Ablation of Amyloid Precursor Protein Increases Insulin Degrading Enzyme Levels and Activity in Brain and Peripheral Tissues

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Abstract

The amyloid precursor protein (APP) is a type-I transmembrane glycoprotein widely studied for its role as the source of β-amyloid peptide, accumulation of which is causal in at least some cases of Alzheimer's disease (AD). APP is expressed ubiquitously and is involved in diverse biological processes. Growing bodies of evidence indicate connections between AD and somatic metabolic disorders related to type-2 diabetes, and *App*$^{-/-}$ mice show alterations in glycemic regulation. We find that *App*$^{-/-}$ have higher levels of insulin-degrading enzyme (IDE) mRNA, protein, and activity compared to wild-type controls. This regulation of IDE by APP was widespread across numerous tissues including liver, skeletal muscle, and brain as well as cell types within neural tissue including neurons, astrocytes, and microglia. RNAi-mediated knockdown of APP in the SIM-A9 microglia cell line elevated IDE levels. Fasting levels of blood insulin were lower in *App*$^{-/-}$ than *App*$^{+/+}$ mice, but the former showed a larger increase in response to glucose. These low basal levels may enhance peripheral insulin sensitivity, as *App*$^{-/-}$ mice failed to develop impairment of glucose tolerance on a high-fat, high-sucrose ("western") diet. Insulin levels and insulin signaling were also lower in *App*$^{-/-}$ brain; synaptosomes prepared from *App*$^{-/-}$ hippocampus showed diminished insulin receptor phosphorylation compared to *App*$^{+/+}$ mice when stimulated *ex vivo*. These findings represent a new molecular link connecting APP to metabolic homeostasis and demonstrate a novel role for APP as an upstream regulator of IDE *in vivo*.

Keywords: Amyloid precursor protein, Insulin degrading enzyme, Alzheimer's disease, insulin, microglia

INTRODUCTION

Alzheimer's disease is characterized in part by the robust accumulation of extracellular plaque aggregates of the amyloid β-peptide (Aβ) in the central nervous system, and multiple lines of evidence indicates that Aβ is a causal factor in AD (24, 77). While familial AD appears to arise from elevated production of longer (42- or 43-amino acid) forms of Aβ, sporadic cases of AD may be more dependent upon compromised clearance of the peptide, which comprises both efflux from the brain into the vasculature and proteolysis. Leading candidates for proteolytic destruction of Aβ include the metalloproteases neprilysin and insulin-degrading peptide (IDE) (5). IDE is a zinc metalloprotease discovered for its ability to hydrolyze insulin, but it has numerous other substrates; in addition to Aβ, it is active against pancreatic amylin (44). All insulin-sensitive tissues degrade insulin, and IDE is established to play an important role in regulating insulin signaling and an essential role in insulin catabolism (16).

Aβ is generated by enzymatic cleavage of the much larger amyloid precursor protein (APP). The genes encoding APP are widely conserved among other species, suggesting the protein performs important or advantageous biological functions (82). APP is part of a three-member family which includes amyloid precursor-like protein (APLP)-1 and -2 (59). While expression of APLP1 is restricted to the central nervous system, both APP and APLP2 are expressed ubiquitously and can be detected in a variety of tissues (49, 78); only APP contains the Aβ sequence. APP and APLPs have diverse and critical roles in development and physiology, both in and out of the CNS (8), highlighted by the neonatal lethality of *App*/*Aplp2* double knockouts (31). Deletion of the *App* gene alone confers a more benign phenotype, including reduced animal

body weight and organ size, changes in grip strength and broad changes in metabolism (58, 71, 92).

Mounting evidence indicates connections between AD and perturbations in insulin/glucose regulation related to type-2 diabetes mellitus (T2DM) (28, 33, 51, 57, 80, 81). APP may be involved in these links, as illustrated by several lines of evidence. For instance, genetic ablation of APP perturbs basal glycemic levels (63). A recent report demonstrates numerous metabolic differences in *App*$^{-/-}$ mice, including the finding that circulating levels of insulin are lower in *App*$^{-/-}$ mice (12). In tissue from obese human subjects, increased expression of APP in adipocytes correlates with the degree of insulin resistance, hyperinsulinemia, and plasma A$\beta_{1\text{-}40}$ levels (47, 48). In wild-type C57BL/6 mice, high-fat diet feeding increases the expression of APP in both hippocampus and adipose tissue (72).

Among its diverse actions, APP contributes to cell adhesion and other aspects of extracellular matrix that appear to involve complex relationships with proteases (85). In addition to its processing by the α-, β-, γ-, and ε-secretases, APP itself modulates protease activity. The splice variants expressed in nonneuronal cells contain a Kunitz-type serine protease inhibitor (KPI) domain (9), and other protease inhibitor domains are universal to all splice variants (30, 34). In our recent work, we found that IDE levels are higher in pancreatic tissue extracts and primary pancreatic islet cultures derived from *App*$^{-/-}$ compared to *App*$^{+/+}$ mice (42). In this study we have expanded upon our initial observation of increased IDE in the *App*$^{-/-}$ pancreas by demonstrating that regulation of IDE by APP occurs in other tissues, including the brain, highlighting implications for metabolism of Aβ.

MATERIALS AND METHODS

Animals

The APP knockout mice (*App*$^{-/-}$) strain B6.129S7-App^{tm1Dbo}/J (https://www.jax.org/strain/004133) and wild-type (*App*$^{+/+}$) C57BL/6 mice, were purchased from the Jackson Laboratory (Bar Harbor ME). APP is knocked out in *App*$^{-/-}$ mice by deletion of the promoter region and Exon 1 of the *App* gene (92). Both male and female mice were utilized in this study. Where indicated, normal mouse chow was replaced with "western diet" (ENVIGO TD.88137): 42% kcal from fat, 34% sucrose by weight. Western diet TD.88137 has the following composition by weight: 17.3% protein, 48.5% carbohydrates and 21.2% fat with a calorie content of 4.5 kcal/g. Two control diets were used (ENVIGO TD.08485 and LabDiet JL Rat and Mouse/Auto 6F FK67) having 17 – 19% protein, 55 – 61% carbohydrate, and 5.2 – 7.3% fat by weight. Control diets had a composition of 13 – 16% kcal from fat and 3.45 – 3.60 kcal/g. Such studies began with experimentally naïve male mice that were 8 weeks of age (first GTT), and diets deviated at 9 weeks of age. Animals were euthanized by CO_2 asphyxiation and cardiac exsanguination and blood was cleared by perfusion with phosphate-buffered saline. Animal use was approved by the University of North Dakota Institutional Animal Care and Use Committee and the Central Arkansas Veterans Healthcare System Animal Care and Use Committee. Mice were provided with food and water *ad libitum* and housed in a 12-hour light/dark cycle. This study conforms to the National Research council of the National Academies Guide for the Care and Use of Laboratory Animals.

