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AMYLOID PRECURSOR PROTEIN AND INSULIN HOMEOSTASIS

by

Joshua Adam Kulas
Bachelor of Science, University of Wisconsin-Madison, (2012)

A Dissertation
Submitted to the Graduate Faculty

of the

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In partial fulfillment of the requirements

for the degree of

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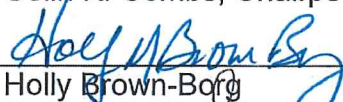
May
2018

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Colin K. Combs, Chairperson



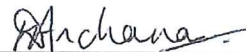
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Title: Amyloid Precursor Protein and Insulin Homeostasis

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Joshua A. Kulas
May, 2018

TABLE OF CONTENTS

LIST OF FIGURES	ix
ACKNOWLEDGEMENTS.....	xi
ABSTRACT	xiii
CHAPTER	
I. INTRODUCTION.....	1
Preface.....	1
History and Discovery of the Amyloid Precursor Protein	2
APP in AD: The Amyloid Hypothesis.....	3
The Physiologic Role of APP.....	4
Structure, Expression and Processing of APP.....	4
The APP Protein Family	5
Functions of APP and APLPs in the Brain and Peripheral Organs.....	6
General Overview of AD.....	8
Metabolic Dysfunction and Alzheimer 's Disease	9
Brain Glucose Hypometabolism in Alzheimer's Disease	9
Insulin and Insulin Receptor in the Brain	11
Insulin and Insulin Resistance in Alzheimer's Disease	13
A β as a Promoter of Insulin Resistance.....	16

	Insulin Degrading Enzyme in Alzheimer's Disease	18
	Intranasal insulin as Therapeutic Agent in Alzheimer's Disease	19
	Dissertation Research Objective	21
II.	METHODS	23
	Animals.....	23
	Animal Use	23
	Human Tissue	24
	Western Blots	24
	Immunohistochemistry/Immunofluorescence	25
	Thioflavin S Staining.....	26
	Antibodies.....	26
	Enzyme Linked Immunosorbent Assays (ELISA)	27
	Pancreatic Islet Isolation and Culture	28
	Min6 Cell Line Culture and Glucose Stimulated Insulin Secretion (GSIS) Assay	29
	Human and Mouse Primary Islet Culture Glucose Stimulated Insulin Secretion (GSIS) Assay	30
	Glucose Tolerance Testing.....	31
	RNA Extraction and RT-qPCR	31
	siRNA Knockdown of APP.....	32
	APP Plasmids and Transfections	32
	Mouse Neuron Primary Culture	33
	Mouse Microglia and Astrocyte Primary Culture.....	33

	IDE Activity Assays	34
	Hippocampus Synaptosome Preparation and Stimulation.....	35
	Statistical Analysis.....	36
III.	RESULTS	37
	Study 1 – Amyloid Precursor Protein in the Endocrine Pancreas	37
	Introduction.....	37
	APP was Expressed within the Endocrine Pancreas	39
	Pancreatic APP was Not Processed to A β	44
	APP/PS1 Mice Showed no Differences in Glucose Tolerance Testing or Pancreatic Insulin Levels.....	49
	APP Expression Modulated Pancreatic BACE2, GLUT4, and IDE Levels in the Transgenic Mice.	51
	BACE2 Immunoreactivity was Localized to Islet Periphery with Decreased Immunoreactivity in APP/PS1 Mice With no Concomitant Increase in A β Immunoreactivity.	53
	Immunohistochemistry for BACE2 Demonstrated Differential BACE2 Localization in α and β Cells in Both Mouse and Human Tissue.....	57
	Primary Murine Islet Cultures Showed Significant Differences in APP and IDE Content.	59
	APP was Processed by α -secretase Activity in Primary Islet Cultures and Recombinant sAPP Potentiated Islet Insulin but not Glucagon Secretion.	61

IV.	RESULTS	65
	Study 2 – Amyloid Precursor Protein and Insulin Degrading Enzyme.....	65
	Introduction.....	65
	IDE Protein and mRNA are Increased in APP-/- Tissues.....	67
	IDE Protein is Increased in APP-/- Neuron, Astrocyte and Microglia Cell Cultures: siRNA Knockdown of APP in Microglia Increases IDE	70
	IDE Activity is Increased in APP-/- Tissues	73
	Insulin Content and Insulin Signaling are Altered in APP-/- Hippocampus tissue and Synaptosomes.....	75
	Aged APP-/- Mice Display Fasting Hypoglycemia Without Changes in Glucose Tolerance	78
	Overexpression of APP or Treatment with Secretase Inhibitors Does Not Alter IDE Levels	80
V.	DISCUSSION.....	83
	Study 1 - Amyloid Precursor Protein in the Endocrine Pancreas	83
	Study 2 - Amyloid Precursor Protein and Insulin Degrading Enzyme.....	91
	Limitations of Work Presented in this Dissertation	97
	Summary Conclusions and Future Directions	99
	REFERENCES.....	101

LIST OF FIGURES

Figure	Page
1. Amyloid precursor protein was detected in mouse and human pancreas including islets of Langerhans.....	41
2. APP immunoreactivity was increased in pancreatic adenocarcinoma	43
3. Amyloid precursor protein was detectable in the insulin producing β cells of the human pancreas and the APP/PS1 mouse	45
4. $A\beta$ was not detectable in mouse pancreas by immunohistochemistry or ELISA	46
5. Thioflavin staining and $A\beta$ immunohistochemistry in human pancreatic tissue and mouse brains	48
6. No difference in glucose tolerance or pancreatic insulin content was observed in APP-/- and APP/PS1 mice, but whole pancreas glucagon content was reduced in APP/PS1 mice	50
7. APP was present in glucagon producing cells in the APP/PS1 mouse	51
8. Differences in 2 month old mouse pancreatic BACE2, IDE and GLUT4 were observed by western blot.....	52
9. Immunohistochemistry of 2 and 12 month old mouse pancreas	54
10. BACE2 immunohistochemistry with epitope blocking peptide control and glucagon immunohistochemistry with optical density quantitation in mouse tissue	55
11. BACE2 was present in glucagon positive cells	58
12. BACE2 immunoreactivity in wild type and transgenic mouse islets	58

13.	Western blots of 12 month old mouse primary islet cultures.....	60
14.	sAPP was released from primary murine and human islet cultures and recombinant sAPP α potentiated islet insulin secretion during glucose stimulated insulin secretion assay	62
15.	IDE is increased in APP-/- tissues	68
16.	IDE is increased in APP-/- cell cultures.....	71
17.	IDE activity is increased in APP-/- tissues	74
18.	Insulin signaling and content are altered in the APP-/- brain	76
19.	Aged APP-/- animals show metabolic changes	79
20.	APP fragments and secretase inhibitors do not alter IDE	81
21.	Model of Pancreatic APP and summarized findings	100

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease in which the brain progressively deteriorates resulting in cognitive decline, language impairment, diminished spatial awareness, memory loss and ultimately, death. AD is the most prevalent neurodegenerative disease and the number of people afflicted by AD is expected to more than double in the next 30 years. AD is characterized in part by the formation of amyloid plaque deposits in the brain. It is known that these plaques are composed of the peptide A β , which aggregates as a function of age and is produced locally within the brain by cleavage of the Amyloid precursor protein (APP). It is now recognized that type 2 diabetes is a risk factor for the development AD and the AD brain has impaired insulin signaling. Transgenic mice lacking the APP gene show broad changes in metabolism, suggesting APP may play an important in regulating metabolic homeostasis. This dissertation consists of two central studies aimed at understanding the relationship between APP and insulin signaling. The first aim is to examine the role APP plays in plaque formation within the pancreas and to understand APP's contribution to endocrine pancreas physiology. The second aim is to examine the role APP plays in the regulation of the Insulin Degrading Enzyme (IDE).

In the first study, APP is found to be present in the pancreas and overexpressed within the insulin producing β cells in the APP/PS1 mouse model of AD. APP presence and overexpression does not result in the formation of the A β peptide or plaques, but instead APP is processed through α -secretase pathway to yield sAPP α . In primary cultures of pancreatic islets, increased APP expression results in increased insulin secretion. sAPP α was detected in the cell conditioned media of pancreatic islet primary cell cultures. Application of recombinant sAPP α to both human and mouse pancreatic islet cultures potentiates glucose stimulated insulin secretion. This effect is observed when sAPP α was applied to the Min6 pancreatic β cell line. The BACE2 enzyme, an APP cleaving α -secretase enzyme, localizes to pancreatic α cells. Additionally, increased protein levels of IDE are observed in both pancreatic tissue lysate and primary islet cultures of mice lacking the APP gene (APP $^{-/-}$).

In the second study, we found IDE protein is found to be increased in numerous tissues including muscle, liver and brain from the APP $^{-/-}$ mouse compared to wild type (WT) controls. IDE mRNA levels are also found to be increased in APP $^{-/-}$ animal brain tissue. IDE protein is ubiquitous in the brain and is detectable in neuron, astrocyte and microglia primary cell cultures. APP $^{-/-}$ primary cultures of these cell types shows increased protein levels of IDE. siRNA knockdown of APP is sufficient to increase IDE protein in a cell line. IDE activity is found to be increased in APP $^{-/-}$ tissues. APP $^{-/-}$ brain tissue shows diminished levels of insulin signaling and reduced insulin content. Synaptosomes stimulated

acutely with insulin from APP^{-/-} animals show diminished insulin signaling compared to WT synaptosomes.

CHAPTER I

INTRODUCTION

Preface

The topic of this dissertation is focused on the role of the amyloid precursor protein (APP) and its contribution to insulin homeostasis. APP is established to play a central role in the pathology of Alzheimer's Disease. Insulin signaling and metabolic homeostasis are important factors in influencing the progression of the disease. Elucidating the precise physiologic role APP plays in metabolic function and insulin signaling is essential for understanding the pathophysiology of this debilitating neurodegenerative disease.

For this dissertation, two separate studies were conducted involving the role of Amyloid precursor protein and its role in the production, secretion and clearance of the insulin peptide. The first study examines the amyloid precursor protein in the pancreas and tests the hypothesis that Alzheimer's disease-like peptide plaques can form in the insulin producing β -cells to cause local cell death, insulin insufficiency and glucose intolerance. The second study builds off of findings in the first and examines the role of amyloid precursor protein in both the central nervous system and peripheral organs to test the hypothesis that APP acts as a negative regulator of the Insulin Degrading Enzyme (IDE).

History and Discovery of the Amyloid Precursor Protein

The Amyloid precursor protein and its role in health and disease is perhaps best understood in the context of its discovery. During the early 19th century the German physician Alois Alzheimer reported the case of a female patient with unusually advanced dementia for her age. The patients' health quickly deteriorated and she soon passed away. Following her death, Alois performed an autopsy of the patient's brain and was surprised to observe what would become known as the hallmarks of "Alzheimer's Disease" (AD). These observations included the formation of aggregated "plaques" and intracellular "tangles" in brain tissue as well as changes in the lipid composition of the brain (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995).

It was not until several decades later that the physical composition of these plaques was elucidated by the chemists Glenner and Wong, who revealed that these seemingly neurotoxic plaques were actually formed of a self-aggregating peptide approximately 40 amino acids in length (Glenner & Wong, 1984). Around this same time it was recognized that the plaques observed in AD brains were chemically identical to the plaques found in the brains of aged patients with Downs Syndrome (Masters et al., 1985).

It was hypothesized that the plaque forming peptide, now termed Amyloid Beta ($A\beta$), may actually be cleaved from a larger protein. The pursuit of this hypothesis lead to the landmark discovery of the Amyloid precursor protein (APP) in 1987 (Kang et al., 1987). The gene encoding APP in humans was confirmed to be located on chromosome 21, thus it is believed that humans

afflicted with Down's Syndrome have an extra copy of the gene which results in increased APP and consequently more A β production (Blanquet et al., 1987; Robakis et al., 1987). The discovery of APP and subsequent research revealed that it is a transmembrane protein resembling a cell surface receptor and is ubiquitous throughout the body, but especially abundant in nervous tissue (Selkoe et al., 1988).

APP in AD: The Amyloid Hypothesis

The theory that Alzheimer's Disease is caused by the buildup of toxic A β in the brain known as the Amyloid Hypothesis. After APP was discovered, it was generally recognized that for the A β peptide to become liberated from the larger Amyloid precursor protein, proteolytic enzymes would likely be required. The locations of the amino acids within APP that must be hydrolyzed to generate A β peptide could be logically deduced from the known sequences of APP and A β . Thus, during the 1990's several independent research groups sought to identify the proteolytic enzymes, termed "secretases" which could cut the APP protein at these defined sites. It was hoped that identification of these enzymes could lead to pharmacological targets. In 1995 the γ -secretase enzyme was identified, and it was revealed that mutations in this enzyme lead to early onset AD (Levy-Lahad et al., 1995; Sherrington et al., 1995). This finding, along with the finding that certain familial mutations in APP drive another form of early onset AD, greatly bolstered the evidence for the Amyloid Hypothesis (Citron et al., 1992). It is now accepted that A β and its aggregated oligomeric forms are generally neurotoxic.

At this present time, the Amyloid Hypothesis remains the dominant theory of AD (Selkoe & Hardy, 2016).

The Physiologic Role of APP

Structure, Expression and Processing of APP

APP is a single pass transmembrane protein with a small intracellular c-terminal region, and a large extracellular n-terminal region (Kang et al., 1987; U. C. Muller & Zheng, 2012). The protein is trafficked to the cell membrane via the conventional secretory pathway, and contains a YENPTY motif on its c-terminus for clathrin-mediated endocytosis (Jacobsen & Iverfeldt, 2009; Muresan & Ladescu Muresan, 2015). Numerous isoforms of the APP gene have been reported, but three isoforms in particular seem to be most abundant (Golde, Estus, Usiak, Younkin, & Younkin, 1990; Pan, Monteggia, & Giordano, 1993; Selkoe et al., 1988). Neurons produce the relatively lower molecular weight 695 amino acid APP, while most other cell types including glial cells produce the 750 to 770 amino acid isoforms. Alternative splicing of APP varies with tissue and cell type (Nalivaeva & Turner, 2013). Unlike the APP695, the heavier molecular weight isoforms of APP contain a Kunitz protease inhibitor domain and heparin binding domain which may be important for their function.

Mature APP protein can be enzymatically processed by a variety of secretases, resulting in the formation of different APP fragments ranging from small peptides to large soluble 100 kilodalton proteins. In general, this processing is described as “amyloidogenic” or “non-amyloidogenic”, meaning producing or not-producing A β respectively. Amyloidogenic processing of APP involves the

cleavage of APP by the BACE1 enzyme (β -secretase) on the extracellular domain, followed by cleavage of APP within the cell membrane by the γ -secretase protein complex to ultimately yield the A β peptide along with a secreted APP fragment sAPP β (Selkoe & Hardy, 2016; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). In non-amyloidogenic processing, the APP protein is cleaved within the A β sequence by one of the many α -secretase enzymes resulting in the release of the large sAPP α fragment extracellularly, where it is thought to act as a ligand for a variety of receptors (Habib, Sawmiller, & Tan, 2017).

In addition to processing mechanisms involving the ectodomain of APP, the intracellular domain of APP can also be cleaved to yield a short peptide fragment known as AICD (APP Intracellular Domain). This AICD region of APP contains the YENPTY signal sequence and interacts with a variety of other proteins. AICD is thought to be involved in the regulation of gene expression (Kimberly, Zheng, Guenette, & Selkoe, 2001).

The APP Protein Family

The APP protein is part of a family of three known proteins including APP, APLP1 and APLP2 (U. C. Muller & Zheng, 2012). Like APP, APLP1 and APLP2 are both single pass transmembrane proteins. APLP1 and APLP2 are processed similarly to APP, but cannot produce A β due to the lack of the amyloidogenic A β sequence within them (Eggert et al., 2004; Li & Sudhof, 2004). APLPs are capable of being processed through both α and β secretase processing mechanisms. Like APP, the extracellular domain of the APLPs is released into

extracellular space when the proteins are cleaved by α -secretase. This protein fragment is termed either sAPLP1 α or sAPLP2 α (Walsh et al., 2007). While APP and APLP2 are ubiquitously expressed, APLP1 expression is limited to the brain (Lorent et al., 1995; Wasco et al., 1993).

Functions of APP and APLPs in the Brain and Peripheral Organs

The APP family of proteins is widely conserved among species and present in numerous multicellular organisms, suggesting the gene is biologically important or advantageous in survival and proliferation (Tharp & Sarkar, 2013). While the role of APP in the generation of A β is now well characterized, the physiologic functions of APP are less certain. Studies in transgenic animals have revealed that the APP family of proteins is required for survival, implying these proteins perform an essential biological function (Heber et al., 2000; Needham et al., 2008). Specifically, studies in transgenic mice reveal that mice must express either the APP or APLP2 gene in order to survive and develop normally past the neonatal stage. Experimental evidence suggests this lethality may be due in part to robust deficits in the development of the neuromuscular junction (Klevanski et al., 2014; P. Wang et al., 2005). Curiously, this fatal double knockout mouse for APP and APLP2 is hypoglycemic before death (Needham et al., 2008). Genetic ablation of just APP alone confers a more benign phenotype, with significant reductions in mouse brain volume, animal size, body weight, serum glucose and changes in whole body metabolism (U. Muller et al., 1994; Needham et al., 2008;

Puig et al., 2017; Zheng et al., 1995). As in mice, in the nematode *C. elegans*, ablation of the APP homologue APL-1 is lethal (Ewald & Li, 2012).

Functional characterization of the APP family of proteins highlights their importance, particularly in the central nervous system. The APP family of proteins have been characterized as synaptic adhesion molecules and upregulated during synaptogenesis (Schilling et al., 2017). Furthermore, the full length APP protein itself is believed to be involved in certain signaling processes and may function as a cell surface receptor for extracellular ligands (Hass & Yankner, 2005; Kang et al., 1987; B. Wang et al., 2017). APP and sAPP α play a role in neurite outgrowth and neuronal migration (Gakhar-Koppole et al., 2008; Hasebe et al., 2013; Ramaker, Swanson, & Copenhaver, 2016). sAPP α has also been shown function as a neurotrophic factor and may play neuroprotective role in the central nervous system (Fol et al., 2016; Hick et al., 2015; Weyer et al., 2011). Additionally, characterization of aged APP $^{-/-}$ mice has revealed that these animals have fewer dendritic spines in addition to learning deficits (K. J. Lee et al., 2010; Tyan et al., 2012). Taken together, these studies suggest APP and its metabolites influence a variety of cellular processes within the brain and are of particular importance in synapse development and function.

Outside of nervous cells, APP has been implicated in a diverse set of biological processes. In immune cells, APP has been demonstrated to be upregulated under pro-inflammatory conditions and may serve roles in cell migration and adhesion as well as regulating general immune cell phenotype (Banati et al., 1993; Manocha et al., 2016; Puig et al., 2017; Sondag & Combs,

2004, 2010). In the skin, APP has a role in regulating cell proliferation and maturation (Herzog, Kirfel, Siemes, & Schmitz, 2004; Hoffmann, Twiesselmann, Kummer, Romagnoli, & Herzog, 2000). In skeletal muscle tissue, sAPP α has been shown to potentiate glucose uptake and APP localizes to the neuromuscular junction with acetylcholine receptors (Hamilton et al., 2014; P. Wang et al., 2005). APP is also present in adipose tissue, where it seems to be upregulated under certain pro-inflammatory conditions and through certain cytokines (Puig, Floden, Adhikari, Golovko, & Combs, 2012; Sommer et al., 2009). APP can be detected in intestinal tissue associated enteric nervous system. Its presence plays a role in nutrient uptake and overall intestinal phenotype (Puig, Lutz, et al., 2015; Puig, Manocha, & Combs, 2015; Puig, Swigost, Zhou, Sens, & Combs, 2012). These studies are but a fraction of the wide array of research documenting the varied and numerous roles of APP in peripheral organs. With increasing research it is becoming clear that the role of APP in each tissue changes depending on the physiological function of the organ and the types of proteins which can both interact with and process APP locally.

General Overview of AD

Alzheimer's disease has become the most prevalent neurodegenerative diseases and a leading cause of death of the elderly. Approximately 5.5 million people in the United States are currently afflicted with AD, and the incidence of the disease is expected to rise to approximately 13.8 million people by 2050 (Taylor, Greenlund, McGuire, Lu, & Croft, 2017). AD is progressive and neurodegenerative, and is characteristic in its progression by affecting particular

regions of the brain more prominently than others. The limbic system and temporal lobes, including the hippocampal formation of the brain are dramatically affected and show pathological changes early in the disease (Braak & Braak, 1997a, 1997b). Pathological hallmarks of AD include A β plaque deposits in the brain and blood vessels, as mentioned above, and the hyperphosphorylated protein Tau which forms intracellular tangles. The regions of the brain affected are critically involved in both the formation and storage of memories, thus their degeneration results in the notorious phenotype of memory which occurs in AD (Teyler & Rudy, 2007). In addition, notable language impairment and spatial memory deficits are observed in AD (Morello, Lima, & Brandao, 2017). At this time there are no available cures for the disease. Therapies which combat the symptoms of AD are available, but limited in their effectiveness (C. Zhang, 2017). Ultimately, affliction with AD results in the death of the patient. In the United States 3.6% of all premature death is due to AD (Taylor et al., 2017).

Metabolic Dysfunction and Alzheimer's Disease

Brain Glucose Hypometabolism in Alzheimer's Disease

Glucose is essential for normal brain function and brain development (Burns, Rutherford, Boardman, & Cowan, 2008; Cornblath & Reisner, 1965). While the brain can utilize ketones as a source of fuel during prolonged fasting and starvation, glucose serves as the primary energy source for the brain. Severe hypoglycemia results in coma and eventually, death. To preserve function and survival, the brain tightly regulates blood glucose levels and food intake (Xu, O'Malley, & Elmquist, 2017). The brains ability to regulate blood sugar

was first demonstrated by the French physiologist Claude Bernard in 1854.

Bernard found that by lesioning the floor of the brain's fourth ventricle in rabbits blood sugar levels would subsequently increase (Tups, Benzler, Sergi, Ladyman, & Williams, 2017).

