The Role of Caveolin-1 in Type 2 Diabetes-Induced Alzheimer's Disease

BY

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THESIS

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Dedicated to my parents, Jeffery and Sharon Bonds,

and to my best friend, Ryann Bunnell.

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CONTRIBUTION OF AUTHORS

Chapter 1 is an overall literature review of each major aspect of my research questions. The focus of the first part of this chapter is the relationship between type 2 Diabetes mellitus and Alzheimer's disease. Portions of this chapter represent a published book chapter (*Bonds et al., "Type 2 Diabetes Mellitus as a Risk Factor for Alzheimer's Disease."* Genes, Environment and Alzheimer's Disease*. By Orly Lazarov and Giuseppina Tesco. London, UK: Elsevier, Academic, 2016. 387-413. Print.*) of which I was the primary author. My collaborators, Peter C. Hart, PhD, Jacob M. Haus, PhD, and Marcelo G. Bonini, PhD, as well as my mentors Richard D. Minshall, PhD, and Orly Lazarov, PhD contributed to the writing of this portion of the chapter. The second portion of chapter 1 represents a more general literature review of neurogenesis and includes figures 2 and 3 which were created by me and were previously published as a part of a literature review (*Tobin et al., Neurogenesis and inflammation after ischemic stroke: what is known and where we go from here. J Cereb Blood Flow Metab, 2014, 34(10)1573-84)* of which I was the second author. Chapter 2 represents all of the materials and methods used in addressing our main research question of the role of Caveolin-1 in type 2 Diabetes induced Alzheimer's disease. Chapter 3 represents a description of my own unpublished experiments directed at addressing our central hypothesis that loss of caveolin-1 in type 2 diabetes leads to increased neuropathology ultimately leading to Alzheimer's disease. Chapter 4 represents the discussion of the results of my experiments, followed by Chapter 5 describing the overall conclusions and future directions. I expect that this line of work will continue in the laboratory after I leave and will ultimately be published as a peer-reviewed manuscript.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ANOVA	analysis of variance
APP	Amyloid precursor protein
APP-CTF	Amyloid Precursor C-terminal Fragment
APPswe	Amyloid Precursor Protein Swedish Mutation
Aβ	Amyloid Beta
BBB	Blood brain barrier
BDNF	Brain derived growth factor
bFGF	basic fibroblast growth factor
BLBP	Brain lipid binding protein
BMPR	Bone morphogenetic protein receptor
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CAM	Cellular adhesion molecule
Cav-1	Caveolin-1
CCP	Clathrin coated pits
CEM	Cholesterol enriched microdomains
CNS	Central nervous system
CPM	Counts per million
CSD	Caveolin scaffolding domain
CSF	Cerebral spinal fluid
DAPI	4', 6-diamidino-2-phenylindole

DCX	Doublecortin
dKO	Double knockout
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate buffered saline
ECM	Endothelial cell media
EDTA	ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunoasorbent assay
eNOS	Endothelial Nitric Oxide Synthase
FBS	Fetal Bovine Serum
FGF2	Fibroblast growth factor-2
FGFR	Fibroblast growth factor recepor
GCL	Granular cell layer
GFAP	Glial fibrillary acidic protein
GLAST	L-glutamate L-aspartate transporter
GLUT	Glucose transporter activated by insulin
HBSS	Hank's Balance Salt Solution
HCl	Hydrochloric Acid
HPC	Hippocampus
ICAM-1	Intracelular adhesion molecule 1
IFN-ɣ	Interferon gamma
IGF1R	Insulin-like growth factor 1 receptor

IL-10	Interleukin-10
IL-6	Interleukin-6
IR	Insulin Receptor-α
IRS	Insulin receptor substrate
kDa	kilodalton
kg	kilogram
Lepr	Leptin receptor
M	Molar
μCi	Micro Curie
MEMα	minimal essential medium alpha
MKR	Diabetic (mutation lysine to argenine)
mL	Milliliter
MLR	Membrane Lipid Raft
NFT	Neurofibrillary tangles
NO	Nitric Oxide
NOTCH	Notch homolog 1
NPC	Neural progenitor cell
NSC	Neural stem cell
P/S	Penicillin-Streptomycin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PS1	Presenilin-1

PS2	Presenilin-2
PSA-NCAM	Polysialated neural cell adhesion molecule
PTEN	Phosphatase and tensin homolog
PVA-DABCO	polyvinyl acetate-1,4-diazabicyclo-[2.2.2]octane
qRT-PCR	Quanitative reverse transcription polymerase chain reaction
RMS	Rostral migratory stream
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SGL	Subgranular Layer
SVZ	Subventricular Zone
T2D	Type 2 Diabetes
TBS	Tris buffered saline
TEER	Trans-endothelial electrical resistance.
TGF-α	Transforming growth factor alpha
TNF-α	Tumor necrosis factor
VCAM-1	vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor
µg	Microgram
µL	Microliter
µM	Micromolar

SUMMARY

Type 2 Diabetes (T2D) is a risk factor for the development of late onset Alzheimer's disease. However, this mechanism underlying induction of AD in T2D is largely unknown. Here we show that increased pro-inflammatory cytokines in diabetic mouse models db/db (*Lepr^{db})* and MKR (*Ckm-IGF1R^{K1003R})*, results in depletion of the endothelial-enriched protein Caveolin-1 (Cav-1). In turn, reduced Cav-1 in the hippocampus of db/db mice causes a decrease in the expression level of insulin receptor leading to reduced, insulin transport into the brains of diabetic mice. Further, depletion of Cav-1 induces the upregulation of amyloid precursor protein (APP) and its amyloidogenic cleavage, as well as upregulation in levels of hyperphosphorylated tau. Moreover, db/db and MKR mice exhibit deficits in recognition memory and hippocampal neurogenesis. Furthermore, our results suggest that Cav-1 is essential for neural progenitor cell proliferation and maintenance in the adult brain and its depletion may compromise neurogenesis via bone morphogenetic protein (BMP) signaling. Taken together, these results suggest that loss of Cav-1 levels is a key step in the escalation of T2D into AD.

Significance

Type 2 diabetes (T2D) is a known risk factor for Alzheimer's disease (AD). According to data collected from the Mayo Clinic Alzheimer's disease Registry, approximately 80% of AD cases also present with T2D or an impaired glucose metabolism disorder. Diabetes currently affects over 29 million U.S. citizens (9.3% of the population) and the healthcare costs associated with treating these patients total $245 billion per year, including $176 billion in direct costs and $69 billion in indirect costs

(disability, work loss, premature mortality). This correlation strongly suggests a mechanistic link between these two devastating diseases, however the current understanding of this link is poorly understood. With the growing population of adults with T2D, the incidence and prevalence of AD will only increase. Understanding the molecular mechanism that underlies the progression of T2D to AD is imperative. This study will provide novel information concerning the mechanism underlying the cognitive deterioration and developing AD-like neuropathology, and may offer new therapeutic targets for the amelioration of AD.

Statement of Hypothesis

The central hypothesis for this dissertation is that loss of caveolin-1 (Cav-1) in type 2 diabetes leads to increased neuropathology ultimately leading to Alzheimer's disease.

Specific Aims

Specific Aim 1: Examine whether reduced expression of Cav-1 in the brain of T2D mice induces neuropathology and compromises brain plasticity, leading to cognitive deficits.

Hypothesis: Inflammation-induced Cav-1 depletion underlies the development of Alzheimer's disease. Experiments will examine the effect of chronic inflammation *in vivo* and *in vitro* on caveolin-1 expression in brain endothelial cells and the hippocampus and assess the effect of reduced Cav-1 on amyloidosis and tau phosphorylation, hippocampal neurogenesis and learning and memory in mouse models of type 2 diabetes.

Specific Aim 2: Examine the role of Cav-1 in insulin transport.

Hypothesis 1a: Endothelial Cav-1 is essential for the metabolism of insulin receptor. Experiments will examine the expression of insulin receptor in the hippocampus and primary brain endothelial cells isolated from global caveolin-1 knockout and reconstituted Cav-1 in endothelial cells only.

Hypothesis 1b: Reduced Cav-1 in the brains of T2D mice compromises insulin supply to the hippocampus which may contribute to loss of cognitive function. To determine the amount of insulin that crosses the blood brain barrier, radiolabeled insulin injected into T2D mice, as well as models with altered endothelial Cav-1 expression, will be utilized and brain regions dissected for analysis.

Specific Aim 3: Examine the role of Cav-1 in hippocampal neurogenesis.

Hypothesis: Cav-1 is essential for the maintenance of hippocampal neurogenic niche. Experiments will examine the effect of Cav-1 loss on neural stem cell proliferation and differentiation both *in vivo* and *in vitro* and determine the effect of Cav-1 on the expression of key neurogenic receptors.

1. INTRODUCTION

Portions of this chapter were previously published in *Bonds et al., "Type 2 Diabetes Mellitus as a Risk Factor for Alzheimer's Disease."* Genes, Environment and Alzheimer's Disease*. By Orly Lazarov and Giuseppina Tesco. London, UK: Elsevier, Academic, 2016. 387-413. Print.* With permission of the publisher (Appendix 2).

1.1. Type 2 diabetes mellitus and Alzheimer's disease

There are several risk factors that contribute to the development of Alzheimer's disease (AD), including insulin resistance and type 2 diabetes mellitus (T2D). According to the data collected from the Mayo Clinic Alzheimer's Disease registry, more than 80% of AD cases also present with T2D or another impaired glucose metabolism disorder. This correlation strongly suggests a mechanistic link between these two devastating diseases, however the current understanding of this link is poorly understood.

T2D is a metabolic disorder that results from reduced or impaired responsiveness to insulin signaling [e.g., insulin resistance]. The reduced glucose uptake by insulin-target organs, along with impaired fatty acid metabolism results in increased free fatty acid in the circulation, causes chronic hyperglycemia, and sustained inflammation which can evolve to cardiovascular and neurological dysfunction (CDC 2017). According to the Centers for Disease Control and Prevention, diabetes currently affects over 30.3 million U.S. citizens (9.4% of the population) and the healthcare costs associated with treating these patients total $245 billion per year, including $176 billion in direct costs and $69 billion in indirect costs (disability, work loss, premature mortality). Given the strong possibility that T2D is a risk factor for AD, which in the United States alone accounts for 11% of all people aged 65 and older and costs approximately $226 billion annually, it is

imperative that more research is done to determine exactly how these two diseases are connected.

To this end, there have been several prospective cohort and case-control studies done to determine the incidences of T2D induced AD patients. For example, one study conducted using a cohort of the Rochester Epidemiology Project demonstrated that T2D increased the relative risk of developing AD over two-fold in men, whereas there was a non-significant trend of an increase in women (Leibson et al. 1997). Surprisingly, there was no significant interaction between T2D and AD when stratified by age despite the fact that 25.9%, or 11.8 million, of individuals age 65 years or older have diabetes (National Diabetes Statistics Report, 2014). However, it was found that 35% of AD patients also present with T2D (Janson et al. 2004). These data suggest that T2D independently confers risk in populations regardless of age and thereby may promote early onset AD. Interestingly, a subset of patients in the Cardiovascular Health Study showed only a slight increase in risk of dementia based on diabetes status alone, whereas there was a marked increase in relative risk when stratified by both diabetes and a variant of apolipoprotein E (ApoEε4) known to be an associated risk factor for early onset sporadic AD (Irie et al. 2008).

Taken together, epidemiological data indicate that T2D and other cardiovascular disease risk factors increase the incidence and reduce the age of onset of AD in human populations.

1.1.1. Type 2 diabetes mellitus pathology

T2D is a chronic disease characterized by a state of persistent hyperglycemia and reduced responsiveness to insulin (i.e., insulin resistance). It is associated with a number

of pathologies of diverse etiology collectively referred to as diabetic complications. These include renal failure, blindness, coagulopathies, impaired wound healing, enhanced cardiovascular disease risk, and neurodegeneration. Insulin resistance is the most significant prognostic indicator of T2D. In fact, T2D is preceded by a phase of hyperinsulinemia and normal plasma glucose levels which indicate that there is a compensatory response by the pancreas to increase insulin levels in order to enhance glucose uptake by target organs. Eventually, pancreatic beta-cells fail to meet the body's need for increased insulin levels in order to maintain glucose within the normal range, which leads to a gradual and sustained increase in plasma glucose and T2D.

Once it develops, T2D has a dramatic impact on whole body metabolism well beyond the handling and utilization of sugars; it also affects lipid processing, protein synthesis, and modifies hormonal and cytokine balance that impact every tissue in the body. Most commonly, T2D presents as numerous associated pathologies that can vary in severity, including hypertension, ketoacidosis, nephropathy, ocular and retinal diseases, and increased risk of stroke. Importantly, T2D has been shown to increase morbidity due to complications from cardiovascular disease and diabetic neuropathy in large part because of its adverse effects on the vasculature (CDC 2017).

Oxidative Stress and Inflammation

Hyperglycemia promotes the glycation of many proteins resulting in enhanced antigenic responses. Associated with hyperinsulinemia and dyslipidemia, glycation also potentiates inflammatory responses by enhancing the production of pro-inflammatory cytokines such as IL-6 and TNFα and repressing the production of anti-inflammatory signals such as IL-10. Therefore, diabetes produces a state of inflammation that

contributes to vascular and neurovascular disease directly and by activating tissue-specific inflammation. For example, it is now well established that chronic or exacerbated systemic inflammation eventually activates microglia, the resident macrophages of the brain (Perry and Teeling 2013). Once activated, microglia-driven inflammation persists for prolonged periods of time. Studies devoted to understanding this mechanism have shown that while peripheral inflammation resolves within days, microgliosis takes weeks to months to fully resolve, thus imposing lasting modifications to the neural landscape.

Indirectly, inflammation is associated with enhanced oxidative stress via increased production of reactive oxygen species (ROS) and diminishing antioxidant responses, with the net result being enhancement of steady state ROS levels. ROS are important participants in the regulation of cell signaling and cell communication, but when produced chronically at high levels, they alter the chemical composition of proteins, lipids, and DNA resulting in their disassembly, dysfunction or fragmentation. Intuitively, the accumulation of dysfunctional or highly altered biomolecules leads to dysfunction at the cellular level, and in the case of endothelial cells, to endothelial dysfunction which is thought to impair the function of blood vessels and alter endothelial cell interactions with other cell types (as detailed below). Therefore, chronic inflammation and progression of oxidative stress are inseparable from and intimately related to the pathogenesis of diabetes. Directly or through their effect on the vasculature, chronic inflammation and oxidative stress also contribute to the many complications associated with diabetes including retinopathy, nephropathy, cardio and neuropathies.

Diabetes and Endothelial Dysfunction

Oxidative stress, accumulation of oxidized and modified lipids in the blood plasma, elevation of inflammatory cytokines, and the generation of electrophiles such as aldehydes, nitroalkenes, and reactive organic compounds impose significant challenges to the endothelium. Reactive species modify electron rich thiol residues and thereby modify proteins with little specificity. They gain access to the cytosol and organelles either though active transport or passive diffusion through membranes. Of the many nonspecific targets of such species, phosphatase and tensin homolog (PTEN) and caveolin-1 (Cav-1) are notorious because the modification of their activities promotes significant changes in cell signaling.

Cav-1 is a scaffold protein present in cholesterol-enriched microdomains (CEM) of the plasma membrane. It is required for formation of caveolae, the small plasmalemmal vesicles produced upon invagination of plasma membrane CEMs. Caveolae are responsible for the transcellular transport (transcytosis) of macromolecules from the plasma through the endothelium. By directly associating with many client proteins, Cav-1 regulates their function and maintains endothelial cell homeostasis. For example, a decrease in Cav-1 expression results in dysregulated (uncoupled) endothelial nitric oxide synthase (eNOS) in that it becomes a primary source of oxidative stress (Minshall et al. 2003). Dysfunctional endothelium results in an inability to maintain systemic blood pressure, deficits in angiogenesis, compromised wound healing, accelerates atherosclerosis, and failure to maintain barrier integrity resulting in the formation of edema and vascular cuffing.

PTEN and Cav-1 appear to be tightly linked in that the loss of PTEN activity can lead to Cav-1 degradation, and vice-versa, by mechanisms that are still under investigation but likely involve oxidative stress. Therefore, loss of function of critical signaling regulators in endothelial cells is thought to contribute to the etiology of cardiovascular disease characterized by vascular hyperpermeability, chronic inflammation, and hypertension while also compromising the supportive role endothelial cells play in maintaining vascular muscle, circulating immune cell, and neural progenitor cell functions dependent on the endothelial cell niche. Therefore, disruption of the ability of the endothelial niche to maintain homeostasis of partner cells may be linked to neuropathologies such as AD (Kreis et al. 2014).

1.1.2 Mouse models of type 2 diabetes mellitus

In addition to diet induced models of T2D, several knockout and transgenic mouse models have been generated that mimic one or more aspects of the very complex T2D condition. This section serves to highlight some of the many models that were created based on their utility in generating insights regarding basic mechanisms of insulin resistance, insulin signaling, and metabolic syndrome associated with the development of T2D. It is important to note that in target organs, insulin signaling is mediated by a family of receptor tyrosine kinases, the two most prominent being the insulin receptor (IR) and the insulin-like growth factor 1 receptor (IGF1R). Both receptors operate through the activation of the same downstream effector kinases, although these signaling pathways exert different biological actions. Many of the animal models are based on manipulation of these receptors, namely genetic deletion of the insulin receptor (IR), insulin receptor

substrate molecule (IRS) and IGF1R. Models were also created in which the major glucose transporter activated by insulin (GLUT4) was deleted globally or in a tissue specific manner. Lastly, two other models that were created via mutation are the obesity independent MKR (mutation of lysine to argenine in skeletal muscle) mouse and obesity dependent mouse carrying a mutation of the leptin receptor ($Lepr^{db}$; db/db).

Insulin Receptor (IR) knockout mouse ($IR^{-/-}$)

Mice lacking the insulin receptor are born resistant to the signaling effects of insulin and therefore rapidly develop diabetes and hyperinsulinemia. These animals are not viable and die within days after birth most likely because of severe diabetic ketoacidosis (Accili et al. 1996). Importantly, it was observed that deletion of the IR leads to rapid failure of pancreatic β-cells to secrete insulin due to chronic hyperinsulinemia-induced negative feedback. Thus, there are few studies conducted using the global $IR^{-/-}$ mouse due to the lethal phenotype of this knockout strain.

Tissue Specific Deletion of IR

In an attempt to overcome the severe phenotype of whole body IR deletion, tissue specific $IR^{-/-}$ mice were created using Cre-lox/P technology. Muscle (Bruning et al. 1998), adipose (both white and brown fat tissue) (Bluher, Kahn, and Kahn 2003), liver (Cohen et al. 2007, Michael et al. 2000), and β-cell (Kulkarni et al. 1999) specific $IR^{-/-}$ mice were generated that revealed varied physiologic and pathologic responses due to the lack of IR-dependent signaling in each cell type. The muscle specific $IR^{-/-}$ mouse exhibited a mild phenotype despite evidence of reduced insulin-dependent glucose uptake by muscle. Contrary to expectations, these mice did not develop either insulin resistance or diabetes because of compensatory signaling by the IGF-1R and by non-insulin dependent AMPK-

driven glucose uptake. IR deletion in adipose cells also did not alter insulin sensitivity, although there was an obvious reduction in fat mass and plasma triglyceride levels observed in these mice. Interestingly mice lacking IR expression in fat cells lived longer.

Deletion of the IR in β-cells generated an unexpected and rather interesting phenotype: impaired glucose-driven insulin release and an increase in appetite. The study by Kulkarni, et al., showed that $IR^{-/-}$ β-cells responded to glucose abnormally and failed to release insulin into the bloodstream immediately following an increase in plasma glucose level implicating the involvement of the IR in regulation of insulin release (Kulkarni et al. 1999).

Interestingly, liver specific $IR^{-/-}$ knockout mice showed the most detrimental phenotype. Severe insulin resistance and hyperglycemia most-likely are related to failure to suppress hepatic gluconeogenesis. The IR was also specifically knocked out in the heart, vascular endothelium (Kondo et al. 2003), and central nervous system (Fisher et al. 2005). In cardiac tissue, IR deficiency led to reduced heart size and contractility, while in endothelial cells, IR deficiency promoted impaired hypoxic-driven neovascularization. IR deficiency in the CNS led to increased weight gain (Bruning et al. 2000) and defective counter-regulatory responses to hypoglycemia, suggesting that insulin signaling in the CNS promotes satiety.

Taken together, these models provide support for the concept that diabetes results from abnormal insulin signaling in canonical and non-canonical target organs, and that ablation of the IR in a particular cell type is useful for obtaining a better understanding of insulin signaling in specific tissue types. Nevertheless, none of the models completely mimicked the systemic metabolic features of diabetes.

Insulin Receptor Substrate (IRS) knockout mice

Many of the most prominent insulin-activated signaling mechanisms are the result of IRS-mediated signaling downstream of insulin binding to the IR or IGF-1R. Mouse models in which one or a combination of IRS-target genes were genetically inactivated revealed a pronounced growth impairment associated with insulin resistance (Ogata et al. 2000, Fantin et al. 2000, Cho et al. 2001). However, knockout of IRS1 only results in a mild diabetic phenotype (Araki et al. 1994). Deletion of IRS2 was shown to lead to diabetes both by impairing insulin action and by promoting insulin deficiency. It was noted that in IRS2 knockout animals, there was significant β-cell death in parallel with a reduction in responsiveness of peripheral tissue to insulin signaling (Withers et al. 1998). IRS3 knockouts presented no discernible phenotype. The reason why the IRS3 knockout showed a lack of phenotypic consequences are not yet clear, although compensation by IRS1 has been suggested (Liu et al. 1999). Knockout of IRS4 resulted in mild phenotypic consequences resembling those observed with IRS1 deficiency. Notably, IRS4 knockout mice were characterized as having reproductive abnormalities (Fantin et al. 2000). In double knockouts of IRS1 and another IRS subtype, a variety of phenotypes were observed ranging from embryonic lethality (in the case of IRS1/IRS2 dKO) to lack of additive effectives in IRS1/IRS4 dKO animals whose phenotype was identical to that of IRS1 knockouts. In the case of IRS1/IRS3 knockouts, insulin resistance with lipoatrophy and reduced intrahepatic and intramuscular deposits of triglycerides was observed.

Glut4 knockout (global and tissue specific)

Insulin-stimulated glucose uptake requires the GLUT4 glucose transporter and therefore genetic mouse models were developed to abolish glucose transport by this

mechanism. GLUT4 knockouts exhibited a severe phenotype characterized by growth retardation, underdeveloped adipose tissue, cardiac hypertrophy, and insulin resistance (Katz et al. 1995). Since GLUT4 is a major facilitator of glucose uptake in skeletal muscle, muscle-specific deletion of GLUT4 phenocopied many of the features of the global knockout including severe insulin resistance and glucose intolerance (Zisman et al. 2000). Interestingly, GLUT4 knockout mice also showed phenotypic gender disparity with males developing hyperglycemia in the postprandial state whereas female knockout mice did not develop hyperglycemia.

Because of the association between insulin resistance and cardiovascular disease, cardiac-specific GLUT4 deficient mice were developed. In these animals, cardiac hypertrophy associated with increased cardiomyocyte size was observed. Although GLUT4 deletion in the heart did not lead to impaired contractile function, recovery from ischemic insult was impaired. This indicates that GLUT4 is essential for insulin-independent glucose uptake in the heart under hypoxic conditions wherein glycolysis compensates for deficits in the oxidative metabolism.

Adipose tissue depletion of GLUT4 generated complex and variable phenotypic consequences in different mouse specimens. Hyperinsulinemia, most likely due to hepatic insulin resistance and a reduced capacity to uptake glucose, indicate that the lack of GLUT4 activity in adipocytes in this model results in prominent and disproportionate effects in distant tissues. Interestingly, despite the hyperinsulinemia and desensitization to insulin signaling in muscle and liver, diabetes does not develop in these mice.

MKR mouse model of insulin resistance (obesity independent)

The functional disruption of IGF1R in transgenic MKR mice was shown to cause T2D (Fernandez et al. 2001). A mutant IGF1R driven by a creatinine kinase promoter (Ckm) encodes for a functionally disabled dominant negative receptor where lysine 1003 is replaced by an arginine residue (L1003R). The expression of mutant receptor (primarily in skeletal muscle and to a lesser extent (~10%) in the heart) allows for insulin binding but disables downstream signaling resulting in insulin resistance. This is because upon binding to insulin, L1003R-IGF1R dimerizes with the endogenous IR and produces a functionally inactive receptor complex. As such, mice expressing mutant IGF1R rapidly develop hyperglycemia and insulin resistance that evolves to β-cell dysfunction and diabetes. Interestingly, MKR mice are not obese but rather show a slight decrease in body weight throughout life as compared to wild type controls. With its close resemblance to the human diabetic state, including loss of β-cell function due to progressive hyperinsulinemia and hyperglycemia, the MKR mouse model has emerged as a powerful and quite unique tool for investigating mechanisms that connect insulin insensitivity to diabetes.