Antibodies

Antibodies against full-length APP (ab32136), IDE (ab32216) were purchased from Abcam (Cambridge, MA) (10, 43). Antibodies p-AKT Thr308 (13038) and AKT (C67E7) were purchased from Cell Signaling Technology, Inc. (Danvers MA). The polyclonal antibody against IDE utilized for immunohistochemistry was purchased from Biolegend product 840301 (San Diego CA) and was formerly available from Covance as product PRB-282C (88). Anti-GAPDH antibody (6C5) and HRP-conjugated secondary antibodies were purchased from Santa Cruz Technologies (Santa Cruz CA). Antibodies used in WES capillary western blots included β-tubulin (Cell Signaling #2146S), insulin receptor (Cell Signaling #3025S), and p-IGF1R-IRβ (Cell Signaling #3024S).

RNA Extraction and RT-qPCR

Total RNA from hippocampus tissue from *App*$^{+/+}$ and *App*$^{-/-}$ mice was isolated using Trizol Reagent (Thermo Fisher Scientific, Waltham MA) according to the manufacturer's instruction. Briefly, the tissue samples preserved in Allprotect Tissue Reagent (Qiagen Inc., Valencia CA) were washed once with PBS and homogenized in Trizol using a Bullet Blender Storm 24 tissue homogenizer (Next Advance, Inc., Averill Park NY) with 5-mm stainless-steel beads (Qiagen). Extracted RNA samples were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). For each sample, cDNA was generated from 1 µg of total RNA using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules CA) per the manufacturer specifications. A previously described primer set for *Ide* (forward: 5'-CTGTGCCCCTTGTTTGATGC-3'; reverse: 5'-GTTCCCCGTAGCCTTTTCCA-3') (43) was purchased from Millipore Sigma (St. Louis MO). qPCR was performed in triplicates using iTaq Universal SYBR Green Supermix and CFX96 Touch™ Real-Time PCR Detection System with

138 CFX Manager 3.0 software (Bio-Rad). Ribosomal protein 18S (PrimePCR SYBR Green Assay
139 Rsp18, Bio-Rad) was used as a reference gene and *Rsp18* Cq values were used to normalize
140 respective *Ide* Cq values (ΔCq). Relative *Ide* mRNA expression was determined as $2^{-\Delta\Delta Cq}$ and
141 shown as mean ± SEM for each mouse strain.
142
143 **Cell lines and cell culture**
144 **Microglia cell line.** The SIM-A9 mouse microglial cell line was previously developed and
145 characterized in our laboratory (60) and is available from ATCC (ATCC CRL-3265). The cells
146 were grown in DMEM/F12 (ThermoFisher Scientific product 12400024) supplemented with
147 10% FBS and penicillin, neomycin, and streptomycin.
148 **Mouse Neuron Primary Culture.** Neurons were cultured from *App*$^{+/+}$and *App*$^{-/-}$ brains on
149 embryonic day 16 (4, 19, 36, 67). Embryos were harvested and brains were placed into sterile
150 dissection media (0.5 mM EDTA, 100 μM EGTA, 5.5 mM glucose, in PBS). Meninges were
151 removed, and cortices were harvested from both hemispheres, minced in dissection media, and
152 treated with 0.25% trypsin. Tissue was digested for 20 min at 37 °C. Digestion was terminated
153 by adding 10 ml DMEM/F12 containing 10% FBS. The cells were then allowed to settle in the
154 bottom of the pipette tip and then added to 10 ml of Neurobasal media supplemented with B27
155 and L-Glutamine, penicillin, streptomycin and neomycin, then triturated approximately 20 times.
156 Cells were cultured in 6-well plates coated overnight with poly-L-lysine. Neuron cultures were
157 grown at 37 °C in 5% CO_2 (54).
158 **Mouse Primary Microglia and Astrocyte Culture.** Microglia and astrocyte cultures were
159 established from 1-day-old *App*$^{+/+}$ and *App*$^{-/-}$ neonatal pups as previously described (54).
160 Meninges were removed from cerebrum, and cortical tissue was harvested in dissection media

(0.5 mM EDTA, 100 μM EGTA, 5.5 mM glucose, in PBS) on ice. Cortices were then digested in trypsin for 15 min at 37 °C. Digestion was terminated using DMEM/F12 media containing 10% FBS. Cortical tissue from each mouse was cultured separately in a T75 flask with 20 ml of DMEM/F12 supplemented with 10% FBS, penicillin, streptomycin and neomycin. Media was supplemented on Day 2 and replaced after one week. After two weeks of culture, microglia were separated from astrocytes by shaking flasks at 200 RPM for 45-60 min. The microglial suspension was collected and plated for microglial culture. Astrocytes adherent to the flasks were removed with trypsin and cultured on 6-well plates for experiments.

Glucose tolerance test (GTT) and Insulin tolerance test (ITT)

Beginning at 7 weeks of age, blood was collected from male mice and blood glucose ($[Glc]_b$) was measured using a glucometer (AlphaTRAK II, Abbott Laboratories). For GTT, mice were fasted 4 h (with access to water); $[Glc]_b$ was determined prior to intraperitoneal injection of Glc (2 mg/g) and at 15, 30, 60, 120 min thereafter. Some GTT results are expressed as incremental area under the curve (AUC); the difference between initial $[Glc]_b$ and subsequent $[Glc]_b$ values ($T_N – T_0$) for each mouse was used for this calculation to remove differences in basal $[Glc]_b$. For ITTs, $[Glc]_b$ was determined in non-fasted mice prior to i.p. injection of insulin (0.75 U/g) and at 15, 30, 60, and 120 min thereafter. GTT and ITT were performed on the same day of the week in alternating weeks. Some ITT results are expressed as incremental area over the curve (AOC); the difference between initial $[Glc]_b$ and subsequent $[Glc]_b$ values ($T_0 – T_N$) for each mouse was used for this calculation to remove differences in basal $[Glc]_b$. For experiments in aged animals, mice were fasted 5 h (with access to water); $[Glc]_b$ was determined prior to intraperitoneal injection of Glc (2 mg/g) and at 15, 30, 60, 120 min thereafter.

184

185 **Insulin ELISA**

186 Blood (25-30 µl) was collected at the 0- and 30-min time points during the GTT performed at
187 Week 6 of the western-diet feeding and centrifuged for 10 min at 2000 *g* and 4° C to extract
188 serum. Insulin concentrations were measured from 10-µl aliquots of serum using an Ultra-
189 Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc.) according to manufacturer's protocol.

190

191 **RNAi knockdown of APP**

192 RNAi-mediated knockdown of the mouse APP gene in SIMA9 microglia was achieved using
193 Dharmacon (Lafayette CO) Accell SMARTpool siRNA (product E-043246-00-0050), with non-
194 targeting RNA (product D-001910-01-50) treated cells used as controls. The siRNA was utilized
195 by combining the Accell siRNA with Accell delivery media per manufacturer's instructions, and
196 cells were treated with 1 µM RNA for 6 days. After 3 days of treatment (72 h), siRNA
197 containing media was supplemented 1:1 with normal DMEM/F12 with 10% FBS. SIMA9 cell
198 protein was harvested on Day 7 for western blot analyses.