In the 1980s, the German physician-scientist Siegfried Hoyer was the first to hypothesize that metabolic dysfunction may play a role in the pathogenesis of Alzheimer's disease and age related cognitive decline. In his time, Hoyer's theories were controversial as they challenged the more traditional "plaques and tangles" view of AD. Specifically, Hoyer observed that glucose metabolism was significantly impaired in the brain of patients with advanced AD, compared to aged matched controls (Hoyer, 1982, 1986). In one patient cohort, he observed more than a 40% reduction in the metabolic rate of glucose of AD patients (Hoyer, Oesterreich, & Wagner, 1988). Hoyer's seminal observations have been further validated over the years by several researchers (Meier-Ruge, Bertoni-Freddari, & Iwangoff, 1994; Sugimoto et al., 2017). This pattern of impaired and regional glucose hypometabolism is not restricted to AD, but is also observed in aged patients with Downs syndrome (Lao et al., 2018). Curiously, this phenomenon in some cases seems to precede, and cannot solely be explained by, changes in gray matter volume in the brain (Wirth et al., 2017). Glucose hypometabolism is also observed in the brains of various animal models of AD (Dodart, Mathis, Bales, Paul, & Ungerer, 1999; Macdonald et al., 2014; Sadowski et al., 2004). In the McGill-R-Thy1-APP rat model of AD, which overexpresses a human mutant APP to produce A β aggregates in the brain, glucose

hypometabolism is observed in aged animal brains along a similar time course with development of A β plaque deposits (Parent et al., 2017). This observation, which is dependent on brain cells taking up the radiofluorinated glucose molecule [^{18}F]FDG, has been shown to be a signal of impaired energy consumption in both neurons and astrocytes (Zimmer et al., 2017). The time course of brain hypometabolism across AD animal models and its detectability in A β driven models of AD suggest A β may be the primary molecular mediator of this phenomenon. However, this does not preclude the possibility that hypometabolism is critically involved in the pathophysiology and development of AD.

Insulin and Insulin Receptor in the Brain

For several decades, it was believed that insulin does not play an important role in brain physiology. This assumption was based on the experimental observation that brain glucose uptake is not robustly stimulated by, or dependent on, insulin *in vivo* (Best, Taborsky, Halter, & Porte, 1981; Goodner, Hom, & Berrie, 1980; Hertz, Paulson, Barry, Christiansen, & Svendsen, 1981). Furthermore, the cells of the brain including neurons were found to utilize different glucose transporters than the insulin sensitive muscle and adipose cells. Neurons primarily utilize the high affinity transporter Glut3, while skeletal muscle and adipose tissue express insulin responsive Glut4 (Stringer, Zahradka, & Taylor, 2015). The brain was thus deemed to be an insulin-insensitive organ. However, more recently, numerous research findings have done much to shift this paradigm and it is now generally accepted that both insulin and the insulin

receptor play a significant role in brain function and brain regulation of peripheral metabolism (Kleinridders, Ferris, Cai, & Kahn, 2014).

While the vast majority of insulin in the human body is synthesized in the β cells in the islets of Langerhans of the pancreas, both the insulin peptide and insulin mRNA are detectable in the brain, suggesting the brain may produce small amounts of insulin locally (Devaskar et al., 1994; Devaskar, Singh, Carnaghi, Rajakumar, & Giddings, 1993). Insulin has also been detected in primary neuron cultures (D. W. Clarke, Mudd, Boyd, Fields, & Raizada, 1986). In addition, insulin is actively transported across the blood-brain barrier (Baura et al., 1993). Increases in peripheral insulin have been shown to activate insulin signaling in specific regions of the brain (Kleinridders et al., 2013). The insulin receptor (IR) is distributed throughout the brain including the olfactory bulb and hypothalamus and is particularly abundant in the hippocampus, cortex and thalamus (Kleinridders et al., 2014). Much of what is known about the role of the insulin receptor in the brain comes from the brain-specific IR knockout mouse generated by the lab of Dr. Ronald Kahn (Bruning et al., 2000). These animals show changes in feeding behavior, become obese and insulin resistant and also develop hypothalamic dysfunction. Thus, brain IR is thought to play an essential role in regulating metabolism in the periphery. Additionally, insulin is thought to participate in the normal function of the brain. Infusion of insulin into the rat brain improves performance in spatial memory tasks (Biessels et al., 1998; Park, Seeley, Craft, & Woods, 2000). The hippocampus is essential for learning and memory and insulin is now believed to play an important role in hippocampal

function. Insulin has been shown to modulate the activity of hippocampal neurons and likely plays an important role in synaptic plasticity and long term potentiation (van der Heide, Kamal, Artola, Gispen, & Ramakers, 2005). This finding has been further validated by the fact that insulin has been shown to have comparable roles in activating signaling cascades and affecting neuron function in other neuronal types (Klockener et al., 2011). In stark contrast to the previous dogma of insulin's role in the brain, recent evidence also suggests insulin plays a surprising role in brain energy utilization. In astrocytes, which are the most abundant cell in the brain, insulin has been shown to enhance glucose uptake and regulate metabolism. Insulin, in cooperation with the related insulin-like growth factor 1 (IGF-1) enhances glucose uptake by regulating the activity and subcellular location of the Glut1 transporter (Fernandez et al., 2017; Hernandez-Garzon et al., 2016). Furthermore, insulin mediated astrocyte glucose uptake is critical for brain regulation of peripheral metabolic processes and also regulates glucose flux across the blood-brain barrier (Garcia-Caceres et al., 2016).

Insulin and Insulin Resistance in Alzheimer's Disease

Given the important roles of insulin in the brain it is not surprising that insulin resistance is associated with cognitive dysfunction and neurodegeneration. Human patients with type 1 or type 2 diabetes have accelerated cognitive decline as they age, in addition to behavioral changes (Biessels, Deary, & Ryan, 2008). Furthermore, epidemiological evidence has implicated type 2 diabetes (T2DB), or insulin resistance, as a risk factor for AD, and T2DB has been shown to more than double the risk of developing late onset

AD (Mittal & Katare, 2016; Vagelatos & Eslick, 2013). Metabolic dysfunction in general, including obesity, also accelerates the progression of cognitive decline and dementia (Beydoun, Beydoun, & Wang, 2008). Some researchers are now considering Alzheimer's disease as a third form of diabetes, or type 3 diabetes (de la Monte, 2014). This hypothesis states that insulin resistance in the brain is a causative factor in AD. The experimental basis of this hypothesis is largely grounded in the work published by Talbot et. al in 2012 (Talbot et al., 2012). Talbot performed human brain tissue slice cultures from both AD patients and age matched controls. These slices were then stimulated with near physiologic levels (1-10 nM) of either insulin or IGF-1, and the induction of the insulin signaling cascade was measured by assessing multiple markers including phosphorylation of the insulin receptor and other downstream markers such as pAKT and pIRS. Talbot observed significant insulin signaling resistance and IGF-1 signalling resistance in the AD brain, including in the hippocampus. This provided the first experimental evidence demonstrating significant insulin resistance in the human AD brain.

The relationship between insulin and AD extends to animals models. In modeling type 1 diabetes, a disease of insufficient insulin production, researchers often utilize the toxin streptozotocin (STZ). STZ selectively kills the insulin-producing β cells of the pancreas, resulting in insulin insufficiency and consequently hyperglycemia. It has been found that monkeys treated with STZ develop AD-like pathology in the brain. This includes increased levels of A β in the hippocampus, changes in Tau phosphorylation and perturbations of several

insulin signaling markers within the brain (Morales-Corraliza et al., 2016). Injections of A β oligomers into the monkey brain also cause insulin resistance directly in the CNS, an effect which is rescued with insulin sensitizing GLP-1 receptor agonists (Bomfim et al., 2012). Hyperglycemia, glucose intolerance and insulin resistance have been noted across several mouse models of AD. These metabolic perturbations occur as a function of age and are exacerbated by high fat diet or STZ-induced insulin insufficiency, suggesting that maintaining peripheral metabolic homeostasis is an important factor in the progression of the AD phenotype (de la Monte, Tong, Schiano, & Didsbury, 2017; Macklin et al., 2017; Vandal et al., 2015; Vandal et al., 2014). Hyperglycemic clamp and microdialysis experiments in rodents demonstrate that hyperglycemia alone is sufficient to drive increased production of A β in the CNS *in vivo* (Macauley et al., 2015). Interestingly, A β alone may not be the sole mediator of insulin dysregulation in the brain. In addition to A β deposits in the brain, intracellular Tau “tangles” are a known pathological feature of the AD brain. Tau has recently been identified to being a potent regulator of insulin signaling in the brain, and deletion or loss of function of Tau drives brain insulin resistance (Marciniak et al., 2017).

In addition to the view that peripheral metabolic defects may drive the pathogenesis of AD, experimental evidence also suggests that increasing A β concentrations in the brain is sufficient to induce diabetes and aberrant whole-body metabolism. Intracerebroventricular injections of A β oligomers into the brains of mice results in peripheral glucose intolerance, possibly due to damage

induced to the hypothalamus. In TNF α receptor knockout mice increasing brain A β does not result in glucose intolerance, suggesting that inflammation plays a necessary role in this phenomenon (J. R. Clarke et al., 2015). Brain-specific knock-in of BACE1 (β -secretase) causes diminished brain glucose uptake and systemic diabetes as a function of age, suggesting that increased brain amyloid burden is the direct mediator of peripheral metabolic dysfunction observed in Alzheimer's (Plucinska et al., 2016). In total, these studies demonstrate that peripheral metabolism and insulin production play a direct role in regulating the progression of AD in the brain, and conversely, that the health of the brain plays a direct role in regulating peripheral metabolism and insulin sensitivity.

A β as a Promoter of Insulin Resistance

In vitro experiments have provided additional mechanistic insight into the role of the A β peptide in disrupting insulin signaling. The A β peptide monomer has been shown to directly bind the insulin receptor. Binding of A β to the IR is competitive with insulin and does not stimulate IR. Increasing concentrations of A β inhibit IR phosphorylation and downstream signaling in the presence of insulin, thus A β acts something like a competitive antagonist. This antagonistic effect of A β for the IR is observed for both the 40 and 42 amino acid lengths of the A β peptide and interestingly, is not observed if the sequence of amino acids in A β is modified (Xie et al., 2002). It is reasonable to question if A β -IR interaction occurs *in vivo*. The estimated K_i of this competitive interaction is in the micromolar range (8 μ M). This far exceeds the normal physiologic concentrations of A β in human cerebral-spinal fluid which are in the nanomolar range or less

(Schirinzi et al., 2017). Micromolar concentrations of A β have also been shown to antagonize the effect of insulin when applied to rat hippocampal and cortical synaptosomes (Heras-Sandoval, Ferrera, & Arias, 2012). Insulin signaling in cultured cells has been shown to be disrupted by intracellular A β , which may have more relevance *in vivo* (H. K. Lee, Kumar, Fu, Rosen, & Querfurth, 2009).

Curiously, A β and sAPP α seem to have antagonistic effects on insulin receptor signaling. Stimulation of muscle or neuronal cultures with sAPP α induces insulin signaling and has neuroprotective effects (Hamilton et al., 2014; Jimenez et al., 2011). Viral mediated sAPP α overexpression has been shown to have pro-cognitive effects in an AD mouse model (Fol et al., 2016). Furthermore, stimulation of cells with insulin or sAPP α shifts APP processing from amyloidogenic processing to non-amyloidogenic processing (Son et al., 2012; X. Wang et al., 2014). sAPP α in particular has been shown to decrease BACE1 activity to reduce A β production (Obregon et al., 2012). However, insulin has been shown to potentiate A β secretion in some cell types, though the effect of this on AD progression is uncertain (Najem, Bamji-Mirza, Yang, & Zhang, 2016; Tharp et al., 2016). A β oligomers have also been shown to inhibit insulin receptor signaling in cultured cells. Treatment of primary hippocampal neuron cultures with A β oligomers has been shown to downregulate IR expression (Liu et al., 2014). It is possible to some extent that A β induced insulin resistance observed *in vivo* is a secondary consequence of inflammation. A β oligomers are potent pro-inflammatory agents, and inflammation has long been known to have a role in inducing insulin resistance in tissues (Hotamisligil, 2006). Indeed, it has been

shown that the some of the deleterious effects of A β oligomers on cognition are dependent on promoting inflammation to impair insulin signaling (Lourenco et al., 2013).

Insulin Degrading Enzyme in Alzheimer's Disease

While mechanisms involving A β production have been the subject of extensive research efforts, much less is known about A β clearance mechanisms and their role in AD. During the 1990's two enzymes were independently identified as having the ability to catabolize the A β peptide; neprilysin (NEP) and insulin degrading enzyme (IDE) (Howell, Nalbantoglu, & Crine, 1995; Kurochkin & Goto, 1994). IDE is of particular interest because of its role in clearing insulin from tissues, thus making it critically important in regulating insulin signaling. In humans, IDE is located on chromosome 10 (Espinosa et al., 1991). Damage to chromosome 10 in the region of IDE has been associated as conferring an increased risk of developing AD (Bertram et al., 2000). Furthermore, tissue samples from human families that are known to develop AD and also have mutations in chromosome 10 have been shown to have decreased IDE activity (Kim et al., 2007). These studies indicate IDE plays an important role in protecting the brain against the development of AD. IDE polymorphisms have also been found to be associated with the development of type 2 diabetes (Karamohamed et al., 2003).

IDE is expressed throughout the body and plays an important role in clearing insulin from traditionally insulin-sensitive tissues (Bondy et al., 1994). When insulin stimulates its canonical receptor, the insulin-receptor complex can

be endocytosed and the bound insulin degraded by IDE, or returned to the plasma membrane and released back into circulation (Levy & Olefsky, 1987). IDE has been shown to play a role in regulating cellular sensitivity to insulin signaling. Degraded insulin peptide fragments still have some ability to bind and activate the insulin receptor (Duckworth, 1988). The majority of IDE is located in the cytosol, but the enzyme is also detected in endosomes, lysosomes and mitochondria and is secreted in exosomes (Duckworth, Bennett, & Hamel, 1998). Interestingly, IDE substrates are not determined by the presence of a particular amino acid sequence, but by relative size and charge. In addition to insulin and A β , IDE has been shown to degrade a wide range of substrates including amylin, glucagon and the AICD fragment of APP (Kurochkin, Guarnera, & Berezovsky, 2018).

IDE is detectable in all of the major cell types of the brain including neurons (Vekrellis et al., 2000). Microglia have also been reported to secrete IDE which may play an important role in A β plaque clearance from the brain (Qiu et al., 1998). Insulin competitively inhibits the degradation of A β by IDE, thus it has been speculated that the hyperinsulinemia observed in type 2 diabetes may slow clearance of A β in brain tissue (Kurochkin & Goto, 1994). IDE is one of the strongest molecular links connecting AD and diabetes to date.

Intranasal Insulin as Therapeutic Agent in Alzheimer's Disease

Given the important role of insulin in normal brain function and the demonstrated insulin resistance observed in AD derived brain tissues, it stands to reason that enhancing insulin signaling in the brain may serve as a therapeutic

strategy to combat AD. However, increasing insulin concentrations in the brain without altering systemic insulin levels and signaling represents a pharmacokinetic challenge. Interestingly, intranasal administering of the insulin peptide represents a possible solution. This possibility has now been tested in humans and animals with remarkable results. In humans, it has been shown that intranasal administration of insulin peptide results in significantly increased concentrations of insulin peptide within the cerebral spinal fluid within 10 minutes and peaks 30 minutes (Born et al., 2002). Importantly, intranasal insulin administration does not robustly change plasma insulin concentrations. This method of dosing insulin directly to the brain has also been demonstrated to work in rodents (Salameh et al., 2015). Intranasal insulin administration has been tested in mouse models of AD and has shown positive effects on memory and cognition. Long term administration of intranasal insulin has shown shown to promote neuron survival and stimulate insulin signaling (Apostolatos et al., 2012). Additionally, in AD mice, long term intranasal insulin promotes neurogenesis and rescues memory defects (Mao et al., 2016; Y. Zhang et al., 2016).

Clinical trials in humans utilizing intranasal insulin to attenuate cognitive and memory deficits are currently ongoing. Evidence to this point has been positive, suggesting that both acute and long-term administration improves cognitive performance (Claxton et al., 2015; Craft et al., 2012; Craft et al., 2017). This includes improved performance in verbal memory scores and selective attention. At this time however, studies in ApoE4 allele patients are mixed.

ApoE4 is a potent risk factor for AD and some evidence suggests intranasal insulin is not effective in these patients (Rosenbloom et al., 2014).

The apparently promising results of these early clinical trials, prevalence of AD in human populations and mounting evidence that insulin signaling and resistance play a prominent role in the pathology of AD warrant the need for additional studies into the biology of insulin in AD mediated neurodegeneration.

Dissertation Research Objective

This dissertation is intended to continue our laboratory's efforts to characterize the physiologic functions of APP and to also gain a greater understanding of the relationship between insulin and Alzheimer's disease. Our lab has focused on understanding the biology of APP in a number of peripheral organs including intestine, immune and adipose tissue in order to gain a greater understanding of APP's biology and its potential role in disease. Our attention is primarily focused on APP function and processing and to look for downstream changes which may be induced by APP loss or overexpression. The objective of this dissertation is to characterize APP's role in insulin homeostasis. We focus on pancreatic dysfunction as it is a well-known factor in insulin resistant diabetes. We also focus on the insulin degrading enzyme because of its established link as

an enzyme linking diabetes and AD. This dissertation is broken into two studies aimed at addressing the following questions:

1. What role does APP play in the function and pathophysiology of the endocrine pancreas?
2. What is the relationship between APP and IDE expression and activity?

CHAPTER II

METHODS

Animals

The APP knockout mice (APP^{-/-}) strain B6.129S7-Apptm1Dbo/J, the APP/PS1 transgenic mouse line, strain 005864 B6.Cg-Tg (APP^{swe},PSEN1^{dE9})85Dbo/ Mmjax and wild type mouse line, C57BL/6, were purchased from the Jackson Laboratory (Bar Harbor, Maine). The APP^{-/-} mice have the APP gene knocked out in the entire organism by the insertion of a neomycin resistance cassette into the promoter region and Exon 1 of the APP gene. The APP/PS1 mice express the Swedish mutation in APP and deltaE9 mutation in the PS1 gene under the control of the mouse prion promoter. APP/PS1 mice have been shown to develop AD-like A β plaques within the brain around 6 months of age. Females from all three strains of mice were collected at 2 and 12 months of age for analysis.

Animal Use

All animal use was approved by the University of North Dakota Institutional Animal Care and Use Committee (UND IACUC) protocols 1505-4 and 0712-1C. Mice were provided food and water ad libitum and housed in a 12 h light/dark cycle. The investigation conforms to the National Research Council of

the National Academies Guide for the Care and Use of Laboratory Animals (8th edition).

Human Tissue

Acetone fixed 10µm healthy and diseased human pancreatic tissue sections were obtained from Bio-Chain (Newark, CA, USA). Isolated pancreatic islets were obtained from Prodo Labs (Aliso Viejo, CA, USA). Tissue use was approved by the UND Institutional Review Board (protocol IRB-200412-198).

Western Blots

Whole pancreas, isolated murine islets and isolated human islets were lysed in RIPA Buffer containing protease inhibitor (Sigma P8340, St. Louis, MO, USA) on ice. Protein concentrations were determined using the Bradford method (Bradford, 1976). In study II, Hippocampus, gastrocnemius muscle, liver and primary cell cultures were lysed in RIPA Buffer containing protease inhibitor (Sigma P8340, St. Louis, MO, USA) on ice. Protein concentrations were determined using the Bradford method 5-10 µg of protein was resolved by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1 hour in 5% BSA-TBST solution and then 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 hours. Luminol chemiluminescence was utilized for visualization of proteins.

Immunohistochemistry/Immunofluorescence

Pancreatic tissue was fixed in 4% paraformaldehyde (PFA) and cut into 10µm serial sections by cryostat. Mouse brains were fixed in 4% PFA, embedded in gelatin, and serial sectioned to a thickness of 40µm using a sliding microtome. The tissue was incubated overnight in primary antibody diluted in a PBS based solution (1% Triton X-100, 3% BSA, 2% horse serum). Vector VIP (Vector Labs, Burlingame, CA, USA) was used as the chromogen for visible light microscopy. For immunofluorescent co-localization, Alexa Fluor 594 and Alexa Fluor 488 conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA) were used with a DAPI counterstain. Nonspecific binding of secondary antibodies was assessed by applying only the secondary antibody in blocking PBS solution. BACE2 epitope blocking peptide studies were conducted by incubating tissue with 1 µg/mL anti-BACE2 antibody with or without 2 µg/mL BACE2 blocking peptide (Abcam ab8392, Cambridge, UK). BACE2 staining was quantified by using Photoshop CS6 software. Images of islets were converted to greyscale and traced around the periphery to assess total islet BACE2 staining intensity. The total islet staining intensity was divided by the total area assessed to normalize for islet size. A total of twelve non-serial sections from 12 month old animals (n=8) from each strain were stained and assessed in this manner. A minimum of twenty islets from each strain of mice was analyzed for its BACE2 staining

intensity. Values obtained from this analysis were normalized to the mean staining intensity value of the wild type strain.

Thioflavin S Staining

Thioflavin S staining was optimized in APP/PS1 mouse brain tissue and then performed in pancreatic tissue by first quenching endogenous fluorescence by incubating slides in 0.2% Sudan Black dissolved in 50% ethanol for 5 minutes. Slides were then washed in PBS and then incubated in 0.02% thioflavin S dissolved in dH₂O for 30 minutes. Slides were washed again in PBS then dehydrated and coverslipped in Permount.

Antibodies

Antibodies against full length APP (ab32136), BACE2 (ab8025), glucagon (ab10988), IDE (ab32216) and the insulin receptor (ab69508) were purchased from Abcam (Cambridge, UK). Antibodies against insulin (L6B10), p-GSK3 β (D3A4), GSK3 β (D5C5Z), p-AKT (193H12) and AKT (C67E7) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The monoclonal 4G8 antibody targeting the A β peptide used in these studies was purchased from Covance (Princeton, NJ, USA) and is now available from Biolegend (San Diego, CA, USA). Antibodies against the N-terminus of APP (MAB348), GLUT2 (07-1402), and GLUT4 (07-1404) were purchased from Millipore (Darmstadt, Germany). Antibodies against GLUT1 (sc-7903), GLUT3 (sc-7682), and ZNT8 (sc-98243) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The antibody for amylin (250470) was purchased from Abbiotec (San Diego, CA, USA). The antibody against human APP (803001) was purchased

from Biolegend (San Diego, CA, USA). The antibody against A β oligomers (11610) was purchased from Cayman Chemical (Ann Arbor, MI, USA). The antibody against neprilysin (MAB1126) was purchased from R&D Systems (Minneapolis, MN, USA).