$Lepr^{db}$ (db/db) model (obesity dependent)

Unlike the other models described above, the db/db model is used to model phases I to III of T2D and obesity. These mice are homozygous for the diabetes spontaneous mutation in the leptin receptor (Lepr) and exhibit morbid obesity beginning at 3-4 weeks of age and by 10 weeks mice weigh an average of 45 g. Further, these mice exhibit chronic hyperglycemia, pancreatic beta cell atrophy, and are hyperinsulemic. The severity of the diabetic phenotype in this model leads to impaired wound healing,

myocardial disease, and most interestingly, peripheral neuropathy. These mice have a lifespan of approximately 10 months.

1.1.3 Alzheimer's disease pathology

Alzheimer's disease can present itself in one of two broad forms. First, familial AD compromises approximately <5% of all AD cases and is inherited. These cases are often early-onset (before the age of 60), and is typically caused by known mutations such as the amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2), with mutations in PS1 being the most common (NIA 2015). Second, and most common, is the sporadic form of AD which compromise >95% of all AD cases. In this form of AD, there is unfortunately no known cause for its development with the greatest risk factor being aging itself. In either case, AD is a devastating disease with pathology beginning before the onset of symptoms. The cognitive symptoms of AD vary case by case but generally include memory impairment, personality changes, emotional disturbances, and language (Cummings 2000).

Given the various known causes for dementia, an AD diagnosis requires post-mortem examination (McKhann et al. 2001). The neuropathologies that are present in the diagnosis are amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs). Plaques are large extracellular deposits of the Aβ peptide which is the result of abnormal processing of APP predominately by PS1. These Aβ plaques are found primarily in the neocortex, the hippocampal formation, and throughout the cortex. NFTs on the other hand are smaller intracellular aggregation of abnormally phosphorylated tau protein. These tangles are found in the brainstem, entorhinal cortex, and throughout the cortex (similar to the Aβ

plaques) (Grudzien et al. 2007, Braak and Braak 1997). Additionally, there are subcortical cholinergic nuclei that undergo degeneration. The most notable of which is the locus coeruleus (LC) (Lyness, Zarow, and Chui 2003). These observations have support the amyloid, tau, and cholinergic hypotheses of AD. However, the main interest of this study is the mechanism by which T2D leads to such a high predisposition for AD. It is the hypothesis that chronic inflammation as a result of T2D which leads to cerebrovascular complications, as well as other pathologies associated with AD that will be discussed here.

1.1.4 Type 2 diabetes mellitus as a risk factor for Alzheimer's disease

Unfortunately, patients with T2D have an increased risk for developing significant cognitive deficits, and furthermore for the development of AD (Allen, Frier, and Strachan 2004). In review of current literature, disruptions in the BBB, prolonged hyperglycemia, as well as chronic inflammation have all been implicated as major risk factors for AD development (Yavuz et al. 2007, Huber 2008, Whitmer 2007).

Cerebrovascular Complications in T2D – Implications for AD development

Microvessels support their surrounding environment via paracrine signaling mediated by trophic factors, such as VEGF, which induces endothelial cell proliferation, stimulates brain plasticity and remodeling, and reduces neuronal degeneration. In the injured brain, newly formed vessels create a niche that can support the migration and neuronal differentiation of neuroblasts (Dobkin 2004, Zhang and Chopp 2009). In turn, neuroblasts reinforce angiogenesis via release of VEGF (Johansson 2007). Under hypoxic conditions, there is an upregulation of hypoxia-inducible factor alpha (HIF-1α)

which promotes the formation of new blood vessels (angiogenesis) via activation of other pro-angiogenic factors such as VEGF, angiopoietins (Ang), and bFGF, to name a few (Ergul et al. 2014). This same process occurs in areas of the uninjured brain and is an important response to the changing metabolic demands of the brain. This is of particular importance in the context of metabolic disorders such as T2D, which has a significant impact on microvascular integrity.

Dysfunctional cerebral neovascularization in T2D

Several studies have shown that there is an increase in cerebral neovascularization in T2D (Prakash et al. 2013, Li et al. 2010, Silvestre and Levy 2006). However, these new vessels do not mature properly thus resulting in an increase of unperfusable vasculature and blood-brain barrier hyperpermeability (Prakash et al. 2012). In turn, this creates a hypoxic environment where the metabolic demands of the surrounding tissue cannot be met. It is important here to take into account that these studies focused on neovascularization in the cerebral cortex and did not focus on the neurogenic areas of the brain that are associated with learning a memory: the subgranular layer (SGL) of the hippocampus and the subventricular zone (SVZ). The vasculature is a critical regulator of the proliferative neural stem cell (NSC) microenvironment. Neurogenesis plays a role in the restorative attempts following an insult to the brain. NSCs are intimately associated with vascular endothelial cells within the niches and their self-renewal, proliferation and early differentiation tightly depend on homeostatic endothelial functions (Eichmann and Thomas 2013). In the SVZ, there is direct access to factors in blood and cerebrospinal fluid, and thus the established vasculature network of the adult SVZ niche is a key component of the specialized microenvironment that supports NSCs

and their progeny (Tavazoie et al. 2008). In the SGL, neurogenesis is closely associated with a process of active vascular recruitment and subsequent remodeling (Palmer, Willhoite, and Gage 2000). Up to 37% of the cells proliferating in the SGZ are endothelial precursors (Abrous, Koehl, and Le Moal 2005). Furthermore, NPCs and angioblasts proliferate together in clusters associated with the microvasculature of the SGL, and cells within these clusters express the VEGF-receptor Flk-1 (Palmer, Willhoite, and Gage 2000). The clustering of neural and endothelial precursors suggests that neurogenesis involves cross-talk with endothelial precursors.

In the context of T2D, Beauquis et al. showed that vascularization of the hippocampus is significantly decreased (Beauquis et al. 2010). This same study also shows that while there is a significant increase in the number of proliferating neuroblasts, these cells do not survive to incorporate into the granular cell layer. Their findings imply that the survival of new neurons depends heavily on the ability of the brain to provide functional vasculature. The formation of new neurons in both the SVZ and the SGL are crucial to brain plasticity, an important function for recovery after trauma (Lazarov et al. 2010). This process of neurogenesis has been shown to be defective in Alzheimer's disease (AD) (Demars et al. 2010, Lazarov et al. 2010).

Interestingly, these findings are similar to what has been shown following an ischemic stroke, another risk factor associated with T2D. It has been observed that depletion of neurogenesis exacerbates stroke outcome leading to increased infarct volume and enhanced neurological deficits (Wang et al. 2012). It has also been shown that following stroke, there is an increase in angiogenesis and the number of NPCs 72 hours and 7 days after injury (Font, Arboix, and Krupinski 2010). However, most NPCs

die and neural replacement does not take place. Taken together, the compromised vascular function observed in T2D not only increases the risk of an ischemic event but can also undermine the regenerative capacity of the brain, which can cause and/or accelerate the onset of AD.

Hyperglycemia and Alzheimer's disease

There are several risk factors that contribute to the development of AD, including insulin resistance and T2D. According to a recent study published in the journal *Diabetes*, more than 80% of AD cases presented with either T2D or an impaired glucose metabolism disorder (data collected from the Mayo Clinic Alzheimer Disease Patient Registry) (Janson et al.). Focusing on the patients with T2D, there was a significant positive correlation between the duration of diabetes and the density of Aβ plaques (adjusting for age) (Janson et al. 2004). One possible explanation for this observation is the presence of hyperglycemia, a common complication in T2D. Hyperglycemia has been observed to result in increased expression of the Receptor for Advanced Glycation End products (RAGE) and RAGE ligands either by exposure to elevated levels of blood glucose, or through increased ROS production (Yan, Stern, and Schmidt 1997, Yao and Brownlee 2010, Smith, Sayre, and Perry 1996). These, as well as other advanced glycation end products, are also observed in Aβ plaques and neurofibrillary tangles present in AD.

Diabetes induced hyperglycemia causes severe deficits in cerebrovascular structure and function. Increased vascular tone/rigidity, decreased cerebral blood flow, and increased expression of matrix metalloproteinases (MMPs) causes a worsening of blood-brain barrier (BBB) function hyperglycemic patients (Ergul et al. 2009). These symptoms are accompanied by degeneration of endothelial cells and pericytes, as well

as increased aggregation and adhesion of platelets to the endothelium (Cheng et al. 2014, Lorenzi, Cagliero, and Toledo 1985, Vinik et al. 2001, Williams et al. 1998). As a result of compromised vascular function, hyperglycemia is a major contributor to neuronal injury, particularly following stroke (Ergul et al. 2012, Capes et al. 2001, Seners, Turc, Oppenheim, et al. 2014, Seners, Turc, Tisserand, et al. 2014, Sasaki et al. 2001). Together, these cerebrovascular complications contribute to the increased risk of develop AD in T2D.

Vascular Inflammation in T2D

A common complication in T2D is the formation of atherosclerotic plaques and impaired endothelium-dependent vasodilation. Increased levels of CRP, as well as the modified lipoproteins within these plaques, potentiate the release and activation of inflammatory molecules, namely IL-1, IL-6, IFN-γ, and TNF-α. Elevated levels of CRP have been shown to decrease the expression of eNOS thereby disrupting NO signaling and interfering with the ability of endothelial cells to respond to changes in blood flow (Venugopal et al. 2002, Verma et al. 2002). Endothelial cell dysfunction combined with elevated levels of pro-inflammatory cytokines induce the expression of endothelial adhesion molecules thereby increasing the influx of inflammatory cells across the endothelium and vessel wall hardening (Davies et al. 1993, Blake and Ridker 2001, De Vriese et al. 2000).

IL-6 and TNF-α activate endothelial cells to synthesize cellular adhesion molecules (CAMs) (Etter et al. 1998). The initial rolling of inflammatory cells along endothelial cells is mediated by selectins. E-selectin is expressed by endothelial cells and P-selectin is also expressed by platelets, as well as endothelial cells. Up-regulation of both selectins

has been associated with T2D and increased serum levels of soluble E-selectin has been shown to predict an ischemic event (Neubauer et al. 2010, Matsumoto et al. 2010). The immunoglobulin family of CAMs mediates the attachment and transendothelial migration of leukocytes. These include the intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) (Davies et al.).

As the plaque continues to build within the vessel wall, monocytes attach and differentiate into macrophages. Micropinocytosis of modified LDLs by macrophages induces formation of foam cells. Formation of these cells increases ROS production and macrophage recruitment contributing further to the impairment of NO signaling and endothelial dysfunction (Rajagopalan et al. 1996, Yorek 2003). In response to inflammatory cell recruitment, the vessel wall attempts to repair itself by inducing the proliferation of smooth muscle cells. These cells surround the foam cells thereby creating a fibrous cap, which further contributes to impaired blood flow. The plaques within the cap become destabilized through the production of ROS and activation of matrix metalloproteases (Rajagopalan et al. 1996, Galis et al. 1994, Shah et al. 1995). The presence of IFN-γ inhibits the production of collagen thereby contributing to weakening of the fibrous cap. Formations of cholesterol crystals erode the endothelium causing encroachment of the plaque into the vessel lumen leading to obstructed blood flow. Rupture of the plaque releases the hardened lipids into the blood stream where they can travel to smaller arterioles to potentially induce an ischemic event and neurovascular uncoupling.

Impairments in cerebral blood flow causes significant injury to neurons and the connections that are made between them, which is a contributing factor to the cognitive

decline observed in AD (Marchant et al. 2012). In an attempt to isolate the effects of cerebrovascular alterations in cognition in the context of AD, Marchant et al. discovered that cerebrovascular disease and Aβ aggregation were independent contributors to cognitive impairment. This study, as well as several others suggests that the cognitive decline observed in cerebrovascular disease and AD may share common pathways (altered endothelial regulation of cerebral blood flow, pathological changes in BBB integrity and function, and neurovascular uncoupling) (Marchant et al. 2012, Girouard and Iadecola 2006, Girouard et al. 2007, Mooradian 1988).

Alzheimer's disease – Inflammation and vascular dysfunction

As presented above, many are the pathways that contribute to change the vasculature in sustained and deleterious ways. How then do functional changes in the vascular network affect the viability of neural tissue impacting cognition, motion and memory? Recent findings indicate that sustained inflammation affects the capacity of endothelium to transport peptides from the circulation to underlying tissues. The endothelium, in fact, plays a critical role in actively transporting a number of macromolecular compounds through the blood vessel wall. This transport (generally referred to as "transcytosis") is mediated by endocytic structures called caveolae that reside on the plasma membrane and internalize macromolecules such as insulin and albumin via c-Src and dynamin-dependent vesicle fission (Sverdlov, Shajahan, and Minshall 2007), which then move across the cell body where they fuse with the basal membrane and exocytose stored contents. Formation of caveolae is dependent on the expression of Cav-1, a 22 kDa protein that assembles into oligomeric membrane-associated chains which promote the invagination of CEMs (Minshall et al. 2000, Vogel

et al. 2001, John et al. 2003). Caveolae also contain receptors, for example for insulin and albumin, which upon activation promote the transcytosis of bound and fluid phase macromolecules from the circulation to the underlying cells (Wang et al. 2015a, Wang, Wang, and Barrett 2011) (Figure 1). Recently, it was demonstrated that Cav-1 is

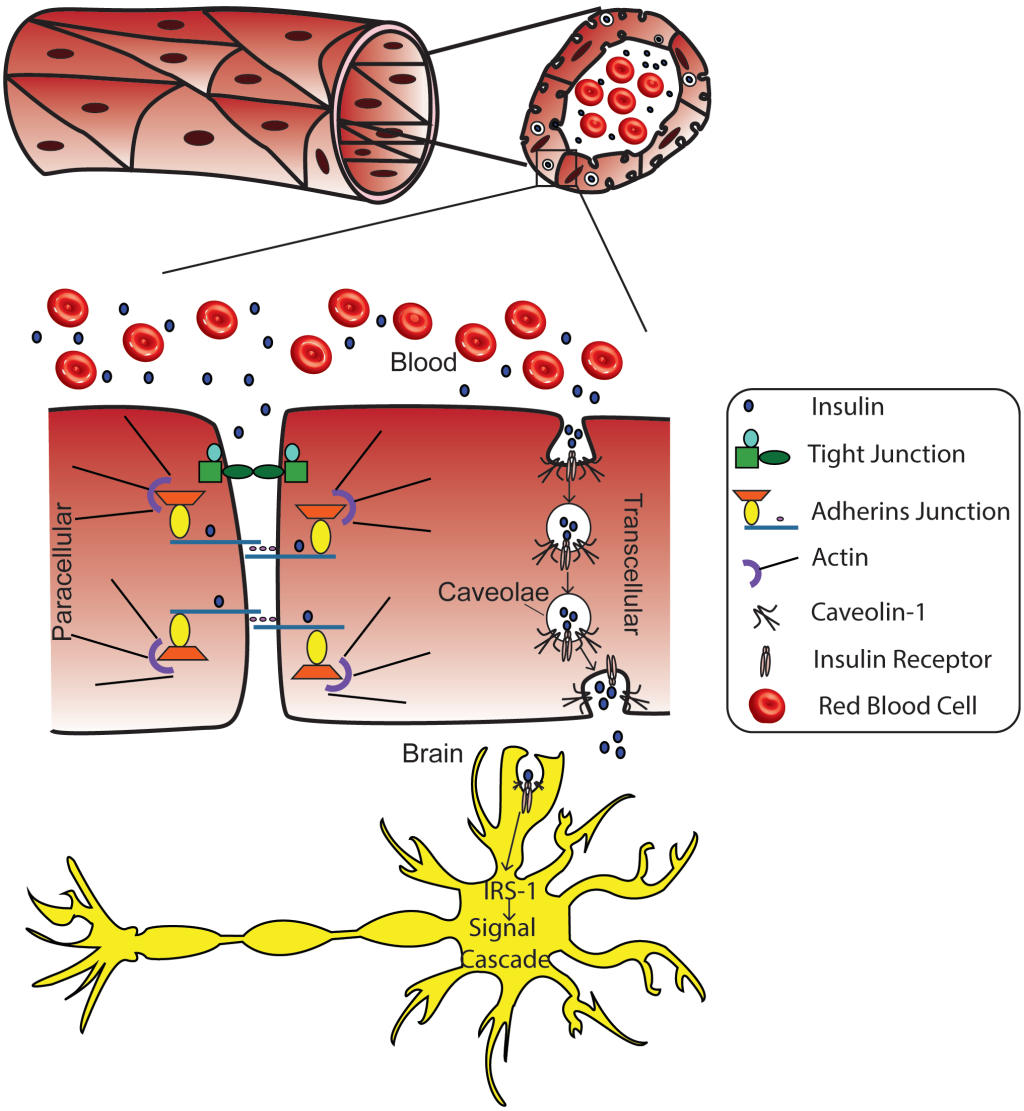

Figure 1. Schematic representing caveolae mediated insulin transport across the BBB. Insulin receptors localized to caveolae bind circulating insulin. Upon binding, caveolae mediated endocytosis occurs to transport the bound insulin across the BBB where it can bind to its receptors on various cell types and induce signal transduction in the brain.

susceptible to nitric oxide and oxidative stress with the chemical modification of a specific cysteine residue (Cys 156) being sufficient to trigger protein degradation and depletion (Bakhshi et al. 2013). Oxidative stress accompanies inflammation and is significantly enhanced in diabetic patients (Giacco and Brownlee 2010, Brouwers et al. 2010, Pi et al. 2009, Kohen Avramoglu et al. 2013, Stadler 2012), providing a link between chronic inflammation and alterations to the endothelium that impact cerebrovascular homeostasis. Depletion of caveolin-1 disrupts caveolae formation and caveolae-dependent transport. Since this mechanism is largely responsible for the uptake and transport of insulin, a 51 amino acid polypeptide (~5.8 kDa), impairment of caveolae formation at the level of the endothelium is likely to negatively impact the access of insulin to the brain (Wang et al. 2006, Wang et al. 2015b, Wang, Wang, and Barrett 2011) (Figure 1).

In the brain, a variety of glucose transporters (GLUT1, GLUT2, GLUT3, GLUT4, GLUT5 and GLUT8) are expressed though at different levels in the various cells of the brain (McEwen and Reagan 2004). GLUT5 for instance is prevalent in microglia whereas GLUT1 predominates in astrocytes. In the cerebellum, neural subpopulations in the cortex, and the hippocampus which largely controls memory, the insulin-activated transporter GLUT4 is particularly important as it controls a large fraction of the glucose uptake by the neurons in these regions. It is therefore not only possible but likely that diminished access of insulin to these areas will produce significant deficits in the ability of these cells to nourish themselves, function normally, and survive stress. The role of Cav-1 and the ability of the neurovascular endothelium to maintain insulin signaling in these

areas of the brain is an active area of research that will likely provide a clearer picture regarding the link between diabetes and risk of developing AD.

In summary, many different mechanisms are likely to contribute to the onset, progression, and severity of neural degeneration and Alzheimer's disease as they relate to vascular disease and diabetes. The mechanisms proposed here are viewed as central etiologic components and likely useful therapeutic targets. Currently, enhancement of insulin signaling in the brain is being pursued as a potential therapeutic for Alzheimer's disease, and both pharmacologic and life style changes that have already been shown to have a positive effect in the management of diabetes itself include healthier diets, exercise, and lower caloric intake. These not only reinforce the epidemiologic connections between diabetes, vascular dysfunction, and Alzheimer's disease, but also reiterate the fact that effective treatments for Alzheimer's disease need to take into consideration vascular health and endocrine interventions. Furthermore, there should be an increased focus on effects of chronic inflammation not only on the vasculature, but also on the health and function on neural stem cells.

1.2 Stem Cells and Neurogenesis

1.2.1 Adult Neurogenesis and the Neurogenic Niche

Until fairly recently, it was thought that there were no new neurons generated in the adult brain. Evidence has since emerged, however, that there exist pockets of neural stem cells (NSC) in the adult brain, known as the neurogenic niche (Fuentealba, Obernier, and Alvarez-Buylla 2012) responsible for generating new neurons and glia, thus maintaining the neurogenerative capacity of the brain throughout life (Yao, Mu, and Gage 2012, Ihrie

and Alvarez-Buylla 2011). These cells reside exclusively in two discrete microenvironments: the subgranular layer of the dentate gyrus (SGL) in the hippocampus, and the subventricular zone (SVZ) located in the walls of the lateral ventricles. During the processes of self-renewal, migration and differentiation, there are distinct morphological and molecular markers which are expressed, allowing for the tracing and classification of various cellular progeny (Lazarov et al. 2010). Within these microenvironments of the SVZ and SGL are several other cell types (ependymal cells in the SVZ, astrocytes and endothelial cells in both) that interact with NSCs to provide the necessary cues critical for their function (maintenance, self-renewal and proliferation) (Lazarov et al. 2010, Lee et al. 2012). Ependymal cells line the walls of the lateral ventricles and are in direct contact with the cerebral spinal fluid (CSF) that fills the ventricles. These cells are often found to surround the apical processes of type B NSCs and provide access to soluble factors and signaling molecules within the CSF. Astrocytes and endothelial cells have been shown to promote NSC proliferation *in vitro* (Lee et al. 2012, Shen et al. 2004). It is thought that these cells provide both structural and trophic support via secretion of vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), epidermal growth factor (EGF), and fibroblast growth factor-2 (FGF2) *in vivo* as well. However, the exact nature of these relationships requires further study.

1.2.2 Neurogenesis in the SVZ

Over the past ten years, it has been established that the NSCs of the SVZ are a population of quiescent glial fibrillary acidic protein (GFAP)-positive cells that share signaling pathways with astrocytes (Suh, Deng, and Gage 2009). The SVZ produces NSCs that migrate along the rostral migratory stream to the olfactory bulb where they can

differentiate into granule cells or periglomerular neurons that are involved in olfaction. Before these cells migrate from the SVZ, they can be classified as Type B NSCs or Type C transit-amplifying cells (NPC). Type B cells express GFAP, the predominantly neural intermediate filament protein nestin (expressed only in dividing cells), sex determining region Y-box 1, 2 (Sox1 and Sox2), brain lipid binding protein (BLBP), and the astrocyte-specific L-glutamate L-aspartate transporter (GLAST) (Lazarov et al. 2010, Capela and Temple 2002, Pastrana, Cheng, and Doetsch 2009, Kriegstein and Alvarez-Buylla 2009). Type B cells give rise to GFAP-negative transit-amplifying type C cells, which then give rise to type A cells (migrating neuroblasts). These cells are PSA-NCAM and DCX expressing neuroblasts that, in mice, migrate radially on “glial tubes” in the rostral migratory stream (RMS) towards the olfactory bulb where they can terminally differentiate into granule cells or periglomerular neurons (Lazarov et al. 2010). In humans, the composition of the SVZ is similar but not identical to that in rodents. The close association of ependymal cells and astrocytes observed in rodents, as well as other primates and dogs, is not observed in humans. There is a layer, known as the hypocellular layer, which is mostly lacking of cell bodies, but is thought be the site where the regulation of neuronal function, metabolic homeostasis, and/or NSC proliferation and differentiation occur (Quinones-Hinojosa and Chaichana 2007). Another main difference is that the human SVZ lacks chains of migrating neuroblasts. Finally, the human SVZ has far fewer proliferating cells than what is observed in the rodent. While these differences in the structure and microenvironments make the rodent a sub-optimal model for the study of neurogenesis, it is important to note the molecular processes of NSCs “stem-cell behavior” is common between both systems (Johansson et al. 1999).