199

200 **Western blot analyses**

201 Hippocampus, gastrocnemius, liver tissues and primary cell cultures were lysed in
202 radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor (Sigma P8340, St.
203 Louis MO) on ice. Protein concentrations were determined using the Bradford method (3). Five
204 to ten µg of protein was resolved by 10% SDS-PAGE and transferred to PVDF membranes.
205 Membranes were blocked for 1 h in Tris-buffered saline containing Tween-20 (TBST) and 5%
206 BSA solution, and then they were incubated overnight in 5% BSA-TBST solution containing the

primary antibody. The next day primary antibodies were washed off the membrane with TBST, and HRP-conjugated secondary antibodies were applied to membranes in 5% BSA-TBST for 2 h. Luminol chemiluminescence was utilized for visualization of proteins with and Aplegen Omega Lum G Gel Documentation System (Gel Company, San Francisco, CA). Western blot analysis was performed using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA). To quantify western blot bands (ie IDE), a box was traced around the protein band of interest. The mean pixel intensity of the area of the box was then measured using the histogram. The box was then moved to additional bands until all bands of interest were quantified. The measured values of bands of interest were then normalized to the mean intensity of the loading control protein for each individual sample. Finally, all normalized protein values were divided by the mean of the $App^{+/+}$ condition to obtain a mean of 1.0 for $App^{+/+}$ samples.

IDE Activity Assays

The IDE activity assays were purchased from AnaSpec (Fremont, CA) (Catalog # AS-72231). The assay utilizes a FRET substrate which emits increased fluorescence when cleaved by IDE. This increased fluorescence is due to liberation of 5-carboxyfluorescein (5-FAM) from a quenching molecule also on the IDE substrate. Hippocampus, liver, and gastrocnemius were collected on ice from $App^{+/+}$ and $App^{-/-}$ mice. Our preliminary experiments utilizing this assay suggested that IDE enzyme activity is negatively affected by sonication. Tissue was homogenized on ice in assay buffer using a Powermasher II (Nippi, Japan). Fifty µl of individual brain homogenate was loaded into a well of a 96-well plate containing 50 µl of substrate solution. The fluorescence intensity was measured on a Biotek Plate reader using an excitation of

485 nm and emission of 528 nm at 5-min increments. Fluorescent units measured were converted to concentrations of 5-FAM. The protein concentration of each tissue homogenate was determined using the Bradford method and 5-FAM concentrations were normalized to the total protein in each sample. Total IDE activity in each sample was calculated using the formula ([Final Concentration] - [Initial Concentration] / Time × Volume) × Dilution Factor described by Kurauti et al. and normalized as described above (43).

Immunohistochemistry

Left hemispheres of mouse brains were fixed in 4% paraformaldehyde/PBS for 48 h and embedded in a 15% gelatin matrix as described previously (61). The brains were cryosectioned into 40-µm serial sections by a sliding microtome (Leica SM 2000R, Leica Biosystems Inc., Buffalo Grove, IL). The free-floating sections were incubated in the anti-IDE antibody diluted 1:200 overnight at 4 °C in PBS containing 1% Triton X-100, 3% BSA, 2% horse serum. Visualization of the antigen was carried out using a VECTASTAIN Elite ABC-HRP Kit with VIP as the chromogen (Vector Laboratories, Burlingame, CA). The brain sections were mounted onto subbed glass slides and dehydrated through an ethanol gradient prior to coverslipping using Permount mounting medium (ThermoFisher).

APP Plasmids and Transfections

pCAX APP 751 and pCAX APP 695 were a gift from Drs. Dennis Selkoe & Tracy Young-Pearse and are available from Addgene as plasmids 30138 and 30137 (89). Plasmids were prepared using Qiagen Endofree Plasmid Giga Kit (product 12391). Primary astrocyte cultures

were transfected using Lipofectamine 3000 (ThermoFisher Scientific) as per the manufacturer instructions.

APP peptides

Bacterial recombinant sAPPα695 was purchased from Sigma Aldrich (Product number S9564). Other sAPP peptides utilized in this study were expressed in an eukaryotic system. HEK293 cells stably transfected with a vector encoding human $APP751_{1-668}$, $APP695_{1-612}$, were grown to subconfluency in T175 flasks and changed to a serum-free medium supplemented with 0.5 mM L-glutamine, 50 μM ethanolamine, and 10 nM sodium selenite. After four days, the conditioned medium was loaded onto a 15-ml DE52 column to concentrate the acidic proteins. The column was eluted with phosphate buffer (pH 7.4) containing 0.75 M NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF); 1-ml fractions were collected. The fractions containing sAPPα (determined by immunodetection on dot blots) were pooled, dialyzed 1 h at 4 °C versus 2 L of PBS (pH 7.4) containing 1 mM EDTA, 1 mM PMSF; the dialysate was loaded onto a 5-ml Supelco Hi-Trap heparin column (GE Healthcare). The heparin column was eluted with an FPLC pump-controlled salt gradient, starting with 20 mM phosphate buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF and finishing with the same buffer containing 1 M NaCl; 0.5-ml fractions were collected. The sAPPα-containing fractions were pooled, dialyzed 1 h at 4 °C versus 2 L of 20 mM Tris-HCl, pH 8, containing 20 mM NaCl, 1 mM EDTA, and 1 mM PMSF; the dialysate was loaded onto a Bio-Scale Q5 anion-exchange column (Bio-Rad). The Q5 column was eluted with an FPLC pump-controlled salt gradient, starting with a 20 mM Tris-HCl buffer (pH 8) containing 20 mM NaCl, 1 mM EDTA, and 1 mM PMSF and finishing with the same buffer containing 1 M

NaCl; 0.5-ml fractions were collected. The sAPPα-containing fractions were further analyzed by SDS-PAGE with silver staining.

Recombinant bacterial-produced APP fragments were applied at a concentration of 20 nM in serum-free DMEM/F12, while eukaryotic produced fragments were applied at a concentration of 200 nM. Fragments were applied for a total of 72 h and refreshed at 24 and 48 h.

Secretase inhibitor treatment

Secretase inhibitors were applied to SIMA9 cells in DMEM/F12 supplemented with 1% FBS, penicillin, streptomycin, and neomycin for one week at a concentration of 1 μM. Fresh inhibitors and media were applied daily to the cells. Neither overt toxicity nor increased LDH activity in cell culture media was observed. LDH activity assays were purchased from Cayman Chemical (Product number 601170) and were performed per manufacturer's instructions. The α-secretase inhibitor, GI254023X, was purchased from Sigma Aldrich. The recently described β-secretase inhibitor, Verubescestat, was purchased from Selleckchem (Houston TX). The widely used γ-secretase inhibitor, DAPT, was purchased from Sigma Aldrich. The dose of 1 μM is well above the IC50 value of Verubescestat, reported at 3.4 nM for murine BACE1. The reported IC50 for DAPT is 0.02 μM. The reported IC50 for GI254023X inhibition of ADAM10 is 5.3 nM (15, 32, 38, 50).