Enzyme Linked Immunosorbent Assays (ELISA)

Human A β ELISA kits (Millipore, Darmstadt, Germany) were utilized for quantitation of tissue and islet culture A β . Whole pancreas tissue was lysed in radioimmunoprecipitation assay buffer (RIPA) to yield detergent soluble A β . A β in islet cultures was assessed by hand picking islets directly from culture and lysing 20 islets of similar size from each animal in 100 μ l SDS-free Triton X-100 buffer. This lysate was then pulse sonicated and the lysate A β immediately measured by ELISA. To assess islets for quantitation of total pancreatic insulin and insulin secretion from murine islets, a mouse insulin ELISA kit (Millipore ZRMI-13K, Darmstadt, Germany) was used. Total pancreatic glucagon content was assessed using a glucagon ELISA kit (R&D Systems, Minneapolis, MN, USA). Tissue samples were normalized by total sample protein as determined by the Bradford method. Human islet insulin secretion was quantified using a human insulin ELISA kit (Invitrogen KQA12511, Carlsbad CA, USA). ELISAs were performed as recommended by the manufacturer's instructions. Hippocampus tissue was lysed in radioimmunoprecipitation assay buffer (RIPA) on ice. The lysate was sonicated on ice. To assess total hippocampus insulin content a mouse insulin ELISA kit (Millipore) was used. Insulin content was normalized to

total sample protein as determined by the Bradford method. ELISAs were performed as recommended by the manufacturer's instructions.

Pancreatic Islet Isolation and Culture

Pancreatic islets were isolated from C57BL/6 and transgenic mice using modifications of prior methods (Stull, Breite, McCarthy, Tersey, & Mirmira, 2012; Szot, Koudria, & Bluestone, 2007). The pancreas was first perfused by cannulating the bile duct with a combination of 1 mg/mL collagenase and thermolysin (products C9407 and P1512, Sigma Aldrich, St. Louis, MO, USA) in a sterile HBSS solution. The hepatic bile duct was closed using silk sutures prior to protease injection to direct fluid into the pancreas. Whole pancreas was then removed and placed in 50mL tubes containing HBSS on ice. To initiate digestion, the tubes were then placed in a 37 degree water bath and digestion was monitored with intermittent mechanical disruption. After approximately 10 to 15 minutes, islets were then isolated from crude tissue extract by density centrifugation along a Histopaque (Sigma Aldrich, St. Louis, MO, USA) gradient before further purification using 70 µm filters. Islets from individual animals were cultured separately in RPMI 1640 (Sigma Aldrich, St. Louis, MO, USA) containing 5 mM glucose, 10% FBS, 1% glycine and 1% penicillin/neomycin/streptomycin. Human cadaveric pancreatic islets were obtained from Prodo Labs (Aliso Viejo, CA, USA) and cultured in RPMI 1640 (Sigma Aldrich, St. Louis, MO, USA) containing 5 mM glucose, 10% FBS, 1% glycine and 1%

penicillin/neomycin/streptomycin for 24 hours before experiments were conducted.

Min6 Cell Line Culture and Glucose Stimulated Insulin Secretion (GSIS) Assay

The murine MIN6 beta cell line was purchased from Addexbio Technologies (San Diego, CA, USA). MIN6 cells were cultured in DMEM/F12 media (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 15% FBS and 0.05 mM 2-mercaptoethanol. MIN6 pseudoislets were produced by seeding MIN6 cells in low-attachment cell culture plates (Product #3471) (Corning Incorporated, NY, USA). Three days after seeding and pseudoislet formation, MIN6 pseudoislets were utilized in a GSIS assay. Pseudoislets were first removed from cell culture media by wheel pipette and placed into Krebs Ringer buffer (137 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃) containing .05% BSA and 3.7 mM glucose for 60 minutes. Following incubation, pseudoislets were placed in fresh Ringer buffer and then ten pseudoislets of similar size were selected by wheel pipette and placed into 1.5 mL tubes containing 300 μ L Krebs Ringer Buffer with 3.7 or 16.7 mM glucose and 0.05% BSA. For sAPP α (Product S9564, St. Louis, MO, USA) stimulation experiments, the glucose solution was supplemented with 1 or 10nM sAPP α . After 1 hour of stimulation, supernatants were collected and frozen at -80 degrees. Insulin concentration was determined in supernatants using mouse insulin ELISA kit (Millipore ZRMI-13K, Darmstadt, Germany).

Human and Mouse Primary Islet Culture Glucose Stimulated Insulin Secretion (GSIS) Assay

Human or murine islets were cultured for 24 hours in RPMI 1640 with 5 mM glucose and 10% FBS. Islets were hand-picked from culture and incubated for 1 hour prior to the GSIS assay in Krebs Ringer buffer (137 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃) containing .05% BSA and 3.7 mM glucose. Following incubation, the islets were placed in fresh Ringer buffer. 8-10 islets/experiment were then hand selected and placed into sterile 1.5 mL tubes containing 300 μ L Krebs Ringer Buffer with glucose and BSA. For sAPP α (Product S9564, St. Louis, MO, USA) stimulation experiments, the glucose solution was supplemented with 10nM sAPP α . For human islet stimulations, a range of glucose concentrations were used (3.7, 10, and 16.7 mM) to span from low to high concentrations. Due to limitations in animal numbers of the WT, APP^{-/-}, and APP/PS1 genotypes as well as the increased time required to hand select islets from the 3 genotypes for multiple conditions in the time sensitive GSIS assay, we chose the 5mM glucose concentration for the murine islet stimulation since it is physiologically relevant and we had observed the strongest and most consistent effect of sAPP stimulation of human islets near this concentration. After one hour, supernatants were frozen at -80 degrees. Samples were thawed and diluted using sterile Ringer's buffer prior to ELISA.

Glucose Tolerance Testing

Study I: Two month old WT, APP^{-/-}, and APP/PS1 animals were fasted for 5 hours before receiving 2g/kg glucose by oral gavage. Prior to gavage and at times 15 minutes, 30 minutes, one hour and two hours after gavage blood glucose was measured by tail clipping and the TRUEresult glucometer (Home Diagnostics, Inc, Fort Lauderdale, FL, USA)

In study II, 12 month old male and female WT and APP^{-/-} were fasted for 5 hours before receiving 2g/kg glucose by IP injection. Prior to injection and at times 15 minutes, 30 minutes, one hour and two hours after gavage blood glucose was measured by tail clipping and the TRUEresult glucometer.

RNA Extraction and RT-qPCR

Total RNA from hippocampal tissues (10mg) were 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 a Bullet Blender Storm 24 tissue homogenizer (Next Advance, Inc., Averill Park, NY) using 5mm stainless-steel beads (Qiagen). Extracted RNA samples were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

For each sample, a cDNA library was generated from 1 µg of total RNA using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA) per manufacturers specifications. A primer set for Ide (forward: 5'-CTGTGCCCCTTGTTTGATGC-3'; reverse: 5'-GTTCCCCGTAGCCTTTTCCA-3')

previously described (Kurauti et al., 2017) and 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 CFX Manager Software 3.0 (Bio-Rad) as the operating platform. Ribosomal protein S18 (PrimePCR SYBR Green Assay Rsp18, Bio-Rad) was used as a reference gene and Rsp18 Cq values were used to normalize respective Ide Cq values (ΔCq). Relative Ide mRNA expression was determined as $2^{-\Delta Cq}$ and shown as mean \pm SEM for each mouse strain.

siRNA Knockdown of APP

siRNA mediated knockdown of the mouse APP gene in SIMA9 microglia was achieved using Dharmacon (Lafayette, CO) Accell SMARTpool siRNA (product E-043246-00-0050) or cells were treated with non-targeting siRNA (product D-001910-01-50) as a control. siRNA was utilized by combining the Accell siRNA with Accell delivery media per manufacturer's instructions and cells were treated with a working concentration of 1 μ M siRNA for 6 days. After 3 days of treatment (72 hours), siRNA containing media was supplemented 1:1 with normal DMEM/F12 with 10% FBS. SIMA9 cell protein was harvested on day 7 for western blot analysis.

APP Plasmids and Transfections

pCAX APP 751 and pCAX APP 695 were a gift from Dennis Selkoe & Tracy Young-Pearse and are available from Addgene as plasmids 30138 and 30137 (Young-Pearse et al., 2007). Plasmids were prepared using Qiagen Endofree Plasmid Giga Kit (product 12391). Primary astrocyte cultures were

transfected using lipofectamine 3000 (ThermoFisher Scientific) as per manufacturer's instructions.

Mouse Neuron Primary Culture

Neurons were cultured from WT and APP^{-/-} embryonic brain tissue on embryonic day 16. Pregnant mothers were killed using CO₂ and cervical dislocation. Embryos were harvested and brains placed into sterile dissection media (.5mM EDTA, 100 μM EGTA, 5.5 mM glucose, in PBS). Meninges were removed and both cortices were harvested and minced in dissection media then added to 0.25% trypsin. Tissue was digested for 20 minutes at 37 degrees centigrade. Digestion was terminated by adding 10 mL DMEM/F12 containing 10% FBS. The cells were allowed to settle in the bottom of the pipette tip and then added to 10 mL of neurobasal media supplemented with B27 and L-Glutamine, then triturated approximately 20 times. Cells were cultured in 6 well plates coated overnight with poly-L-lysine. Neuron cultures were grown at 37 degrees in 5% CO₂.

Mouse Microglia and Astrocyte Primary Culture

Microglia and astrocyte cultures were grown from 1 day old WT and APP^{-/-} neonate pups. Meninges were removed from pups and cortical tissue was harvested in dissection media (.5mM EDTA, 100 μM EGTA, 5.5 mM glucose, in PBS) on ice. Cortices were then digested in trypsin for 15 minutes. Digestion was terminated using DMEM/F12 media containing 10% FBS. Each individual's cortical tissue was cultured separately in a T75 flask with 20 mL of DMEM/F12 supplemented with 10% FBS. Media was supplanted on day two and replaced

after 1 week. After two weeks of culture, microglia were separated from astrocytes by shaking flasks at 200 RPM for 45 minutes to 1 hour. Supernatant was collected for microglia, while astrocytes adhered to flask and were removed with trypsin. Astrocytes were then passaged and cultured on 6 well plates for experiments.

IDE Activity Assays

The IDE activity assays were purchased from AnaSpec (Fremont, CA) (Catalog # AS-72231). The IDE activity assay utilizes a FRET substrate, which emits increased fluorescence when cleaved by IDE. Hippocampus tissue, liver, and gastrocnemius muscle was collected on ice from WT and APP^{-/-} animals. 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 an automated mortar and pestle. 50uL of each individual animal's tissue homogenate was loaded into a well of a 96 well plate containing 50uL of substrate solution. The fluorescent intensity was measured on a fluorescent plate reader (BioTek) using an excitation of 485 nm and emission of 528 nm at 5 minute 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 total protein in each sample. Total IDE activity in each sample was calculated using the formula (Final Substrate Concentration – Initial Substrate

Concentration / Time x Volume) x Dilution factor described by Kurauti et al. and normalized to each samples total protein.

Hippocampus Synaptosome Preparation and Stimulation

Synaptosomal insulin responsiveness was evaluated by *ex vivo* stimulation of isolated synaptosomal preparations as previously described (Franklin & Tagliatela, 2016). Briefly, frozen WT and APP^{-/-} mouse hippocampi were homogenized using SynPER reagent (Thermo Scientific) with 1 % protease inhibitor cocktail and phosphatase inhibitor cocktail and centrifuged at 1230 \times g for 10 min at 4°C. The supernatant was collected and centrifuged once more at 15000 \times 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 MgSO₄, 1.2-mM CaCl₂, 20-mM HEPES, 0.1-mM NaH₂PO₄, 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 10nM or 200nM of diluted U-100 insulin. All tubes were incubated at 37 °C for 15 min. Samples were pelleted at 10000 \times g for 10 min at 4°C and resuspended in 1 \times RIPA (75-mM NaCl, 25-mM Na₂PO₄, 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). Data was collected using the area

under peaks for the specified proteins and analysis performed using a Student's t-test.

Statistical Analysis

Statistical analysis was performed using SigmaPlot 12.0 software. Values were averaged +/-SD and statistical significance was determined via one-way ANOVA, two-way ANOVA or t-test, as appropriate. In the case of statistical significance, the Turkey-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 for WT and APP^{-/-} 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.

CHAPTER III

RESULTS

Study 1 – Amyloid Precursor Protein in the Endocrine Pancreas

Introduction

Several lines of evidence indicate that metabolic dysfunction, insulin resistance and perturbations in cerebral glucose utilization may play a critical role in the disease process (Bedse, Di Domenico, Serviddio, & Cassano, 2015; Biessels, Staekenborg, Brunner, Brayne, & Scheltens, 2006; De Felice & Ferreira, 2014; de la Monte, 2014; Hoyer, 1991). Type 2 diabetes, or insulin resistance, is reported to be a risk factor for developing dementia and evidence suggests insulin signaling dysfunction occurs in the brains of AD patients (Crane et al., 2013; Talbot et al., 2012).

One protein thought to be critical in the development of AD is A β , a self-aggregating peptide cleaved from the larger amyloid precursor protein (APP) (Goldgaber, Lerman, McBride, Saffiotti, & Gajdusek, 1987; Selkoe et al., 1988). APP is a single pass transmembrane protein of uncertain function highly expressed in the CNS and detectable in many other cell types (Arai et al., 1991; Konig et al., 1992; Schlossmacher et al., 1992). It is cleaved by proteases such as beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) and gamma secretase to release N-terminal fragments (sAPP α and sAPP β) and varying size A β fragments (A β 1-40 and A β 1-42). Much of the research focused on APP

biology has been in the context of A β plaque deposition in the central nervous system. However, it is known that higher molecular weight isoforms of APP are present in many peripheral organs (Selkoe et al., 1988). Our recent work has focused on characterizing APP expression and function in several peripheral tissues including intestinal and adipose tissue (Puig & Combs, 2013; Puig, Floden, et al., 2012; Puig, Lutz, et al., 2015; Puig, Swigost, et al., 2012; Sondag & Combs, 2010).

The pancreas has an essential role in the regulation of blood glucose levels, primarily through the secretion of the hormone insulin from pancreatic β cells. Pancreatic dysfunction is a well-known consequence of both type 1 and type 2 diabetes (Ashcroft & Rorsman, 2012). Numerous studies in both primates and mice suggest that damaging or stressing the endocrine pancreas may promote changes in the central nervous system associated with the pathogenesis of AD (Ho et al., 2004; Julien et al., 2010; Morales-Corraliza et al., 2016; Takeda et al., 2010; X. Wang et al., 2010). Injections of insulin into a transgenic mouse model of AD have been shown to improve the memory of these mice as well as alter circulating concentrations of A β (Vandal et al., 2014). Administration of insulin into the brain intranasally is being explored as a cognitive enhancing therapeutic strategy in patients with AD (Claxton et al., 2015; Holscher, 2014; Reger et al., 2008).

Like Alzheimer's disease, diabetes has its own plaque pathology, in which the self-aggregating peptide amylin, or IAPP, is co-secreted with insulin and damages pancreatic islets (Lorenzo, Razzaboni, Weir, & Yankner, 1994;

Westermarck, Wilander, Westermarck, & Johnson, 1987). Whether A β itself influences pancreatic IAPP aggregation through cross-seeding, or injures islets independently of IAPP remains uncertain. There are conflicting reports of the detection of amyloid beta within human islets (Miklossy et al., 2010; Oskarsson et al., 2015). Increased levels of A β or A β aggregates have been detected within the pancreas of some transgenic mouse models of AD (Shoji et al., 1998; Shoji et al., 2000; Vandal et al., 2015).

There is evidence that suggests that the endogenous functions of APP may be linked to the regulation of insulin secretion and signaling or whole body metabolism. The detection of APP has been reported in the exocrine and endocrine pancreas by immunohistochemistry and in-situ hybridization, respectively (Beer, Masters, & Beyreuther, 1995; Figueroa, Shi, Gardell, & Austin, 2001). Additionally, a rigorous analysis of mice lacking APP (APP^{-/-}) and/or the related APLP2 gene shows changes in circulating blood glucose concentrations (Needham et al., 2008). Another report has suggested APP may directly regulate the process of insulin secretion from pancreatic islets (Tu et al., 2012). In this study we examine murine and human pancreatic tissue to test the hypothesis that APP or A β play a role in pancreatic function or pathophysiology respectively.

APP was Expressed within the Endocrine Pancreas

To begin studying APP in the pancreas, we examined whole pancreatic tissue collected from two month old wild type C57B6 (WT) , APP^{-/-}, and APP/PS1 mice. The APP^{-/-} animals have the APP gene knocked out in all

tissues. APP/PS1 mice overexpress both human APP and the γ -secretase complex protein Presenilin-1 protein under the control of the prion promoter. This leads to high transgene expression in the central nervous system and results in A β plaque deposition in the brain with age. Pancreatic protein lysates were resolved by 10% SDS-PAGE and immunoblotted to compare expression of APP (Fig 1A). APP was detected in wild type pancreas, absent in APP^{-/-} tissue, and overexpressed in APP/PS1 mice. However, the human APP695 transgene migrated at an apparent lower molecular weight than endogenous mouse APP, consistent with reported expression of higher molecular weight isoforms in peripheral tissues (Selkoe et al., 1988).

In order to determine which cell types were expressing APP, immunohistochemistry was next performed. Pancreas from both two and twelve month old WT, APP^{-/-} and APP/PS1 mice was serial sectioned and stained using antibodies against the c-terminus of APP (Fig 1B). Endogenous APP immunoreactivity localized to both endocrine and exocrine pancreas, with clear immunoreactivity in pancreatic islets at 12 months of age. APP immunoreactivity in the APP/PS1 transgenic mice robustly localized to islets at either age. Following our investigation of murine tissue, we determined whether human pancreatic islets also expressed APP. Immunohistochemistry was performed on healthy, type 2 diabetes, and cancerous human pancreatic tissue using antibodies directed against the c-terminus of APP. Similar to the mouse tissue findings, we observed clear APP immunoreactivity within the endocrine pancreas

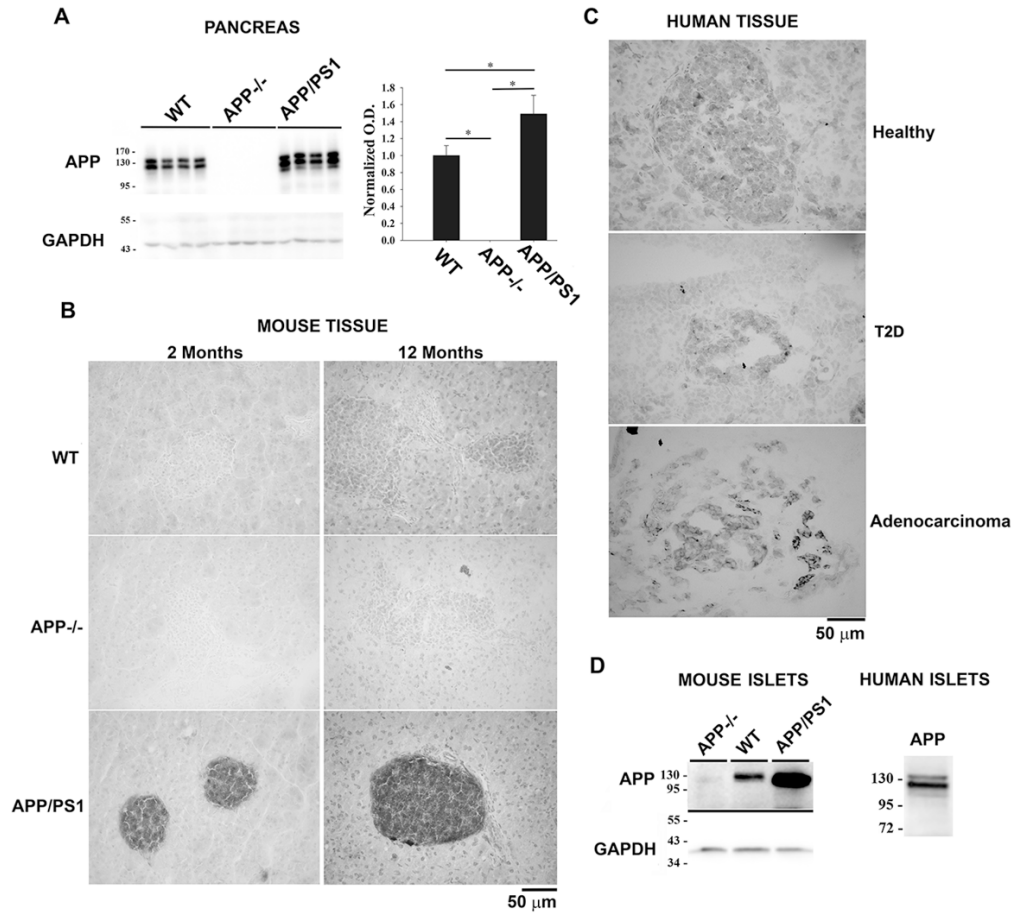
Figure 1. Amyloid precursor protein was detected in mouse and human pancreas including islets of Langerhans.

(A) Whole pancreas was collected from C57BL/6 (WT), APP^{-/-}, and APP/PS1 mice (n=7) and lysed in RIPA buffer. 20 µg of protein was resolved by 10% SDS-PAGE and western blotted using anti-APP (Y188) antibody or anti-GAPDH antibody as a loading control. Normalized optical density was averaged +/- SD, *p<0.05.

(B) Pancreas was harvested from C57BL/6 (WT), APP/PS1, and APP^{-/-} at 2 months (n=7) and 12 months (n=4) of age. Tissue was fixed in 4% paraformaldehyde and cryosectioned into 10 µm serial sections. Immunohistochemistry was performed using anti-APP (Y188) antibody. Vector VIP was used as the chromogen.

(C) Immunohistochemistry was performed on human pancreatic tissue. Pancreas from a healthy 77 year old male donor, 68 year old male type 2 diabetic donor and a 66 year old male with pancreatic adenocarcinoma was probed using anti-APP antibody (Y188) with vector VIP used as the chromogen.

(D) Representative western blot of protein lysates from cultured pancreatic islets from WT, APP^{-/-}, and APP/PS1 mice. Islet protein was resolved by 10% SDS-PAGE, transferred to PVDF, and immunoblotted using anti-APP antibody (Y188). Islets from a healthy 55 year old male donor were cultured then lysed in RIPA buffer and resolved using 10% SDS-PAGE then transferred to PVDF membrane and western blotted using anti-APP antibody, 22C11.



of all three tissue types (Fig 1C). Pancreatic adenocarcinoma samples showed particularly robust APP islet immunoreactivity (Fig 1C, Fig 2). This is in agreement with a number of studies demonstrating upregulated APP in pancreatic cancer where it may play a role in the disease process (Hansel et al., 2003; Woods & Padmanabhan, 2013).