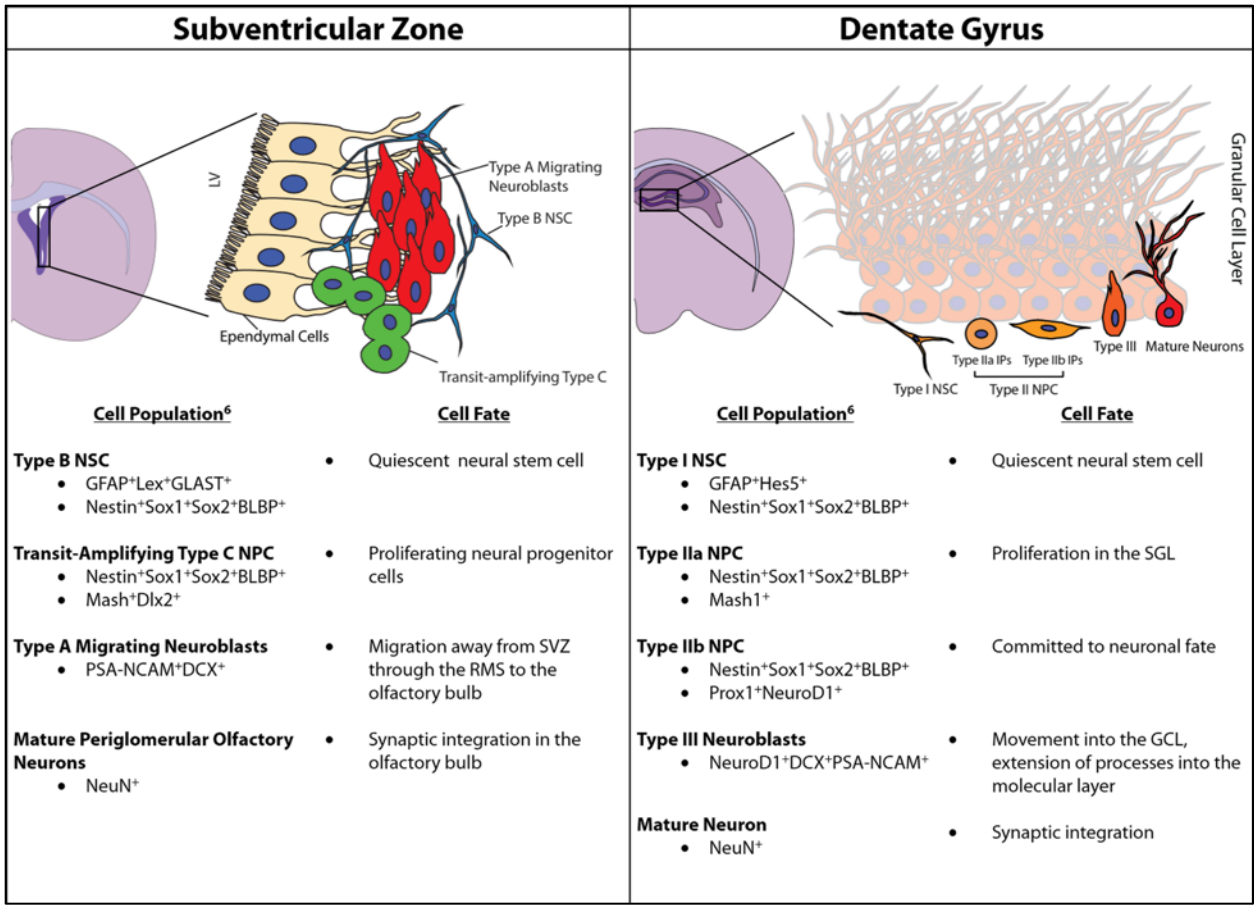

Figure 2. Adult neurogenesis in rodents. Coronal sections of the rodent show the neurogenic environments of the adult brain: the subventricular zone (SVZ; left) and the subgranular layer (SGL; right). The SVZ contains Type B NSCs which give rise to transit-amplifying Type C cells followed by Type A migrating neuroblasts. These cells migrate along the rostral migratory stream (RMS) towards the olfactory bulb before terminal differentiation. The SGL contains Type I NSCs which are similar to the Type B cells in the SVZ. Type I NSCs give rise to Type II NPCs (or intermediate progenitors; IP) which can be further classified as Type IIa and Type IIb. These Type IIb cells are early-committed neuronal progenitor cells which give rise to Type III neuroblasts which migrate into the granular cell layer where they exit the cell cycle and become mature neurons. All cell types described here can be identified based on a unique set of molecular and morphological markers, as described in the Cell Population column. From Tobin et al., 2014, reproduced with permission (Appendix 3; Tobin, Bonds et al. 2014)

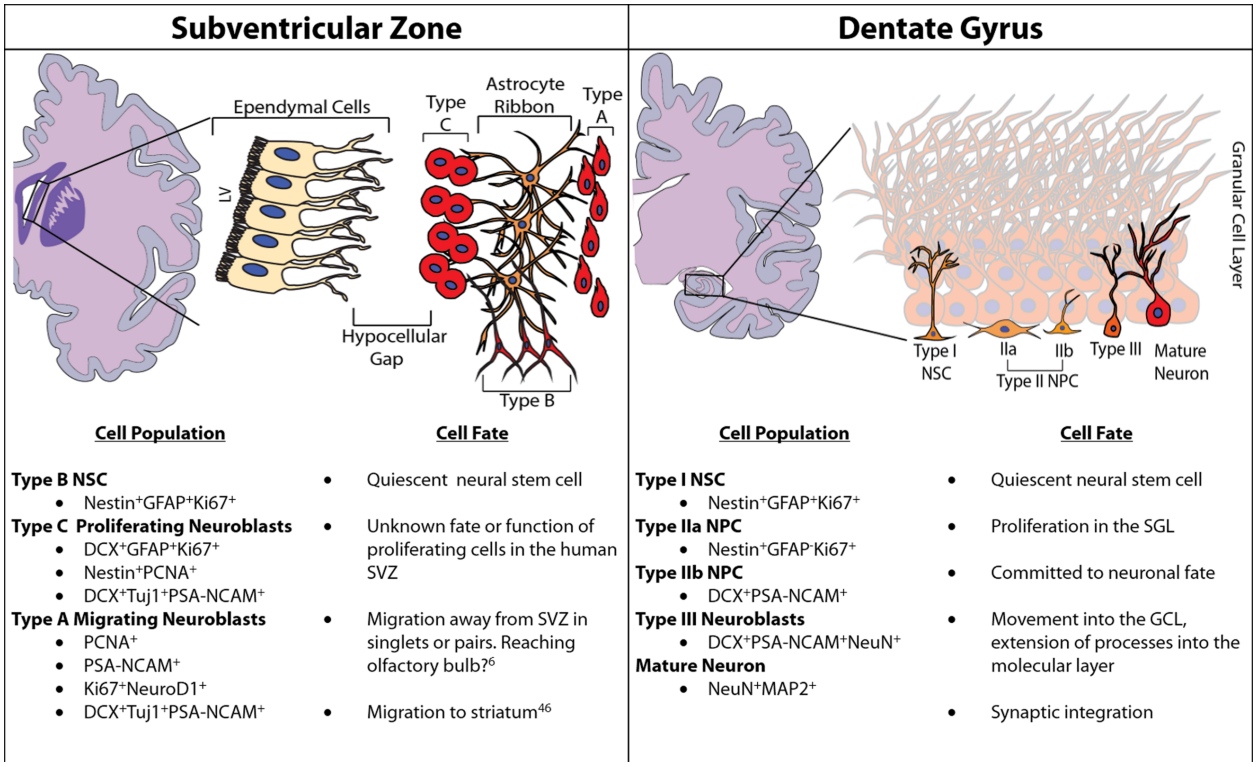

Figure 3. Adult neurogenesis in humans. Coronal sections of the human brain show the neurogenic environments of the adult brain: the subventricular zone (SVZ; left panel) and the dentate gyrus of the hippocampus (DG; right panel). Cell types unique to these regions as well as the fate of these cells are listed. Cell types described here can be identified based on a unique set of molecular and morphological markers, as noted in the Cell Population column. Abbreviations: LV – lateral ventricle; SGL – subgranular layer; GCL – granular cell layer. From Tobin et al., 2014, reproduced with permission (Appendix 3; Tobin, Bonds et al. 2014)

1.2.3 Neurogenesis in the SGL

The SGL gives rise to NSCs that differentiate into granule cell neurons in the hippocampus (Ihrie and Alvarez-Buylla 2011, Lazarov et al. 2010). This population of cells is very similar to the SVZ with a few exceptions. The newly formed neurons of this region populate the granule cell layer and thus only migrate a short distance. Further, these cells that arise are classified as being from two separate cell populations (type I and type II) based on the expression of unique sets of molecular markers. Type I cells resemble the type B NSCs that are found within the SVZ and are known to express nestin, GFAP, mammalian hairy and Enhancer-of-split homologues (Hes5), BLBP and Sox1/2 (Lazarov et al. 2010). Type II cells are more similar to the type C cells (NPCs or intermediate progenitors) and are GFAP-negative. These cells are classified into two subpopulations: Type IIa, which express Mash1 (Ascl1) and Sox2, and Type IIb, expressing the transcription factors Prospero homeobox protein 1 (PROX1), Neurogenic differentiation 1 (NeuroD1), as well as Doublecortin (DCX). These intermediate progenitor cells continue to proliferate and give rise to Type III cells, also known as migratory neuroblasts, which express DCX and polysialated neural cell adhesion molecule (PSA-NCAM) (Lazarov et al. 2010). At this point the cells exit the cell cycle after a limited number of divisions and become mature neurons that incorporate into the GCL send axonal projections into the CA3 region of the hippocampus and spineless dendrites into the molecular layer (Figure 2) (Zhao et al. 2006).

1.2.4 Role of astrocytes, endothelial cells, and pericytes within the neurogenic niche

As described above, there are several other cell types within the neurogenic niche that may exert an effect on NPCs. Astrocytes, ECs, and pericytes each have varying

effects on the proliferation and differentiation of NPCs. An *in vitro* study revealed that astrocytes and ECs enhanced NPC proliferation and neurosphere formation, however exactly what these other soluble factors were that the astrocytes and/or endothelial cells are providing in the niche is unknown (Ehret, Vogler, and Kempermann 2015). According to this study, pericytes did not seem to have a significant effect on the proliferation and/or survival of NPCs. The functions of caveolae and Cav-1 in astrocytes are similar to that of other cell types, it is crucial for signaling and trafficking (Parat and Riggins 2012). Specifically, Cav-1 in astrocytes localizes with P-glycoprotein (P-gp) which is critical for BBB function (Ronaldson et al. 2004).

1.2.5 Role of vasculature in the Stem Cell Niche

The vasculature is a critical regulator of the NSC microenvironment. NSCs are intimately associated with vascular endothelial cells within the neurogenic regions and their self-renewal, proliferation and early differentiation tightly depend on homeostatic endothelial functions (Eichmann and Thomas 2013). Within the SVZ, there is direct access to soluble factors in the blood and CSF and thus the established vascular network of the adult SVZ niche is a key component of the specialized microenvironment that supports NSCs and their progeny (Tavazoie et al. 2008). In the SGL, neurogenesis is closely associated with a process of active vascular recruitment and subsequent remodeling. Furthermore, NPCs and angioblasts proliferate together in clusters associated with the microvasculature of the SGL, and cells within these clusters express the VEGF-receptor, Flk-1 (Palmer, Willhoite, and Gage 2000). In fact, up to 37% of the cells proliferating in the SGL are endothelial precursors (Abrous, Koehl, and Le Moal 2005).

The clustering of neural and endothelial precursors suggests that neurogenesis involves cross-talk with endothelial precursors. In fact, in culture, endothelial cells have been shown to enhance proliferation of NPCs, as well as promote differentiation into neurons (Ehret, Vogler, and Kempermann 2015). Understanding the signals mediating the intimate interaction between endothelial cells and NSCs/NPCs will provide new insight into potential mechanisms of brain repair.

Microvessels and NPCs support each other via paracrine signaling mediated by trophic factors (such as VEGF) which induces endothelial cell proliferation, stimulates brain plasticity and remodeling, and reduces neuronal degeneration. In the penumbra, newly formed vessels create a niche that can support the migration and neuronal differentiation of neuroblasts (Dobkin 2004, Zhang and Chopp 2009). In turn, neuroblasts reinforce angiogenesis via release of VEGF (Johansson 2007).

1.2.6 Critical roles of growth factor receptor expression

As discussed, the neurogenic niche and the growth factors generated by resident cells modulate stem cell properties of NSCs and NPCs. These properties include self-renewal, proliferation, differentiation, and cell death. Thus, expression of specific growth factor receptors and their proper localization in the membrane is critically important.

Epidermal growth factor receptor (EGFR) is an essential transmembrane protein that responds to the epidermal growth factor (EGF) and transforming growth factor α (TGFα). These growth factors stimulate cell growth, proliferation, and differentiation. Importantly, EGFR has been found to be associated with lipid rafts and its localization to this region of the membrane is vital for its ability to bind its specific ligands (Freeman et al. 2007).

Another important receptor in the neurogenic niche is the bone morphogenetic protein receptor 1α (BMPR1α) and bone morphogenetic protein receptor 2 (BMPRII). These receptors are part of the transforming growth factor β superfamily. BMP signaling promotes the quiescence of NSCs and NPCs and is thus a negative regulator of neurogenesis. Inhibition of BMP signaling induces these cells to enter the cell cycle and proliferate (Bond et al. 2014, Meyers et al. 2016). Ligand binding to these receptors leads to the phosphorylation of SMAD1/5/8 which translocates to the nucleus to induce gene expression. Both BMPR1α and BMPRII are localized to specialized regions of the membrane, such as lipid rafts and clathrin coated pits (CCP). Knockdown of Cav-1 has been shown to enhance the SMAD-dependent signaling pathway in an immortalized mouse myoblast cell line (Saldanha et al. 2013). However, interactions between BMPRs and Cav-1 in neural stem cells has not been well described.

1.3 Caveolin

Caveolin-1 (Cav-1) is the primary “coat” protein of caveolae, the omega-shaped invaginations of the plasma membrane that provide cholesterol-enriched platforms that anchor membrane proteins and serve as important endocytic vesicles in many cell types, including NSCs and ECs, as well as regulating the activity of several signaling molecule and proteins (Minshall et al. 2003, Peffer et al. 2014, Cohen et al. 2004). Cav-1 is not only required for caveolae formation and trafficking, but also serve both structural and metabolic roles, and furthermore are critical for maintaining feedback inhibition loops that control cell signaling, such as endothelial nitric oxide synthase (eNOS). There are also two other isoforms of caveolin that are expressed – Cav-1α and Cav-1β. The α-isoform

contains residues 1-178 (full length) and the β-isoform only contains residues 32-178 which results in a smaller form of Cav-1 (Cohen et al. 2004). While both isoforms are capable of generating caveolae, the exact functional differences between them is unknown. Within Cav-1 there is a scaffolding domain (CSD; residues 82-101) which acts both to anchor proteins within caveolae, as well as to regulate cell signaling. It is thought that by concentrating signaling molecules to this region of the membrane enables rapid modulation of cell signaling events (Cohen et al. 2004).

Cav-2 is another member of the caveolin family which also has multiple isoforms – Cav-2α (full length), -β and -γ. There is little known about the functional significance of Cav-2 and its isoforms, however Cav-2 is generally coexpressed with Cav-1.

Cav-3 is thought to be mainly associated with striated muscle cells (skeletal muscle, diaphragm, and heart) but has also been described to be expressed to some extent in astrocytes (Badaut et al. 2015, Nishiyama et al. 1999). In fact, a study by Nishiyama et al. described the role of Cav-3 in the development of AD in that it directly interacts with APP in astrocytes and furthermore may provide a platform for APP and presenilin-1 to associate (Nishiyama et al. 1999).

Given that chronic neuroinflammation is exhibited in AD, it is important to understand the cell types that contribute to this state, as well as the role of caveolin isoforms in these cell types. Microglia, cells which are important for the generation of inflammatory cytokines in the CNS, express both Cav-1 and Cav-3 (Niesman et al. 2013) Microglial Cav-1 in is mainly expressed in the membrane where caveolae and MLRs are present, while Cav-3 is mainly expressed in the cytoskeleton. In a study by Niesman et al., Cav-1 is described to act as a negative regulator of microtubule stability, whereas

Cav-3 acts to regulate cellular structure while having no discernable effect on cell signaling in the CNS (Niesman et al. 2013, Ikezu et al. 1998). For the purpose of this study, Cav-1 will be the main focus.

1.3.1 Mouse models regulating expression of caveolin

Global caveolin-1 knock-out (Cav-1KO)

Mice lacking Cav-1 throughout the body are viable, fertile and do not have any observable physical abnormalities. Additionally, Cav-1KO mice show signs of enhanced neuronal aging and degeneration, including reduced expression of synaptic markers. Cav-1KO animals also exhibit reduced cerebrovascular volume, as well as AD-like pathology such as altered APP metabolism and increased Aß deposits (Head et al. 2010). It has also been described that the Cav-1KO have deficits in endothelial cell health and function in the heart and lung, and that re-expression of Cav-1 in the vasculature can rescue these phenotypes (Sun, Minshall, and Hu 2011, Murata et al. 2007, Head et al. 2010, Head et al. 2011). Cav-1 has also been implicated in a number of cancers, including breast and gastrointestinal cancer. However, Cav-1KO mice do not develop spontaneous tumors, and its role in different cancers is debated.

Cav-2 knockout (Cav-2KO)

With the exact functional role of Cav-2 to yet be determined, Cav-2KO mice were generated in order to attempt to discern its function (Razani, Wang, et al. 2002). It was found that Cav-2 is not necessary for caveolae formation or for the localization of Cav-1 to the MLR. Additionally, Cav-2KO mice did not display any of the vascular abnormalities or other central abnormalities as described in the Cav-1KO mouse. However, Cav-2KO mice did display an increase in lung pathology that is similar to what is described in the

Cav-1KO mouse. This evidence suggests that Cav-2, although not necessary for the formation of caveolae, is important in lung disease development.

Cav-3 knockout (Cav-3KO)

Generation of the Cav-3KO mouse has served to identify its role in the development cardiomyopathy and other muscular dystrophies. These mice were found to have normal levels of Cav-1 and -2 and normal caveolae in non-muscle tissue. However, unlike Cav-2, Cav-3 expression was found to be adequate to drive caveolae formation (Li et al. 1998). Muscle degeneration was observed in these mice at only 8 weeks old, and diaphragm degeneration was observed between 8-30 weeks of age (Hagiwara et al. 2000).

Cav-1/3 double knockout (Cav-1/3 dKO)

The Cav-1/3 dKO was created by breeding the global Cav-1KO with a global Cav-3KO mouse. This mouse is also null for Cav-2, as it is unstable without the presence of Cav-1. Interestingly these mice did display a combined phenotype of the Cav-1KO and Cav-3KO coupled with the development of a severe cardiomyopathy at 2 months of age, (Park et al. 2002, Cohen et al. 2004). However, these mice did not reveal any loss of viability as a result of total loss of all 3 forms of caveolin compared to the single knockout animals.

Endothelial cell specific caveolin-1 knock-out (EC-Cav-1KO) and Caveolin-1 reconstitution specifically in endothelial cells (Cav-1RC)

The vascular, cardiac, and pulmonary defects in Cav-1KO mice have been well studied and defects have been mainly ascribed to loss of Cav-1 in endothelial cells. However, because there is global knockout of Cav-1, the defects could also be cause by

other cell types. Thus, it became crucial to evaluate the contribution of endothelial Cav-1 to the defects observed in the global knockout. To unravel the effects of caveolin expression specifically in endothelial cells, both the endothelial cell specific Cav-1KO (EC-Cav-1KO) and reconstitution specifically in endothelial cells (Cav-1RC) were developed as previously described (Murata et al. 2007). The EC-Cav-1KO was created using the Cav-1 floxed mouse crossed with Tie-2-Cre carrying mouse. These mice have no observable phenotype, are fertile, and have a normal lifespan. Upon further investigation, the EC-Cav-1KO does display altered vascular defects which is rescued in the Cav-1RC mouse (Murata et al. 2007).

1.3.2 Role of Caveolin-1 in health and disease

It has been well described that Cav-1 expression in the brain decreases with age (Head et al. 2010) and that loss of Cav-1 in various mouse models is implicated in numerous diseases, including pulmonary fibrosis, cardiovascular disease, diabetes, and AD (Cohen et al. 2004, Head et al. 2010, Sun, Minshall, and Hu 2011). Cav-1 has been suggested to regulate neurogenesis, but the nature of this regulation remains controversial and inconclusive (Jasmin et al. 2009, Li et al. 2011). The data provided in this study aims to elucidate its role in this process.

Cav-1 has also been classified as both a tumor suppressor and an oncogenic factor in various cancers (Williams and Lisanti 2005, Chen and Che 2014, Pucci et al. 2015, Sugie et al. 2015, Chatterjee et al. 2015). These conflicting roles of Cav-1 likely depends on the type and state of the cancer, and also adds to the confusion of its role in promoting or inhibiting cell cycle progression in dividing cells.

Cav-1 has also been implicated in the development of T2D since it was discovered that insulin ligand was concentrated within caveolae (Cohen et al. 2004). A study performed by Razani et al. showed that Cav-1KO mice were resistant to diet-induced obesity (Razani, Combs, et al. 2002). In a follow up study, Cav-1KO mice were bred with db/db mice which led to the observation that the db/db-Cav1KO mouse only gained approximately half the weight compared to the db/db mouse with normal Cav-1 expression (Cohen et al. 2004). It has also been shown that loss of Cav-1 leads to a nearly complete loss of insulin receptor expression in adipose tissue (Cohen et al. 2003). These findings show that while loss of Cav-1 itself, and the subsequent decrease in insulin receptor expression is not enough to produce a diabetic phenotype, the regulation of Cav-1 expression plays an instrumental role in the development of T2D. The data presented in this thesis aims to extend these findings to the brain.

Lastly, Cav-1KO mice have shown a number of vascular abnormalities both peripherally and centrally. Cav-1KO mice have been documented to develop hypertrophic cardiomyopathy, decreased cerebrovascular volume, and increased NO production via upregulation of eNOS (Cohen et al. 2004, Head et al. 2010, Minshall et al. 2003). Each one of these abnormalities have been observed in both T2D and AD, thus Cav-1 is a prime candidate linking these two diseases.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

For a list of chemicals, reagents, and primary antibodies used reference Table 1.

Viruses were obtained from Dr. Hemal Patel's laboratory at the University of California at San Diego. Viruses used were an Ad-CMV-GFP (control) and Ad-CMV-Cav1 to overexpress caveolin-1, as well as a LV-shRNA-scrambled control and AAV9-shRNA-Cav-1.

2.1.2 Animals

All animal experiments were approved by the University of Illinois Institutional Animal Care and Use Committee, ACC Protocol # 17-123 (Lazarov) and # 16-204 (Minshall).

2.2 Methods

2.2.1 Neurosphere Culture

Isolation. 8-12 weeks old mice were euthanized via isoflurane overdose and brains dissected into HBSS stored on ice followed by dissection of the subventricular zones and hippocampus. Tissue was minced with a sterile scalpel and transferred to 3mL warm media (DMEM/F-12, 20mM KCl, 2μg/mL heparin, 1% penicillin-streptomycin, 20% B27 supplement, 10% N2 supplement). After tissue was settled, media was removed, and tissue was dissociated with 0.1% trypsin-EDTA and incubated with agitation at 37°C for 7 minutes. Trypsin inhibitor (139 μg/mL plus 10 μg/mL DNase I in $HBSS^{-/-}$) was added and samples spun at 700 rpm for 5 minutes. Tissue pellet was resuspended in 1mL warm media supplemented with 20 ng/mL EGF and 10 ng/mL bFGF and pipetted 20X with a P1000 pipet and cultured at 37°C, 5% CO_2 and passaged every 7 days.

Passaging. Media is collected and spun at 1000 rpm for 5 minutes. Neurospheres are dissociated with 0.05% trypsin-EDTA and incubated in a 37°C water bath for 7 minutes. Trypsin inhibitor was added, and cells spun at 1000 rpm for 5 minutes. Cells are counted and plated at 10,000 cells/cm^2.

Viral Infection. Cells were incubated with virus and respective control for 72 hours in neurosphere media supplemented with EGF and bFGF. Cells were then passaged as described and resuspended in neurosphere media supplemented with EGF and bFGF and cultured according to experimental requirements.

2.2.2 Primary Endothelial Cell Culture

Preparation of solutions. Prior to isolation a 0.005% collagen solution is prepared (Sigma Aldrich, Table 1). A 25cm^2 flask (4 brains/flask) or a 6 well plate (1-2 brains/well) is coated with 0.005% collagen by incubation for 1 hour at room temperature. A papain solution (Worthington Biochemical, Table 1) by reconstitution of one vial in 5 mL of MEMα containing 1% P/S (each brain requires 833.3 μL of papain solution). A DNase solution (Worthington Biochemical, Table 1) is prepared by reconstituting one vial in 0.5 mL of MEMα (each brain requires 41.7 μL of DNase solution). Digestion buffer was prepared by mixing the papain and DNase solution (833.3 μL papain and 41.7 μL DNase per brain) and incubated at 37°C. A heparin solution was prepared by resuspending 10,000 units of heparin sodium salt per 1 mL (10,000 U/mL) of culture grade water and passed through a 0.22 μm syringe filter. Complete endothelial cell media (ECM; Cell Biologics, Table XX) was prepared by thawing the supplement kit and adding a full vial of each supplement (VEGF, ECGS, EGF, Hydrocortisone, L-Glutamine, Antibiotic-Antimycotic solution, FBS) to 500 mL of endothelial cell media which is then stored at 4°C and warmed to 37°C prior

to isolation. A heparin-ECM solution was prepared by diluting 27.5 μL of the heparin solution to 50 mL of ECM (excess media stored at 4°C). A 25% BSA solution is prepared by addition of 12.5 g BSA (Sigma, Table 1) to 50 mL $HBSS^{-/-}$. Once BSA is dissolved it is passed through a 0.22 μm vacuum filter. Lastly, a 4 μg/mL puromycin solution is prepared. Prior to isolation, all solutions (except collagen) are warmed to 37°C.