Hippocampus synaptosome preparation and stimulation

Synaptosomal insulin responsiveness was evaluated by *ex vivo* stimulation of isolated synaptosomal preparations as previously described (21). Briefly, frozen $App^{+/+}$ and $App^{-/-}$ mouse

hippocampi were homogenized using SynPER reagent (Thermo Scientific) with 1 % protease inhibitor cocktail and phosphatase inhibitor cocktail, and homogenates were centrifuged at 1230 g for 10 min at 4 °C. The supernatant was collected and centrifuged once more at 15,000 g for 20 min at 4 °C. The pellet was resuspended in a physiological buffer, HEPES-buffered Krebs-like (HBK) buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, 20 mM HEPES, 0.1 mM NaH_2PO_4, and 10 mM D-glucose, pH 7.4), and aliquoted into tubes of equal protein for unstimulated and insulin-stimulated samples. All tubes received 8 mM ATP, and insulin stimulation was performed with 10 nM or 200 nM of diluted U-100 insulin. All tubes were incubated at 37 °C for 15 min. Samples were pelleted at 10000 g for 10 min at 4 °C and resuspended in RIPA buffer (75 mM NaCl, 25 mM Na_2PO_4, 1 mM EDTA, 0.5% NP-40, and 0.5% TritonX-100) plus 1% protease inhibitor cocktail and phosphatase inhibitor cocktail. The bicinchoninic acid assay method was used to prepare samples of equal protein concentration for WES capillary western blot technology (ProteinSimple, San Jose CA). Data is reported as area under the peak for the specified proteins.

Statistical Analysis

Statistical analysis was performed using SigmaPlot 12.0 software. Values were averaged ± SEM and statistical significance was determined via Student's t-test or one-way ANOVA as appropriate. In the case of statistical significance, the Tukey-Kramer *post hoc* test or Holm-Sidak multiple pair-wise comparisons were used where applicable. The difference in the mean relative *Ide* mRNA expression values was statistically analyzed by performing unpaired t-test with Welch correction factor using GraphPad Prism 7.03 software (GraphPad Software, Inc., La Jolla, CA). P-values less than 0.05 were considered statistically significant.

RESULTS

IDE protein and mRNA were increased in *App*$^{-/-}$ tissues

We began examining the potential role of APP in regulating IDE in skeletal muscle from two-month-old female *App*$^{+/+}$ C57BL/6 and *App*$^{-/-}$ mice via western blot analysis of homogenates. As expected, APP was not detectable in gastrocnemius tissue collected from *App*$^{-/-}$ mice but was detectable in *App*$^{+/+}$ controls (Figure 1A). Interestingly, IDE protein levels were found to be significantly higher in *App*$^{-/-}$ muscle (n=6, $P<0.001$) compared to the controls. Next, we examined liver tissue collected from *App*$^{+/+}$ and *App*$^{-/-}$ animals (Figure 1B). Similar to the skeletal muscle, we observed higher IDE protein levels in liver tissue extracts from *App*$^{-/-}$ mice compared to *App*$^{+/+}$ controls (n=7, p=0.023). These data, along with our previous observations of elevated IDE protein in pancreatic extracts from *App*$^{-/-}$ mice (42), suggested that APP negatively regulates IDE levels in numerous peripheral tissues.

Due to the potential role of IDE in Aβ clearance, we next sought to examine if ablation of the APP gene altered IDE protein levels in the brain. To identify which cell types may be most abundantly expressing IDE, we performed immunohistochemistry to detect IDE in *App*$^{+/+}$ and *App*$^{-/-}$ brain tissue. IDE was diffusely detectable throughout the brain, including within the white matter. Although neurons within the hippocampus were stained to a greater extent, IDE immunostaining did not specifically label a single cell type in the CNS. This is in agreement with previous research demonstrating the expression of IDE in multiple cell types of the brain

including neurons, astrocytes and microglia (see http://www.brainrnaseq.org/) (91). Because of this high expression, as well as the critical roles in normal memory and AD pathology played by the hippocampus, we assessed IDE protein levels in this region from *App*$^{+/+}$ and *App*$^{-/-}$ brains by western blot (n=6-7) (Figure 1C). IDE protein levels were found to be almost three-fold higher in *App*$^{-/-}$ hippocampus tissues compared to *App*$^{+/+}$ controls ($p<0.001$). This suggested that APP regulation of IDE extends to the central nervous system and is not limited to peripheral organs. We then examined mRNA levels to examine whether APP may be regulating IDE at the transcriptional or post-translational levels. When relative abundance of *Ide* transcripts were determined using RT-qPCR (n=4/5), we observed a significantly higher level of *Ide* mRNA in *App*$^{-/-}$ hippocampus tissue compared to *App*$^{+/+}$ controls ($p=0.002$) (Figure 1D). Similar to protein levels of IDE, we observed a difference in *Ide* mRNA expression in *App*$^{-/-}$ hippocampus tissue of greater than three-fold compared to *App*$^{+/+}$ controls. Collectively, these data suggested that APP acts as a negative regulator of IDE levels and that ablation of APP increases IDE at the levels of transcription and protein. Furthermore, our immunohistochemistry suggests that IDE is produced throughout the brain in many cell types, and IDE expression is particularly increased in hippocampal neurons in *App*$^{-/-}$ mouse brain.

IDE expression was dependent on APP levels in cultured neurons, astrocytes and microglial cells

Following our observation that mRNA and protein levels for IDE were robustly increased in the hippocampus tissue of *App*$^{-/-}$ mice, we next sought to determine which cell type(s) found in the brain are responsible for the difference in IDE content. (Figure 2A). As expected, APP was

abundant in *App*$^{+/+}$ cultures of cortical neurons and not detectable in *App*$^{-/-}$ cultures. Consistent with our observations in homogenized hippocampus, total IDE protein levels were significantly higher in *App*$^{-/-}$ neuronal cultures compared to *App*$^{+/+}$ controls (n=4, $p<0.010$). We also established cultures of astrocytes and microglia from *App*$^{+/+}$ and *App*$^{-/-}$ mice to determine if APP regulates IDE protein in these glial cell types in a manner similar to neurons (n=3/condition). The APP detected in cultures of astrocytes and microglia had lower mobility, predicted from the differential expression of KPI-containing splice variants in these various cell types. IDE protein levels were found to be significantly higher in both astrocyte (Figure 2B; p=0.009) and microglia (Figure 2C, p=0.009) cultures relative to *App*$^{+/+}$ controls. These data suggested that APP regulates IDE in both neurons and glia and is not restricted to a specific isoform of APP expressed in limited cell types. Furthermore, this suggests that multiple cell types are responsible for increased total IDE levels found in *App*$^{-/-}$ hippocampus extracts.

Given that we observed increased IDE in *App*$^{-/-}$ microglia and that microglial IDE may be particularly important for degrading Aβ, we next tested the hypothesis that knockdown of APP with siRNA in the SIMA9 microglial cell line will increase IDE levels *in vitro* (Figure 2D). Knockdown of APP protein was performed over 1 week with either an anti-*App* siRNA pool or a scrambled RNA control (n=6/condition). As expected, treatment with anti-*App* siRNA significantly reduced APP as measured by western blot (p=0.003). IDE protein levels were robustly and significantly increased in microglia ($p<0.001$) treated with anti-*App* siRNA, suggesting that knockdown of APP *in vitro* is sufficient to increase IDE protein levels in cultured cells. This also suggests that APP has a direct role in regulating IDE and that the increased levels

of IDE observed in the *App*$^{-/-}$ mice is not a consequence of overall differences in the physiology of the animals.