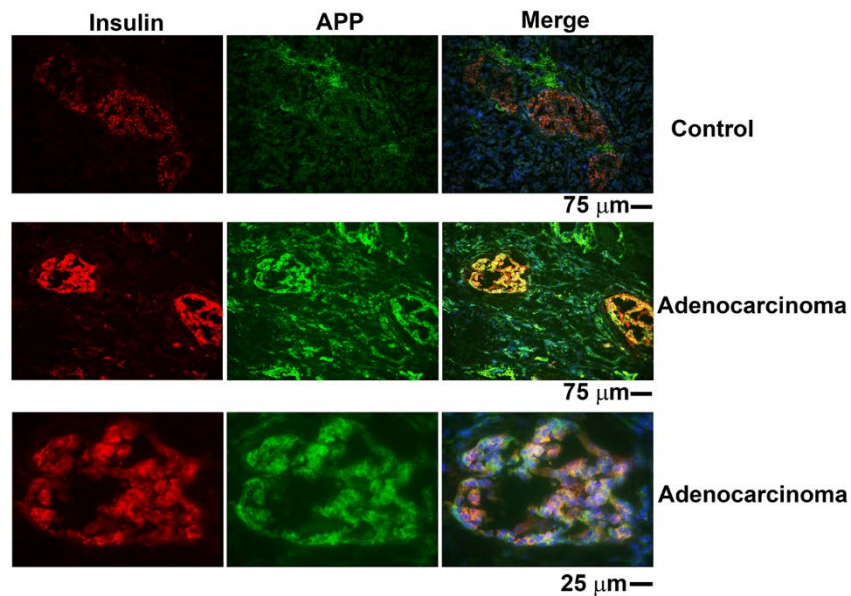


Figure 2. APP immunoreactivity was increased in pancreatic adenocarcinoma. Cryosections (10µm) from human healthy pancreas and pancreatic adenocarcinoma were immunostained for APP and insulin. Alexa Fluor 488 and 594 conjugated secondary antibodies were used for visualization.

The islet enriched APP immunoreactivity was validated by western blot analysis from both murine and human islet cultures. Islets grown from WT, APP^{-/-}, and APP/PS1 mice demonstrated the expected increase in APP expression in the APP/PS1 line with no detectable APP in the APP^{-/-} mice (Fig 1D). Human islet cultures also demonstrated robust APP levels via western blot analysis (Fig 1D).

Pancreatic islets are primarily composed of insulin producing β cells. To determine whether APP localized within these specialized cells we next examined pancreas from the APP/PS1 mouse by immunofluorescence using primary antibodies against APP and insulin. APP immunoreactivity was highly localized to the insulin producing cells in the APP/PS1 mice (Fig 3A) Using the same approach, we confirmed that APP localized to β cells of the human pancreas (Fig 3B).

Pancreatic APP was Not Processed to A β

The presence of A β containing plaques has been reported in the pancreas of some strains of transgenic mice although evidence of A β in human pancreas is conflicting (Miklossy et al., 2010; Oskarsson et al., 2015). To determine whether A β was present in our transgenic samples we utilized two methods, ELISA and immunohistochemistry, to detect A β in mouse and human pancreas. Human A β 1-40 and 1-42 was quantified by ELISA from WT, APP^{-/-} and APP/PS1 hippocampus, pancreas and primary islet culture lysates from twelve month old animals (Fig 4A). Interestingly, while high concentrations of human A β were detectable in the brain lysates of APP/PS1 transgenic animals, we did not detect

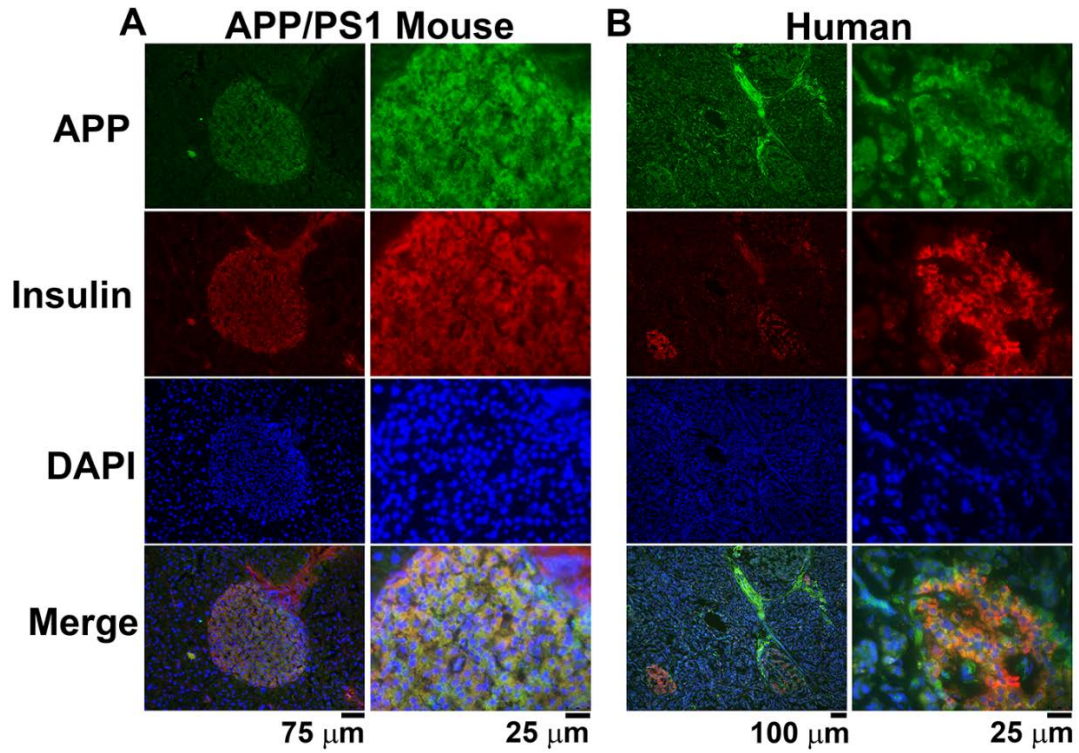


Figure 3. Amyloid precursor protein was detectable in the insulin producing β cells of the human pancreas and the APP/PS1 mouse. Pancreatic APP was not processed to $A\beta$. Immunofluorescent staining was performed using (A) APP/PS1 transgenic mouse and (B) human tissue cryosections (10 μ m). Anti-APP (Y188) and insulin primary antibodies with Alexa Flour 488 and Alexa Flour 594 fluorophore conjugated secondary antibodies were used for visualization with DAPI counterstain.

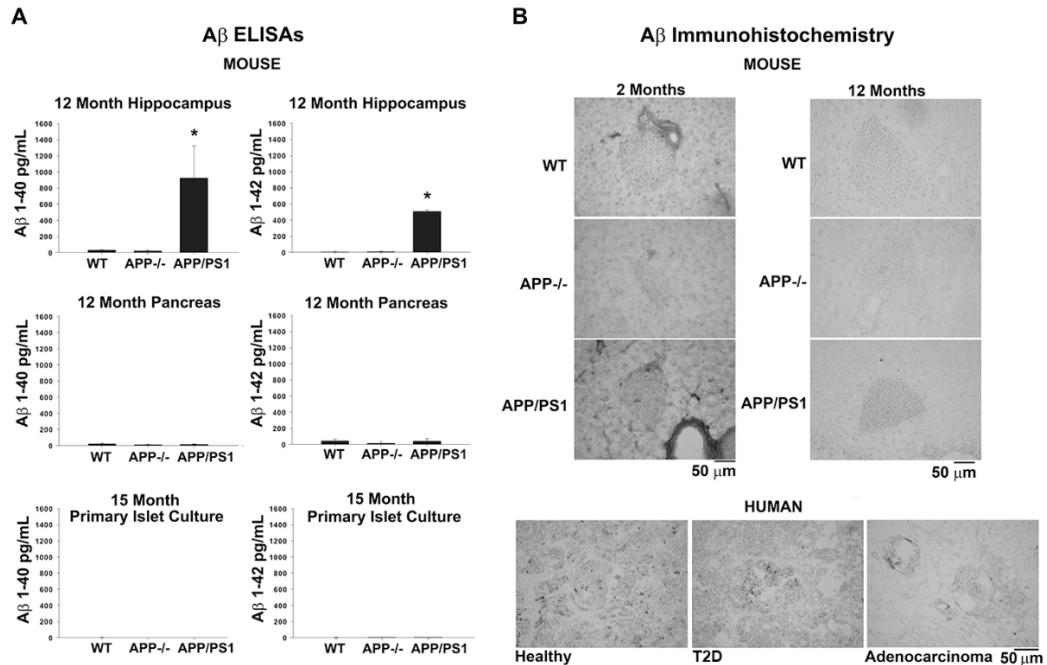


Figure 4. A β was not detectable in mouse pancreas by immunohistochemistry or ELISA. (A) Mouse pancreas and hippocampus tissue was lysed in RIPA buffer (n=4/strain), while islets (n=6/strain) were pelleted and lysed in Triton X-100 buffer to yield detergent soluble A β extract. Extract was used to quantify both human A β 1-40 and human A β 1-42 by ELISA. APP^{-/-} animals served as a negative control to measure the background of the assay. Data is expressed as mean values +/-SD, *p<0.05 from WT. (B) Immunohistochemistry was performed on 10 μ m cryosections of WT, APP^{-/-}, and APP/PS1 murine pancreas as well as human healthy control, type 2 diabetes (T2D), and adenocarcinoma pancreas tissue. Anti-A β antibody (4G8) was used with Vector VIP as the chromogen.

a significant increase in A β in the pancreas or islet lysate of these animals. Taken together, these data suggest that APP is processed by alternative mechanisms in the pancreas as compared to brain.

To further investigate the possibility of pancreatic A β production, we utilized immunohistochemistry to stain for A β . Since we and others have previously observed that the APP/PS1 line accumulates brain A β deposits in an age dependent manner (Gordon et al., 2002; Manocha et al., 2016), WT and transgenic mouse pancreas were immunostained to detect A β at two and twelve months of age (Fig 4B). A faint amount of immunoreactivity was observed in 12 month old APP/PS1 islets relative to the APP^{-/-} and WT mice likely due to the anti-A β antibody, 4G8, cross-reacting with full length APP. However, there were clearly no distinct signs of A β plaque deposition in either the two or twelve month old mouse pancreas. We also examined healthy, type 2 diabetes, and adenocarcinoma human pancreas tissue for A β immunoreactivity as well as for nonspecific amyloids by thioflavin staining (Fig 4B, Fig 5). While thioflavin S stained several areas of both healthy and type two diabetic pancreas, no detectable A β plaque-like deposits were observed although some scattered puncta which may correspond to A β or sAPP were noted in all conditions, including within pancreatic islets. Following these negative results, we sought to determine if the methodology we employed was sufficient to detect amyloid deposits. We performed immunostaining for A β in addition to thioflavin S staining in 12 month WT and APP/PS1 mouse brain tissue sections (Fig 5). As expected, APP/PS1 brains showed abundant plaque deposition using both methods without

detectable plaques in WT brains (Fig 5) in contrast to a clear lack of any plaque-like deposits in the pancreas (Fig 4B).

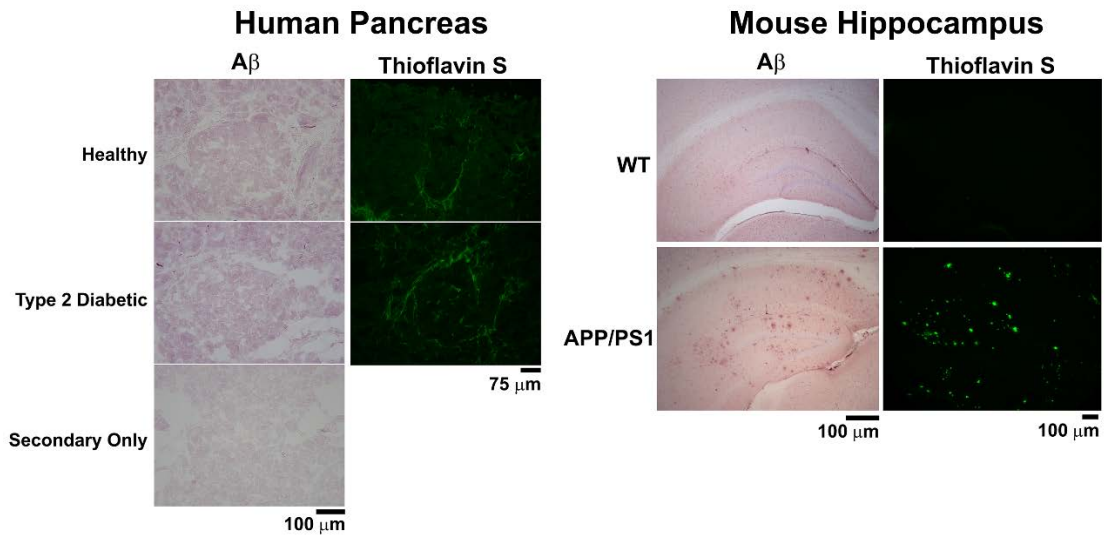


Figure 5. Thioflavin staining and Aβ immunohistochemistry in human pancreatic tissue and mouse brains. Cryosections (10μm) of healthy human pancreas and type 2 diabetic pancreas or WT and APP/PS1 brain tissue sections (40 μm) were immunostained for Aβ or stained with thioflavin S. Vector VIP was used as the chromogen.

APP/PS1 Mice Showed no Differences in Glucose Tolerance Testing or Pancreatic Insulin Levels but had Significantly Decreased Pancreatic Glucagon Compared to WT and APP^{-/-} Mice.

An essential function of the pancreas is to regulate blood glucose levels. In order to test if differences in pancreatic islet APP content caused changes in whole body glucose tolerance we subjected WT and transgenic animals to 2g/kg glucose challenge by oral gavage. Blood glucose measurements were taken from tail clipping after four hours of fasting and at 15, 30, 60 and 120 minutes after glucose challenge (Fig 6A, B). Surprisingly, no differences were observed across genotypes. In addition to assessing glucose tolerance, we also homogenized whole pancreas from 2 month old animals and assessed their insulin and glucagon content by ELISA (Fig 6C, D). Glucagon concentrations from APP/PS1 pancreas were significantly lower than WT and APP^{-/-} mice although no changes in insulin content were detected across genotypes. Based upon the glucagon differences, APP/PS1 pancreas tissue was fluorescently double labeled with anti-APP and glucagon antibodies. Although there were relatively few glucagon immunoreactive cells it was clear that they colocalized with APP immunoreactivity suggesting that transgene expression alters the biology of these cells (Fig 7).

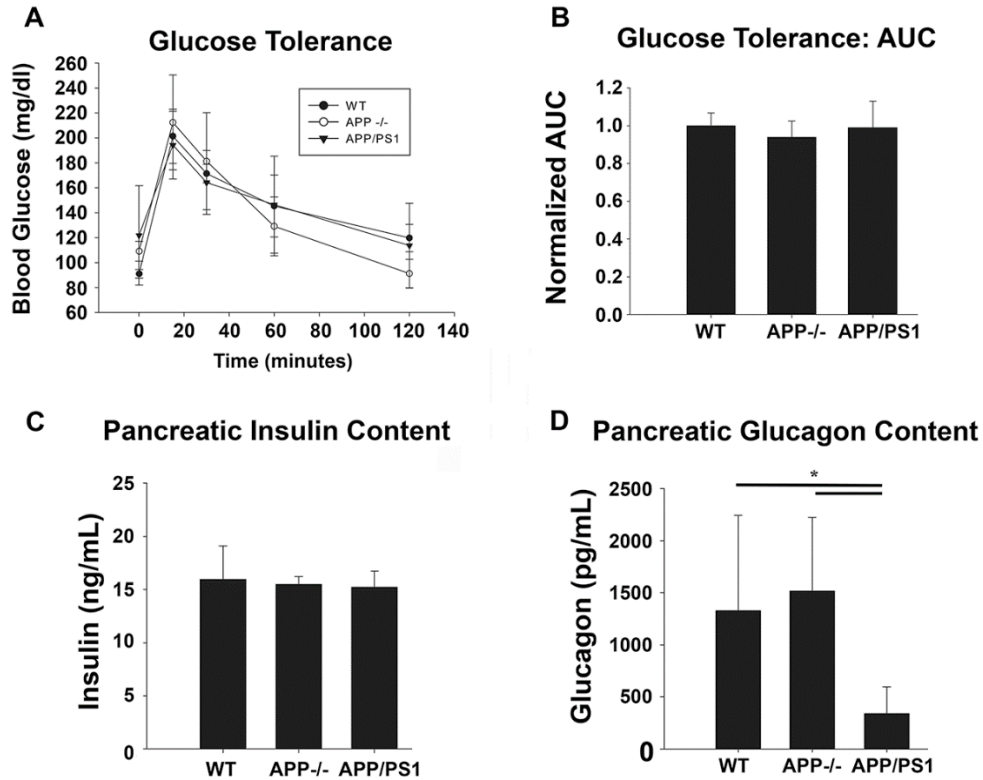


Figure 6. No difference in glucose tolerance or pancreatic insulin content was observed in APP^{-/-} and APP/PS1 mice, but whole pancreas glucagon content was reduced in APP/PS1 mice. 2 month old WT, APP^{-/-}, and APP/PS1 mice were subjected to glucose tolerance testing. Prior to testing, mice were fasted four hours and then received 2g/kg glucose by oral gavage. (A) Blood glucose was measured at intervals by blood glucose meter (n=4-7). (B) The area under the curve (AUC) from glucose tolerance testing of each mouse was measured and the results from each strain are graphed. Each strain was normalized to the mean AUC of the WT strain. Whole mouse pancreas was lysed using RIPA buffer. (C) Insulin (n=6-7) and (D) glucagon (n=6-7) content were assessed by ELISA. Data is shown as mean values +/-SD, *p<0.05.

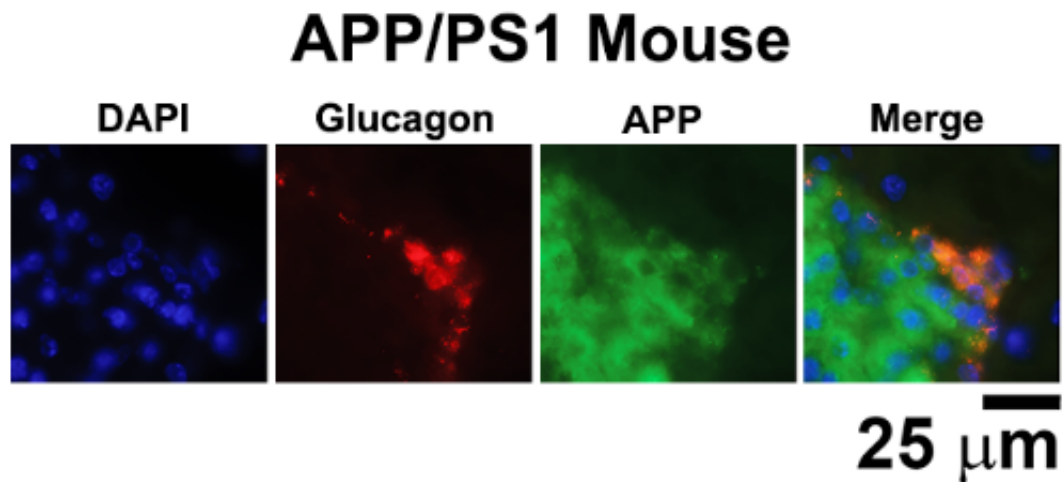


Figure 7. APP was present in glucagon producing cells in the APP/PS1 mouse. Cryosections (10 μ m) of APP/PS1 mouse pancreas were immunostained for APP and glucagon. Alexa Fluor 488 and 594 conjugated secondary antibodies were used for visualization.

APP Expression Modulated Pancreatic BACE2, GLUT4, and IDE Levels in the Transgenic Mice.

To better understand the contribution of APP expression to pancreatic physiology, we quantified protein levels from homogenized murine pancreas of WT and transgenic animals by western blot with a particular focus on proteins involved in insulin signaling, glucose uptake and A β degradation (Fig 8).

Although no changes in protein levels of the glucose transporters GLUT1, GLUT2, or GLUT3 were observed across genotypes, protein levels of GLUT4 were significantly elevated in APP^{-/-} mice compared to WT, and APP/PS1 mice. No differences in insulin receptor (IR), pAkt or pGSK3 β levels were observed. Similarly, total protein levels of zinc transporter 8 (ZNT8) did not differ across

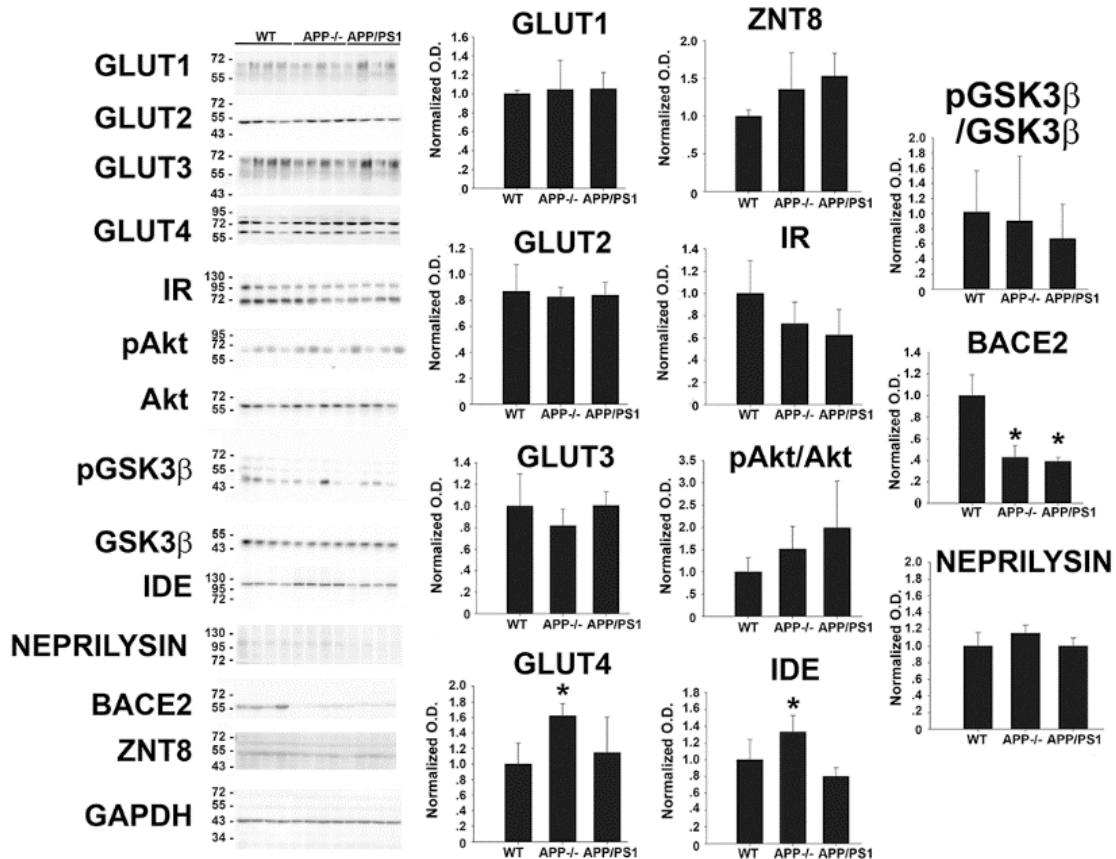


Figure 8. Differences in 2 month old mouse pancreatic BACE2, IDE and GLUT4 were observed by western blot. Whole pancreas from WT, APP^{-/-}, and APP/PS1 mice was lysed in RIPA buffer and resolved using 10% SDS-PAGE. Protein was transferred to PVDF membrane and western blotted for multiple proteins using GAPDH as a loading control. Optical density was measured and normalized to mean WT optical density (n=7). Data is expressed as mean values +/-SD, *p<0.05.

species. BACE2 has been suggested to both interact with APP in vitro as an α -secretase and play a role in pancreatic insulin secretion through the cleavage of its substrate, TMEM27 (Esterhazy et al., 2011; Yan, Munzner, Shuck, & Bienkowski, 2001). We assessed BACE2 protein levels using an antibody against an amino acid sequence within the prodomain of BACE2 that is not found in BACE1 (B. D. Bennett et al., 2000). Strikingly, total protein levels of BACE2

were significantly reduced in APP^{-/-} and APP/PS1 compared to WT indicating that APP may influence the expression or activity of BACE2 in the pancreas. Since both A β and insulin are degraded by a common enzyme, insulin degrading enzyme (IDE) (Qiu et al., 1998), we quantified IDE protein levels to observe a significant increase in APP^{-/-} mice compared to all other genotypes. We also quantified protein levels of another A β degrading enzyme, neprilysin, but detected no differences across genotypes. This suggested that APP has a role in regulating the expression of GLUT4, IDE and BACE2 in the pancreas.