Isolation Procedure. Mice were euthanized via isoflurane overdose and decapitated. Heads were placed into a petri dish, sprayed liberally with ethanol and transferred into a cell culture hood. Brains were dissected out and carefully rolled onto Whatman filter paper to remove meninges followed by removal of the cerebellum. Tissue was stored in sterile petri dish containing warm MEMα until dissection is complete. Once complete, brains were transferred to lid of petri dish and chopped with a sterile razor blade. Tissue was then collected and centrifuged and 1000g for 10 minutes at 4°C. Supernatant was aspirated, and pellet resuspended in appropriate amount of digestion buffer. Tissue is then digested for 15 minutes at 37°C. Following digestion tissue is triturated 2-3 times through 19- and 21-gauge needles sequentially. Digested tissue was transferred to a 15 mL (maximum of 4 brains/vial) conical vial and 2 volumes of 25% BSA was added, thoroughly mixed and centrifuged at 3880g for 15 minutes at 4°C. Myelin and supernatant are aspirated. Pellet is resuspended in 1 mL of ECM + heparin, transferred to a clean vial, and centrifuged at 1000g for 5 minutes at 4°C. Supernatant is discarded and pellet is resuspended in the appropriate amount of ECM + heparin (8 mL/25cm^2) and cells are plated. The following morning, growth surfaces are washed vigorously with F12 Nutrient Mix (Gibco, Table 1) 3-5x to dislodge any loosely attached cells. After washing, ECM + puromycin (4μg/mL; Invivogen, Table 1) for 48 hours to select for endothelial cells. After

48 hours media containing puromycin is removed and cells are washed twice with $HBSS^{+/+}$ and replaced with ECM (8mL/25cm^2). Media is then changed daily until confluent and ready for experiments.

Splitting. Cells were rinsed once with $HBSS^{-/-}$ (Gibco, Table 1), followed by incubation with $HBSS^{-/-}$ for 3 minutes at 37°C. Next, the cells were incubated with 0.25% trypsin-EDTA (Gibco, Table 1) for 2 minutes at 37°C. Trypsin was neutralized with 2 volumes of complete ECM. Cells were collected and spun at 1000 x g for 5 minutes, followed by resuspension in complete ECM and plating.

2.2.3 bEnd.3 Culture

Brain endothelial cells (bEnd.3) were purchased from ATCC (Table 2). Vials were rapidly thawed at 37°C with gentle agitation for approximately 2 minutes. Vial contents were transferred to a tube containing 9 mL of complete culture medium (DMEM, 10% FBS, 1% P/S) and spun at 125 x g for 5 minutes. Cells were resuspended in complete culture medium and plated according to experimental requirements.

Splitting. Media is removed, and cells are rinsed with warm PBS to remove all traces of serum which contains trypsin inhibitor. Cells are then incubated in 0.25% trypsin-EDTA for 3-5 minutes until cells have detached. Trypsin was neutralized with 3 volumes of complete growth medium, cells collected and spun at 125 x g for 5 minutes. Supernatant is discarded, and cells resuspended in complete culture medium. Cells are subcultured at a ratio of 1:5.

Freezing. Complete culture medium as described above is supplemented with 5% (v/v) DMSO and stored on ice. Cells are dissociated as described above and resuspended in 1 mL freezing medium.

2.2.4 Quantitative reverse transcription polymerase chain reaction

RNA was extracted from cultured cells using the ISOLATE II RNA Mini Kit (Bioline USA) as detailed in the manufacturer's protocol. RNA was quantified using a Nanodrop and stored at -80°C until used. cDNA synthesis was performed using the SuperScript™ III First-Strand Synthesis SuperMix (ThermoFisher Scientific) as detailed in the manufacturer's protocol starting with 1 µg total RNA per sample and using oligo(dT) priming. cDNA was stored at -20°C until used. qPCR was performed using SensiFAST™ SYBR® & Fluorescein Kit (Bioline USA). Each reaction was done using 1 µL of starting cDNA. Relative expression was calculated using the ΔΔCt method using Actin as the housekeeping gene. Primers sequences used are listed in Table 4.

2.2.5 HEK Cell Culture

HEK cells with WT (751) APP and with APPswe were cultured in DMEM containing GlutaMAX (Gibco, Table 1), 10% FBS, 1% P/S, and Geneticin (20 µL per 10 mL added prior to plating; Gibco, Table 1). Media was replaced every 48-72 hours.

Splitting. Cells were rinsed twice with sterile PBS to remove all traces of FBS. Cells were incubated in 0.25% trypsin-EDTA for 2 minutes at 37°C. Trypsin was neutralized with 2 volumes of warm media. Cells were collected and spun at 1000 rpm for 5 minutes. Cells were resuspended in media and plated at a ratio of 1:5.

Viral Infection. Cells were split as described above, however cells were counted and resuspended in Opti-MEM (Gibco, Table 1) containing virus (2×10^9 viral particles per 10 mL media), 1% FBS, and 1% P/S for 24 hours at 37°C. Viral media was then replaced with growth media for another 48 hours.

Amyloid-β *Collection*. Cells were cultured as described above. Cells cultured in a 10 cm dish were incubated with 1 mL media for 2 hours at 37°C. Media was collected and prepared for western blot by adding tricine sample buffer containing ß-mercaptoethanol at a ratio of 1:2. Samples were boiled for 10 minutes at 95°C and stored at -20°C.

2.2.6 Novel Object Recognition

This test was performed over 4 days. On Days 1 and 2, mice were placed in the arena for 10 minutes of habituation. On Day 3 (familiarization phase), mice were placed in the arena with two identical objects and were allowed 20 minutes to explore the objects for 30 seconds (once the animal explored for 30 seconds, they were removed from the arena). On Day 4 (novel object test phase), animals were reintroduced to the arena containing a familiar and novel object. Mice were allowed to explore the objects as described above. Identical and novel objects were similar in size but differed in color and shape. The location of the novel object was placed on the non-preferred side (as determined during familiarization phase) to avoid side preference. Object exploration was recorded manually, and the percentage of exploration time of each object was computed. *Statistical Analysis*. Exploration time of the familiar vs. novel object during the test phase was compared and analyzed by paired t-test, *P<0.05.

2.2.7 Radiolabeled Insulin Injection (*in vivo*)

Mice were anesthetized with a ketamine/xylazine mixture. 1µCi of radiolabeled ^{125}I-Insulin (Perkin Elmer, Table 1) was injected via the jugular vein and allowed to recover for 60 minutes. Following recovery, mice were re-anesthetized (if necessary) and the vena cava was isolated to collect whole blood from each animal. 100 µL of blood was used for CPM measurements. Following blood removal, the brain was dissected out and the cortex,

hippocampus, subventricular zones, and cerebellum separated. Each sample was then quantified for ^{125}I-Insulin CPM on a gamma counter (Perkin Elmer, Wizard 2 gamma counter).

2.2.8 BrdU Preparation and Injection

BrdU Preparation. 5'-bromo-2'-deoxyuridine (BrdU; Sigma, Table 1) was dissolved in physiological saline at 20 mg/mL and was through a 0.22 μm syringe filter (Millipore) attached to a 10cc syringe into a red top vacutainer tube (BD Biosciences). The BrdU solution was protected from light until administration.

BrdU Administration. A single dose of BrdU was administered intraperitoneally at 100 mg/kg in physiological saline. 24 hours following BrdU injection, animals were sacrificed via isoflurane overdose and transcardially perfused with cold PBS. Brains were then removed and post-fixed in 4% PFA for 48 hours at 4°C, followed by cryoprotection in 30% sucrose for 72 hours at 4°C.

2.2.9 ELISA

Mouse ELISA assays were performed with brain tissue lysate as detailed in the provided protocols. IL-1β (BD, Catalog # 555603), IL-6 (BD, Catalog # 555240), TNF (BD, Catalog # 555268), IL-10 (BD, Catalog # 555252), Aβ-40 (ThermoFisher, Catalog # KMB3481, Aβ-42 (ThermoFisher, Catalog # KMB3441).

2.2.10 Western Blotting

Cells or tissue were collected and homogenized in lysis buffer (150mM Na_2CO_3, 1mM EDTA, pH 8) containing protease inhibitor cocktail (1:100, Sigma, Table 1) and sonicated 3X for 15 seconds at 40% amplitude. Tissue was then spun at 10,000 rpm for 5 minutes (this step was not performed for cell lysate). Supernatants were collected, and protein

quantified via microplate BCA method and normalized to equal concentrations with lysis buffer and 4X sample buffer (0.35M Tris-HCl, pH 6.8, 30% glycerol, 5% β-Mercaptoethanol, 10% SDS, bromophenol blue). Excess protein lysate (with no sample buffer) was stored at -80°C. Samples were then boiled for 10 minutes at 95°C. 10-15μg of each sample was loaded into a 10% Tris-glycine gel and run in 1X Tris/Glycine/SDS buffer at 115V for 1 hour and 25 minutes followed by transfer in 1X Tris/Glycine buffer to a 0.45μm nitrocellulose membrane (Bio-Rad) at 125mV for 1 hour. Membranes were blocked for 30 minutes in 5% non-fat dry milk in PBST (PBS + 0.1% Tween-20), then incubated in primary antibody overnight at 4°C with shaking (for primary antibodies and dilutions, see Table 3). Membranes were washed 3X for 5 minutes in PBST and placed in appropriate secondary antibody (Table 3) for 1 hour at room temperature with shaking. Membranes were washed 3X for 15 minutes in PBST and images with an Amersham ECL chemiluminescence substrate (GE Healthcare). Levels of protein were quantified using densiometric measurements from ImageJ Software (La Jolla, CA). All protein levels were normalized to levels of actin unless otherwise noted.

Amyloid-β detection. Samples were prepared in tricine sample buffer (1:2) and separated on a 16.5% Tris-Tricine gel (Bio-Rad, Table 1). Running voltage was set at 75 V for 30 minutes followed 100 V for 2 hours, or until samples were adequately separated. Western blot transfer was done as described above. Membranes were then incubated in boiling PBS and microwaved for 5 minutes, followed by incubation with room temperature PBS for 5 minutes. Membranes were incubated in blocking buffer (5% non-fat dry milk in PBST) for 30 minutes. Membranes were then incubated with anti-mouse 6E10 antibody (Biolegend, Table 3) overnight at 4°C with shaking. Secondary antibodies, developing,

and densitometry analysis were done as described above. Levels of amyloid-beta were compared between conditions as the same number of cells were plated prior to amyloid-beta collection.

Statistical Analysis. Samples were subjected to densitometry analysis (ImageJ) and analyzed using unpaired student's t-test, *P<0.05.

2.2.11 Membrane Lipid Raft Fractionation

Cells and tissue were lysed in 1 mL of lysis buffer (150mM Na_2CO_3, pH 11) containing protease inhibitor cocktail (1:100, Sigma) followed by sonication 3X at 40% amplitude for 15 seconds on ice with 60 second incubation on ice in between cycles. Equal protein concentrations of homogenate were added to 1 mL of 80% sucrose followed by careful layering of 6 mL of 35% sucrose and 4mL of 5% sucrose, sequentially. The samples were centrifuged at 175,000 g with a SW41Ti rotor (Beckman Coulter) for 3 hours at 4°C. Samples were collected in 1 mL aliquots with the MLRs found at the 5/35% interface (fractions 4-6) and analyzed via western blot as described above.

Statistical Analysis. MLR blots were analyzed by calculating the percent of each target protein in each buoyant (fractions 4-6) and heavy fractions (10-12).

$$\frac{Bouyant\ Fraction}{(Bouyant + Heavy\ Fraction)}$$

Percentages were then analyzed by unpaired students t-test, *P<0.05.

2.2.12 Immunohistochemistry

Brains were sagittally sectioned at 50 μm using a freezing microtome (Leica Biosystems) and stored in cryoprotectant at -20°C in a 96 well plate. Every sixth section was used for IHC staining. Floating sections were rinsed 3 times in TBS for 5 minutes. For BrdU antigen retrieval, sections were pretreated with 2N HCl for 30 minutes at 37°C. Sections were

neutralized by incubation in 0.1M borate buffer for 10 minutes at room temperature. Sections are rinsed 6 times in TBS for 5 minutes (Note: this process was only done when staining for BrdU). Sections were blocked with TBS^{++} (TBS containing 0.3M glycine, 0.25% TritonX-100, 5% normal donkey serum) for 2 hours at room temperature. Sections are then incubated in primary antibodies (Table 3) diluted in TBS^{++} for 72 hours at 4°C. Following primary antibody incubation, sections are rinsed 3 times for 5 minutes at room temperature. Sections are then incubated with biotinylated species-specific anti-IgG (as needed) in TBS^{++} for 1 hour. Sections were then incubated in a fluorophore conjugated secondary antibody (Table 3) diluted in TBS^{++} for 2 hours. A final incubation in TBS containing DAPI was done for 5 minutes was performed before sections were mounted onto SuperFrost glass sides (Fisher) and cover slipped using PVA-DABCO (polyvinyl acetate-1,4-diazabicyclo-[2.2.2]octane). Slides were allowed to set overnight at room temperature, followed by long-term storage at 4°C protected from light.

2.2.14 Stereology

Quantification of immunostained sections were done using StereoInvestigator (MBF Biosciences). Sections were counted using the following parameters: Counting frames, as well as the sampling grid were set to 100 μm x 100 μm. Section thickness was averaged at 35 μm.

2.2.15 Statistical Analyses

All statistical analyses were performed in GraphPad Prism (Version 7.0a; GraphPad Software Inc). Data shown are ±SEM and a probability of less than 0.05 was considered significant. Individual statistical analyses as appropriate are described in the figure legends.

Chemical/Reagent	Company	Catalog Number
^{125}I-Insulin	Perkin Elmer	NEX420010
16.5% Tris-tricine gel	Bio-Rad	4563064
BrdU	Sigma	B5002
BSA	Sigma	7030
Collagen from calf skin	Sigma	C8919
Complete mouse endothelial cell medium with kit	Cell Biologics, Inc.	M1168
DMEM containing GlutaMAX	Gibco	10569-010
DNase	Worthington Biochemical	LK003172
ECL Amersham	GE Healthcare	RPN2232
F12 nutrient medium	Gibco	11765-054
Fibronectin	Sigma	F1141
Geneticin	Gibco	10131-035
HBSS -/-	Gibco	14175-095
Opti-MEM	Gibco	31985062
Papain	Worthington Biochemical	LK003178
Protease inhibitor cocktail	Sigma	P8340
Puromycin	Invivogen	ant-pr
Trypsin -EDTA	Gibco	25300-054
Trypsin Inhibitor	Sigma	T6522

Table 1. Chemicals and Reagents

Cell Line	Company/Lab	Catalog #
bEnd.3 [BEND3]	ATCC®	CRL2299™
HEK WT (751) APP	Ella Zeldich	Boston University School of Medicine
HEK APPswe	Ella Zeldich	Boston University School of Medicine

Table 2. Cell Lines

Primary Antibody	Company	Catalog number
6E10	Biolegend	803001
Actin	Millipore Sigma	MA511869
APP-CTF	Sigma	A8717
AT8	ThermoFisher	MN1020
Cav-1	BD Biosciences	610060
Cav-1	Cell Signaling	3238S
CP13	Laboratory of Peter Davis, PhD	
DA9	Laboratory of Peter Davis, PhD	
EGFR	Santa Cruz	sc-03
Insulin Receptor 1α	Abcam	ab5500
pSMAD1/5/8	Cell Signaling	9516

Secondary Antibody	Company	Catalog number
Biotin anti-Goat	Jackson Immuno	805-065-180
Biotin anti-Mouse	Jackson Immuno	715-065-150
Biotin anti-Rabbit	Jackson Immuno	711-065-152
Biotin anti-Rat	Jackson Immuno	712-065-150
Dky anti-Mouse Cy3	Jackson Immuno	715-165-150
Dky anti-Mouse Cy5	Jackson Immuno	715-175-150
Dky anti-Rabbit Cy3	Jackson Immuno	711-165-152
Dky anti-Rabbit Cy5	Jackson Immuno	711-175-152
Dky anti-Rat Cy3	Jackson Immuno	712-166-150
Streptavidin Cy2	Jackson Immuno	016-220-084

Table 3. Antibodies. List of primary (top) and secondary (bottom) antibodies used. Abbreviations: 6E10 – Amyloid-β; APP-CTF – Amyloid precursor protein C-terminal fragments; AT8 – phosphorylated Tau epitope (Ser202, Thr205); Cav-1 – Caveolin-1; CP13 – phosphorylated Tau epitope (Ser202); DA9 – total Tau; EGFR – Epidermal growth factor receptor; pSMAD – phosphorylated SMAD1/5/8.

Gene	Gene ID	Forward (5' - ->3')	Reverse (5' --> 3')
Actin	11461	CAACCGCGAGAAGATGAC	AGGAAGGCTGGAAGAGTG
BMI-1	12151	AAATCCCCACTTAATGTGTGTCC	CTTGCTGGTCTCCAAGTAACG
BMPR1α	12167	TGGCACTGGTATGAAATCAGAC	CAAGGTATCCTCTGGTGCTAAAG
BMPRII	12168	TTGGGATAGGTGAGAGTCGAAT	TGTTTCACAAGATTGATGTCCCC
EGFR	13649	GCCATCTGGGCCAAAGATACC	GTCTTCGCATGAATAGGCCAAT
FGFR	14182	AGCTGTCCAGATAAAGACACTGT	ATTTTACCGAGTCCCGTTTCC
IGF1R	16000	CTGGACCAGAGACCCTTTGC	GGACGGGGACTTCTGAGTCTT
Insulin Receptor 1α	16337	CTCCTGCTAACATCCACCTTG	AGCTCGCTAACTGAGATAGTCAT

Table 4. Primer sequences for qRT-PCR. All primer sets were designed against mouse genes. Abbreviations: BMPR1α and II – Bone morphogenetic protein 1α and 2; EGFR – Epidermal growth factor receptor; FGFR – Fibroblast growth factor receptor; IGF1R – Insulin-like growth factor 1 receptor.

3. Results

3.1 Caveolin-1 modulates the amyloidogenic pathway

HEK cells containing the APPswe mutation were transfected with a lentivirus containing a Cav-1 siRNA to downregulate Cav-1. Following 3 days of infection, media was collected for Aβ analysis. Results show that as a direct result of Cav-1 downregulation there is a significant increase in Aβ (6E10) (Figure 4A). Furthermore, cells were lysed to investigate levels of FL-APP, a transmembrane protein which, in neuropathological states, is cleaved to produce Aβ as well as other pathological CTFs. Both levels of FL-APP and β-CTF which were found to be significantly increased. These results indicate unequivocally that loss of Cav-1 enhances the amyloidogenic pathway.

To determine if overexpression of Cav-1 could reverse these effects, HEK cells containing the APPswe mutation were transfected with an adenovirus to overexpress Cav-1 (Figure 5A). Following 3 days of infection, media was collected for Aβ analysis. Results show that levels of both FL-APP and APP-CTF were returned to levels comparable to control levels (Figure 5B). Most significantly, levels of Aβ were no longer elevated and were returned to levels comparable to control (Figure 5B).

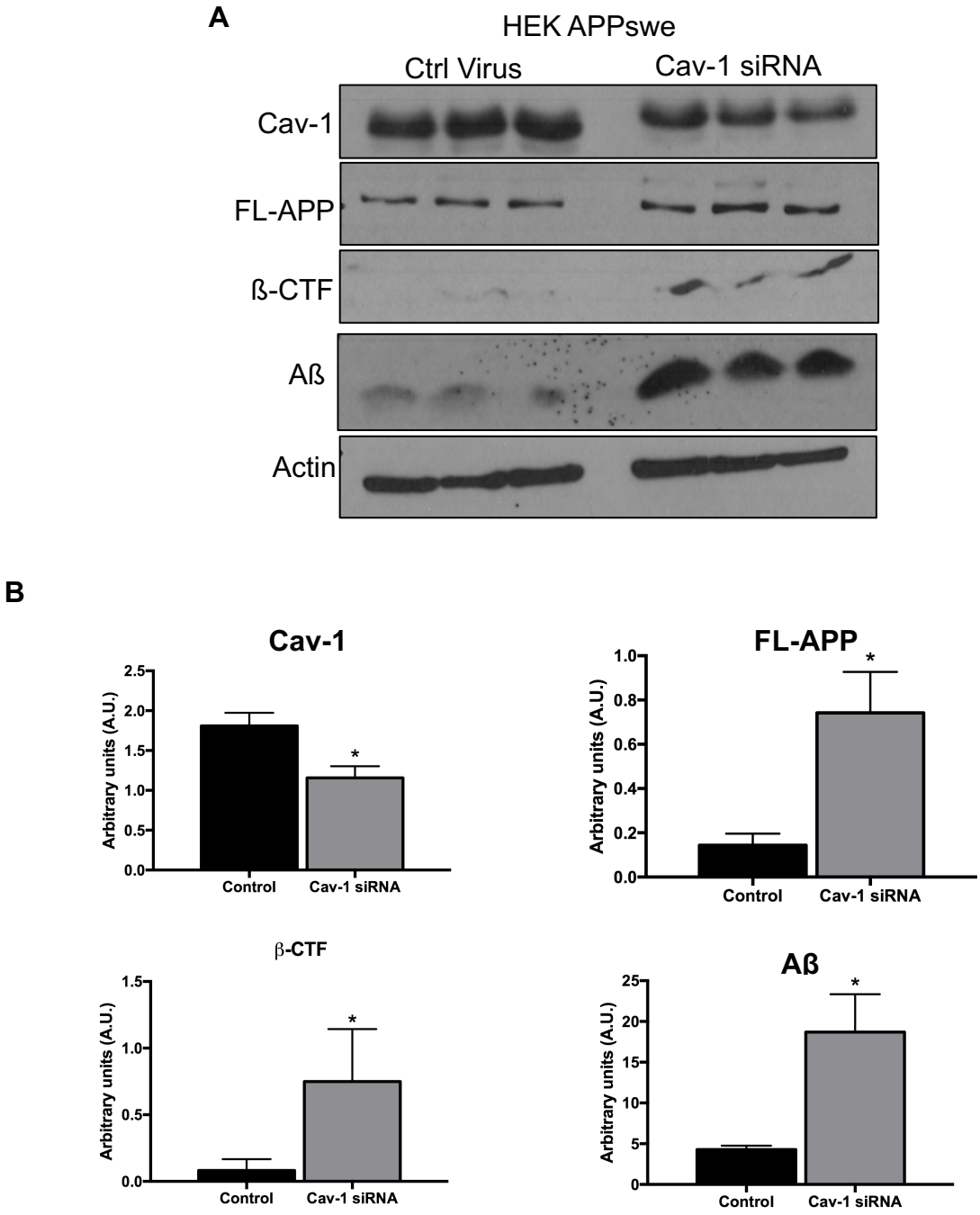

Figure 4. Loss of Cav-1 enhances amyloidosis in HEK cells containing the human APPswe mutation. A. HEK cells treated with an adenovirus containing an siRNA to Cav-1 causes a significant decrease in Cav-1 protein expression leading to significant increases in FL-APP, β-CTF, and Aβ. **B.** Quantification of panel A. (Unpaired t-test, *P<0.05).