IDE activity was increased in *App*$^{-/-}$ tissues

To confirm a potential impact on substrates of the differential levels of IDE protein, we also compared the enzymatic activity of IDE in *App*$^{+/+}$ and *App*$^{-/-}$ mouse tissues. To measure IDE activity we utilized a commercially available IDE activity assay. This assay uses a FRET peptide IDE substrate that emits increased 5-FAM fluorescence when hydrolyzed by the IDE enzyme. Hippocampus, gastrocnemius, and liver tissue was collected from 2-month-old *App*$^{+/+}$ and *App*$^{-/-}$ mice (n=6 per condition) and homogenized. Each tissue homogenate was then mixed with the IDE substrate, and fluorescence was measured over time (Figure 3). The IDE activity of each tissue extract was calculated and normalized to the total protein content of the sample. Significantly higher IDE activity was observed in *App*$^{-/-}$ hippocampus (p=0.016) and liver extracts (p=0.003), indicating that the higher IDE protein likely leads to differences in the stability of IDE substrates in these tissues. Unlike hippocampus and liver tissue, no significant differences in IDE activity were observed between *App*$^{+/+}$ and *App*$^{-/-}$ gastrocnemius tissue extracts (p=0.746).

Insulin levels and insulin signaling were altered in *App*$^{-/-}$ hippocampus tissue and synaptosomes

We next sought to probe for consequences of increased IDE protein and enzyme activity on insulin levels and insulin signaling markers in hippocampus tissue. We measured the insulin levels of 2-month-old *App*$^{+/+}$ and *App*$^{-/-}$ hippocampus extracts by ELISA (n=11/13). Hippocampus extracts from *App*$^{-/-}$ mice had significantly lower hippocampal insulin levels compared to *App*$^{+/+}$ animals (p=0.032), suggesting that total insulin levels in the brain is reduced in these animals (Figure 4A). We next examined hippocampus extracts from *App*$^{+/+}$ and *App*$^{-/-}$ mice for changes in insulin signaling markers. While we were unable to clearly detect phosphorylated insulin receptor in these lysates (Figure 4B), we also examined the T308 residue of AKT, as it is well-established to be phosphorylated in response to insulin signaling (1). We observed significantly lower (p=0.025) levels of pAKT T308 in *App*$^{-/-}$ hippocampal lysates compared to *App*$^{+/+}$ controls, suggesting that *App*$^{-/-}$ animals have diminished insulin signaling in brain tissue.

To further examine insulin signaling in the brains of these animals, we prepared synaptosomes from *App*$^{+/+}$ and *App*$^{-/-}$ brain tissues and stimulated them acutely with insulin *ex vivo*. Stimulated synaptosome lysates were then analyzed for protein and phosphorylated protein content by the WES capillary western blot. We have previously utilized this method to examine insulin signaling in the brains of other transgenic mice (73), and the details of this method have been expanded upon in recent literature (21). *App*$^{+/+}$ and *App*$^{-/-}$ synaptosomes were stimulated with either 10 or 200 nM insulin (n=4-6/condition). Interestingly, *App*$^{-/-}$ synaptosomes showed diminished phosphorylation of the insulin receptor compared to *App*$^{+/+}$ synaptosomes when stimulated with either 10 nM (p=0.0315) or 200 nM (p=0.0265) insulin. (Figure 4C). No differences in the ratio of phosphorylated insulin receptor (IR) to total insulin receptor were

observed in the unstimulated synaptosomes. These data suggest that *App*$^{-/-}$ animals have impaired insulin signaling in the brain at the level of the insulin receptor.

App$^{-/-}$ mice had low basal levels of circulating insulin and were protected from impairment of glucose tolerance by western diet

While our data suggest *App*$^{-/-}$ animals have changes in brain insulin signaling, their higher levels of IDE in peripheral tissues suggested they might exhibit somatic effects as well. Paradigms of diet-induced obesity have been documented to elevate markers of metabolic syndrome, such as impaired glucose tolerance secondary to insulin resistance. We fed *App*$^{+/+}$ and *App*$^{-/-}$ littermates a "western" diet (WD, high fat and high sucrose) for 13 weeks. Consistent with previous studies, *App*$^{+/+}$ mice on the WD showed impaired glucose tolerance in glucose tolerance tests (GTT). At Week 0 (7 weeks of age), prior to the introduction of WD, *App*$^{+/+}$ and *App*$^{-/-}$ showed nearly identical responses to glucose challenge (not shown). By Week 8 (7 weeks of diet manipulation; 15 weeks of age), *App*$^{+/+}$ mice showed blood glucose concentrations ($[Glc]_b$) that were higher on WD compared to normal diet (ND) controls (Fig. 5A). Conversely, the $[Glc]_b$ in *App*$^{-/-}$ mice fed WD was not different from ND controls. To ascertain that the differences in GTT involved differences in glucose excursion, independent of differences in initial $[Glc]_b$, the GTT profiles were integrated as area under the curve (AUC) using at each time point $[Glc]_b$ values from which the initial $[Glc]_b$ had been subtracted individually for each animal. The mean of these values over weeks 14 – 20 (13 – 19 weeks on diet) showed an overall impairment of glucose tolerance in *App*$^{+/+}$ mice fed WD, but *App*$^{-/-}$ mice failed to reach this state on either diet (Fig. 5B). In addition, WD raised fasting $[Glc]_b$ in *App*$^{+/+}$ but not *App*$^{-/-}$ mice.

An insulin tolerance test (ITT) was used to evaluate insulin sensitivity on weeks alternating with the GTT. At Week 1 (7 weeks of age; prior to dietary manipulation) *App*$^{+/+}$ and *App*$^{-/-}$ mice had similar insulin sensitivity, with *App*$^{-/-}$ mice trending toward a more robust response (data not shown). By Week 9 (16 weeks of age; 8 weeks on WD), there was a significant difference in the WD-fed mice as a function of genotype (Fig. 5C). To ascertain that the difference between *App*$^{+/+}$ and *App*$^{-/-}$ mice reflected differences in insulin resistance and was independent of differences in initial $[Glc]_b$, the area of the deflection from the initial $[Glc]_b$ over time was integrated. In contrast to the GTT calculations, ITTs were integrated as area over the curve (AOC): For this calculation, the $[Glc]_b$ at each time point was subtracted from the initial $[Glc]_b$, in effect setting the ceiling for the curve at the initial $[Glc]_b$ for each animal. The mean of these values over weeks 13 – 19 (12 – 18 weeks on diet) showed insulin resistance in *App*$^{+/+}$ mice fed WD, but *App*$^{-/-}$ mice were protected from this outcome (Fig. 5D).

The above results indicate that *App*$^{-/-}$ mice retained greater insulin sensitivity than their *App*$^{+/+}$ counterparts on a WD. We measured blood insulin levels in a fasted state and 30 min after a glucose injection. While the *App*$^{+/+}$ mice fed WD clearly exhibited hyperinsulinemia, *App*$^{-/-}$ mice maintained normal basal insulin levels on this diet (Table 1). In fact, *App*$^{-/-}$ mice on normal and western diet had lower fasting insulin levels than their *App*$^{+/+}$ counterparts, potentially implicating a role for elevated IDE in regulating circulating insulin levels. Due to the low basal levels of insulin observed in the fasting state of *App*$^{-/-}$ mice, the change in insulin levels in response to glucose was much greater in these animals.