BACE2 Immunoreactivity was Localized to Islet Periphery with Decreased Immunoreactivity in APP/PS1 Mice With no Concomitant Increase in A β Immunoreactivity.

Following our observation of changes in pancreatic BACE2 and IDE protein levels, we performed immunohistochemistry on 2 month old WT and transgenic mouse pancreatic serial sections to determine where changes in protein expression localized (Fig 9A). Increased immunoreactivity for IDE was observed in APP^{-/-} tissue, with the higher levels of IDE observed in the acinar tissue of the pancreas. Immunohistochemistry for BACE2 using antibodies directed against the N-terminal prosequence revealed BACE2 expression within the endocrine pancreas, with the highest levels of immunoreactivity at the periphery of islets. In agreement with the western blots, BACE2 immunoreactivity was notably less robust in APP/PS1 mice compared to WT. To validate this pattern of BACE2 tissue expression and further investigate APP processing within pancreatic islets, we performed immunohistochemistry on 12 month old WT and transgenic mouse pancreas sections using primary antibodies against

BACE2, IAPP, CD68, A β Glucagon (Fig 9B, Fig 10) Immunohistochemistry for BACE2 revealed a similar pattern of protein expression at 12 months of age as was observed at 2 months, with the highest levels of BACE2 detected at the periphery of the islets. We further validated our BACE2 immunohistochemistry results by performed peptide blocking studies in mouse tissue to demonstrate the specificity of the BACE2 islet staining (Fig 10). Antibody pre-incubation with the BACE2 epitope blocking peptide completely abolished islet BACE2 staining at the islet periphery demonstrating our immunohistochemistry results are specific

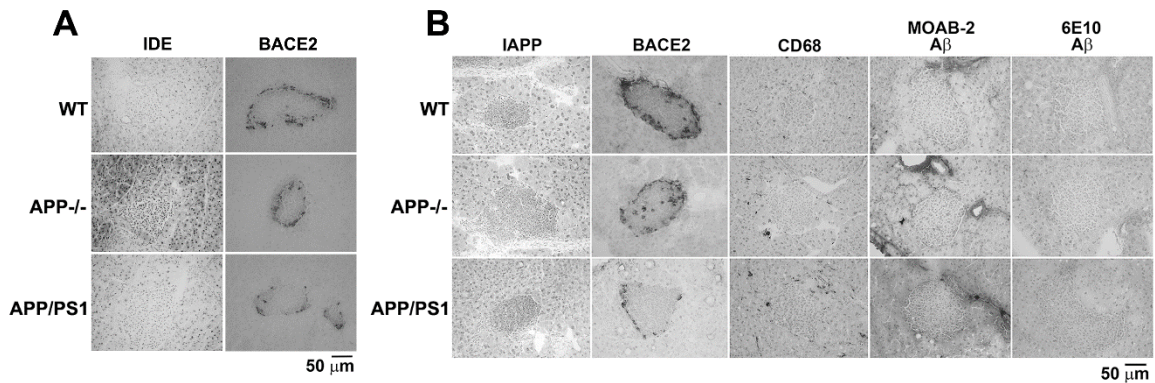


Figure 9. Immunohistochemistry of 2 and 12 month old mouse pancreas. (A) Immunohistochemistry was performed on WT, APP^{-/-}, and APP/PS1 mouse 2 month old 10 μ m pancreas cryosections using primary antibodies against IDE and BACE2 (n=7). Tissue was fixed with 4% paraformaldehyde and Vector VIP chromogen was used as the chromogen. (B) Immunohistochemistry was performed on 12 month old murine 10 μ m pancreas cryosections (n=4). Primary antibodies against amylin/IAPP, BACE2, CD68, A β oligomers (MOAB-2) and A β were utilized to detect proteins. Vector VIP was used as the chromogen.

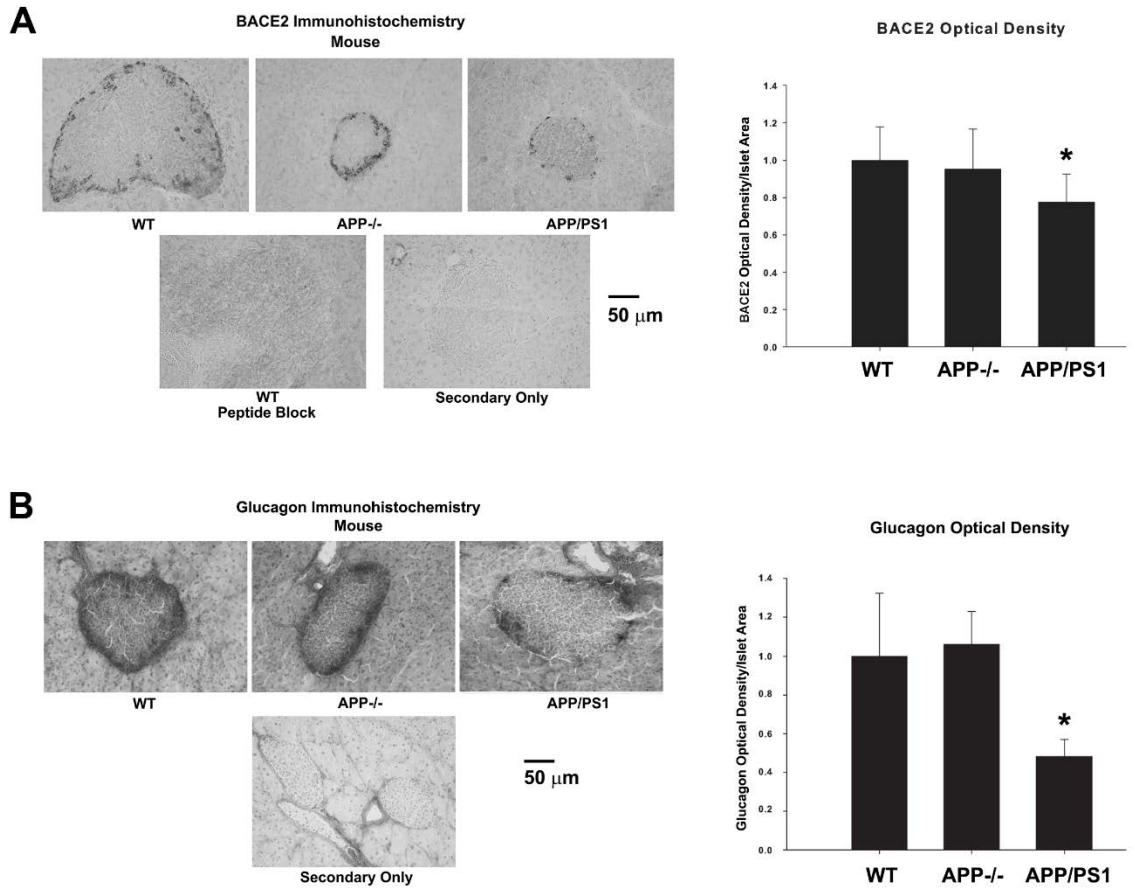


Figure 10. BACE2 immunohistochemistry with epitope blocking peptide control and glucagon immunohistochemistry with optical density quantitation in mouse tissue. (A) Cryosections (10 μ m) of WT and transgenic mouse pancreas were immunostained for BACE2 in the presence or absence of a BACE2 epitope blocking peptide. Vector VIP was used as the chromogen. Densitometric analysis of BACE2 islet immunostaining was conducted in 12 month old mice (n=8). Staining intensity values were normalized to WT levels and averaged \pm SD, *p<0.05. (B) Cryosections (10 μ m) of WT and transgenic mouse pancreas were immunostained for glucagon with Vector VIP as the chromogen. Densitometric analysis of glucagon islet immunostaining was conducted in 12 month old mice (n=5-6). Staining intensity values were normalized to WT levels and averaged \pm SD, *p<0.05.

to the BACE2 epitope. We also quantified the intensity of BACE2 and glucagon immunoreactivity in mouse islets and observed significantly less BACE2 and glucagon staining in APP/PS1 mouse islets (Fig 10). No differences in IAPP/amylin aggregation were observed across mouse genotypes, which is as expected as mouse amylin is not known to form amyloid plaques. No A β plaque-like structures were detectable using antibodies against monomeric or oligomeric A β . Staining with anti-CD68 antibody to detect macrophages did not reveal any differences in islet macrophage infiltration suggesting local overexpression of APP within islets did not result in local A β -mediated immune activation as is known to occur in the central nervous system (Dhawan, Floden, & Combs, 2012; Jimenez et al., 2008). As expected, mouse glucagon was localized primarily at the islet periphery.

***Immunohistochemistry for BACE2 Demonstrated Differential
BACE2 Localization in α and β Cells in Both
Mouse and Human Tissue.***

To determine the identity of the pancreatic islet cells stained positive for BACE2, we performed immunofluorescent double labeling of tissue from WT 2 month old animals for BACE2 and either insulin or glucagon (Fig 11). Surprisingly, BACE2 immunoreactivity was highest in cells staining positive for glucagon suggesting BACE2 may be differentially active or expressed in pancreatic α compared to β cells. The staining of α cells at the periphery of the islet is consistent with the known anatomical organization of murine pancreatic islets of Langerhans (Cabrera et al., 2006). Using this approach, we stained WT, APP^{-/-}, and APP/PS1 mouse pancreas for both BACE2 and insulin, and obtained complimentary results indicating the BACE2 enzyme localized within the islets and was more highly immunoreactive outside the insulin producing cells at the periphery of the islets (Fig 12). We then co-labeled human pancreatic tissue for BACE2 and glucagon in the absence or presence of the BACE2 epitope blocking peptide to determine if BACE2 was also present in human α cells (Fig 11). Interestingly, we again observed robust BACE2 immunoreactivity in glucagon positive cells, indicating this pattern of BACE2 expression is conserved across mouse and human α cells. Pre-incubation with the BACE2 epitope blocking peptide eliminated BACE2 immunoreactivity in α cells, indicating this antibody staining was specific to the BACE2 protein epitope.

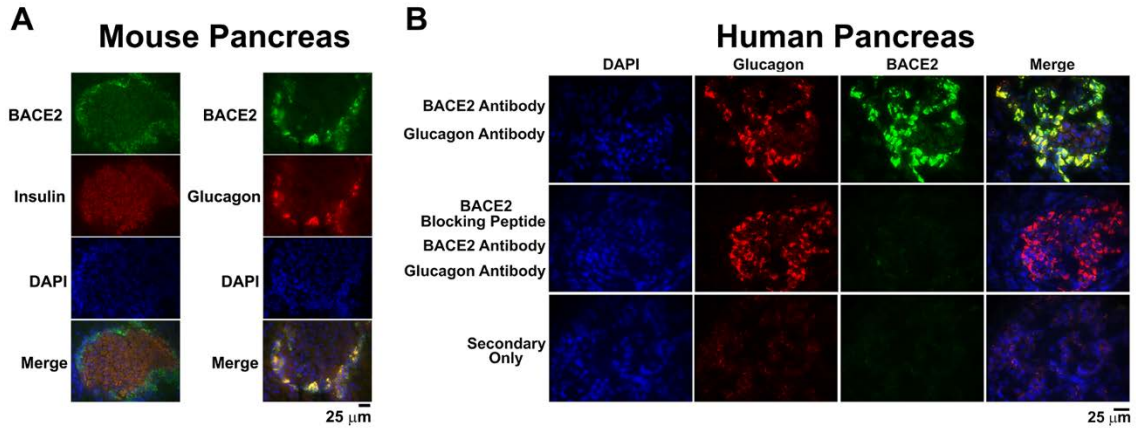


Figure 11. BACE2 was present in glucagon positive cells. Immunohistochemistry was performed on (A) WT mouse and (B) human 10μm pancreas cryosections using antibodies against BACE2 and insulin or glucagon with or without pre-incubation with BACE2 epitope blocking peptide. Fluorescent secondary antibodies were used for visualization (anti-rabbit Alexa-Fluor 488 and anti-mouse Alexa Fluor 594). DAPI staining was used to visualize cell nuclei.

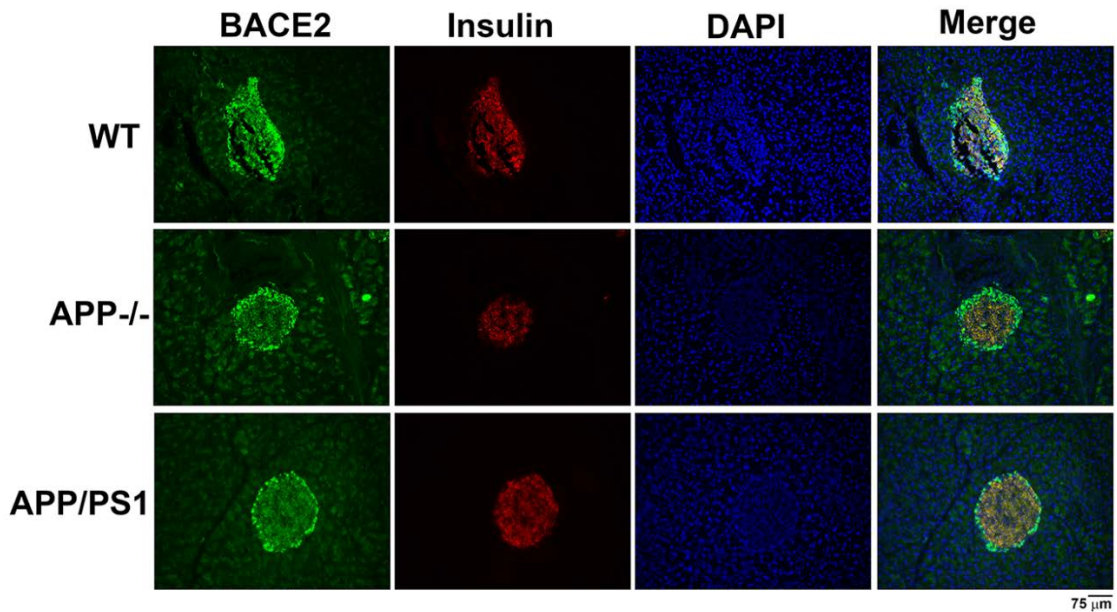


Figure 12. BACE2 immunoreactivity in wild type and transgenic mouse islets. Cryosections (10μm) of WT and transgenic mouse pancreas were immunostained for BACE2 and insulin. Alexa Fluor 488 and 594 conjugated secondary antibodies were used for visualization with a DAPI counterstain.

Primary Murine Islet Cultures Showed Significant Differences in APP and IDE Content.

Only a small fraction of the pancreas is composed of hormone secreting endocrine cells. To determine if protein differences observed in the whole pancreas lysate were due in part to differences in pancreatic islets, we cultured pancreatic islets isolated from WT, APP^{-/-}, and APP/PS1 animals. We cultured these islets for 24 hours and selected healthy islets of similar size for lysing and western blot analysis (Fig 13). We examined islet lysate for differences in APP, IDE, GLUT4 and BACE2 as differences in the levels of these proteins were observed in whole pancreas lysate. GAPDH was utilized as a loading control. As expected, APP was not detectable in APP^{-/-} islets while APP/PS1 islets had high levels of APP. Surprisingly, like our whole pancreas extracts, IDE was significantly increased in APP^{-/-} islets compared to WT. We were unable to detect GLUT4 in islet lysates. Islet BACE2 protein levels were not significantly different in transgenic animals compared to wild type controls, in contrast to our immunostaining results. One possibility for this discrepancy is perhaps due to loss of peripheral islet cell integrity during the isolation or culture conditions we employed suggesting that further optimization is required in future work.

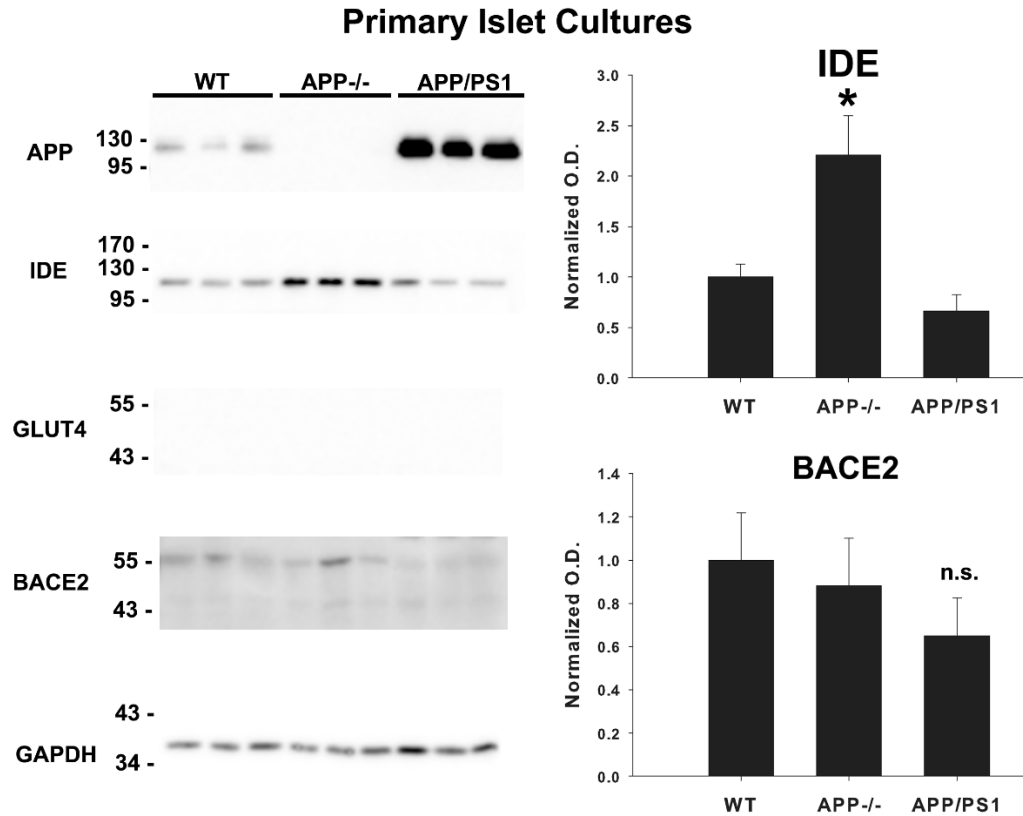


Figure 13. Western blots of 12 month old mouse primary islet cultures. WT, APP^{-/-}, and APP/PS1 pancreatic islets (n=3) were cultured overnight and the next day 50 islets from each animal were selected, pelleted, and lysed in RIPA buffer. Protein lysate was resolved by 10% SDS-PAGE then transferred to PVDF membranes and western blotted with antibodies against APP (Y188), IDE, GLUT4, and BACE2. Anti-GAPDH was used as a loading control. Optical density was measured for IDE and BACE2 and was normalized to WT optical density. Data is expressed as mean values +/-SD, *p<0.05.

APP was Processed by α -secretase Activity in Primary Islet Cultures and Recombinant sAPP Potentiated Islet Insulin but not Glucagon Secretion.

The presence of BACE2, as well as the lack of detectable A β , suggested that pancreatic APP was processed by a non-amyloidogenic pathway. It is known that sAPP is detectable in the conditioned media of cell cultures from a number of cell types (Weidemann et al., 1989). To determine if processing of pancreatic APP resulted in the release of sAPP into conditioned media, we grew primary cultures of both mouse and human pancreatic islets and collected the cells and conditioned media after 24 hours for western blot analysis (Fig 14A). Both murine and human mutant APP were processed through the α -secretase pathway resulting in the release of sAPP into culture media, including the human mutant APP expressed in APP/PS1 islets. Human sAPP α from APP/PS1 islets was detected using the 6E10 monoclonal antibody, which has been demonstrated to bind human sAPP α (Bailey et al., 2008).

To better understand whether secreted sAPP α may have autocrine effects, we assessed insulin and glucagon secretion from primary cultures of isolated islets. A glucose stimulated insulin secretion (GSIS) assay demonstrated significantly attenuated insulin secretion from APP $^{-/-}$ islets compared to WT and APP/PS1 while APP/PS1 islets had significantly higher insulin secretion compared to both WT and APP $^{-/-}$ cultures (Fig 14B). In order to determine whether the differences in insulin secretion could be related to sAPP α , WT islet cultures were stimulated with recombinant sAPP α during the GSIS assay.