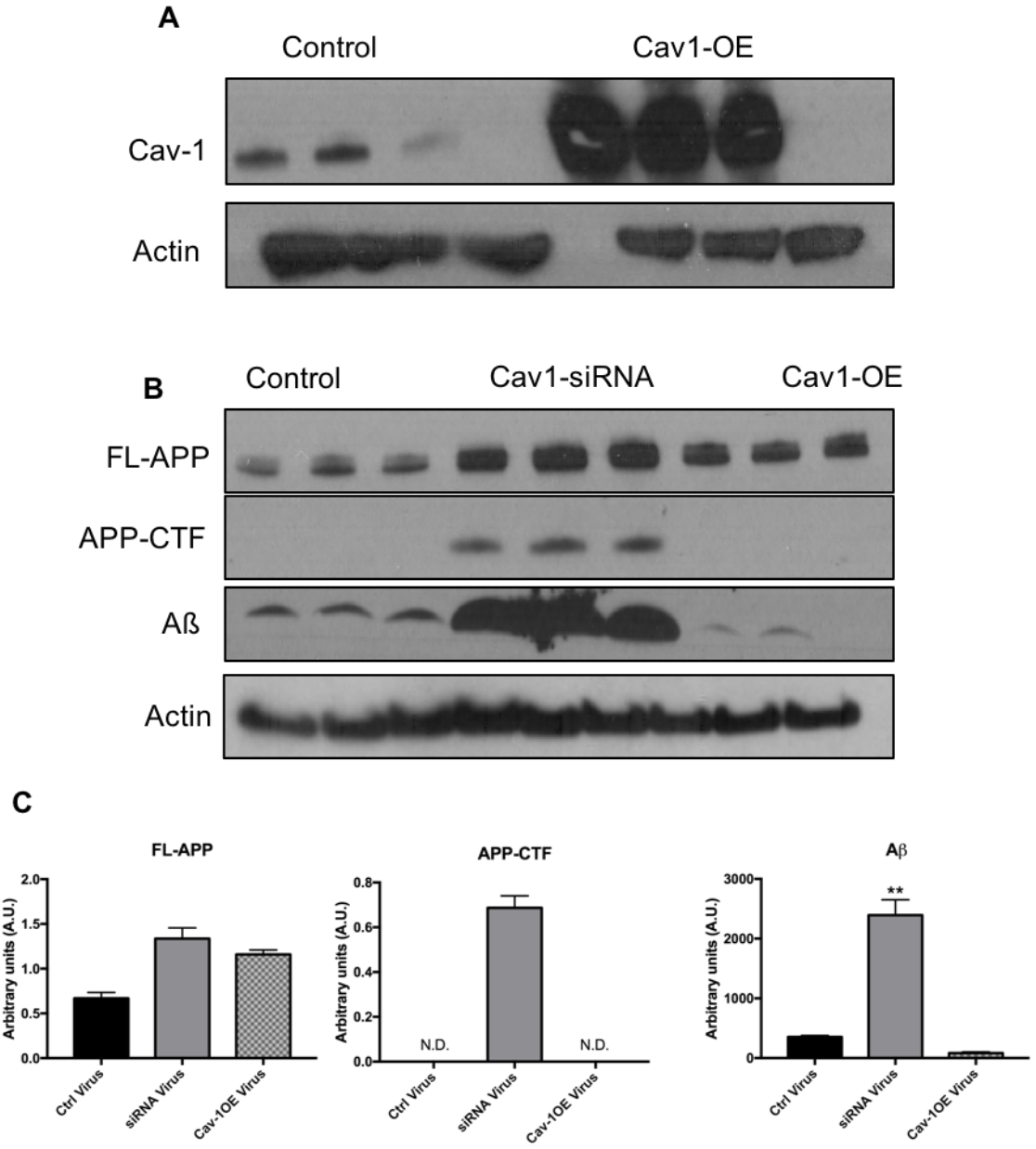

Figure 5. Expression of Cav-1 in HEK-APPswe cells modulates Aβ production. A. HEK-APPswe cells were treated with a lentivirus to induce overexpression of Cav-1. **B.** Comparison of HEK-APPswe cells containing the control, Cav-1 siRNA, or overexpressing virus (Cav1-OE). Downregulation of Cav-1 induces increases in FL-APP, APP-CTF, and Aβ, while overexpression of Cav-1 does cause a significant increase in FL-APP, there is no longer detectable levels of APP-CTF and levels of Aβ are returned to nearly below control levels. **C.** Quantification of panel B. (N = 3, Unpaired t-test, *P<0.05).

3.2 Chronic Inflammation induces depletion of Cav-1 in the brain of MKR diabetic mice.

Our next step was to determine if there is any loss of Cav-1 in the brains of diabetic mice, and furthermore what could cause this loss. It is known that in T2D, patients experience chronic inflammation, therefore we set out to determine if exposure to pro-inflammatory cytokines induced loss of Cav-1. To this end, bEnd.3 cells were cultured and exposed to TNFα and IL-6 and analyzed for Cav-1 expression. We found that as a result, Cav-1 was significantly decreased (Figure 6A, B).

To confirm that there is loss of Cav-1 in MKR brains, hippocampi were isolated and analyzed for total Cav-1 expression. Results show that indeed there is a significant loss of Cav-1 in the brains of these animals (Figure 6E). We further analyzed levels of pro- and anti-inflammatory cytokines in the brains of these mice by ELISA. Using the SOCS1-KO mouse as a positive control for chronic inflammation, we found that the MKR mice do display chronically increased levels of pro-inflammatory cytokines IL-6 and IL-1β, and a significant decrease of the anti-inflammatory cytokine IL-10 (Figure 6F).

Furthermore, we observe that there is a significant decline of Cav-1 as the MKR mice age (Figure 7A) and that there is a corresponding increase of IL-6 and TNFα in the brain (compared 2 vs. 6 months; Figure 7B). These results show that as a consequence of chronic inflammation there is a significant decline of Cav-1 in the brains of diabetic mice.

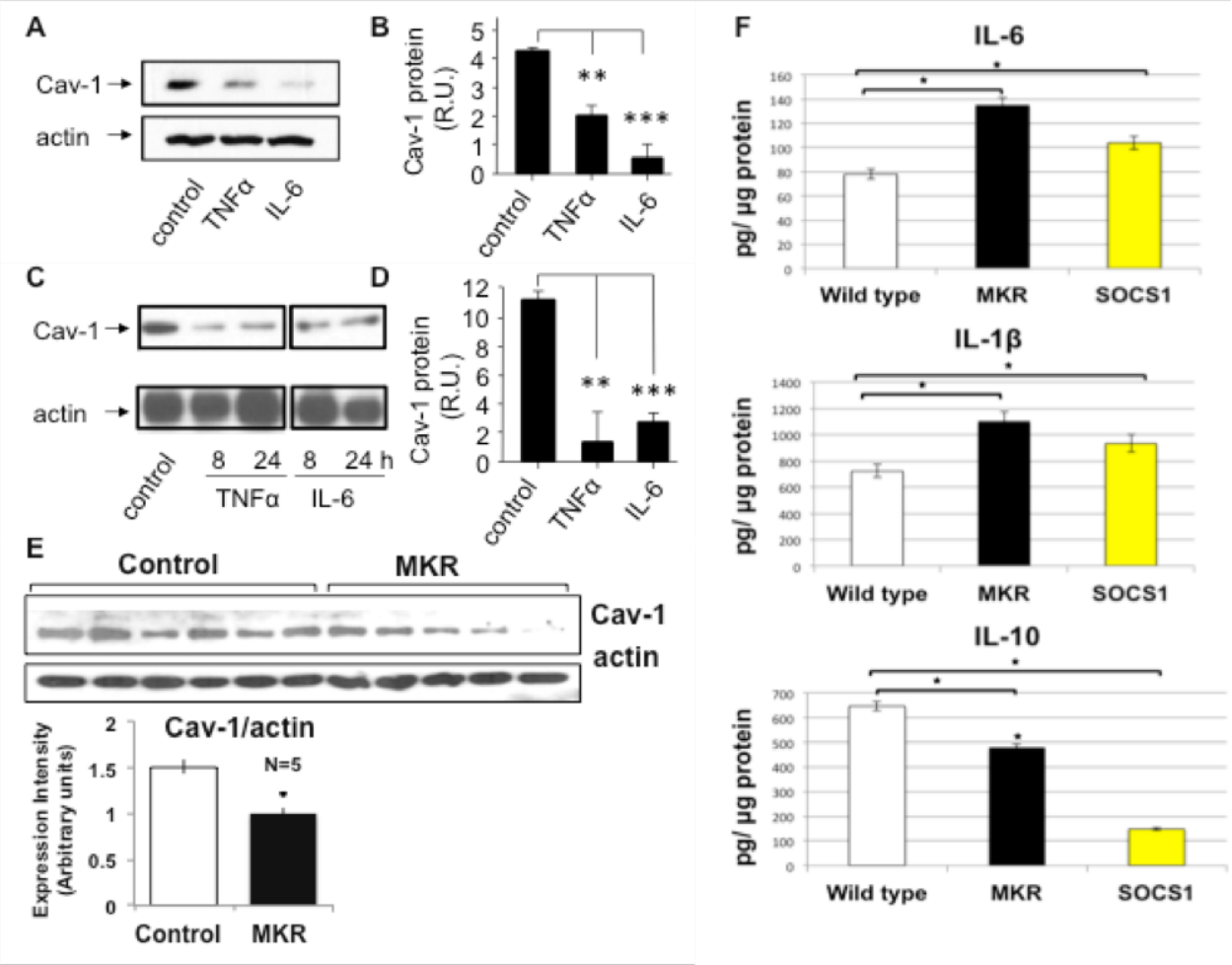

Figure 6. Chronic Inflammation induces depletion of Cav-1. A. Exposure of pro-inflammatory cytokines TNFα and IL-6 induces Cav-1 depletion in bEnd.3 cells (brain endothelial cells – ATCC). **B.** Quantification of (A) (Unpaired t-test, *P<0.05). **C.** bEnd.3 cells were exposed to TNFα and IL-6 for 8 and 24 hours. **D.** Quantification of (C) (N = 3; Unpaired t-test, *P<0.05). **E**. Comparison of Cav-1 expression in hippocampal lysate of WT control (FVB) and MKR diabetic mice show significant depletion of Cav-1 at 4-5 months of age (N = 5; Unpaired t-test, *P<0.05). **F.** Analysis of pro-inflammatory cytokines (IL-6 and IL-1β) and anti-inflammatory cytokines (IL-10) in the brains of WT control, MKR, and SOCS1 mice. MKR and SOCS1 mice show significant increases in pro-inflammatory cytokines and decreases in anti-inflammatory cytokines compared to WT control (ELISA, unpaired t-test, *p<0.05).

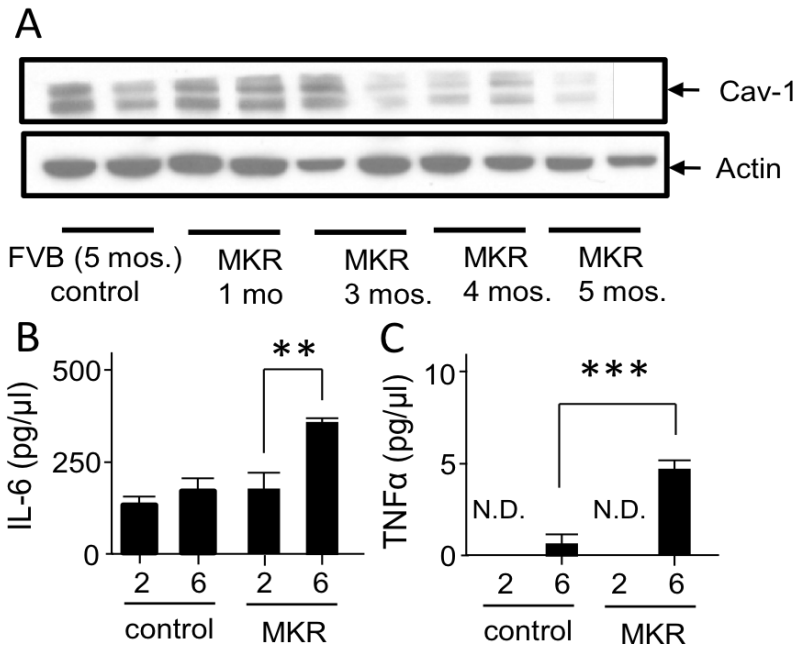

Figure 7. Progressive loss of Cav-1 expression and increase in pro-inflammatory cytokines in diabetic MKR mice. A. Skeletal muscle isolated from FVB control (5 months) and MKR (1, 3, 4, and 5 months) mice were analyzed for Cav-1 expression. Results show a reduction in Cav-1 as the MKR mice age compared to control. Brain, lung, and heart tissue were also analyzed (not shown) and showed a similar degree of reduction in Cav-1 expression. **B, C.** Brain tissue analyzed by ELISA for levels of IL-6 (B) and TNFα (C) at 2 and 6 months of age. (B) Levels of IL-6 were significantly elevated in MKR brains at 6 months of age. (C) Levels of TNFα were detected at 6 months of age and found to be significantly elevated in MKR mice compared to FVB control. (Unpaired t-test, *P<0.05).

3.3 Increased neuropathology in diabetic mice.

Following analysis of chronic inflammation, we set out to determine if there were any changes in levels of phosphorylated Tau. Tau is a microtubule associated protein which is necessary to build and maintain the microtubules which is essential for axonal transport. Abnormal hyperphosphorylation of tau and subsequent formation of neurofibrillary tangles (NFT) are classical hallmarks of AD pathology. There are a variety of antibodies that recognize different abnormal tau epitopes. Here, we utilized AT8 which recognizes phosphorylated tau at residues Ser202 and Thr205, and CP13 which recognizes phosphorylated tau at residue Ser202. Accumulation of these epitopes represents pre-tangle stage cells (Luna-Muñoz et al. 2007). Thus, to determine if there are early signs of NFT formation we analyzed levels of AT8 (pTau) in the hippocampus of MKR mice. Results show a significant increase in pTau, as well as a significant decrease of Cav-1 (Figure 8).

To follow up with the db/db model, we investigated if there were any changes in Cav-1, Tau (both phosphorylated and total), as well as FL-APP. Results show a significant decrease in Cav-1 which corresponded with a significant increase in pTau (CP13) and T-Tau (DA9), as well as the ratio of pTau/T-Tau (Figure 9). This ratio is likely important for correct cell function, and an increase may indicate an imbalance of the isoforms which could be responsible for levels of hyperphosporlyated tau. It is apparent that there is an increase in phosphorylation processes (e.g., due to enhanced kinase activity), in addition to an increase in tau production. We also looked at levels of FL-APP and found levels to be significantly increased (Figure 9). Taken together, these results indicate an increase in neuropathology in both models of diabetes.

3.4 Trending increase in levels of Aβ 42/40 in hippocampi of type 2 diabetic mice.

To determine if there are also elevated levels of mouse Aβ 42/40, hippocampi from 10-12 week old WT control and db/db mice were isolated and processed for Aβ extraction as described. Levels of Aβ 42 and 40 were analyzed by ELISA. Results show a trending increase in the ratio of Aβ 42/40 indicating that the diabetic phenotype results in a pathological state associated with AD (Figure 10). Of note, these mice are relatively young and thus any elevation should be taken into account.

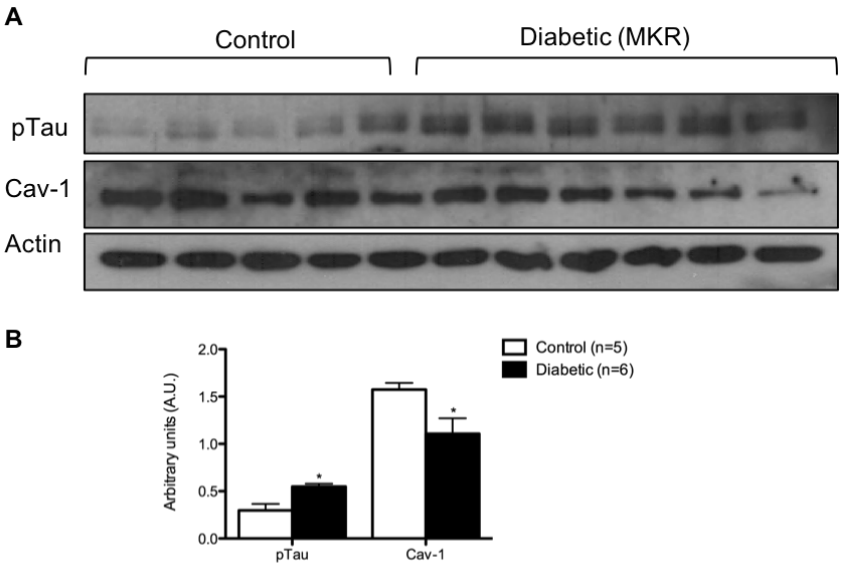

Figure 8. Increased levels of pTau and decreased Cav-1 in the hippocampus of MKR mice. A. Hippocampi of MKR mice show increased levels of pTau (AT8) and decreased levels of Cav-1. **B.** Quantification of panel A. (N = 5 (FVB control) and 6 (MKR diabetic); unpaired students t-test, *P<0.05)

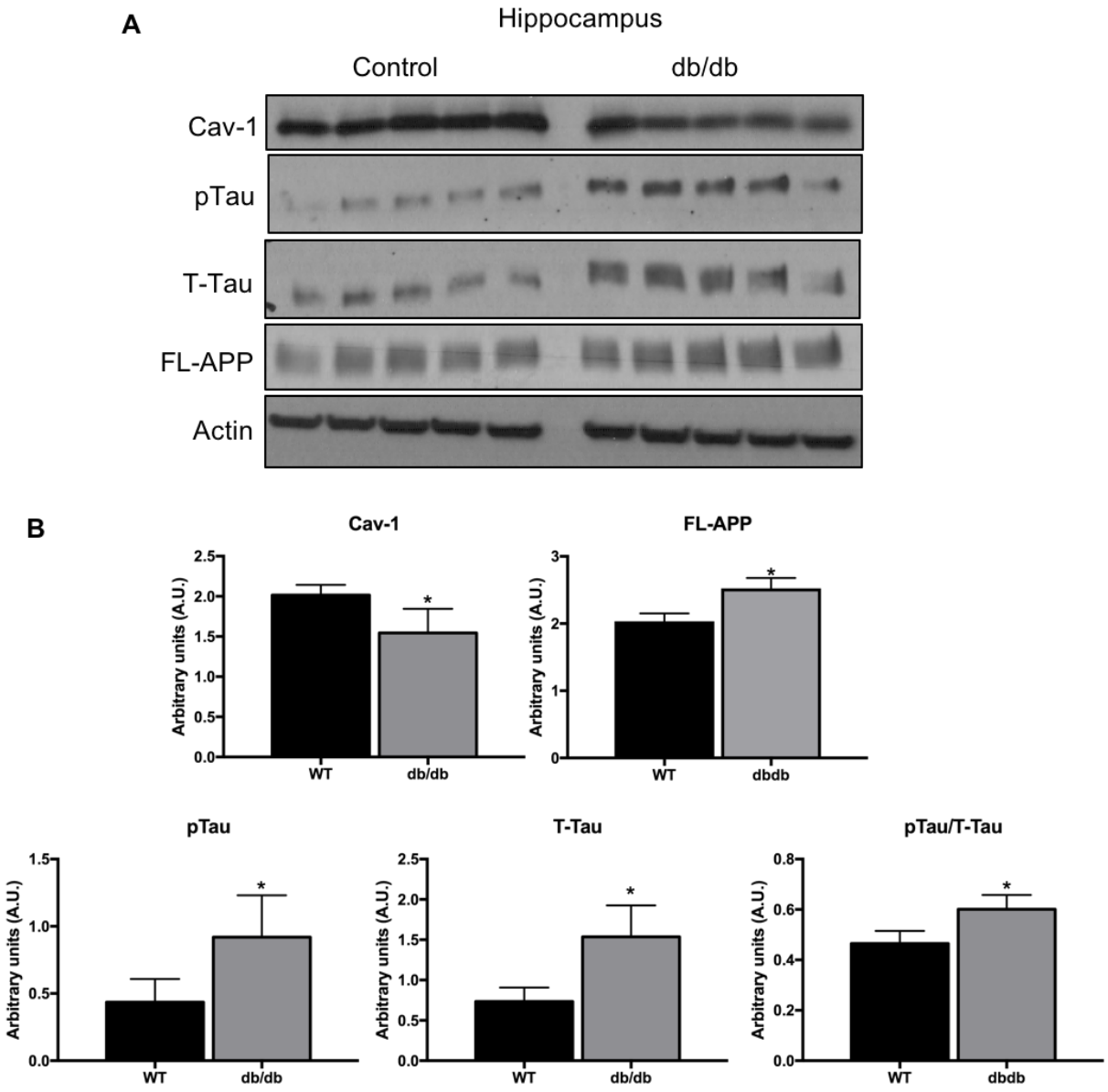

Figure 9. Neuropathological alterations in diabetic db/db mice. A. Comparison of hippocampal lysate from WT control (C57/Bl6) and db/db mice show a significant decrease in Cav-1 expression. Diabetic mice also show a significant increase in FL-APP, as well as in the levels of both pTau (CP13) and T-Tau (DA9), and the ratio of pTau/T-Tau was significantly increased. C. Quantification of panel B. (N=5, Unpaired t-test, *P<0.05).

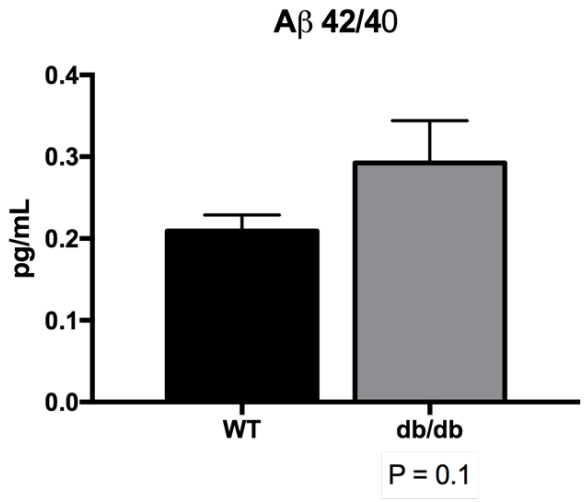

Figure 10. Diabetic mice display a trending increase in ratio of Aβ42/40 in the hippocampus. Analysis of levels of Aβ 42 and 40 were performed by ELISA. The ratio of Aβ 42/40 were calculated for WT and diabetic mice (db/db). The db/db mice show a trending increase in this ratio indicating an increase in Aβ production in the hippocampus. (N = 4, Unpaired t-test, *P<0.05)

3.5 Hippocampal learning and memory impairments in type 2 diabetic mice.

3.5.1 MKR and db/db diabetic mice display deficits in novel object recognition and increased neuropathology.

Object recognition memory is largely dependent on healthy hippocampal function. Thus, hippocampal functioning can be probed using the novel object recognition (NOR) test by taking advantage of the fact that rodents generally display a preference for novel stimuli as compared to familiar stimuli. Here, overall preference is expressed as a percentage of time spent exploring each object.

When testing the control (FVB) and MKR diabetic mice, the FVB displayed a clear and significant preference for the novel object whereas the MKR group did not (Figure 11A). This indicates that the MKR mice are experiencing deficits in hippocampal learning and memory. These results led us to further examine if there were any neuropathological hallmarks in the hippocampi of these mice.

Following our findings in the MKR model, we further examined hippocampal function in the db/db diabetic mice. Results again show that the WT control (C57Bl/6) group display a clear preference for the novel object compared to the familiar object. Expectedly, the db/db group did not show any preference for the novel object indicating some loss in hippocampal function (Figure 11B).

3.5.2 Compromised neurogenesis in the hippocampus of diabetic MKR and db/db mice.

Given the results of the behavioral test, we set out to determine if there were any deficits in neurogenesis that could explain this observation. We thus investigated the quiescent neural stem cell pool ($Nestin^{+}GFAP^{+}$) in both MKR (Figure 12A) and db/db (Figure 13A) mice. Results show that while the MKR mice do not display a deficit in this

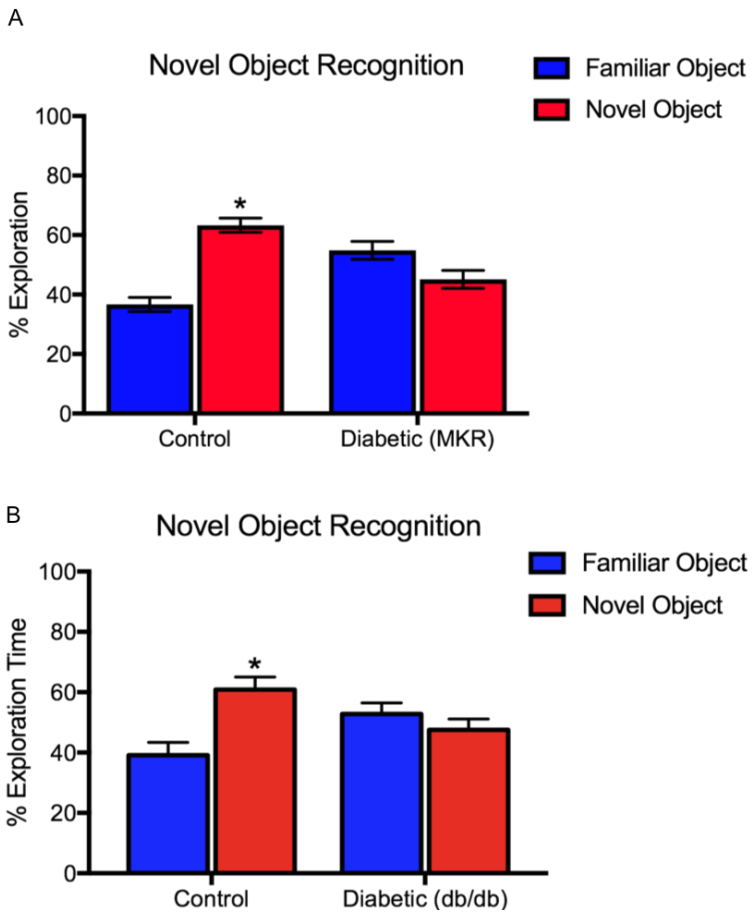

Figure 11. Learning and memory deficits and neuropathological alterations in diabetic mice. A. MKR mice fail the Novel Object Recognition test at 4 months of age, while control mice perform it successfully (N=13, paired t-test, *P<0.05). **B.** db/db mice fail the Novel Object Recognition test at 10-12 weeks of age, while control mice perform it successfully (N=12, paired t-test, *P<0.05).