Aged *App*$^{-/-}$ mice have normal glucose tolerance but are hypoglycemic when fasted

Aging is a well-established risk factor for both diabetes and Alzheimer's disease (37, 66)**.** While, as stated above, we found no differences in glucose tolerance in young *App*$^{-/-}$ animals on a normal chow diet compared to *App*$^{+/+}$ controls, we also tested 12-month old cohorts of both *App*$^{+/+}$ and *App*$^{-/-}$ mice (n=11/13) on normal chow to examine the potential consequences that may result from the increased levels of IDE while aging (Figure 6). Animals were fasted for 5 hours on the day of the experiment and then administered 2g/kg body weight glucose by intraperitoneal injection. Blood glucose was monitored just prior to injection and at 15, 30, 60 and 120 minutes after injection. Data was graphed as blood glucose versus time and the area under the curve was assessed. No significant difference was observed in overall glucose tolerance (p=0.12) between *App*$^{+/+}$ and *App*$^{-/-}$ mice. However, we did observe a robust and significant reduction in the fasting blood glucose levels of *App*$^{-/-}$ mice (p=0.002) compared to *App*$^{+/+}$ controls. Blood glucose was also measured in free-fed *App*$^{+/+}$ and *App*$^{-/-}$ mice (n=5/condition) and was not significantly different (p=0.221). These data suggest aged *App*$^{-/-}$ mice on normal chow remain glucose tolerant but are hypoglycemic when fasted, potentially indicating that increased IDE levels at older ages may have consequences on normal glucose homeostasis.

Overexpression of APP or treatment with secretase inhibitors did not alter IDE levels

APP is cleaved by a variety of enzymes termed secretases, which result in the production of various APP fragments. To examine if IDE regulation occurs in an APP fragment-specific manner and if selective inhibition of secretase isotypes differentially alter levels of IDE, SIM-A9

503 microglia cells were treated for 7 days in DMEM with 1% serum in the presence or absence of
504 GI254023X (α-secretase inhibitor), Verubecestat (β-secretase inhibitor), or DAPT (the γ-
505 secretase inhibitor) (Fig. 7A). SIM-A9 cells were chosen for this experiment as they are
506 amenable to culturing in low-serum conditions and had shown an increase in IDE protein levels
507 when APP was knocked down with siRNA (Figure 2D). No overt toxicity or change in LDH
508 activity in the media was observed from this treatment (data not shown), indicating the secretase
509 inhibitors did not cause robust cell death. No significant difference was found in the IDE protein
510 content of SIM-A9 cells treated with inhibitors compared to untreated or vehicle controls
511 (n=3/treatment, p=0.45) (Figure 7A).
512
513 Next, to further examine if a specific APP metabolite may be capable of changing IDE levels, we
514 tested if recombinant sAPP fragments would reduce protein levels of IDE in *App*$^{-/-}$ astrocyte
515 primary cultures. sAPP is thought to exert effects on cells by inducing signaling through a
516 variety of receptors (26). Cells were treated for 3 days with 20 nM of prokaryotically expressed
517 recombinant sAPPα, sAPPβ, or mutant sAPPα lacking N-terminal amino acids 304-612;
518 eukaryotically expressed sAPPα695 or sAPPα751 were also tested at 200 nM. These sAPP
519 fragments did not alter IDE levels in cultures, indicating that the full-length APP protein or
520 another APP fragment is required for differences in IDE levels observed in *App*$^{-/-}$ tissues (Fig.
521 7B).
522
523 Finally, we tested if overexpression of the APP695 isoform or the APP751 isoform in *App*$^{-/-}$
524 astrocytes was sufficient to reduce levels of IDE. Primary astrocyte cultures were either mock
525 transfected or transfected with plasmids coding for APP695, APP751 or green fluorescent

protein (GFP). Cellular protein was collected after 5 days and IDE was measured by western blot. Surprisingly, overexpression of APP in *App*$^{-/-}$ astrocytes did not significantly change IDE levels (p=0.56) (Fig. 7C).

DISCUSSION

Glucose hypometabolism in the brain is observed in advanced Alzheimer's disease, in animal models of AD, and in Down syndrome patients (45, 65, 68, 87). AD patients exhibit type-2 diabetes or impaired glucose tolerance at twice the rate of age-matched controls (86). A plethora of evidence shows an association between type-2 diabetes and cognitive decline, but recent studies have challenged the hypothesis that AD is promoted by type-2 diabetes, as the latter is better correlated with vascular dementia (34, 35, 41, 55). Moreover, diabetes has not been found to exacerbate Aβ deposition in all studies (69, 76). The converse, however, may be true, as indicated by the perturbation of peripheral insulin/glucose regulation in AD models (52, 53, 62, 64). Most of these models involve overexpression of APP and evidence suggests the precursor participates in metabolic homeostasis. For example, *App*$^{-/-}$ knockout mice, in addition to having lower body weight, show reduced weight gain compared to *App*$^{+/+}$ controls when placed on a high-fat diet (71). In muscle tissue, sAPPα potentiates glucose uptake (27). Our data demonstrates that ablation of the APP gene in mice or prolonged knockdown of APP in cell culture results in higher levels of IDE compared to *App*$^{+/+}$ controls. Higher levels of IDE protein were observed in muscle, liver, and hippocampal tissue extracts from *App*$^{-/-}$ animals. In agreement with this, primary cell cultures of neurons, astrocytes and microglia from *App*$^{-/-}$ mice all had elevated IDE levels compared to *App*$^{+/+}$ cultures. Furthermore, *Ide* mRNA levels were

significantly increased in hippocampal tissue from *App*$^{-/-}$ animals compared to controls, suggesting that increased IDE protein was due to increased transcription. The effect seen *in vivo* did not appear to result from indirect effects of developmental abnormalities because knockdown of APP in cultured cells was sufficient to acutely elevate protein levels of IDE.

We observed robust and significantly higher IDE activity in both hippocampus and liver extracts from *App*$^{-/-}$ animals compared to *App*$^{+/+}$ controls. IDE activity was not statistically different in skeletal muscle tissue extracts despite increased total IDE proteins levels. This could be due to the nature of the activity assay, which uses cleavage of a FRET peptide as an indicator of IDE activity. Muscle tissue may contain other enzymes capable of cleaving the FRET substrate. Interestingly, muscle tissue lysates had additional, lower-molecular-weight IDE bands on western blots compared to other tissues, suggesting there may be alternative forms of IDE in muscle or that the enzyme is subject to an unusually higher rate of proteolysis in this tissue.

Although the brain is traditionally viewed as an insulin-insensitive organ, recent literature demonstrates insulin and insulin signaling are involved in the normal physiology of the brain (18, 23, 40). Moreover, impaired insulin signaling may play a role in AD (75, 81). Indeed, intranasal insulin is under investigation as a therapeutic intervention for AD (7). We observed significantly lower hippocampal insulin levels in *App*$^{-/-}$ animals suggesting that higher IDE activity is tantamount to greater insulin catabolism. This concept is in general agreement with previous research showing IDE$^{-/-}$ mice have impaired tissue insulin degradation (17).