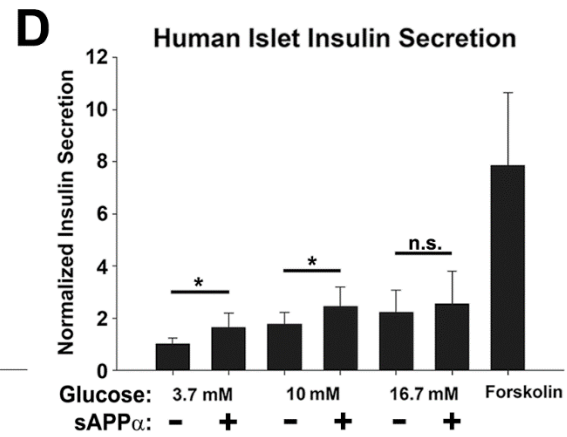
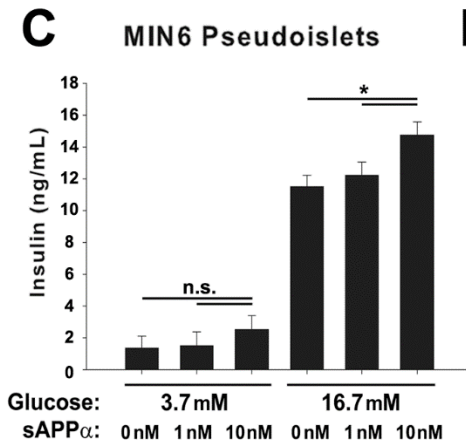
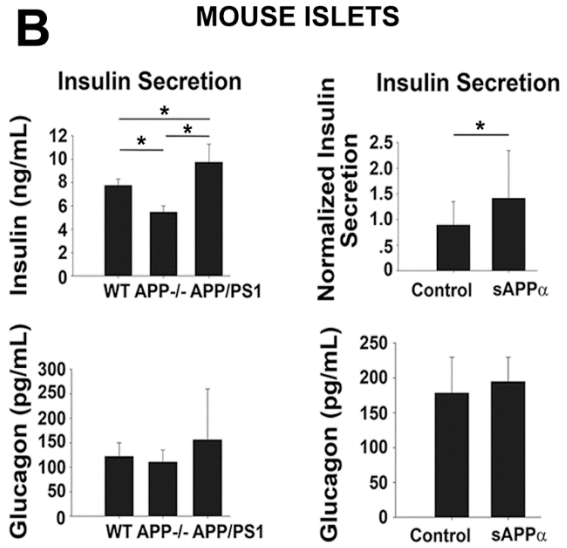
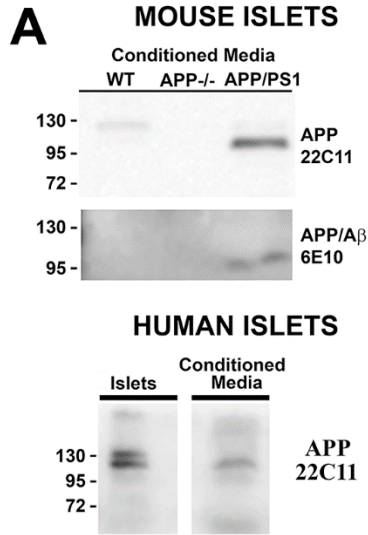
Figure 14. sAPP was released from primary murine and human islet cultures and recombinant sAPP α potentiated islet insulin secretion during glucose stimulated insulin secretion assay.

(A) Primary islet cultures from WT, APP $^{-/-}$, and APP/PS1 mice were grown for 24 hours. Conditioned media was collected and protein content was assessed by western blot using antibodies against N-terminal APP. 22C11 detects both mouse and human APP while 6E10 is specific to human APP and detects sAPP α . Human islets were cultured for 24 hours and both conditioned media collected for analysis by western blot using anti N-terminal APP antibody (22C11).

(B) Primary cultures of mouse islets were assessed for differences in insulin and glucagon secretion at 5mM glucose. Wild type islets were stimulated with 10 nM recombinant human sAPP α and supernatant was collected for insulin (n=8) and glucagon (n=4) ELISA. Insulin secretion data from multiple sAPP stimulation experiments were pooled by normalizing to the mean of the unstimulated control from each experiment.

(C) The MIN6 insulin secreting cell line was grown as pseudo-islets and was stimulated with 0, 1nM, or 10nM recombinant sAPP α in GSIS assay at either 3.7mM or 16.7 mM glucose (n=6-8/condition). Data is expressed as the mean +/- SD, *p<0.05.

(D) Human islets were treated with 10 nM recombinant sAPP α during the GSIS assay in 3.7, 10, and 16.7 mM glucose. After one hour of treatment, islet insulin secreted into the supernatant was assessed by ELISA. Data was pooled from five donors and normalized to basal insulin secretion at 3.7 mM. Data is expressed as the mean +/- SD, *p<0.05. 1 μ M forskolin was used with 16.7 mM glucose to potentiate insulin secretion as a positive control.



In these studies, we utilized 10 nM sAPP α for stimulations as this concentration has been shown to initiate signaling events in neuronal cells and is above the estimated EC50 of 2.3nM (Jimenez et al., 2011). Stimulation with sAPP α resulted in a significant but modest potentiation of islet insulin but not glucagon secretion (Fig 14B).

This suggested that differences in basal insulin secretion from primary cultures of islets across genotype may be due, in part, to autocrine stimulation with sAPP α . It also suggests that sAPP α has a role particularly in insulin biology of pancreatic islets or may support islet phenotype and survival in vitro.

Following our observation that sAPP α potentiates insulin secretion in cultures of primary mouse islets, we sought to further validate this finding by utilizing the MIN6 mouse β -cell line to test the effect of different doses of sAPP α and different concentrations of glucose on insulin secretion from MIN6 pseudoislets (Fig 14C). Similar to our primary islet cultures, we observed that treatment with sAPP α modestly but significantly potentiated insulin secretion from MIN6 pseudoislets at 16.7mM glucose concentration. This effect was dose dependent, with 10 nM but not 1 nM recombinant sAPP α having a significant effect.

In order to further validate the findings from the mouse islets and MIN6 cells, we performed similar GSIS assays on human donor islets. Treatment of human islets with 10 nM recombinant sAPP α during GSIS assays demonstrated a significant potentiation of insulin secretion using a range of glucose concentrations (Fig 14D).

CHAPTER IV

RESULTS

Study 2 – Amyloid Precursor Protein and Insulin Degrading Enzyme

Introduction

Mounting evidence suggests that perturbations in brain metabolism and insulin signaling play a role in the pathology of Alzheimer's disease (AD) (Harris et al., 2016; Hoyer, 1991; Macauley et al., 2015; Mosconi et al., 2006; Talbot et al., 2012). Histologically, AD is characterized in part by the robust accumulation of extracellular “plaque” aggregates of the A β peptide in central nervous system (Selkoe & Hardy, 2016). A β is generated in the brain by the enzymatic cleavage of the much larger Amyloid precursor protein (APP). Amyloid precursor protein or APP is a transmembrane glycoprotein encoded on the 21st chromosome in humans (Blanquet et al., 1987; Robakis et al., 1987). APP is part of a three member family of proteins which also includes APLP1 and APLP2 (U. C. Muller & Zheng, 2012). While expression of APLP1 is largely restricted to the central nervous system, both APP and APLP2 are expressed ubiquitously and can be detected in a variety tissues (Lorent et al., 1995). Of this protein family, only APP generates the A β peptide. Interestingly, the APP family of proteins seems to be required for life, or is at least critical for normal animal development. Knockout of APP and APLP2 genes in mice has been shown to be postnatally lethal, as is knockout of both APLP1 and APLP2 (Heber et al., 2000). Deletion of just the

APP gene confers a more benign phenotype, including reduced animal body weight and organ size, changes in grip strength and broad changes in metabolism (Puig et al., 2017; Zheng et al., 1995). Mouse knockouts of APLP1 have also been studied and show similar reductions in animal body weight (Heber et al., 2000). The APP gene is widely conserved among other species, suggesting it performs important or advantageous biological functions (Tharp & Sarkar, 2013).

Despite the known phenotypic changes in APP^{-/-} mice, the physiologic function(s) of APP is less certain. However, over the last three decades numerous studies have investigated the APP family and much is now known. The structures of APP, APLP1 and APLP2 are similar (U. C. Muller & Zheng, 2012). All three proteins are membrane spanning with a small intracellular domain and a large n-terminal ectodomain. The mature proteins are processed similarly by proteases, resulting in the release of soluble extracellular domain APP/APLP fragments termed sAPP and sAPLPs (Walsh et al., 2007). Research on the APP family of proteins highlights their importance, particularly in the central nervous system. The APP family of proteins have been characterized as synaptic adhesion molecules and upregulated during synaptogenesis (Schilling et al., 2017). Furthermore, the full length APP protein itself is believed to be involved in certain signaling processes and may function as a cell surface receptor for extracellular ligands (Hass & Yankner, 2005). It has also been reported that APP and its metabolites play a role in neurite outgrowth and neuronal migration (Gakhar-Koppole et al., 2008). Taken together, these studies

suggest APP and its metabolites can influence a variety of cellular processes and may be particularly important in the CNS and at the synapse.

In our other work, we observed higher levels of insulin degrading enzyme (IDE) protein in pancreatic tissue derived from APP^{-/-} animals compared to control animals (Kulas, Puig, & Combs, 2017). IDE is a zinc metalloprotease widely recognized for its role in hydrolyzing insulin in addition to numerous other substrates including the plaque forming peptides A β and pancreatic amylin or IAPP (Kurochkin et al., 2018). In this study we sought to expand on this observation and have examined the APP^{-/-} mice to test the hypothesis that deletion of the APP gene alters the protein levels of IDE.

IDE Protein and mRNA are Increased in APP^{-/-} Tissues

We began examining the role of APP in regulating IDE by collecting liver tissue from two month old female C57BL/6 and APP^{-/-} mice. This tissue was chosen in particular because of the physiologic importance of insulin signaling in regulating liver gluconeogenesis and glycogenolysis. The transgenic APP knockout mouse has the APP gene knocked out in all tissues via deletion of the promoter region and first exon. Mouse liver was homogenized on ice and protein content was assessed by western blot. (Fig 15A). Interestingly, IDE protein levels were found to be significantly increased in APP^{-/-} muscle (n=7, P<0.023) compared to controls. Next, we examined muscle tissue collected from WT and APP^{-/-} animals (Fig 15B). Like in liver, we observed increased IDE protein content in liver tissue

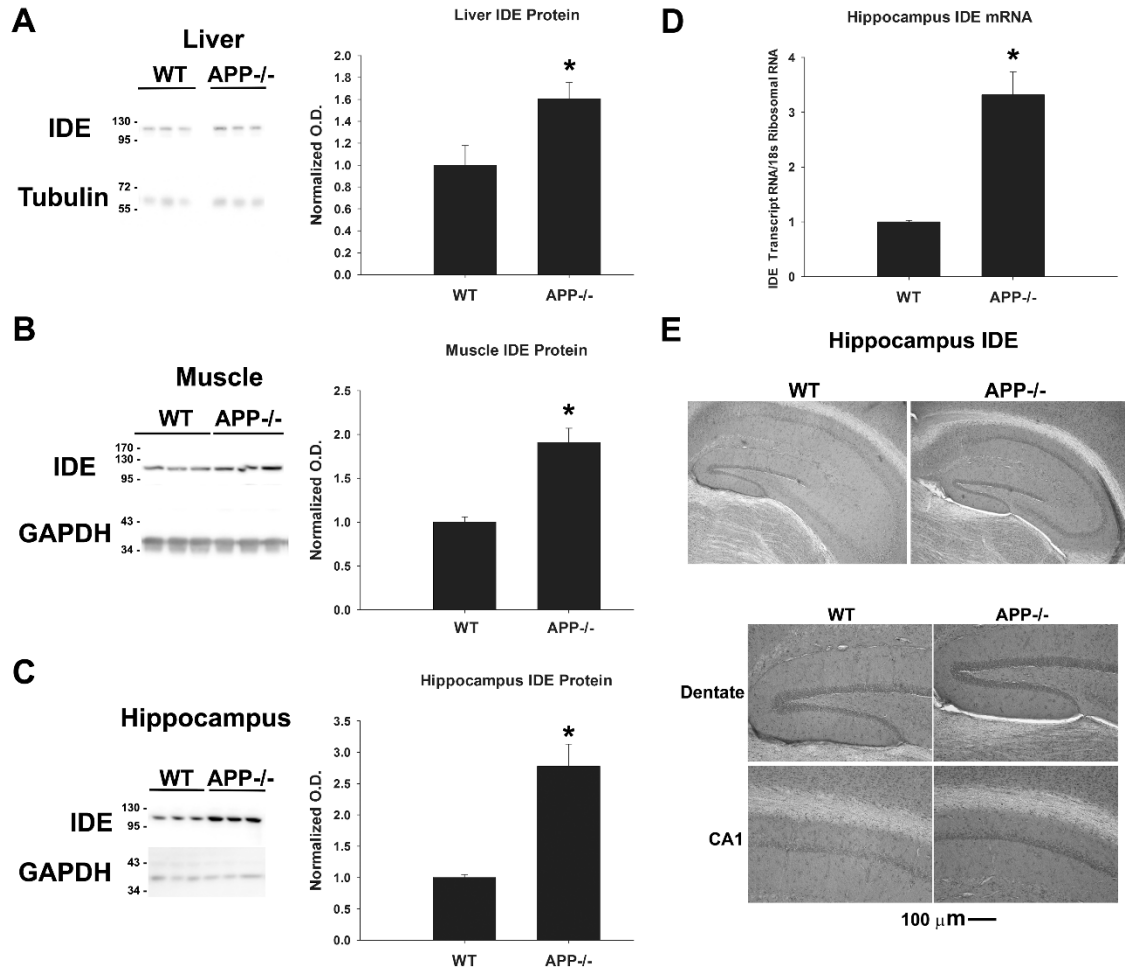


Figure 15. IDE is increased in APP^{-/-} tissues. (A-C) IDE protein was measured in liver (n=7), gastrocnemius muscle (n=6) and hippocampus tissue (n=6-7) by western blot and normalized to GAPDH as a loading control.

(D) IDE mRNA levels were measured in hippocampus extracts (n=4-5) and normalized to ribosomal 16S RNA as a loading control. Statistical significance $p < 0.05^*$ was determined by students t-test.

(E) Immunohistochemistry for IDE protein was performed on WT and APP^{-/-} brain tissue sections (n=4-6). Data is graphed as \pm SEM.

extracts from APP^{-/-} mice compared to WT controls (n=7, p=0.001). These data, along with our previous observations of elevated IDE protein in pancreatic extracts from APP^{-/-} mice, suggested to us that APP negatively regulates IDE levels in numerous peripheral tissues.

It has been suggested that IDE degrades a number of small amyloidogenic peptides, including A β , which may in turn have important implications in the pathogenesis of Alzheimer's Disease. Because of this seemingly important role of IDE in peptide clearance, we next sought to examine if ablation of the APP gene altered IDE protein levels in the CNS. Because the hippocampus serves a critical role in memory and is damaged early in the progression of AD, we collected hippocampus tissue from WT and APP^{-/-} brain hemispheres and extracted protein for western blot (n=6-7). As expected, APP was not detectable in APP^{-/-} brain tissue homogenate (Fig 15C). Surprisingly, IDE protein levels were found to be significantly increased and almost three fold higher in APP^{-/-} hippocampus tissues compared to WT controls (p<0.001). This suggested that APP regulation of IDE extends to the central nervous system. We then examined mRNA levels to determine if APP is regulating IDE at the level of transcription or if it is a post-translational phenomenon. RNA was extracted from WT and APP^{-/-} hippocampus tissues and converted to cDNA and the relative abundance of IDE transcripts were determined relative to 18s ribosomal RNA (n=4/5). Interestingly, we observed a significantly increased abundance of IDE mRNA in APP^{-/-} hippocampus tissue compared to WT controls (p=0.002) (Fig 15D). Similar to protein levels of IDE, we observed a greater than 3 fold

average increase in IDE expression in APP^{-/-} hippocampus tissue compared to WT controls. To gain a greater understanding of which cell types may be most abundantly expressing IDE, we performed immunohistochemistry to detect IDE in WT and APP^{-/-} brain tissue. IDE was detectable throughout the brain including in white matter. Neurons within the hippocampus were stained to a greater extent than other cell types, though IDE immunostaining did not robustly label a single cell type compared to others in the CNS (Fig 15E). Collectively these data suggested that APP acts as a negative regulator of IDE levels and that ablation of APP increases IDE at the level of transcription. IDE is produced throughout the brain and across many cell types.

IDE Protein is Increased in APP^{-/-} Neuron, Astrocyte and Microglia Cell Cultures. siRNA Knockdown of APP in Microglia Increases IDE.

Following our observation that IDE mRNA and protein levels are 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 increased IDE content. We began by culturing embryonic day 16 neurons from WT and APP^{-/-} mouse embryos (Fig 16A). As expected, APP was abundant in WT neurons and not detectable in APP^{-/-} cultures. Consistent with our observations in whole tissues, IDE protein levels were significantly increased in APP^{-/-} neuronal cultures compared to WT controls (n=4, p<0.010). Unlike neurons, astrocytes and microglia, two other abundant cell types found in the CNS, produce a higher molecular weight isoform of APP which may have functional consequences for APP biology. Astrocytes are the most abundant cell type found in the CNS and

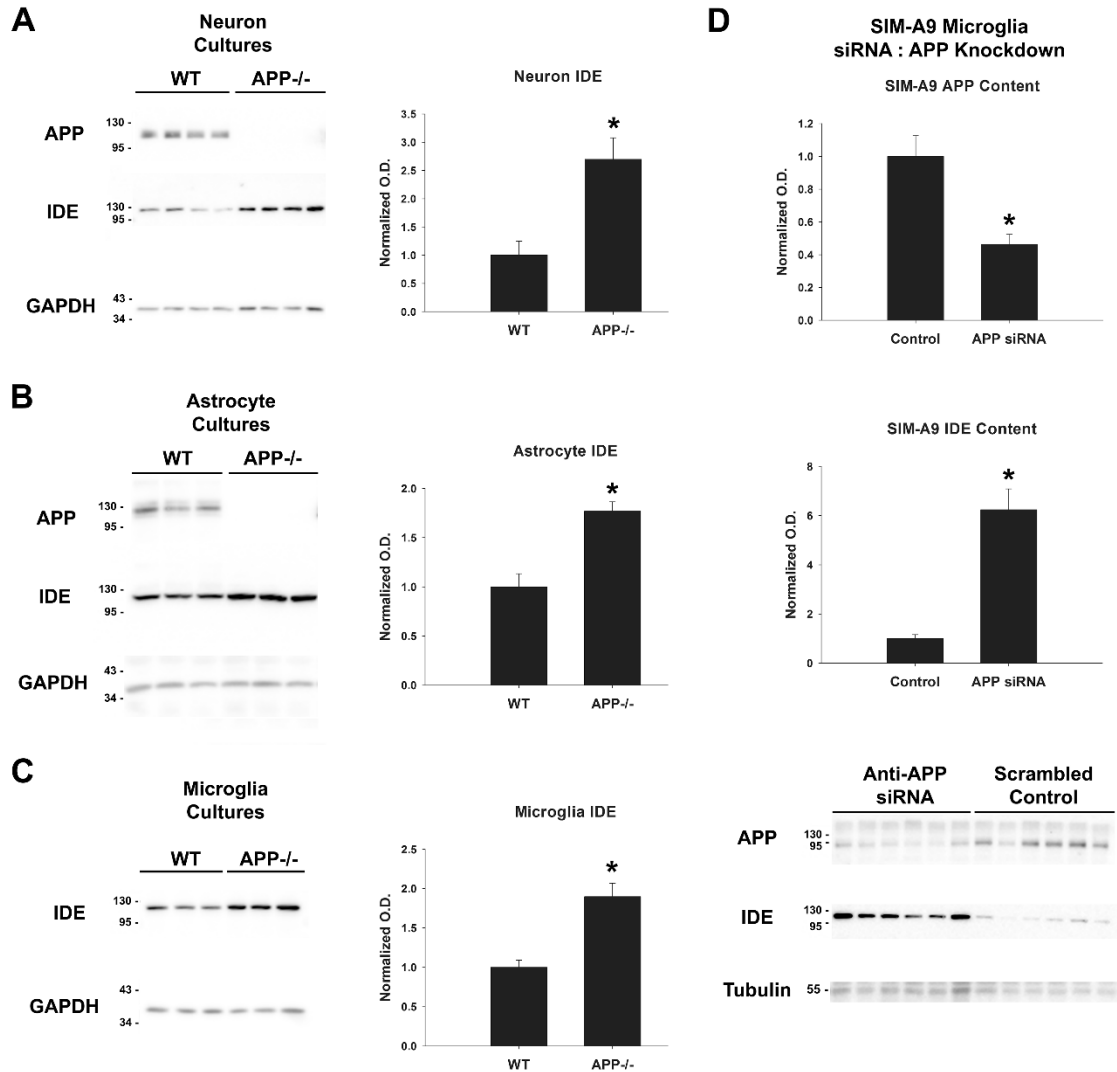


Figure 16. IDE is increased in APP-/- cell cultures.

(A-C) Primary cell cultures of neurons (n=4), astrocytes (n=3), and microglia from WT, 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 students t-test.

(D) Cultures of SIM-A9 mouse microglia cells (n=6/condition) were treated with either scrambled siRNA or anti-APP siRNA for one week. After treatment, total cell protein content was assessed by western blot with α -tubulin as a loading control. Significance $p < 0.05^*$ was determined by students t-test. Data is graphed as \pm SEM.

play an important role in brain metabolism among many other functions. Thus, we cultured astrocytes and microglia to examine if APP regulates IDE protein in these glial cell types in a manner similar to neurons (n=3/condition). We observed that APP migrated at higher molecular weight in astrocytes relative to neurons, consistent with known higher molecular weight isoforms of APP in these cells (Figure 16B). IDE protein levels were found to be significantly increased in both astrocyte (p=0.009) and microglia (Figure 16C, p=0.009) cultures relative to WT controls. This data suggested APP regulates IDE in both neuron and glia and that APP regulation of IDE is not specific to the isoform of APP expressed in diverse 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 A β , we next tested the hypothesis that knockdown of APP with siRNA in the SIMA9 microglial cell line will increase IDE levels *in vitro* (Figure 16D). Knockdown of APP protein was performed over 1 week with either an anti-APP siRNA pool or a scrambled siRNA control (n=6/condition). As expected, treatment with anti-APP siRNA significantly reduced APP as measured by western blot. (p=0.003). Surprisingly, 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 the increased levels of IDE observed in the

APP^{-/-} mouse may not be a consequence of general differences in the physiology of the animals.