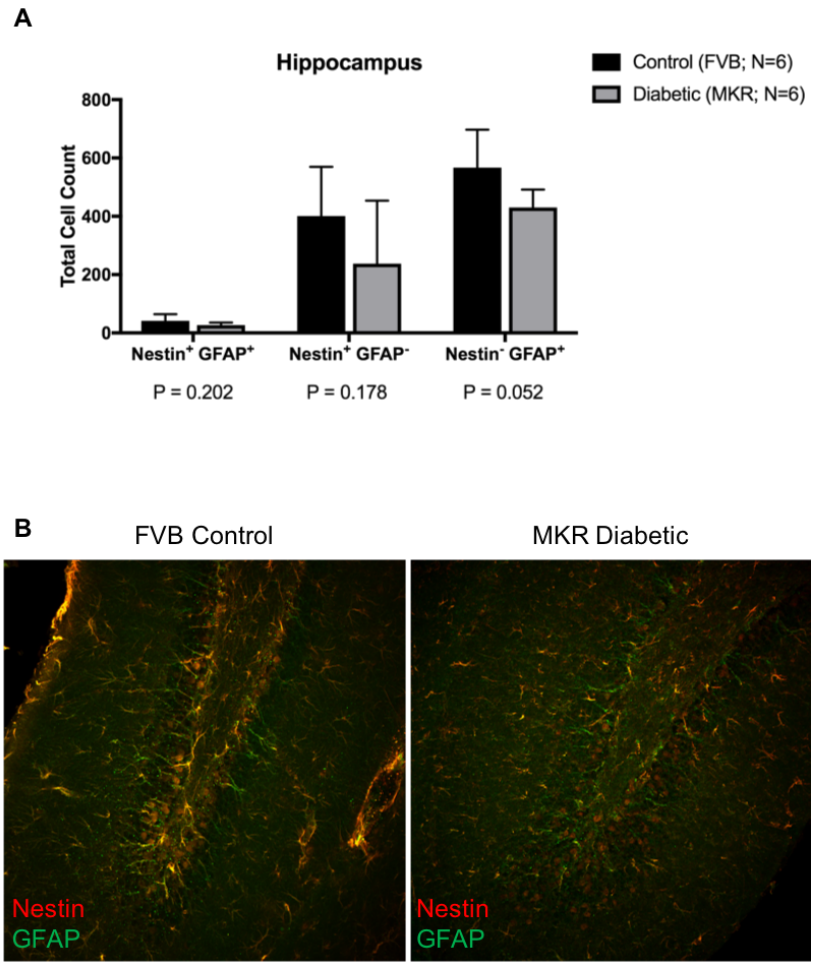

Figure 12. MKR mice display a loss of mature astrocytes in the hippocampus. A. Stereological analysis of the hippocampal quiescent neural stem cell ($Nestin^+$ $GFAP^+$) and $Nestin^+$ $GFAP^-$ populations show no significant declines. However, MKR mice display a trending decrease in the number mature astrocytes ($Nestin^-$ $GFAP^+$). **B.** Representative confocal images (25X). (Unpaired t-test; *P<0.05)

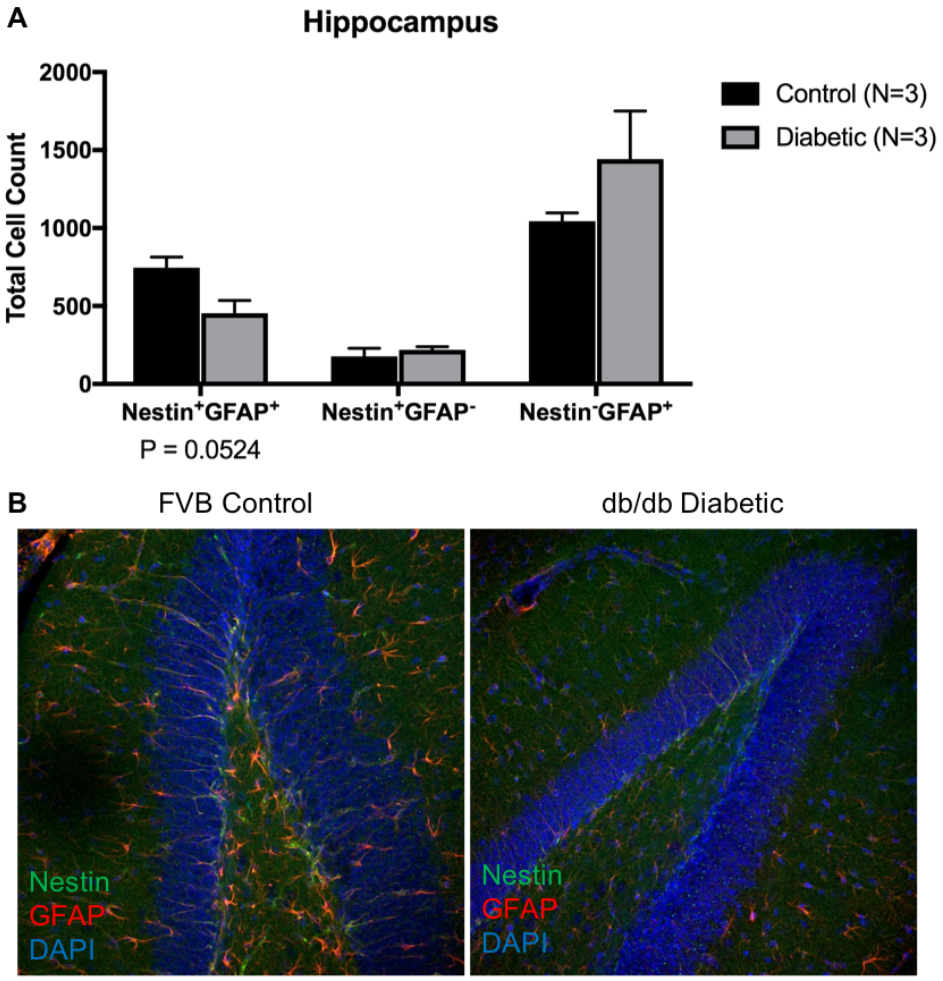

Figure 13. Compromised hippocampal neurogenesis in db/db mice. A. Stereological analysis of the hippocampal quiescent neural stem cell (Nestin^{+} GFAP^{+}) in db/db mice shows a trending decrease (p = 0.0524). No significant decreases in Nestin^{+} GFAP^{-} or mature astrocytes (Nestin^{-} GFAP^{+}) were detected. **B.** Representative confocal images (25X) (Unpaired t-test; *P<0.05)

population, the db/db mice do show a trending decrease in this cell population. Interestingly, the MKR mice showed a trending decrease in the number of mature astrocytes, while the db/db showed a trending increase.

We further analyzed the proliferating and immature neuroblast populations of both MKR and db/db mice. Interestingly, in the MKR group we observe a trending decrease in the number of proliferating neuroblasts (Figure 14A; $Nestin^{+}$ DCX^{+}, $p = 0.056$) and a significant decrease in the number of immature neuroblasts (Figure 14A; $Nestin^{-}$ DCX^{+}, $p = 0.015$). While we do not observe a difference in the proliferating neuroblast group in the db/db mice, we do see a trending reduction in the immature neuroblast population (Figure 15A; $Nestin^{-}$ DCX^{+}, $p = 0.1$). Given that DCX^{+} cells generally go on to become neurons, these results indicate there may be some neuronal loss in diabetic mice.

3.6 Alterations in proliferative capacity of db/db and Cav-1KO neurospheres *in vitro*

Intrigued by the previous results, we set out to determine if we could observe this phenomenon *in vitro*. To this end, we performed a clonogenic assay and measured the number of spheres formed, diameter, area, and number of cells present. Our results interestingly show a decrease in the number of neurospheres formed in the db/db condition compared to both WT and Cav-1KO (the Cav-1KO shows an increase relative to WT; Figure 16A). Measurements of the diameter, area, and number of cells showed that the db/db and Cav-1KO conditions were significantly decreased compared to WT (Figure 16B-D). These results indicate that there may be a contribution of Cav-1 to the compromised ability of db/db cells to proliferate.

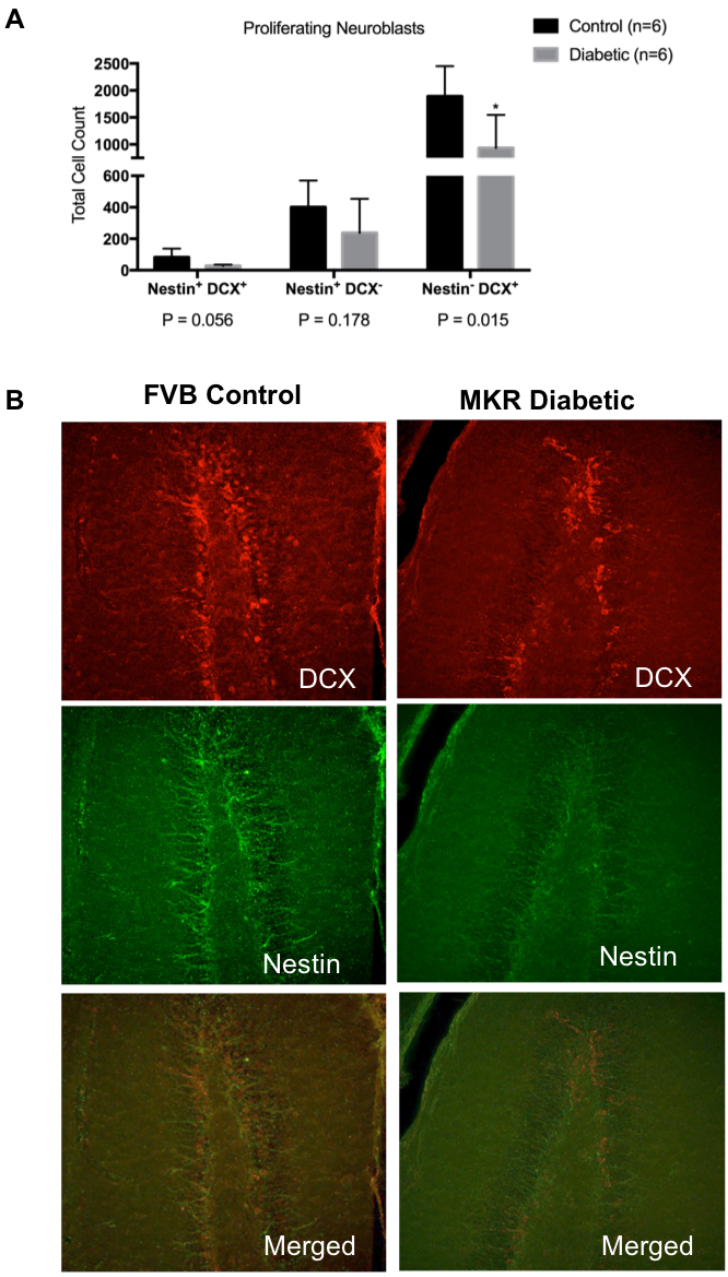

Figure 14. Compromised hippocampal neurogenesis in hippocampus of MKR mice. **A.** Stereological analysis of proliferating neuroblasts in the hippocampus (SGL) of MKR and FVB mice. Results show a trending decrease in proliferating neuroblasts (Nestin^{+} DCX^{+}; p = 0.056) and a significant decrease of immature neuroblasts (Nestin^{-} DCX^{+}) in MKR mice compared to FVB control. **B.** Representative confocal images (25X). (Unpaired t-test, *p<0.05)

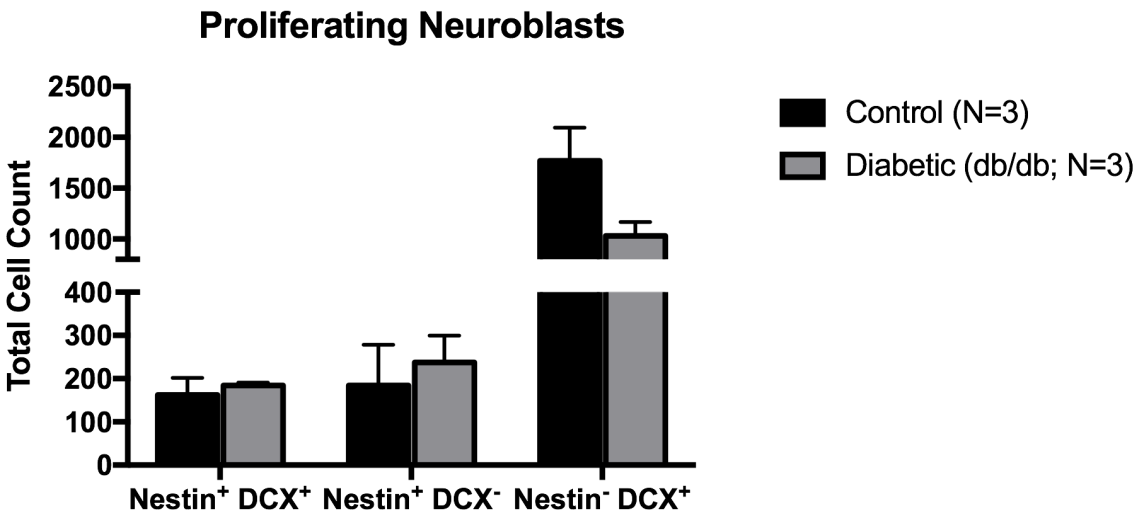

Figure 15. Analysis of proliferating and immature neuroblasts in the hippocampus of dbdb mice. Stereological analysis of proliferating neuroblasts in the hippocampus (SGL) of WT and db/db mice. (Unpaired t-test, *P<0.05)

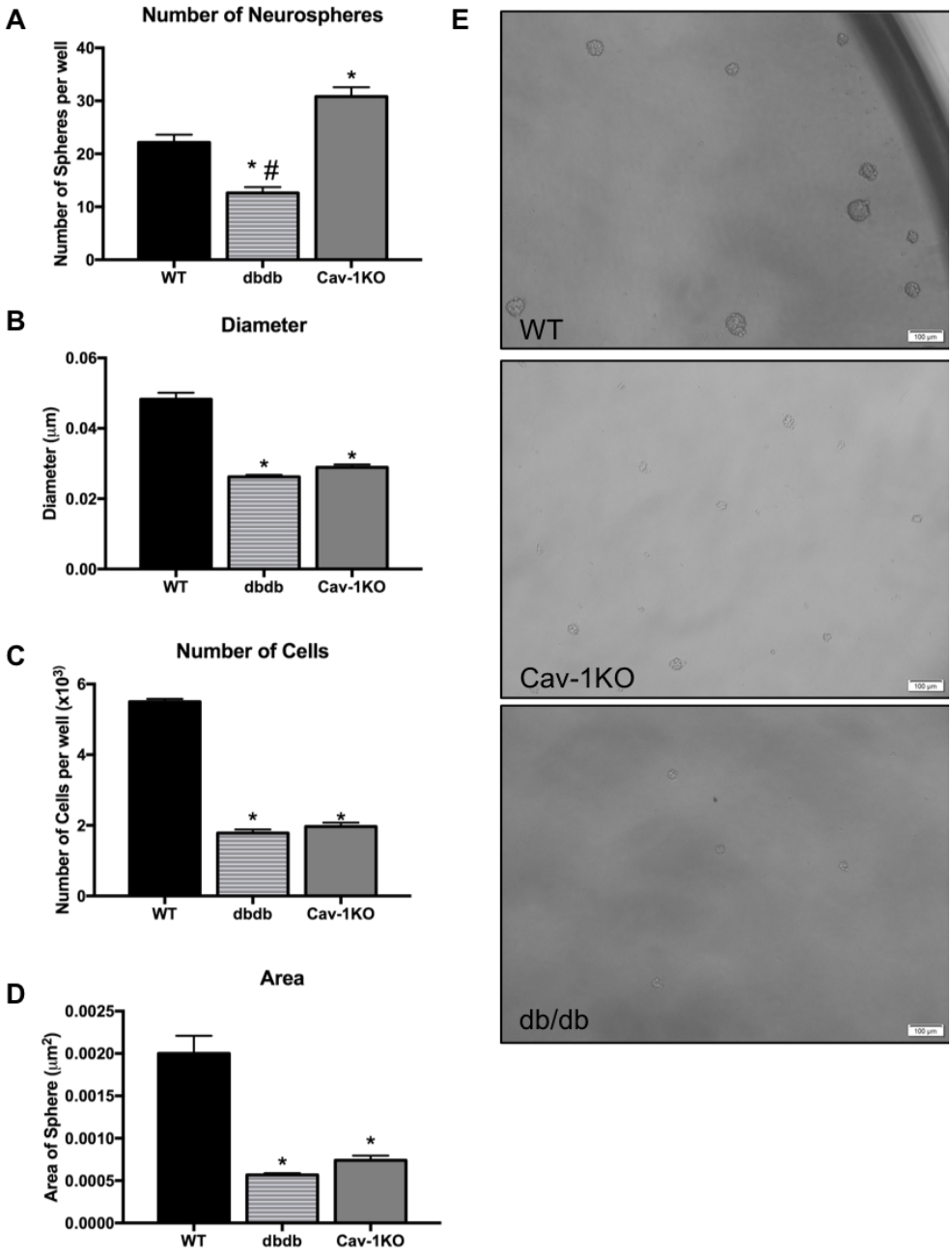

Figure 16. Compromised proliferative capacity of diabetic (db/db) and Cav-1KO hippocampal neural progenitor cells *in vitro*. Clonogenic assay of primary NPCs from WT, db/db, and Cav-1KO. **A.** Analysis of the number of neurospheres formed show a significant decrease in db/db compared to both WT and Cav-1KO. Cav-1KO, however, display a significant increase compared to WT control. Analysis of the diameter **(B)**, number of cells **(C)**, and area **(D)** show that db/db and Cav-1KO neurospheres are comparable and are significantly decreased compared to WT. **E.** Representative images from WT, Cav-1KO, and db/db wells. (One-way ANOVA with multiple comparisons, *P<0.05)

To determine if this is the case, db/db cells were infected with a virus to overexpress Cav-1 as described. Measurements of the same parameters as mentioned above, overexpression of Cav-1 in neurospheres did not have any significant impact on the ability of the db/db cells to regain proliferative capacity (Figure 17A-D). Taken together, these results show that while loss of Cav-1 may contribute to loss of proliferative capacity, rescue of its expression levels alone is not enough to restore it.

3.7 Loss of Cav-1 compromises insulin transport and receptor expression in diabetic (db/db) mice.

Given the results thus far, we were interested to see what other effects Cav-1 loss may have on neurospheres and whether or not this could negatively impact db/db cells. To this end we investigated levels of insulin receptor 1α. Insulin is not generated in the brain itself, it must be transported across the BBB from the periphery. The expression of insulin receptor (IR) in endothelial cells is critical for effective insulin transport into the brain. Once it crosses the BBB it is critical that cells express the receptor for insulin so that it may properly respond. Evidence suggest that caveolae play a critical role in this process as the insulin receptor localizes to the MLR. Thus, we hypothesized that loss of Cav-1 leads to decreased insulin receptor expression, both *in vivo* and *in vitro* and therefore insulin transport across the BBB.

To this end, we investigated mRNA levels of insulin receptor 1α in WT, Cav-1KO, and db/db neurospheres. Results show a significant loss in the Cav-1KO and db/db condition compared to WT (Figure 18A). Furthermore, mRNA levels of insulin receptor 1α in the db/db were significantly decreased compared to Cav-1KO. These results support our earlier data that while Cav-1 loss contributes to the alterations observed in

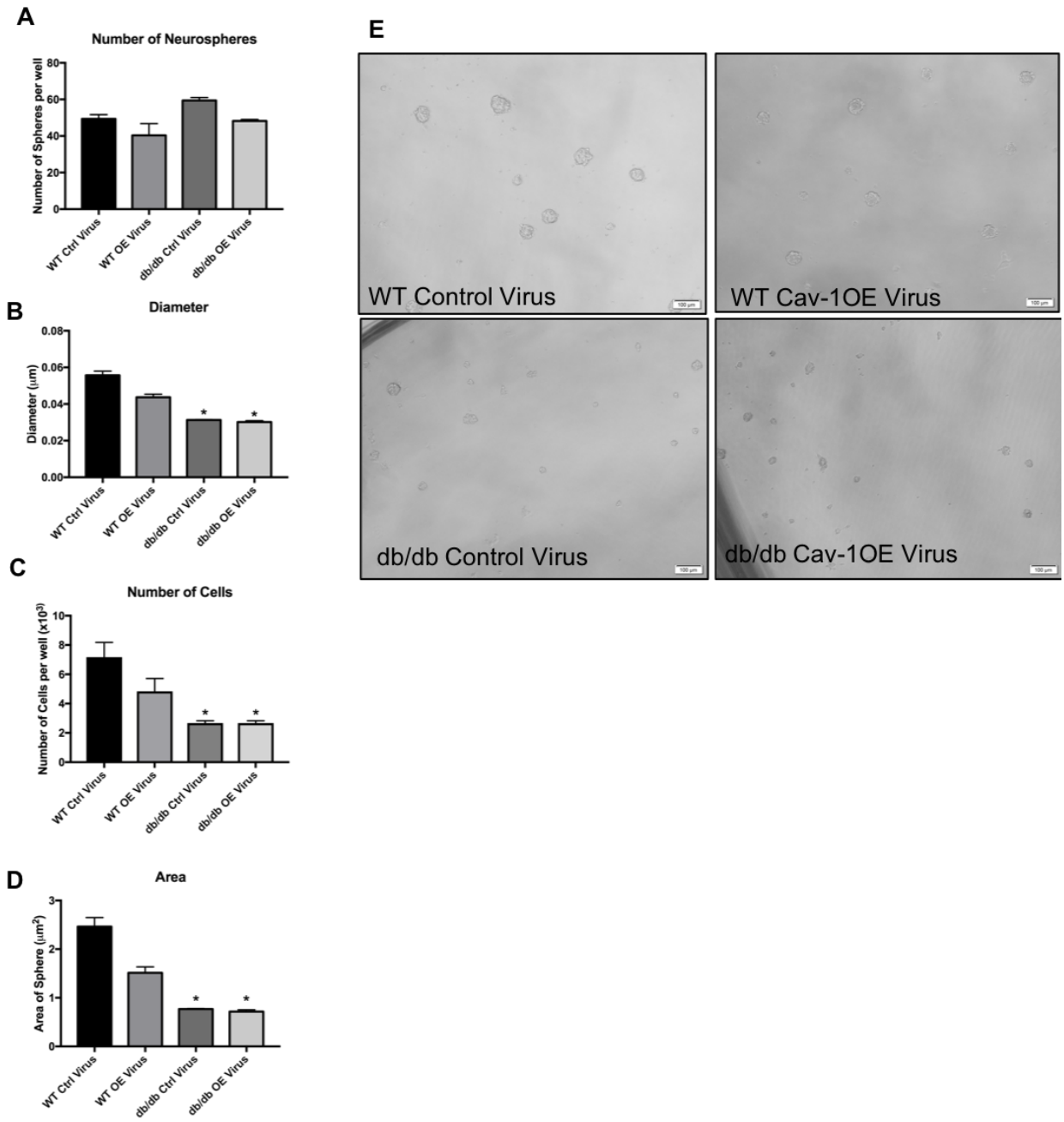

Figure 17. Overexpression of Cav-1 in db/db neurospheres does not rescue proliferation capacity. Clonogenic assay of primary NPCs from WT and db/db infected with a control (ctrl virus) or Cav-1 over-expressing virus (OE virus). **A.** Analysis of the number of neurospheres formed show no differences among groups. However, analysis of the diameter **(B)**, number of cells **(C)**, and area **(D)** show that db/db neurospheres are significantly smaller compared to WT control virus. **D.** Representative images from each condition. (Two-way ANOVA with multiple comparisons, *P<0.05)

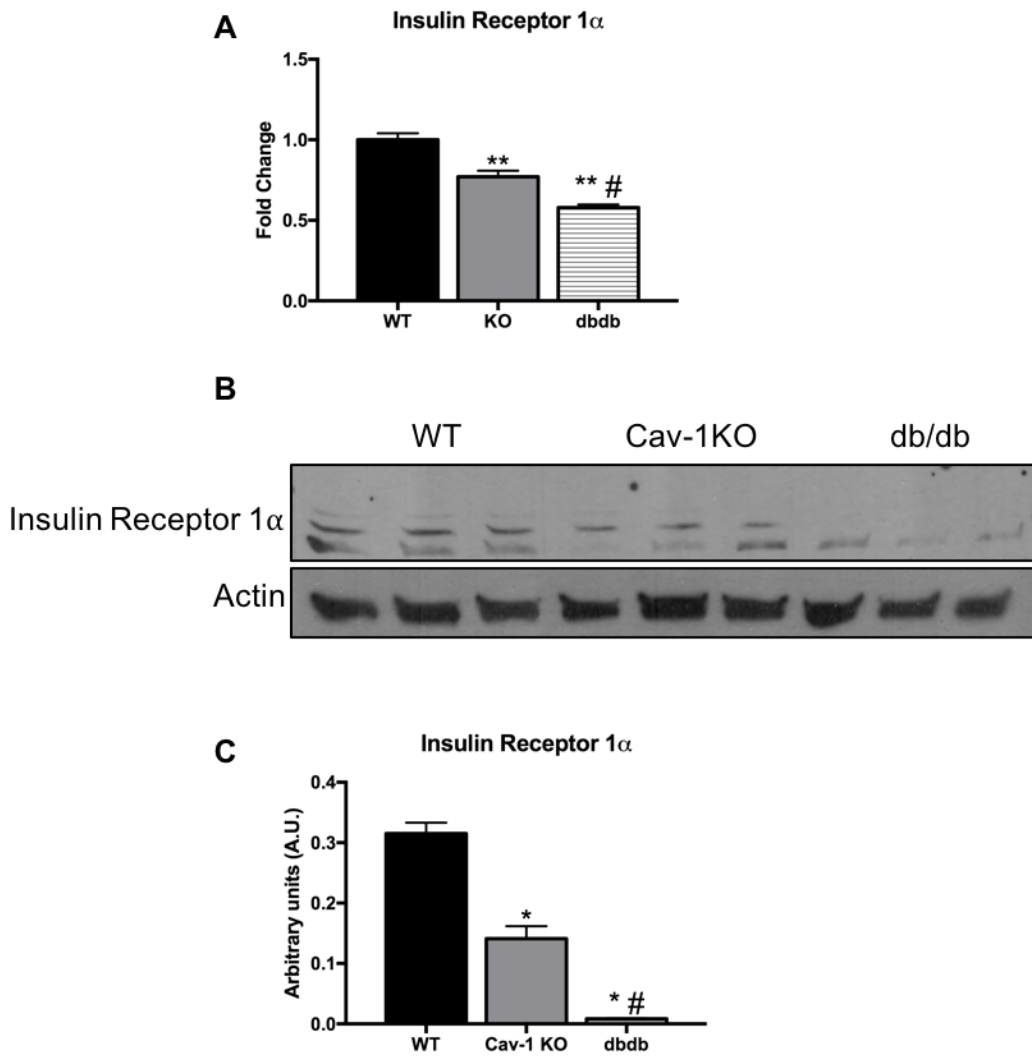

Figure 18. Significant decreases in Insulin receptor in Cav-1KO and db/db mice *in vitro*. Neurospheres isolated from the hippocampi of WT, Cav-1KO, and db/db mice were analyzed for mRNA expression of insulin receptor 1α **(A)**. Results show a significant decrease in Cav-1KO compared to WT, and in db/db mice compared to both WT and Cav-1KO. **B.** Total protein expression of insulin receptor 1α is significantly decreased in Cav-1KO compared to WT*, and in db/db compared to both WT and Cav-1KO$^{\#}$. **C**. Quantification of panel B. (N = 3; Unpaired t-test, * $^{\#}$ P<0.05).

the db/db, there are likely other mechanisms in action. These results were further supported by western blot analysis of insulin receptor 1α protein expression (Figure 18B, C). We further investigated mRNA levels of IGF1R and found that it was also significantly decreased in Cav-1KO and db/db neurospheres (Figure 19).