571 Our data also indicates that increased IDE protein and activity alter brain insulin signaling.
572 While we did not clearly detect phosphorylated insulin receptor in our hippocampus
573 homogenates, possibly due to the transient nature of insulin receptor phosphorylation, we did
574 observe modest but significantly reduced levels of pAKT(T308) in the *App*$^{-/-}$ hippocampus. This
575 suggested that insulin signaling may be impaired in *App*$^{-/-}$ brain tissues, perhaps due to lower
576 levels of insulin itself. However, we also observed diminished phosphorylation of the insulin
577 receptor following acute stimulation of *App*$^{-/-}$ synaptosomes compared to *App*$^{+/+}$ controls. This
578 suggests that differences in signaling in *App*$^{-/-}$ brains may occur via receptor dysfunction as well.
579
580 To examine physiologic consequences of increased tissue levels of IDE in *App*$^{-/-}$ mice, we
581 assessed insulin in serum. Six weeks of western diet produced hyperinsulinemia in *App*$^{+/+}$ mice
582 but not *App*$^{-/-}$ mice. Pharmacologic inhibition of IDE *in vivo* has been shown to alter insulin
583 levels and impair glucose tolerance (14). Consistent with this finding, elevated IDE in the *App*$^{-/-}$
584 mice correlated with protection against insulin resistance and glucose intolerance. It is possible
585 that suppression of circulating insulin permits the maintenance of greater insulin sensitivity in
586 *App*$^{-/-}$ mice, which may manifest in the lower serum insulin levels observed in *App*$^{-/-}$ mice.
587 Indeed, this in agreement with a recent report showing that reduced pancreatic beta cell mass in
588 *App*$^{-/-}$ mice challenged with a high fat diet (13). It should be noted that while levels of serum
589 insulin were reduced in fasted *App*$^{-/-}$ mice, their pancreatic response to glucose showed a greater
590 dynamic response, achieving levels similar to those in *App*$^{+/+}$ mice. This exaggerated
591 responsiveness appears to be consistent with the phenotype of isolated pancreatic islets in a
592 previous report (83). In addition to impacts on insulin, IDE may alter peripheral glucose
593 regulation through its degradation of glucagon (79). In agreement with our study, recent work by

Czeczor et al. observed lower plasma levels of insulin in fasted *App*$^{-/-}$ mice fed a high fat diet for 14 weeks (12). However, they observed that high fat diet caused glucose intolerance while insulin tolerance remained normal in the *App*$^{-/-}$ mice. The incongruity is perhaps due to the large difference in sucrose composition in the diets which was 34% by mass in our "western diet" study and 20% in the study by Czeczor et al.

It is interesting to note that while *App*$^{-/-}$ mice retained insulin sensitivity in the periphery when placed on a western diet, they had impaired insulin signaling in the brain. The reason for this is unclear. However, insulin sensitivity in tissues is affected by numerous factors, including phosphorylation status of the insulin receptor substrate 1 (IRS-1), downstream of the insulin receptor (11, 56). Surprisingly, hippocampal synaptosomes from *App*$^{-/-}$ animals demonstrated acute insulin resistance at the level of the insulin receptor suggesting a complex response to elevated IDE in the brain. Since the brain is exposed to much lower levels of insulin than peripheral organs it is not surprising that elevated IDE activity in the brain may have differential consequences on tissue insulin signaling compared to the periphery. Furthermore, it is known that the adult brain expresses a different isoform of the insulin receptor which has a greater affinity for both insulin and IGF-II compared to the insulin receptor predominantly found in peripheral tissues (2, 22, 25). This difference in central and peripheral insulin receptor isoforms may further account for the apparent differential effects of increased IDE in the brain compared to periphery.

To further examine the potential functional consequences of increased tissue levels of IDE in aging animals, we performed glucose tolerance testing in 12 month old male and female WT and

App$^{-/-}$ animals. Pharmacological inhibition of the IDE *in vivo* has been shown to alter insulin levels and affect glucose tolerance (14). While we did not observe a significant difference in overall glucose tolerance between WT and *App*$^{-/-}$ animals, we were surprised to observe that when aged *App*$^{-/-}$ animals were fasted, they became hypoglycemic. This is in contrast to our prior analysis of glucose levels in 2 month *App*$^{-/-}$ animals, which did not reveal this phenomenon despite increased levels of pancreatic IDE (42). This finding, however, is in general agreement with work by Needham et al. which demonstrated lower plasma glucose in both *App*$^{-/-}$ and APLP2$^{-/-}$ mice (63). Other groups have shown that aged *App*$^{-/-}$ animals have impaired spatial learning with age (58, 70). It can be speculated that aging may reveal phenotypes present in *App*$^{-/-}$ mice not observed at earlier ages. Indeed, it has been shown that aged *App*$^{-/-}$ mice, but not young *App*$^{-/-}$ mice, show fewer dendritic spines and changes in spine morphology in addition to impaired LTP (46, 84). The compromised metabolic homeostasis we observed in aged animals may reveal the potentially deleterious consequences of increased IDE in tissues, despite normal overall glucose tolerance. IDE has been implicated in the degradation of the glucose elevating hormone glucagon, which may account in part for this the hypoglycemia we observed in fasting conditions (79).

The mechanism by which ablation of APP results in increased IDE transcription remains to be elucidated. The APP intracellular domain (AICD) can interact with other proteins such as Fe65, Dab1, and the histone acetyltransferase Tip60 to transduce intracellular signals (6, 39). One of these accessory proteins is NEDD8, which activates ubiquitin-proteasome degradation of substrates in the Cullin pathways (74). However, little is known about degradation of IDE via the ubiquitin-proteasome system, and our analysis of mRNA suggests a pretranslational site of

action. Treatment of the SIM-A9 microglia cell line with secretase inhibitors did not significantly change IDE levels, suggesting that APP processing is not necessary for the impact on IDE. Previous research has indicated that APP may be able to affect transcription independently of γ-secretase activity (29). Regardless, a reliance on holo-APP would not be inconsistent with schemes in which both the intact precursor and one of its fragments are involved. It has been suggested that sAPPα requires the APP holoprotein for its normal signaling functions (90). A recent report has shown that viral overexpression of sAPPα in the brain increases IDE and restores memory deficits in a mouse model for AD (20). Perhaps the increased IDE observed in the *App*$^{-/-}$ mice results from loss of normal sAPPα-APP signaling. In our study, *in vitro* treatment of *App*$^{-/-}$ astrocytes with recombinant sAPPα or sAPPβ did not alter IDE protein levels; nor did treatment of the SIM-A9 microglia cell line with α- or β-secretase inhibitors. It was also somewhat surprising that overexpression of human APP695 or APP751 was not sufficient to change IDE levels in *App*$^{-/-}$ astrocyte cultures. This may be due to abnormal processing or trafficking of the exogenous, overexpressed APP. Regardless, long-term siRNA knockdown of APP *in vitro* was sufficient to robustly change IDE levels supporting our observations in the *App*$^{-/-}$ mice. It is also possible that changes in IDE occur indirectly from loss of APP instead of APP being directly involved in IDE transcription.