IDE Activity is Increased in APP^{-/-} Tissues

While numerous APP^{-/-} mice tissues had increased IDE content compared to WT controls, we next examined if the enzymatic activity of IDE was also increased in APP^{-/-} mouse tissues. To measure IDE activity, we utilized a commercially available IDE activity assay. This assay utilizes a FRET peptide IDE substrate that emits increased 5-FAM fluorescence when hydrolyzed by the IDE enzyme. Hippocampus tissue, gastrocnemius muscle and liver tissue were collected from 2 month old WT and APP^{-/-} mice (n=6 condition) and homogenized. Each tissue homogenate was then mixed with the IDE substrate and fluorescence was measured over time (Fig 17). The IDE activity of each tissue extract was calculated and normalized to the total protein content of the sample. Significantly increased IDE activity was observed in APP^{-/-} hippocampus (p=0.016) and liver extracts (p=0.003), indicating that increased IDE protein content in these tissues leads to functional differences in IDE activity. Unlike hippocampus and liver tissue, no significant differences in IDE activity were observed between WT and APP^{-/-} gastrocnemius muscle tissue extracts.

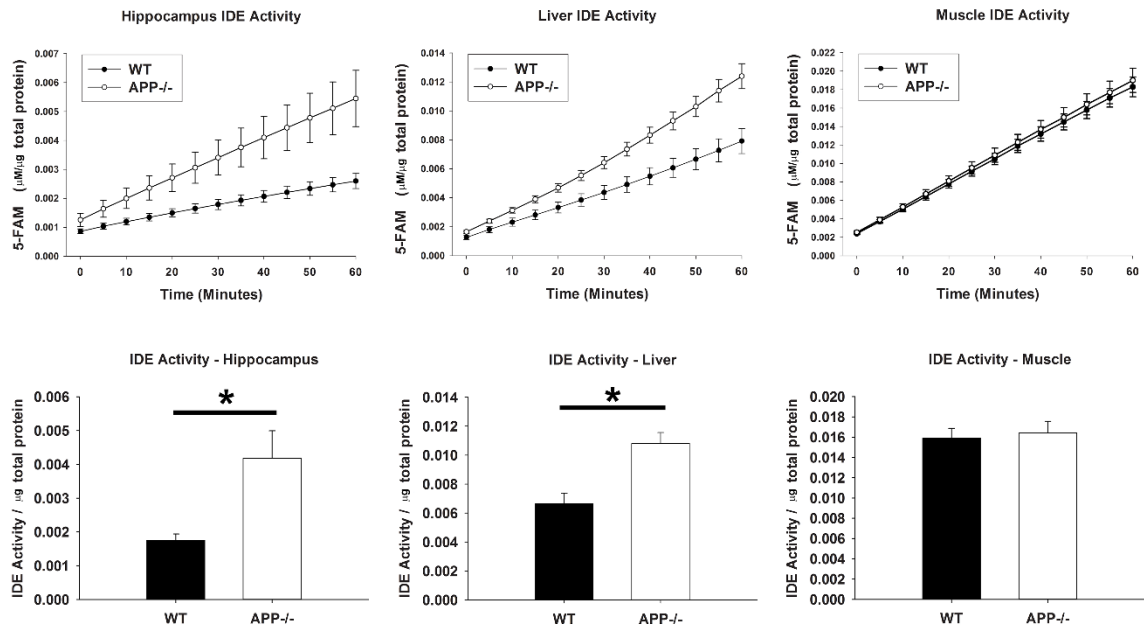


Figure 17. IDE activity is increased in APP-/- tissues. WT and APP-/- tissues (n=6) were harvested on ice and rapidly assayed for IDE activity over the course of 1 hour. The IDE activity assay uses a FRET peptide which is liberated from a quencher and exhibits increased fluorescence when cleaved by IDE. Relative fluorescent units are converted to concentrations of FAM via standard curve. The data is 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 students t-test. Data is graphed as \pm SEM.

Insulin Content and Insulin Signaling are Altered in APP^{-/-} Hippocampus tissue and Synaptosomes

We next sought to probe for consequences of increased IDE protein content and enzyme activity on insulin levels and insulin signaling markers in hippocampus tissue. We measured the insulin content of 2 month old WT and APP^{-/-} hippocampus extracts by ELISA (n=11/13) and normalized to total protein (Fig 18A). Hippocampus extracts from APP^{-/-} mouse had significantly reduced hippocampal insulin content compared to WT animals (p=0.032), suggesting that insulin content of the brain may be reduced in these animals. Following this observation, we next examined hippocampus extracts from WT and APP^{-/-} mice for changes in insulin signaling markers. While we were unable to clearly detect phosphorylated insulin receptor in these lysates (Fig 18B), we also examined markers of insulin signaling downstream of the receptor which serve as a positive indicator of insulin signaling. We observed significantly reduced (p=0.025) levels of pAKT S308 in APP^{-/-} hippocampal lysates compared to WT controls, suggesting that APP^{-/-} animals have diminished or impaired insulin signaling in brain tissue.

To further examine insulin signaling in the brains of these animals, we prepared synaptosomes from WT and APP^{-/-} tissues and stimulated them acutely with insulin *ex vivo*. Stimulated synaptosome lysate was then analyzed for protein and phosphor protein content by WES capillary western blot. We have previously utilized this method to examine insulin signaling in the brains of other transgenic mice (Puig et al., 2016) and the details of this method have been

Figure 18. Insulin signaling and content are altered in the APP^{-/-} brain.

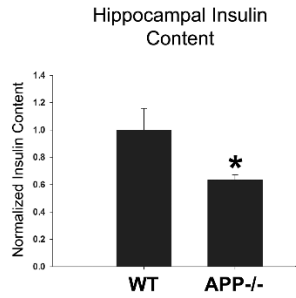
(A) Total hippocampal insulin content was measured in RIPA extractions of WT and APP^{-/-} hippocampus tissue by ELISA. (n=13)

(B) Insulin signaling cascade markers were assessed in two-month old WT and APP^{-/-} hippocampal tissue. (n=5-6/condition)

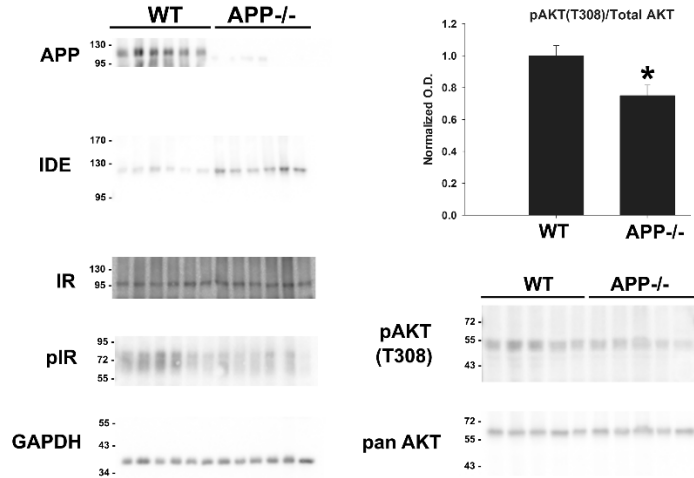
(C) Hippocampal synaptosomes from 2 month old WT and APP^{-/-} animals were prepared and stimulated with 10 nM insulin (n=5/5) or 200 nM insulin (n=6/4) pIR, total IR and β tubulin protein levels were measured using the WES system. Data was collected using the area under peaks for the specified proteins and analysis performed using a Student's t-test with significance $p < 0.05^*$.

Data is graphed as \pm SEM.

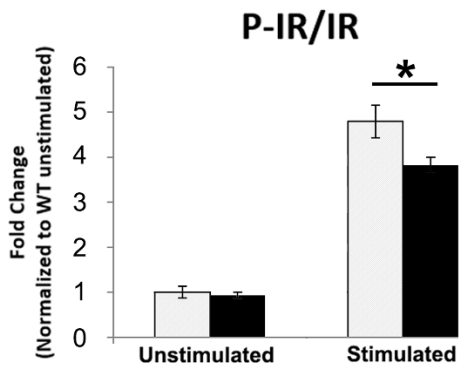
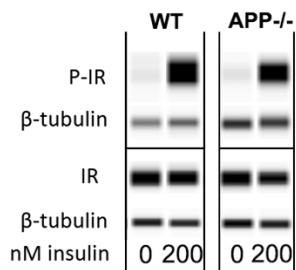
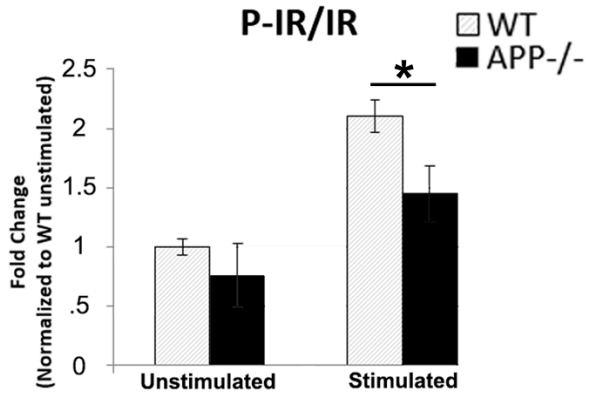
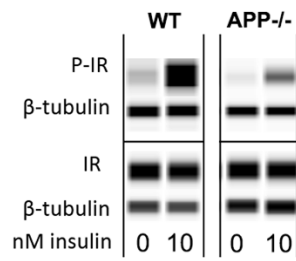
A Insulin Content



B Hippocampus Lysate



C Synaptosome Stimulations



expanded on in recent literature (Franklin & Taglialatela, 2016). WT 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 WT synaptosomes when stimulated with either 10 nM (p=0.0315) or 200 nM (p=0.0265) insulin. (Fig 18C). No differences in the ratio of phosphorylated IR to IR 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.

***Aged APP^{-/-} Mice Display Fasting Hypoglycemia
Without Changes in Glucose Tolerance.***

While our data suggests APP^{-/-} animals have changes in brain insulin signaling, the potential effects of elevated peripheral IDE on metabolic homeostasis remains uncertain. To determine if APP^{-/-} animals have abnormal glucose homeostasis we performed glucose tolerance testing on 12 month WT and APP^{-/-} (n=11-13/condition). 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 by glucometer 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 (Fig 19). No significant difference was observed in overall glucose tolerance (p=0.12) between WT and APP^{-/-} animals. However, we did observe a robust and significant reduction in the fasting blood glucose levels of APP^{-/-} animals (p=0.002) compared to WT controls. Blood glucose was also measured in free fed WT and APP^{-/-} mice (n=5/condition) and was not significantly different

($p=0.221$). These data suggest APP^{-/-} animals are glucose tolerant but have metabolic abnormalities when fasted.

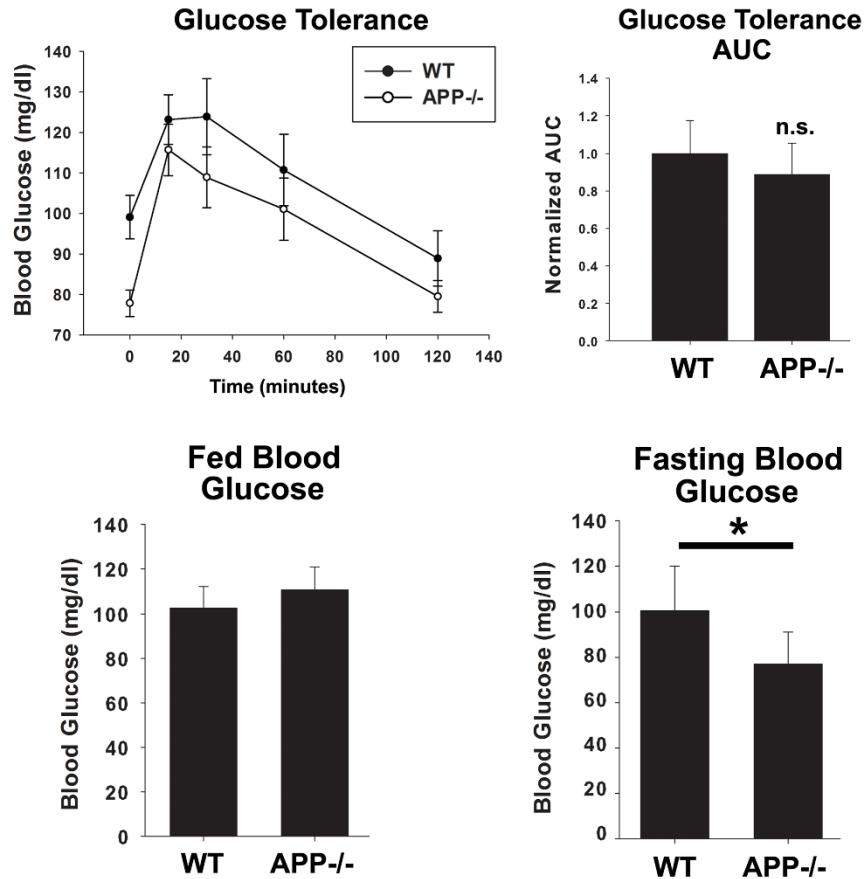


Figure 19. Aged APP^{-/-} animals show metabolic changes. WT and APP^{-/-} animals (n=11-13) were fasted and subjected to glucose tolerance testing with IP injections glucose. Area under the curve (AUC) was measured for each animal's blood glucose over time, and WT and APP^{-/-} animals were compared by t-test with $p<0.05^*$. Blood glucose was measured and graphed in fasted or in free fed (n=5) WT and APP^{-/-} mice. Significance $p<0.05^*$ was determined by students t-test. Data is graphed as \pm SEM.

Overexpression of APP or Treatment with Secretase Inhibitors Does 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 inhibition of secretases through selective inhibitors is sufficient to alter levels of IDE, SIMA9 microglia cells were treated for 7 days in 1% serum solution with the alpha secretase inhibitor GI254023X, the beta secretase inhibitor Verubecestat, or the gamma secretase inhibitor DAPT (Fig 20A). No significant difference was found in the IDE protein content of SIMA9 cells treated with inhibitors compared to untreated or vehicle controls (n=3/treatment, p=0.45) by one-way ANOVA.

Next, to further examine if an APP metabolite may be capable of changing IDE levels, we tested if the application of recombinant sAPP fragments to APP^{-/-} astrocyte primary cultures would reduce the protein levels of IDE. Cells were treated for 3 days with 20 nM of bacterial derived recombinant sAPP α , sAPP β or mutant sAPP α , or with 200 nM eukaryotic derived sAPP α 695 or sAPP α 751 (Fig 20B). These sAPP fragments did not alter IDE levels in cultures, further suggesting the full length APP protein or another APP fragment may be upstream of changes in IDE levels observed in APP^{-/-} tissues.

Finally, we tested if overexpression of the APP695 isoform or the APP751 isoform in APP^{-/-} astrocytes is sufficient to reduce levels of IDE (Fig 20C). Primary astrocyte cultures were transfected using lipofectamine with plasmids coding for APP695, APP751, Green fluorescent protein (GFP), or mock transfected as a control. Cellular protein was collected after 5 days and IDE was

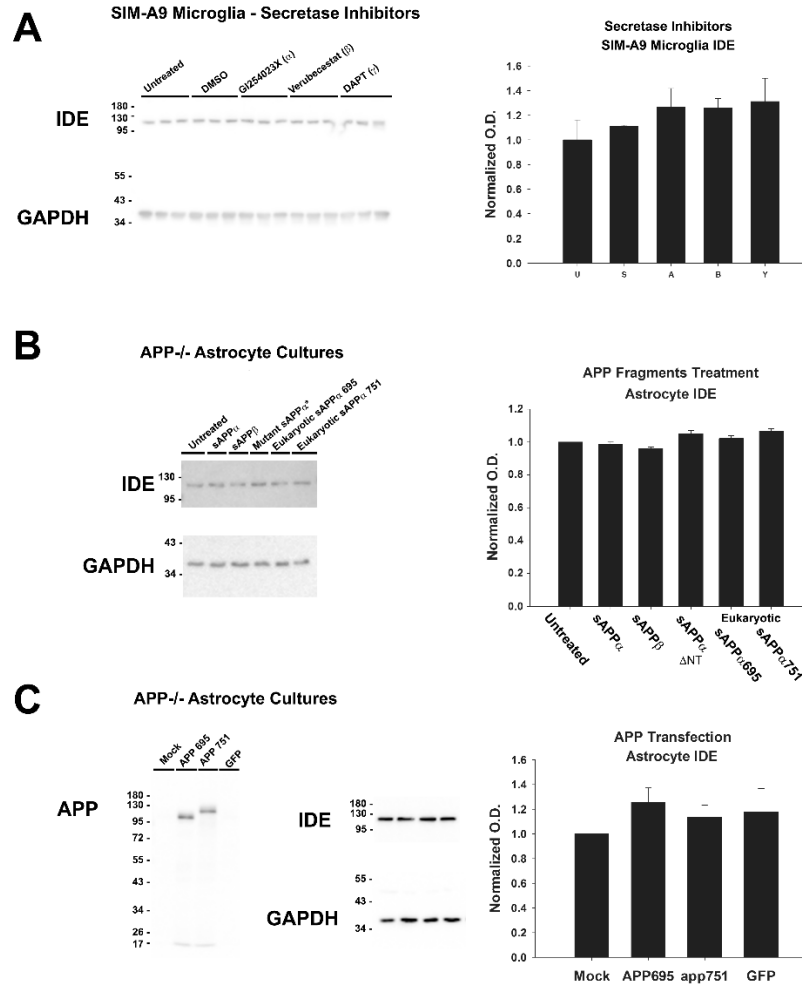


Figure 20. APP fragments and secretase inhibitors do not alter IDE. Overexpression of APP does not alter IDE levels in APP^{-/-} cells. (A) SIMA9 microglia (n=3/condition) were treated with secretase inhibitors, DMSO vehicle, or left untreated for one week and IDE protein content was measured. Significance $p < 0.05^*$ was determined by one-way ANOVA. (B) APP^{-/-} primary astrocyte cultures (n=4/condition) were treated with bacterial recombinant sAPP α , sAPP β , mutant n-terminal truncated sAPP α or euaryotic derived sAPP α 695 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 \pm SEM.

measured by western blot. Surprisingly, overexpression of APP in APP^{-/-} astrocytes did not significantly change IDE levels ($p=0.56$) (Fig 20C). This suggests that overexpression of APP may not be capable of restoring the normal functions of APP, possibly due to aberrant processing of the transgene protein product. Alternatively, overexpression of APP may not allow for normal protein-protein interactions of the APP proteins, or that overexpressed APP may not be trafficked appropriately to perform its normal functions within the cell.

CHAPTER V

DISCUSSION

Study 1 - Amyloid Precursor Protein in the Endocrine Pancreas

In this study we report the presence of APP in pancreatic tissue, including the detection of APP within insulin producing pancreatic β cells. We have confirmed the presence of pancreatic islet APP expression in both C57B6 mice and human samples, demonstrating this pattern of APP expression is confirmed across species. Interestingly, we observed APP is overexpressed within the pancreatic islets of the APP/PS1 mouse model of AD. This mouse model of AD uses the mouse prion promoter to drive human APP transgene expression. This finding indicates that the prion promoter itself may be a useful tool for driving robust gene expression of target genes specifically within the endocrine compartment of the pancreas. It has recently been reported that a 3xTg-AD model of Alzheimer's disease, which uses Thy 1.2 promoter, also overexpresses human APP within pancreatic islets (Vandal et al., 2015). Because of the similarity in gene expression and phenotype between pancreatic islets and neurons, it is likely that numerous genetic elements which drive high expression of genes in the central nervous system will also result in robust gene expression in pancreatic islets (Atouf, Czernichow, & Scharfmann, 1997; Martens et al., 2011; Moller et al., 1992).

Despite the robust overexpression of human mutant APP within the pancreatic islets of the APP/PS1 mouse, we were unable to detect the presence of monomeric human A β , A β oligomers, or fibrils within APP/PS1 pancreatic tissue or islet cultures by ELISA or immunohistochemistry. This is in agreement with previous reports suggesting β -secretase activity is not present in pancreatic tissue extractions despite the presence of BACE1 RNA (Ehehalt et al., 2002; Mowrer & Wolfe, 2008; Sinha et al., 1999). In 2002, Ehehalt et al. described a shorter isoform of BACE1 RNA termed BACE1C in the pancreas along with the more well-known BACE1A isoform. BACE1C lacked detectable β -secretase activity in their assays, suggesting that pancreatic tissue may not produce A β , in part, because of the presence of this enzymatically inactive splice variant. However, BACE1A is still the dominant isoform of BACE1 found in the pancreas so alternative splicing cannot be the sole reason for this phenomenon. In 2008, Mowrer and Wolfe described similar alternative splicing of BACE1 in the pancreas relative to the brain, but also noted that this alone was not sufficient to explain the lack of BACE1 activity in pancreatic tissue. These authors postulated that pancreatic BACE1 is inactive due to post-translational processing mechanisms. Indeed, it has been shown that BACE1 in the pancreas and other tissues undergoes differential proteolytic processing compared to the brain resulting in lower or undetectable levels of the BACE1 holoprotein (Huse et al., 2003). Instead of β -secretase mediated APP processing, our data suggests that APP in pancreatic islets is processed by α -secretase activity to release sAPP α . This is clearly in contrast to the CNS where both forms of APP processing occur.

We were also unable to detect plaque deposition in human pancreas sections using the anti-A β antibody, 4G8. It is possible that pancreatic islets could be affected by circulating concentrations of A β rather than locally produced A β .

To study the significance of APP in pancreatic islet function, we examined two month old WT, APP^{-/-}, and APP/PS1 animals for differences in glucose tolerance by oral gavage. This time point was important for assessing the role of islet APP in glucose tolerance because it is well before A β deposits are known to form in the CNS of the APP/PS1 mouse. A β oligomers or over-expression of the A β producing enzyme BACE1 in the brain have recently been demonstrated as sufficient to induce peripheral metabolic dysregulation (J. R. Clarke et al., 2015; Plucinska et al., 2016). We detected no significant differences in glucose tolerance across strain, indicating ablation or overexpression of mutant APP does not severely compromise glucose homeostasis mechanisms or severely impair islet function at two months of age. Multiple organ systems such as liver and kidney gluconeogenesis and muscle glucose uptake are involved in glucose homeostasis, thus the effect of overexpression or ablation of pancreatic islet APP on blood glucose levels during glucose tolerance testing may be masked by glucose-regulating compensatory mechanisms in other organs. In addition to glucose tolerance testing, we also measured the pancreatic insulin and glucagon content by ELISA. Our results indicate that while total insulin content was unchanged, total pancreatic glucagon levels and glucagon immunostaining were significantly attenuated in animals expressing the APP/PS1 transgenes, suggesting that overexpression of mutant APP or presenilin in pancreatic islets

may alter the development of α cells or affect their secretory functions. BACE2 levels were also diminished in the APP/PS1 animals, further suggesting that the APP/PS1 mice may have fewer pancreatic α cells. Future work requiring, perhaps stereologic assessment, may provide a clearer indication of changes in total α cell numbers in the APP/PS1 compared to WT mice. One possible mechanism for a change in α cell number could be through alterations in Notch biology. Presenilin is part of the γ -secretase complex which cleaves the Notch protein to liberate the biologically active NICD fragment. NICD then enters the nucleus where it affects transcription (Chan & Jan, 1999). Notch signaling is thought to play a critical role in the normal development of the endocrine pancreas suggesting that mutant presenilin expression in the pancreas may indeed influence this biology (Hald et al., 2003; Jensen et al., 2000; Murtaugh, Stanger, Kwan, & Melton, 2003).