To confirm our findings in insulin receptor 1α loss *in vivo*, the hippocampus from WT and db/db were isolated and probed for levels of Cav-1 and insulin receptor 1α. Results show a significant loss of the expression in both proteins (Figure 20).

Given our previous finding *in vitro*, we were interested to see if the effects we were observing *in vivo* is due to the loss of insulin receptor 1α as a result of Cav-1 loss. Thus, we investigated if there was any loss of insulin transport across the BBB in the db/db mouse and if this could be restored when Cav-1 is reconstituted in endothelial cells. First, to determine if this resulted in compromised insulin transport, we injected ^{125}I-Insulin into the jugular veins of db/db mice. Brains were removed and dissected after 60 minutes and analyzed for ^{125}I (results are normalized to weight of tissue). Our results show decreased transport of radiolabeled insulin into the HPC of db/db mice (Figure 21). Taken together, we show that as a result of Cav-1 down-regulation and subsequent decrease of IR there is a significant inability to transport insulin across the BBB. Next, we set out to determine if this was a result of Cav-1 loss in endothelial cells. To this end, primary brain endothelial cells were cultured from WT, Cav-1KO and Cav-1RC brains. Results here show that knockout of Cav-1 expression results in insulin receptor 1α levels at levels that were non-detectable. When Cav-1 is reconstituted in endothelial cells specifically (Cav-1RC), the expression of insulin receptor 1α is restored to above WT levels (Figure 22).

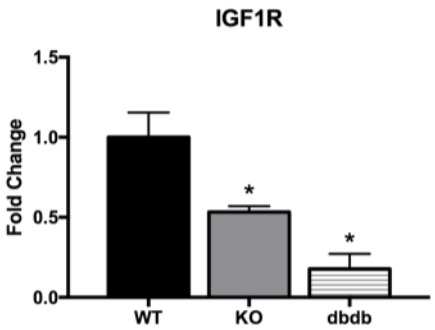

Figure 19. mRNA expression of IGF-1R from primary neurospheres are significantly decreased in Cav-1KO and db/db groups. IGF1R mRNA levels are significantly decreased in Cav-1KO and db/db neurospheres compared to WT. (N= 3; Unpaired t-test, *P<0.05)

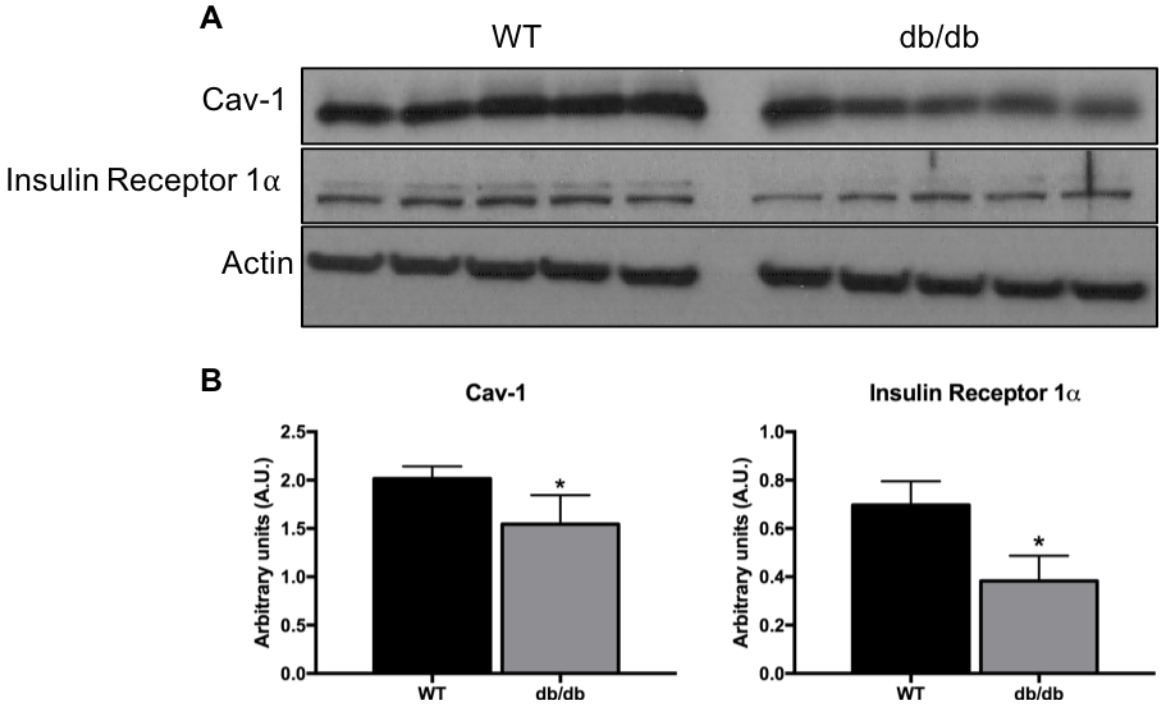

Figure 20. Loss of Cav-1 and Insulin Receptor 1α in Diabetic Mice. A. Western blot analysis of diabetic (db/db) mice show significant decreases in Cav-1 and insulin receptor 1α in hippocampal lysate. **B.** Quantification of **A**. (N = 5; Unpaired t-test, *P<0.05)

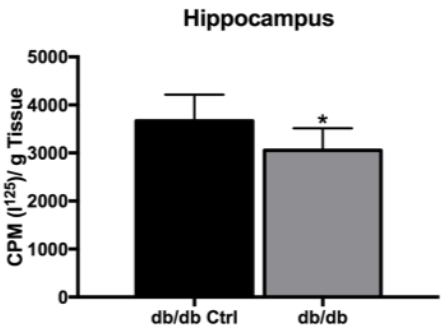

Figure 21. Compromised insulin transport across the BBB in diabetic mice *in vivo*. Radiolabeled insulin injected into the jugular vein and was measured in isolated hippocampi of WT control (db/db control) and db/db mice. Db/db mice show a significant in measured I^{125} counts normalized to weight of tissue (N = 6; Unpaired t-test, *P<0.05)

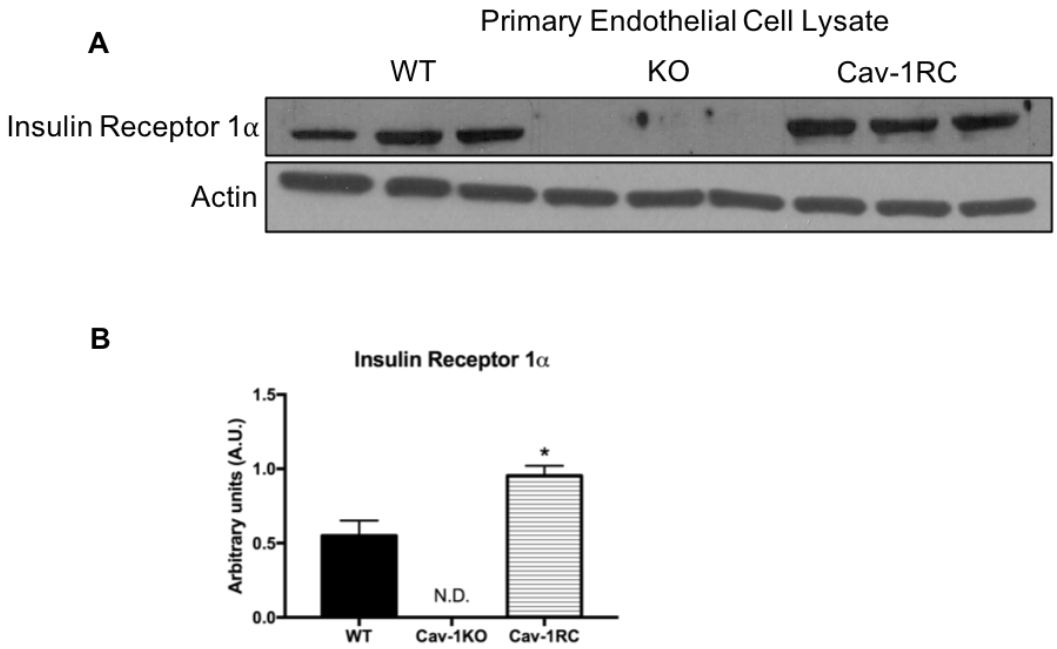

Figure 22. Restoration of Cav-1 in endothelial cells rescues expression of insulin receptor 1α. Primary endothelial cells were isolated from brains of WT, Cav-1KO, and Cav-1RC brains. Western blot analysis reveals nearly non-detectable levels of insulin receptor 1α in endothelial cell lysate. Rescue of Cav-1 restores levels of insulin receptor 1α above WT levels. **B.** Quantification of panel A. (N = 3; Unpaired t-test, *P<0.05)

To determine if this also rescued insulin transport across the BBB, we injected ^{125}I-Insulin as described (results are normalized to weight of tissue and to amount in the blood to give percent injected dose). Results show that rescue of Cav-1 in endothelial cells significantly increases the amount of ^{125}I-Insulin that is able to reach the hippocampus, cortex, and cerebellum (Figure 23A-C).

3.8 Compromised neural stem cell pool in hippocampus of global Cav-1KO: Evidence for a cell autonomous effect.

The role of Cav-1 in the regulation of neurogenesis has not been well described. The following study was performed in order to determine if it is the expression of Cav-1 in either neural stem cells themselves or endothelial cells, or both, regulates neurogenesis. To determine this, we took advantage of several animal models in which Cav-1 has been globally deleted (Cav-1KO), deleted from endothelial cells only (EC Cav-1KO), or reconstituted in endothelial cells only (Cav-1RC). Analysis of the quiescent neural stem cell pool ($Nestin^{+}GFAP^{+}$) in all of these models showed a significant decline in only the Cav-1KO and Cav-1RC (Figure 24). It is important to emphasize here that the Cav-1 RC animals do not express Cav-1 in any other cell type but endothelial cells (Figure 25). Thus, the results indicate that it is expression of Cav-1 in the neural stem cells specifically that regulates this neural stem cell pool.

3.9 Decreased proliferative capacity of Cav-1KO neurospheres *in vitro*.

Given the results we observed *in vivo*, it was critical to understand how loss of Cav-1 affected neurosphere proliferation. To this end, a clonogenic assay performed as described (Demars et al. 2013). Interestingly, we observed that there are significantly more spheres that are formed at the end of the assay (Figure 26A). However, upon further

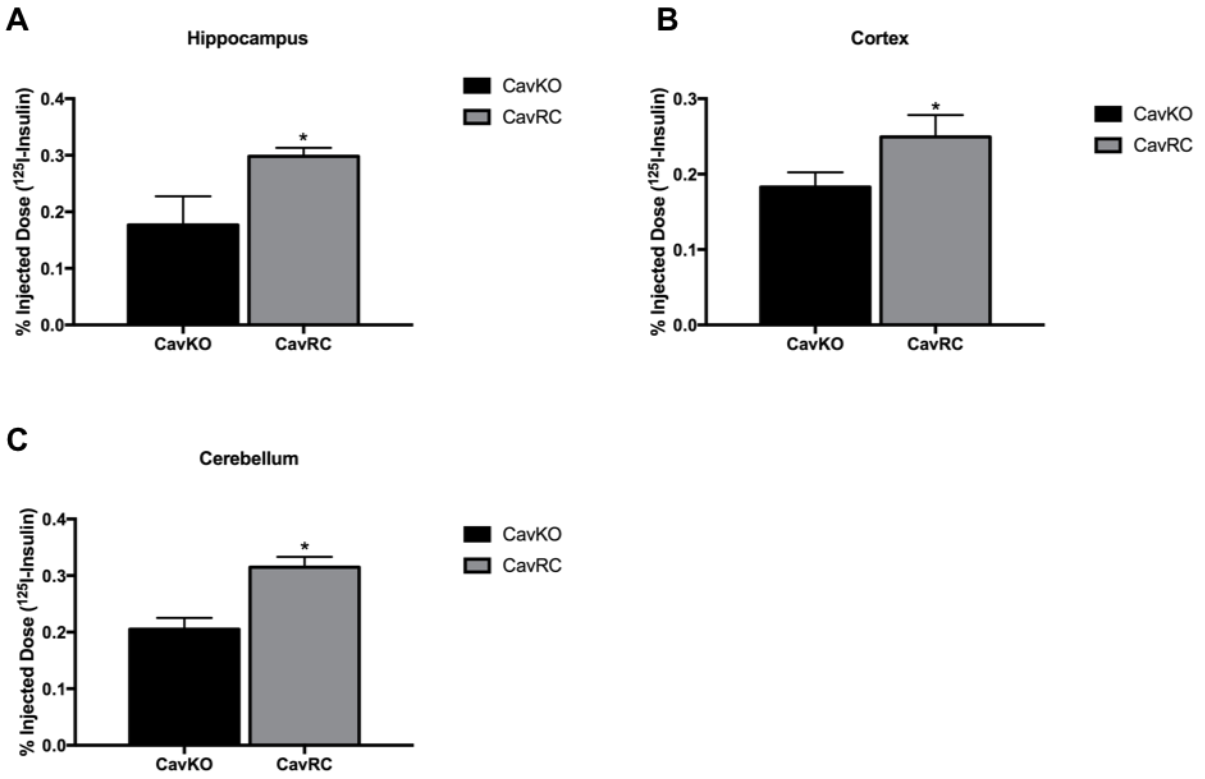

Figure 23. Rescue of Cav-1 specifically in endothelial cells increases insulin transport across the BBB. Radiolabeled insulin injected into the jugular vein was measured 60 minutes after injection. Levels of radiolabeled insulin in the hippocampus (A), cortex (B), and cerebellum (C) were significantly increased in the Cav-1RC group compared to Cav-1KO. (N = 6; unpaired t-test, *P<0.05)

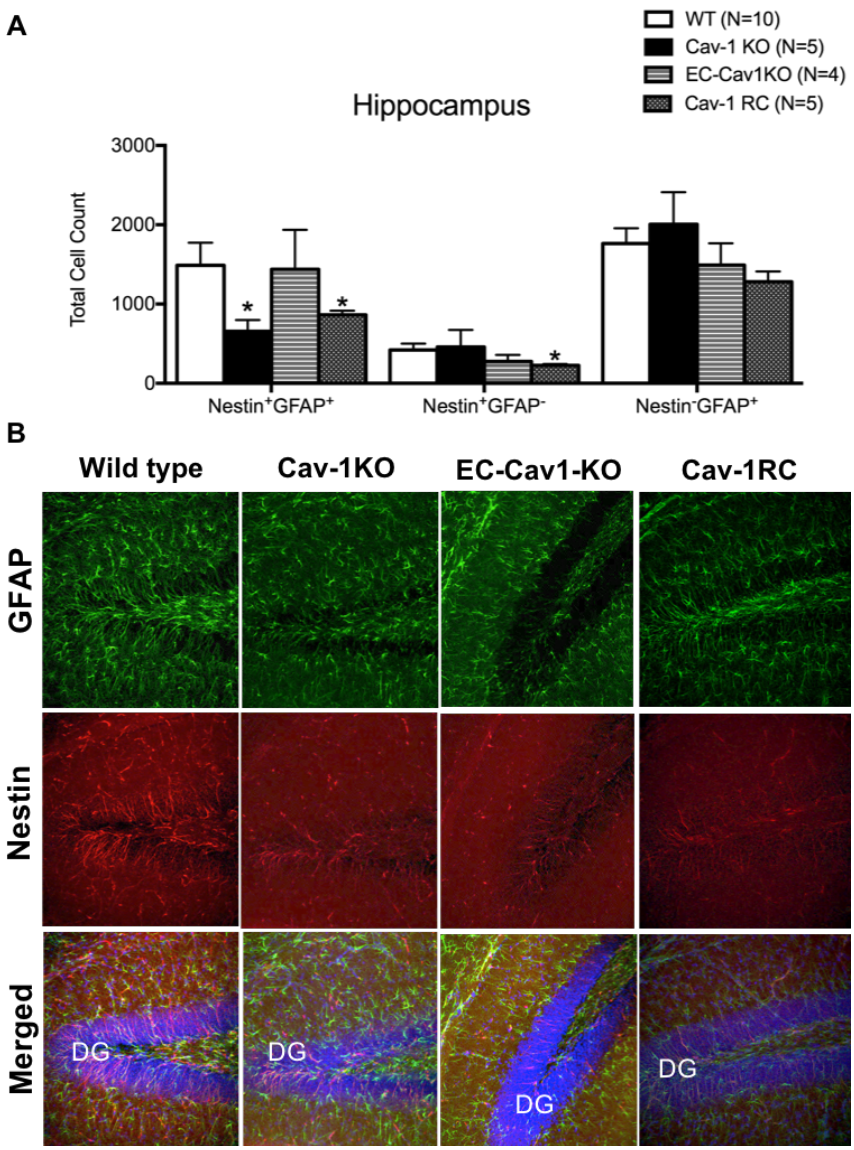

Figure 24. Decreased stem cell pool in Cav-1KO: Evidence for a cell autonomous effect. A. Stereology analysis of Type I neural stem cells ($Nestin^{+}GFAP^{+}$) in the subgranular layer (SGL) of the dentate gyrus in the hippocampus of WT, Cav-1KO, EC-specific Cav-1 KO, and RC-Cav1 mice. Results show significant decreases in the global Cav-1KO and Cav-1RC compared to WT control, while the EC-Cav1KO showed no significant decline in this population. Type II neural progenitor cells ($Nestin^{+}GFAP^{-}$) show a significant decrease only in the Cav-1RC group, and there were no observable changes in mature astrocytes ($Nestin^{-}GFAP^{+}$) among any of the groups. **B.** Confocal images (25x) of neural stem cells ($Nestin^{+}GFAP^{+}$) in the dentate gyrus of the hippocampus of wild type, global caveolin-1 knockout, and endothelial cell specific caveolin-1 knockout mice. (Unpaired ttest with Welch's correction, *$p<0.05$ compared to WT)

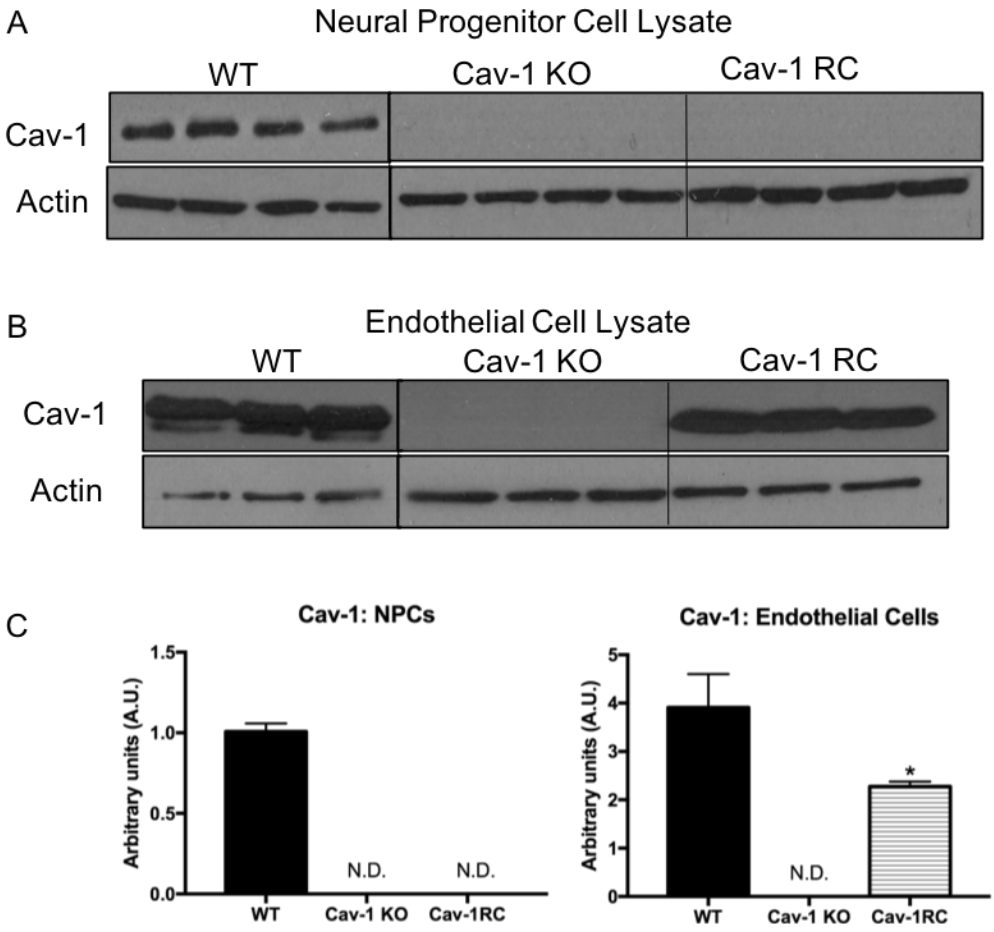

Figure 25. Caveolin-1 expression in primary NPCs and ECs. Western blot analysis of Cav-1 expression in primary (**A**) NSCs and (**B**) ECs from WT, Cav-1KO, and Cav-1RC. Cav-1 expression is not restored to WT levels in the Cav-1RC. **C.** Quantification of panel **A** and **B**. (Unpaired t-test, *P<0.05)

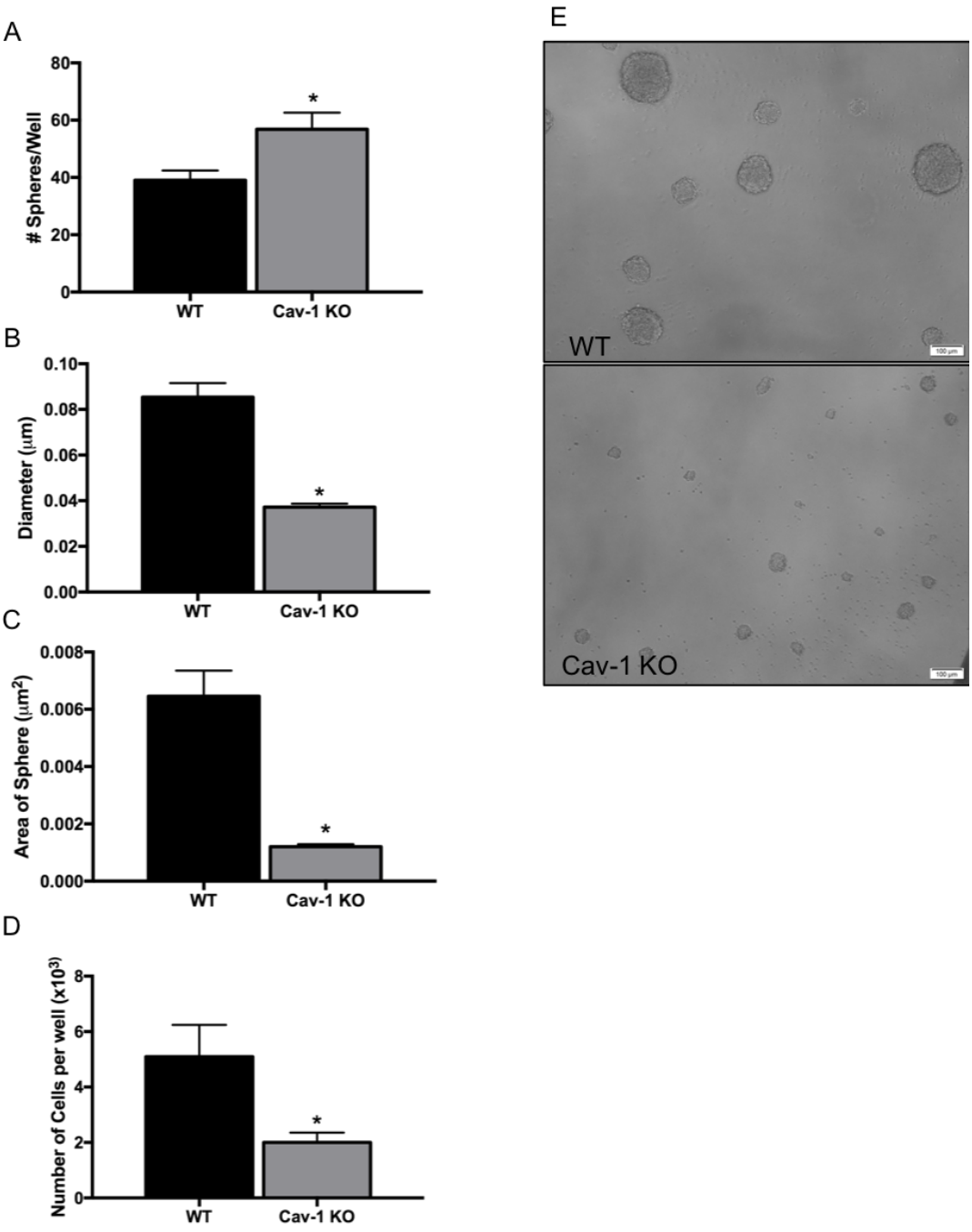

Figure 26. *In* vitro effects of Cav-1 loss on proliferative capacity of NPCs. Clonogenic assay of primary NPCs from WT and Cav-1KO. Cav-1KO show increased number of spheres (**A**), however they are smaller (**B, C**) and contain fewer cells (**D**). **E.** Representative images from each condition. (N = 5; Unpaired t-test, *P<0.05)

investigation we found that these spheres are significantly smaller in diameter and have fewer cells per well (Figure 26B-D). These results suggest that NPCs derived from Cav-1KO mice have a compromised ability to proliferate *in vitro*.