In conclusion, we present new evidence that APP suppresses the levels of IDE mRNA, protein, and enzymatic activity, both in the brain and in peripheral organs. These findings represent a new link connecting APP to metabolic homeostasis and expand on the already substantial connections between Alzheimer's disease and metabolism. Further, these findings may have

relevance for situations—empirical or otherwise—in which holo-APP is overexpressed, wherein greater restriction of IDE levels may alter Aβ accumulation.

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Table 1: Insulin levels at 6 weeks on western diet

Mouse group		[insulin] (ng/ml)		
Genotype	**Diet[1]**	**T_0**	**T_{30}[2]**	**%Δ[3]**
$App^{+/+}$	ND	0.355 ± 0.0823	0.680 ± 0.0930	111.7 ± 78.04
	WD	1.990 ± 0.107	2.077 ± 0.0947	7.07 ± 33.34
$App^{-/-}$	ND	0.111 ± 0.0354	0.466 ± 0.0386††	699.7 ± 292.0*
	WD	0.289 ± 0.0567*	0.627 ± 0.0591†	160.0 ± 55.18

[1]ND (Normal Diet), WD (Western Diet)

[2]30 min after challenge with glucose (2 g/kg body wt.)

[3]Group mean of the percent changes calculated for each mouse

$^{†}p \leq 0.05$, $^{††}p \leq 0.01$ vs. T_0; $^{*}p \leq 0.05$ vs. $App^{+/+}$ counterpart

FIGURE LEGENDS

Figure 1. IDE was increased in *App*$^{-/-}$ tissues. ***A-C,*** IDE protein was measured in liver (n=7), gastrocnemius (n=6), and hippocampal tissue (n=6-7) by western blot and normalized to GAPDH as a loading control. Normalized optical density is graphed as mean values ±SEM. ***D***, *Ide* mRNA levels were measured in hippocampus extracts (n=4-5) and normalized to ribosomal 18S RNA as a loading control. Statistical significance $p<0.05$* was determined by Student's t-test. ***E***, Immunohistochemistry for IDE protein was performed on *App*$^{+/+}$ and *App*$^{-/-}$ brain tissue sections (n=4-6). Representative images at 10X magnification are shown.

Figure 2. IDE was increased in *App*$^{-/-}$ cell cultures. ***A-C***, Primary cell cultures of neurons (n=4), astrocytes (n=3), and microglia from *App*$^{+/+}$, *App*$^{-/-}$ and the APP/PS1 mouse model of AD were grown and assessed for IDE protein content by western blot with GAPDH as a loading control. Significance $p<0.05$* was determined by Student's t-test. ***D***, Cultures of SIM-A9 mouse microglia cells (n=6/condition) were treated with either scrambled RNA or anti-APP siRNA for one week. After treatment, total cellular IDE content was assessed by western blot with α-tubulin as a loading control. Significance $p<0.05$* was determined by Student's t-test. Normalized optical density is graphed as mean values ± SEM.

Figure 3. IDE activity was increased in *App*$^{-/-}$ tissues. *App*$^{+/+}$ and *App*$^{-/-}$ tissues (n=6) were harvested on ice and rapidly assayed for IDE activity over the course of 1 h. The IDE activity assay uses a FRET peptide which is liberated from a quencher and exhibits increased fluorescence when cleaved by IDE. Relative fluorescent units are converted to concentrations of

FAM via standard curve. The data was normalized to the amount of total protein collected from each tissue. IDE activity was calculated from each animal tissue and significance $p<0.05$* was determined by Student's t-test. Data is graphed as mean values ± SEM.

Figure 4. Insulin signaling and total insulin levels were altered in the *App*$^{-/-}$ brain. ***A***, Total hippocampal insulin levels were measured in RIPA extractions of *App*$^{+/+}$ and *App*$^{-/-}$ hippocampus tissue by ELISA (n=13). ***B***, Insulin signaling cascade markers were assessed by western blot in two-month old *App*$^{+/+}$ and *App*$^{-/-}$ hippocampal tissue (n=5-6/condition). ***C***, Hippocampal synaptosomes from 2 month old *App*$^{+/+}$ and *App*$^{-/-}$ animals were prepared and stimulated with 10 nM insulin (n=5/5) or 200 nM insulin (n=6/4). pIR, total IR and β tubulin protein levels were measured using the WES system. Data was collected using the area under the peak for the specified proteins, and analysis performed using a Student's t-test with significance $p<0.05$*. Data is graphed as mean values ± SEM.

Figure 5. *App*$^{-/-}$ mice maintain glucose tolerance on a western diet. *App*$^{+/+}$ or *App*$^{-/-}$ mice were maintained on normal (ND) or western diet (WD). ***A***, GTT at 7 weeks on diet (**$p<0.01$, ***$p<0.001$, *App*$^{+/+}$ WD vs. *App*$^{-/-}$ WD at the indicated time points; ##$p<0.01$, *App*$^{+/+}$ WD vs. *App*$^{+/+}$ ND). ***B***, Mean of GTTs performed during weeks 13-19 on diet, expressed as area under the curve, zeroed to the initial $[GTT]_b$ for each mouse.. (***$p<0.001$, **$p<0.01$). ***C***, $[Glc]_b$ in response to insulin at 8 weeks on diet (*$p<0.05$, **$p<0.01$, *App*$^{+/+}$ WD vs. *App*$^{-/-}$ WD at the indicated time points; ##$p<0.01$, *App*$^{+/+}$ WD vs. *App*$^{+/+}$ ND). ***D***, Mean of ITTs performed during

weeks 12-18 on diet, expressed as area over the curve, capped at the initial $[Glc]_b$ for each mouse (*p<0.05).

Figure 6. Aged *App*$^{-/-}$ animals are hypoglycemic when fasted. Twelve-month-old *App*$^{+/+}$ and *App*$^{-/-}$ mice (n=11-13) were fasted and subjected to glucose tolerance testing with IP injections glucose. Area under the curve (AUC) was measured for each animal's blood glucose over time, and *App*$^{+/+}$ and *App*$^{-/-}$ animals were compared by Student's t-test with p<0.05*. Blood glucose was measured and graphed in free fed (n=5) *App*$^{+/+}$ and *App*$^{-/-}$ mice. Significance p<0.05* was determined by Student's t-test. Data is graphed as mean values ± SEM.

Figure 7. APP fragments, APP overexpression, and secretase inhibitors did not alter IDE in *App*$^{-/-}$ cells. ***A***, SIMA9 microglia (n=3/condition) were treated with secretase inhibitors, DMSO vehicle, or left untreated for one week and IDE protein content was measured. Significance p<0.05* was determined by one-way ANOVA. ***B***, *App*$^{-/-}$ primary astrocyte cultures (n=4/condition) were treated with bacterial recombinant sAPPα, sAPPβ, mutant N-terminal sAPPα or eukaryotic-derived sAPPα695 and 751. IDE protein was measured and significance p<0.05* was determined by one-way ANOVA. ***C***, *App*$^{-/-}$ astrocyte primary cultures (n=6/condition) were transfected with APP695, APP751 or GFP plasmids or mock transfected. IDE protein was measured and significance p<0.05* was determined by one-way ANOVA. Data is graphed as mean values ± SEM.