Our examinations of pancreatic protein content in two month old animals revealed changes in the levels of IDE, GLUT4 and BACE2. Animals lacking the APP gene showed increased levels of IDE and GLUT4, while APP^{-/-} and APP/PS1 animals showed reduced BACE2 levels compared to wild type in western blot. IDE has been identified as an enzyme capable of degrading A β and amylin in addition to insulin. These functions may be important in the pancreas, as inhibition of IDE has been shown to enhance toxicity of islet amylin (R. G. Bennett, Hamel, & Duckworth, 2003). GLUT4 is considered the insulin responsive glucose transporter and is well known for its role in muscle and fat tissue. Recent studies have presented evidence that both sAPP and A β may

influence the cellular localization of GLUT4 (Hamilton et al., 2014; Oliveira et al., 2015). BACE2 has been previously implicated as both an APP cleaving enzyme and regulator of β cell function through the cleavage of the transmembrane protein TMEM27 (Esterhazy et al., 2011). It has been proposed that full length TMEM27 supports beta cell growth and insulin secretion, thus blocking its cleavage by inhibiting BACE2 activity may represent a therapeutic strategy to control blood glucose in diabetic patients (Southan, 2013).

We also examined protein content of primary islet cultures from 12 month old WT, APP^{-/-}, and APP/PS1 animals. Similar to whole-pancreas extracts, IDE was present at higher levels in APP^{-/-} compared to WT controls. This indicates that APP may play a role in negatively regulating the expression of IDE, or that differences in the APP^{-/-} animals lead to upregulated IDE expression. We could not detect GLUT4 in our islet lysates, however GLUT4 is not considered a major glucose transporter in the pancreatic endocrine cells and differences observed in whole pancreas were likely from other cell types. BACE2 levels were not significantly different in islets from transgenic animals compared to controls, which is in contrast to both our immunohistochemistry and whole pancreas western blot results. This could be in part due to the process of pancreatic islet isolation in which the pancreas is digested with proteases to liberate the islets from exocrine tissue. It is likely that this isolation process would affect the cells at the islet periphery expressing BACE2. Alternatively some BACE2 in whole pancreatic extracts may be due to the contribution of other cell types.

Although higher glucose culture concentrations are reported to improve islet culture and reduce apoptosis, we chose to avoid high glucose levels that may influence APP expression or processing. Several lines of evidence suggest that culturing cells in high glucose concentrations can alter APP processing and affect APP protein levels (H. J. Lee et al., 2016; Yang, Wu, Zhang, & Song, 2013). Additionally, a recent study utilizing glucose clamps to sustain hyperglycemia demonstrates that increasing glucose concentrations in vivo alters APP processing to influence A β levels in the CSF (Macauley et al., 2015). We considered the implications of these studies and thus 5mM glucose was selected for our paradigm. We avoided larger islets with necrotic centers in an attempt to minimize artifacts induced by cell death from lower glucose concentrations. However, since islet glucose concentration exposure in vivo may be well above 5mM, there was a concern that our primary islet sAPP α stimulation paradigm was superimposed upon a glucose deficient condition. To verify that sAPP α stimulated insulin secretion at higher glucose concentrations, we employed the MIN6 cell line to demonstrate that insulin secretion was still potentiated at 16.7 mM glucose concentration. Future studies will require further optimization of islet culture conditions including higher glucose concentrations. In addition, it is important to remember that although the wild type mice are littermate controls of the APP/PS1 mice in our study, the APP $^{-/-}$ mice were derived from a homozygous colony and comparison to the other two lines is potentially limited by this. Nevertheless, these data suggest changes in pancreatic APP content or

processing may result in alterations in the levels of proteins important to pancreatic physiology and influence the pathology of pancreatic diseases.

Interestingly, our immunohistochemistry data indicated that primary antibodies directed against BACE2 showed increased binding in pancreatic α cells compared to β cells in both mouse and human tissue. This is particularly interesting as BACE inhibitors are actively being explored as a therapeutic strategy in AD (Kennedy et al., 2016). Our detection of BACE2 in α cells is in contrast to reports indicating the highest pancreatic BACE2 expression is found within pancreatic β cells (Esterhazy et al., 2011; Finzi et al., 2008). A likely reason for this discrepancy is the binding site of the antibody used to detect pancreatic BACE2. BACE1 and BACE2 share a high degree of sequence similarity, and several alternatively spliced transcripts of BACE1 are known to be present in the pancreas (Ehehalt et al., 2002; Mowrer & Wolfe, 2008). In agreement with our findings, a recent study by Segerstolpe et al. also observed higher expression of BACE2 in α cells relative to β cells through single cell transcription profiling (Segerstolpe et al., 2016). In this study we utilized a polyclonal antibody raised against the BACE2 pro-domain sequence not present in the BACE1 sequence. Pro-domain cleavage is thought to be an essential step in the activation of the BACE2 proenzyme, thus our immunohistochemistry results could indicate differences in BACE2 activation in α and β cells (Hussain, Christie, Schneider, Moore, & Dingwall, 2001; Hussain et al., 2000).

Our assessments of isolated pancreatic islets demonstrate both endogenous murine APP and transgenic human APP are released as sAPP into culture media from WT and APP/PS1 mouse islets respectively. We also observed modest but significant differences in the insulin secretion properties of islets overexpressing or lacking APP compared to WT controls, suggesting the presence of APP may influence the basic functional properties of β cells in vitro. However, a lack of detectable differences in APP^{-/-} and APP/PS1 animals compared to WT controls in glucose tolerance testing at two months of age suggests APP is not essential for normal islet insulin secretion or glucose homeostasis in vivo.

sAPP has been shown to exert effects on a variety of cell types. In the central nervous system, sAPP is thought to promote neurite outgrowth and influence the differentiation of neural stem cells (Baratchi, Evans, Tate, Abraham, & Connor, 2012; Freude, Penjwini, Davis, LaFerla, & Blurton-Jones, 2011; Jin et al., 1994; Porayette et al., 2009). sAPP has also been implicated as having growth enhancing effect in pancreatic cancer, in addition to altering the proliferation rate of other cell types (Demars, Bartholomew, Strakova, & Lazarov, 2011; Hansel et al., 2003; Woods & Padmanabhan, 2013). Our data indicate that recombinant sAPP α modestly potentiates insulin secretion in pancreatic islets from both mice and humans in vitro. This suggests that sAPP may exert effects on β cell function, potentially by acting on cell surface receptors in an autocrine fashion. The mechanism through which sAPP exerts its biological effects is uncertain and may be due to interactions with several proteins. Several studies

have identified putative cell surface receptors for sAPP. These include the P75 neurotrophic receptor, LRP1, as well as APP itself (Hasebe et al., 2013; Kounnas et al., 1995). The putative receptors and biological effects of sAPP signaling have recently been extensively reviewed (Habib, Sawmiller, & Tan, 2016). Stimulation of these cell surface receptors by sAPP may influence the survival or phenotype of islets in culture. Indeed, recombinant sAPP has been shown to induce cell signaling cascades which may account for its effects on cultured islets (Bodles & Barger, 2005; Deng et al., 2015; Jimenez et al., 2011). Our future work will focus on identifying signaling cascades and downstream effects induced in pancreatic endocrine cells by both APP and sAPP.

To our knowledge, our study is the first report showing sAPP is released from both primary mouse and human islet cultures into culture media. We also present new data indicating expression of the BACE2 protein in pancreatic α cells. Findings from this study contribute to the basic understanding of amyloid precursor protein and pancreatic islet physiology.

Study 2 - Amyloid Precursor Protein and Insulin Degrading Enzyme

Glucose hypometabolism in the brain is a known component of advanced Alzheimer's disease and is also observed in animal models of AD and in Down syndrome patients (Lao et al., 2018; Oh, Madison, Baker, Rabinovici, & Jagust, 2016; Parent et al., 2017). Type 2 diabetes is also among the risk factors for the development of AD (Wijesekara, Goncalves da Silva, De Felice, & Fraser, 2017). Interestingly, growing research indicates that a normal physiologic function of APP and its associated fragments are regulators metabolic homeostasis. APP-/-

knockout mice, in addition to having lower body weight, show reduced weight gain when placed on a high fat diet (Puig et al., 2017). In muscle tissue, sAPP α has been shown to potentiate glucose uptake (Hamilton et al., 2014). APP's role in metabolism may also be conserved across species. APL-1, the *C. elegans* homologue of APP, is both essential for survival and may play an important role in insulin homeostasis and metabolism (Ewald, Cheng, et al., 2012; Ewald & Li, 2012; Ewald, Raps, & Li, 2012; Puig et al., 2017). Our data presented in this study indicates that ablation of the APP gene in mice or prolonged knockdown of the APP gene in cell culture results in increased levels of the insulin degrading enzyme (IDE) and changes in insulin signaling compared to controls. Increased IDE protein levels were found to be present in muscle, liver and in hippocampal tissue extracts from APP^{-/-} animals. In agreement with this finding, primary cell cultures of neurons, astrocytes and microglia from APP^{-/-} mice were all found to have higher IDE content than WT control primary cultures. Furthermore, mRNA levels of IDE were found to be significantly increased in hippocampal tissue extracts from APP^{-/-} animals compared to controls, suggesting that increased IDE protein is the result of increased transcription of the IDE gene. The phenomenon of increased IDE was most robust in brain tissue and neurons relative to other tissues and cell types assessed in this study, which is interesting because APP, although expressed ubiquitously, is known to be particularly abundant in neuronal cells and generally abundant in the CNS. In further support of this observation treatment of SIMA9 microglial cells with anti-APP siRNA for one week was sufficient to increase protein levels of IDE. This suggests that loss

of APP directly mediates this effect, and it is not a consequence of broad changes in APP^{-/-} animal development and metabolism.

To determine if increased levels of IDE protein resulted in functional differences in enzyme activity, we measured the activity of IDE in liver, muscle and brain tissue. In agreement with our western blot data, we observed robust and significantly increased IDE activity in both hippocampus and liver extracts from APP^{-/-} animals compared to WT controls. IDE activity was surprisingly not statistically different in skeletal muscle tissue extracts, despite increased total IDE proteins levels. The lack of difference in muscle could be due to the nature of the activity assay, which uses cleavage of a FRET peptide as an indicator of IDE activity. It is possible that muscle tissue contains other enzymes capable of cleaving the FRET substrate, or that our protocol of homogenization was not sufficiently rigorous to liberate all of the IDE from the gastrocnemius muscle tissue. Alternatively, we observed additional lower molecular weight bands on western blots for IDE from muscle tissue lysates not present in other tissues, suggesting a there may be alternative forms of IDE in muscle compared to other tissues.

The ability of an enzyme present in tissue extracts to degrade insulin has been known for decades, and an insulin degrading enzyme was purified and further characterized by Duckworth et al. in 1972 (Duckworth, Heinemann, & Kitabchi, 1972; Mirsky & Broh-Kahn, 1949). In 1988, the IDE was cloned and definitively identified (Affholter, Fried, & Roth, 1988). While the brain was traditionally viewed as an insulin insensitive organ, increasing literature

demonstrates insulin and insulin signaling are involved in the normal physiology of the brain (Fernandez et al., 2017; Garcia-Caceres et al., 2016; Kleinridders et al., 2014). Moreover, evidence suggests that impaired insulin signaling may play a role in the pathology of AD (Ribe & Lovestone, 2016; Talbot et al., 2012).

Intranasal insulin is currently under investigation as a therapeutic intervention for AD (Chapman, Schioth, Grillo, & Benedict, 2017). To determine if increased IDE in brain tissue results in lower levels of tissue insulin content we measured the total insulin content in lysates from WT and APP^{-/-} hippocampal extracts by ELISA. We observed significantly reduced insulin levels in the hippocampus of APP^{-/-} animals, suggesting that increased IDE activity in these animals may indeed lower tissue insulin content or increase insulin catabolism in homogenates. This is in general agreement with previous research showing IDE^{-/-} mice have impaired tissue insulin degradation (Farris et al., 2003).

Our data indicates that increased IDE protein and activity alter brain insulin signaling. We examined hippocampus protein extracts for markers of insulin signaling. While we could not clearly detect phosphorylated insulin receptor in our hippocampus homogenates, likely due to the transient nature of insulin receptor phosphorylation, we did observe modest but significantly reduced levels of pAKT(T308) in APP^{-/-} hippocampal lysates compared to WT controls. This indicated that insulin signaling may be impaired or altered in APP^{-/-} brain tissues. To further examine this possibility, we prepared synaptosomes from WT and APP^{-/-} hippocampal tissues and stimulated them acutely using 10 nM or 200 nM of insulin. At both concentrations of insulin tested, we observed

diminished phosphorylation of the insulin receptor in APP^{-/-} synaptosomes compared to WT controls. This finding further indicates either loss of APP or increased IDE may in turn impair insulin signaling in the brain and this may be particularly robust at the level of the synapse.

To examine potential functional consequences of increased tissue levels of IDE in APP^{-/-} 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 (Deprez-Poulain et al., 2015). 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 (Kulas et al., 2017). This finding is, however, in general agreement with work by Needham et al. which demonstrated lower plasma glucose in both APP^{-/-} and APLP2^{-/-} mice (Needham et al., 2008). Other groups have shown that aged APP^{-/-} animals have impaired spatial learning in advanced age (U. Muller et al., 1994; Phinney et al., 1999). It can be speculated that aging may reveal phenotypes in present in APP^{-/-} mice not observed at earlier ages. Furthermore, this compromised metabolic homeostasis may reveal the potentially deleterious consequences of increased IDE in tissues, in spite of normal overall glucose tolerance. IDE has been implicated in the degradation of the glucose

elevating hormone glucagon, which may account for this phenomenon in part (Shen, Joachimiak, Rosner, & Tang, 2006).

The mechanism by which ablation of APP results in increased IDE transcription remains to be elucidated. It has been demonstrated that the intracellular c-terminal fragment can interact with other proteins like Fe65 and the histone acetyltransferase Tip60 to effect transcription (Cao & Sudhof, 2001; Kimberly et al., 2001). However, treatment of SIMA9 microglia with secretase inhibitors for one week was not sufficient to significantly change IDE levels in SIMA9 cells, potentially implicating full length APP or an alternative secretase as the primary mechanistic regulator upstream of changes in IDE. Previous research has indicated that APP may be able to affect transcription independently of γ -secretase activity (Hass & Yankner, 2005). Furthermore, in vitro treatment of APP^{-/-} astrocytes with recombinant sAPP α or sAPP β , or SIMA9 cells with α and β secretase inhibitors did not alter IDE levels, further implicating a mechanism involving the full length APP protein. In spite of this, overexpression of human APP695 and APP751 was not sufficient to change IDE levels in APP^{-/-} astrocyte primary cultures. However, this may be due to abnormal processing or trafficking of overexpressed APP. It is also possible that overexpression results in failure of the endogenous signaling machinery present under WT conditions. Nonetheless, long-term siRNA knockdown of APP in vitro was sufficient to robustly change IDE levels in support of our observations in the

APP^{-/-} mouse. It is possible that changes in IDE occur as an indirect effect of loss of APP instead of APP being directly involved in IDE transcription.

IDE has previously been linked to APP in that IDE is known to degrade both the A β and AICD peptide fragments of APP. In this study we present new evidence that ablation of APP is sufficient to increase IDE transcription, protein levels and 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 number of links suggesting aberrant metabolic processes may play a critical role in Alzheimer's Disease.

Limitations of Work Presented in this Dissertation

The research presented in this dissertation indicates a role for APP in insulin homeostasis and animal metabolism. The limitations of the work discussed in this study are presented as follows.

Data presented in these studies primarily report on differences observed across various strains of mice. Each mouse strain in this study, while originally developed on a C57BL6 background, likely has unique attributes due to both inbreeding of animals and having different origins. It is well established that various behavioral, metabolic and biochemical differences occur across mouse strains (Avila, Kim, & Massett, 2017; Linder, 2006; Vaillant et al., 2014). Thus, differences in animal background may account for at least some of the metabolic and protein changes observed in APP^{-/-} and APP/PS1 animal tissues. The role of sex as a factor of APP in influencing metabolic homeostasis was not rigorously examined in these studies, and most experiments here utilized female animals.

However, differences in IDE and fasting blood glucose reported here in APP^{-/-} females were also observed in male mice.

sAPP α stimulation of pancreatic endocrine cells may not represent physiologic conditions. In this dissertation recombinant sAPP α was applied at concentrations of 10-20 nM. This concentration was chosen based on the known effects of sAPP α in stimulating signaling and neuroprotective effects in neurons (Jimenez et al., 2011). The true concentration of sAPP in the blood is likely different than this concentration. It has been shown that mice which express human sAPP α have concentrations of approximately 200 nM in plasma, while humans have been shown to have approximately 10-20 ng sAPP α /mg plasma protein (Bailey et al., 2012; Ray, Long, Sokol, & Lahiri, 2011). Moreover, the effect of recombinant sAPP α on cultures pancreatic islet insulin secretion was modest and no difference in glucose tolerance was detected across strain. This could indicate sAPP may only play a minor role in affecting islet function *in vivo*, or that its effects on islets involve biological parameters not measured in these studies.

Regarding the role of APP in regulating IDE, all experiments in this study utilized either mouse tissues or mouse-derived cell lines and primary cultures. Thus, APP regulation of IDE is not demonstrated to be conserved in humans here. The mechanism of APP regulation of IDE remains uncertain and may be due to downstream dysfunction following APP ablation, rather than APP directly mediating transcription of the gene. Thus, the mechanistic relationship between APP and IDE awaits more investigation.

Summary Conclusions and Future Directions

The work presented in this study suggests the APP protein plays a role in insulin homeostasis independent of its role in A β production (Summary Fig 21). APP and its metabolites are detectable in the pancreas, where they may influence pancreatic physiology and pancreatic insulin release. Furthermore, loss of APP increases IDE levels and activity, suggesting a role for APP in whole body insulin turnover and signaling. Ablation of APP in mice leads to broad metabolic changes including hypoglycemia which occur as a function of age and are revealed by fasting.

Future work to expand on these studies will examine APP in the Min6 cell line. We have begun to characterize APP in this pancreatic β cell line and observe similar processing and release of APP into conditioned media as observed in the primary pancreatic islet cultures presented in this dissertation.

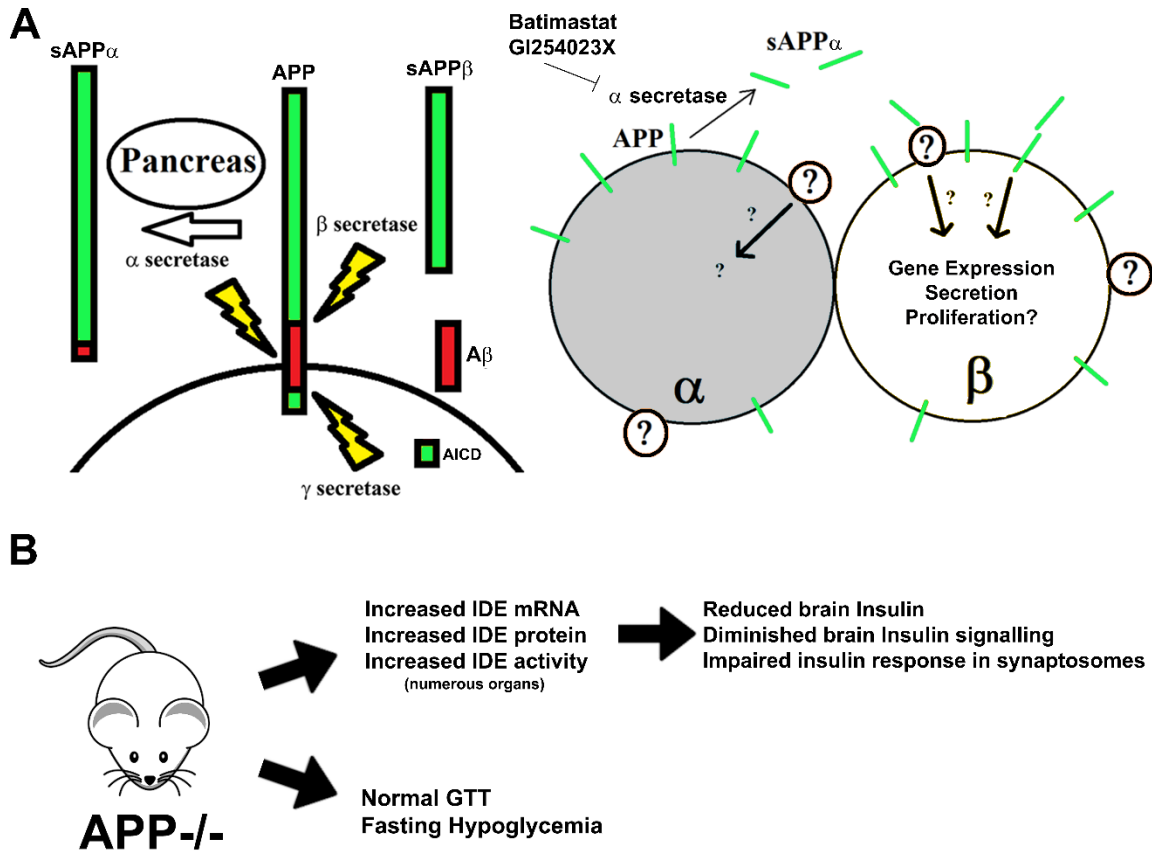


Figure 21. Model of Pancreatic APP and summarized findings. (A) Pancreatic α and β cell APP is processed through the α -secretase pathway to yield sAPP α which is detected in pancreatic cell culture media. sAPP α exerts effects on cells, possibly by acting on cell surface proteins in an autocrine or paracrine manner to effect pancreatic β cell phenotype. Full length APP itself may serve as a receptor for sAPP or drive downstream events independently of sAPP α . (B) APP $^{-/-}$ mice show elevated IDE protein and activity in numerous tissues including the brain. These mice also display low hippocampal insulin content and impaired insulin signaling. APP $^{-/-}$ animals also show impaired glucose homeostasis when fasted.

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