To further assess if this effect could be rescued, neurospheres were infected for 3 days with an adenovirus which increases Cav-1 expression (Ad-Cav-1). Cells were also infected with a control virus containing GFP to track viral infection (Ad-GFP). Following infection, neurospheres were singly dissociated and the clonogenic assay performed. While we observed that the number of spheres formed in the Cav-1KO conditions showed a trending increase compared to the WT controls ($P = 0.0598$), we found that the diameter and of these spheres was also trending toward a significant increase ($P = 0.1$). This result indicates that there the Cav-1KO cells begin to regain the ability to proliferate when Cav-1 is re-expressed (Figure 27). It is important to note that in our control conditions, we observed that overexpression of Cav-1 in the WT condition caused a significant decrease in the diameter of the spheres, as well as a decrease in the number of cells per well. This result indicates that perhaps overexpression of Cav-1 in the WT condition induces some degree of apoptosis (Figure 27).

3.10 Loss of Cav-1 compromises critical neurogenic receptors

Given that there are deficits in the proliferative capacity of NPCs, we next wanted to investigate if there were any changes in the expression and localization of critical neurogenic receptors. First, we examined mRNA levels of the epidermal growth factor receptor (EGFR) and found that they are significantly increased (Figure 28A, B). Intrigued by this result, we next examined if there were any changes in the localization of EGFR by

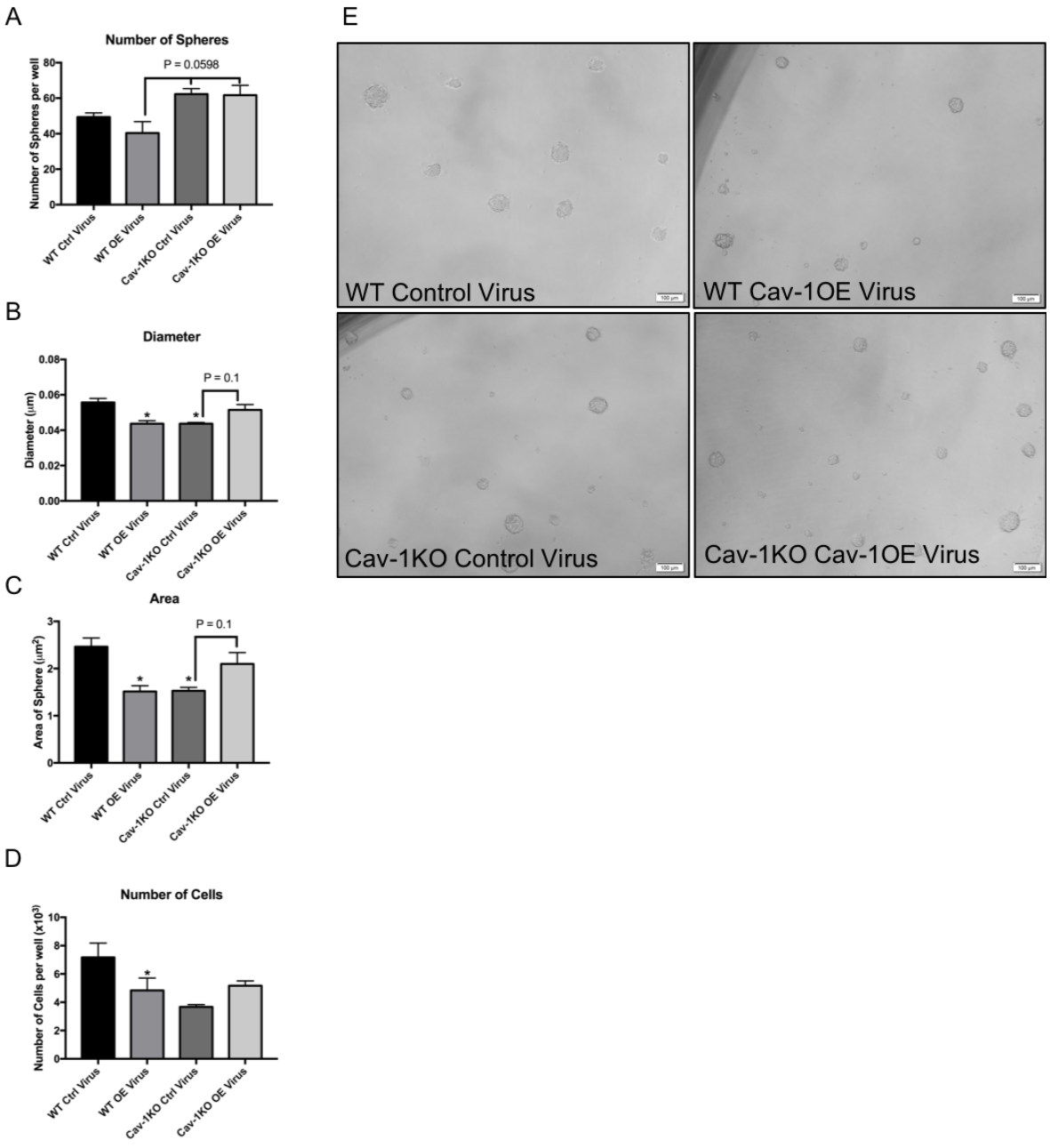

Figure 27. Overexpression of Cav-1 in primary Cav-1KO neurospheres partially rescues proliferation capacity. Clonogenic assay of primary NPCs from WT and Cav-1KO infected with a control (ctrl virus) or Cav-1 over-expressing virus (OE virus). **A**. Analysis of the number of neurospheres formed show a trending increase in the Cav-1KO neurospheres infected with both the control and OE virus. However, analysis of the diameter and area **(B,C)** shows a significant decrease in the WT OE virus condition and a trending increase in the Cav-1KO OE virus condition. **D.** Analysis of the number of cells per well shows a significant decrease in the WT OE virus condition. **E.** Representative images from each condition. (Two-way ANOVA with multiple comparisons, *P<0.05)

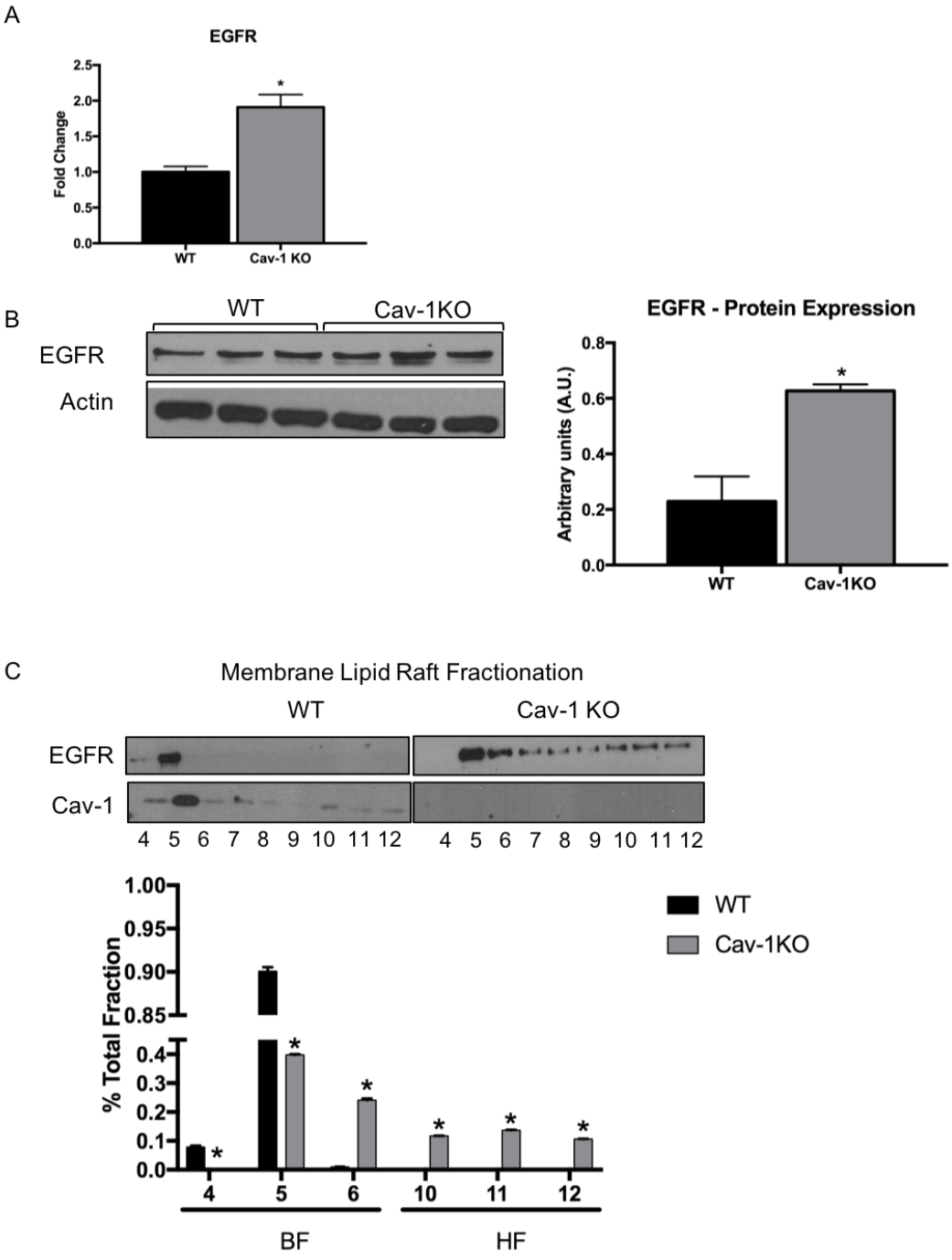

Figure 28. Loss of Cav-1 induces changes in EGFR expression and localization in the membrane. Analysis of EGFR mRNA expression **(A)** and total protein expression **(B)** shows increased levels in Cav-1KO primary neurospheres. **C.** Membrane lipid raft fractionation of WT and Cav-1KO hippocampi analyzed for EGFR expression shows that EGFR is localized mainly to the MLR buoyant fractions (BF 4-6) where Cav-1 is also localized. Loss of Cav-1 induces a significant change in the localization of EGFR to the heavy fractions (HF 10-12) with a significant loss in the buoyant fractions. (N = 3, Unpaired t-test, *P<0.05)

membrane lipid raft fractionation. Results show that in the WT condition, EGFR is tightly localized to the MLR fraction 4-6 (Figure 28C). However, in the Cav-1KO condition, EGFR localization is detected in all fractions indicating that loss of Cav-1 compromises the localization of EGFR. Thus, there seems to be a compensatory mechanism by which EGFR expression in increased as a result of not being localized in the membrane correctly.

There is mounting evidence which suggests there is significant interaction between Cav-1 and the bone morphogenetic proteins (BMPs) (Nohe et al. 2004). BMP levels must be tightly regulated as they are critical negative regulators of neurogenesis. Given that there is a tight interaction between Cav-1 and BMPs, we investigated whether there were any alterations in BMPR1α. Excitingly, we observe an increase in its mRNA expression, as well is in BMPR2, suggesting significant changes in BMP signaling (Figure 29A, B). Given that there is an upregulation of this negative regulator of neurogenesis, we also examined levels of BMI1 (another cell cycle inhibitor gene) and found it to also be significantly increased (Figure 29C). Furthermore, we investigated levels of FGFR to determine if there are any changes in this critical receptor. We found these levels to be significantly decreased (Figure 29D). Taken together, these results suggest that loss of Cav-1 increases negative regulators of neurogenesis thus causing deficits in the proliferative capacity of NPCs, as well as altering the expression and localization of critical growth factor receptors.

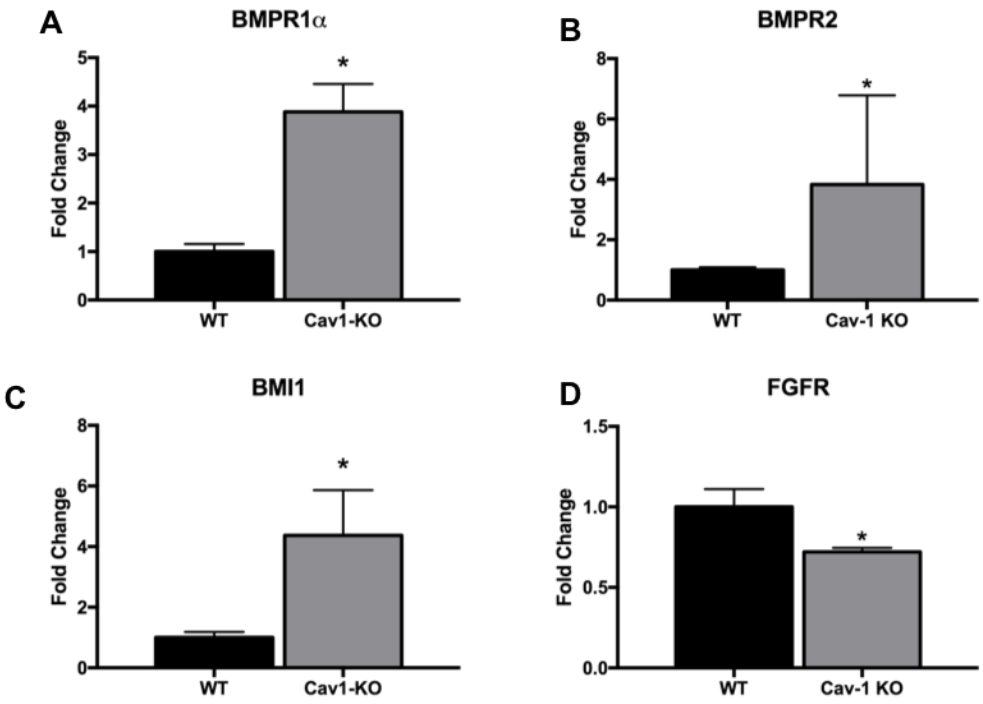

Figure 29. Loss of Cav-1 induces significant alterations in growth factor receptor mRNA expression. Analysis of mRNA expression of **(A)** FGFR shows significantly reduced levels in Cav-1KO neurospheres. However, there are significant increases in cell cycle inhibitors **(B)** BMPR1α, **(C)** BMPR2, and **(D)** BMI1. (N = 3; Unpaired t-test, *P<0.05)

3.11 Treatment with Noggin, a BMP inhibitor, partially rescues proliferative phenotype of Cav-1KO neurospheres

Given our observations that BMPR1α is significantly increased in Cav-1KO neurospheres, we hypothesized that alterations in neurogenesis are being mediated through the BMP pathway. To determine if the phenotype can be rescued, we treated neurospheres with 250ng/mL of noggin, a BMP inhibitor, and performed a clonogenic assay. We found that while there were no differences in the number of spheres, diameter, or area in noggin conditions, we did observe a significant increase in the number of cells in the noggin treated Cav-1KO condition. These results show that the BMP pathway is a significant regulator of proliferation in Cav-1KO, however it is likely not the only pathway that is altered.

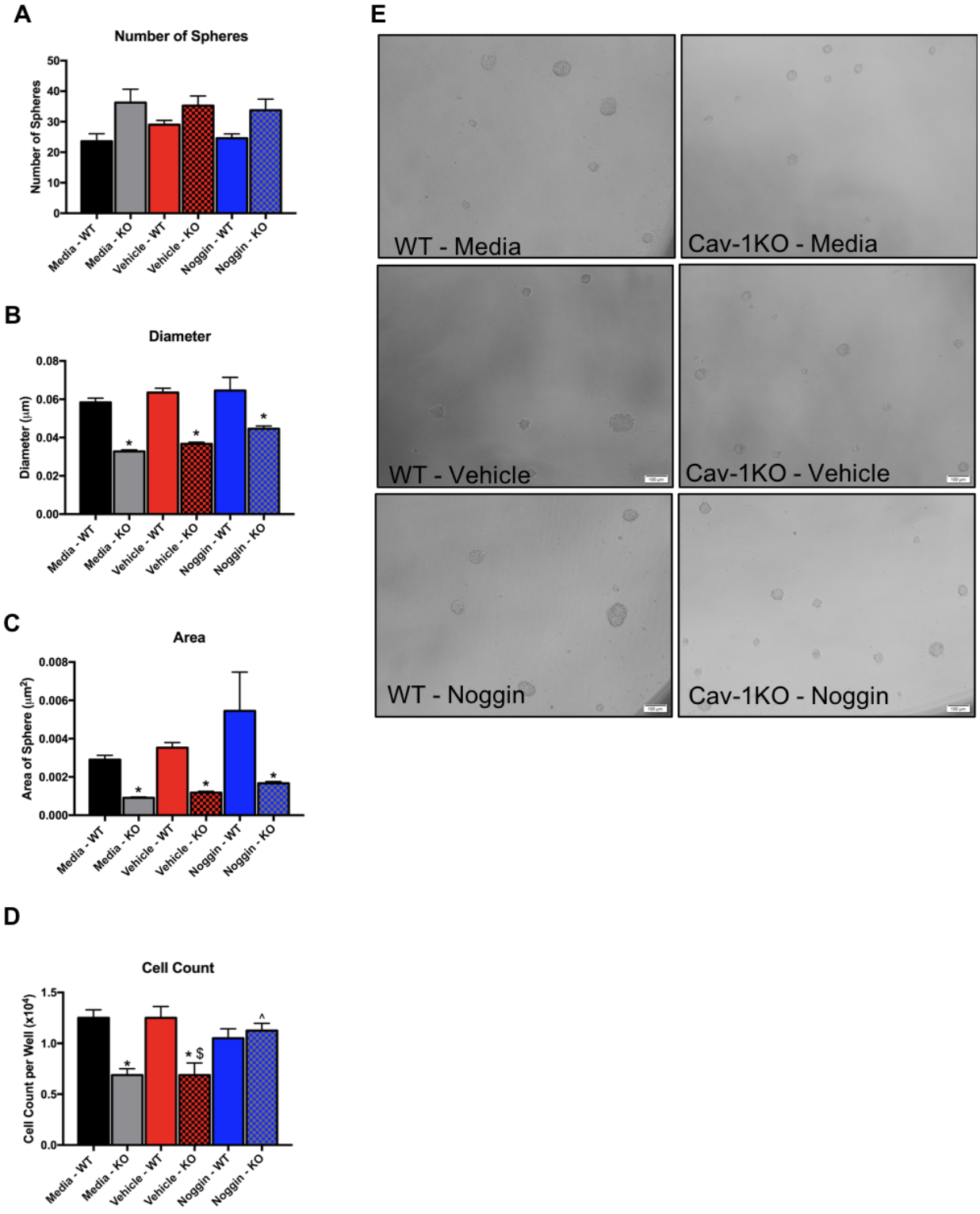

Figure 30. Partial rescue of Cav-1KO phenotype via BMP inhibition with noggin. Primary neurospheres isolated from WT and Cav-1KO hippocampus were cultured under normal conditions (media) or with 250 ng/mL of vehicle (BSA) or noggin. **A,B,C.** The number of neurospheres were analyzed in each condition. There were no significant differences in

the number of neurospheres, diameter, or area compared to respective WT control. **D.** Analysis of the number of cells per well showed that there were fewer cells in the normal and vehicle treated Cav-1KO group. However, in the Cav-1KO noggin treated group, the number of cells were restored to WT levels (*compared to respective WT control, $compared to Noggin WT, ^compared to vehicle KO). D. Representative images from each group and condition. (N = 3/group; Two-way ANOVA with multiple comparisons, * # ^ P<0.05).

4. DISCUSSION

4.1 Chronic inflammation in T2D induces loss of Cav-1 leading to increased AD pathology and compromised neurogenesis.

Many studies regarding AD have focused mainly on the pathology after the onset of cognitive deficits, however it is becoming widely accepted that AD likely begins long before this occurs. In fact, the mechanisms leading to cognitive deficits and the development of sporadic AD are poorly understood. Given the fact that T2D is a major risk factor for AD, it is crucial that the mechanism connecting them be understood. The work presented in this study has provided several critical observations to address this. First, we show that chronic inflammation is present in T2D and negatively affects the expression of Cav-1. Chronic inflammation is known to be elevated in normal aging, as well as in a variety of aging-related diseases, including AD. Our observation that increased pro-inflammatory cytokines depletes endothelial Cav-1 suggests that peripheral mechanisms can initiate an escalating cascade that leads to AD. That means, that in contrast to the previous dogma, dysfunction does not necessarily start in neurons. While increased inflammation in T2D is likely causing multifaceted damage, loss of Cav-1 is one mechanism that causes T2D-induced AD. Thus, this work provides new avenues for therapeutics, by targeting Cav-1 depletion.

We found that loss of Cav-1 is sufficient to accelerate the production of Aβ *in vitro* and *in vivo*, a hallmark of AD neuropathology. This observation indicates that as a result of changes in membrane lipid raft organization there is an increase in APP metabolism. It may be possible that loss of Cav-1 is increasing the activity and/or availability of APP

to its processing enzymes, α-, β- and γ-secretase. To determine if this is the case, experiments designed to measure and/or inhibit secretase activity would be informative.

We found that in the obesity-dependent diabetic model (db/db), there was a trending increase in the ratio of Aβ 42/40 (which reflects a state of pathology) at only 12 weeks of age. The fact that we could almost detect a significant elevation in Aβ 42/40 at this early time point suggests to us that as these mice age they are likely to exhibit amyloid buildup. Aging studies of diabetic mice to follow up with this observation would be critical. Additionally, experiments to further down-regulate Cav-1 in these mice to see if this accelerated any observable neuropathology would perhaps establish a more causal role for Cav-1 in this process. Furthermore, our observation that not only is Cav-1 decreased in the hippocampus of these mice, but that this decrease is progressive with age and disease stage strongly suggests a causal relationship between Cav-1 depletion and disease progression. With age being one of this most prevalent risk factors for the development of AD, if this is coupled with the pathological state observed in T2D then it follows that T2D can accelerate the onset of AD neuropathology via Cav-1 degradation.

We also have observed an increase in phosphorylated tau in both models of diabetes. Tau is phosphorylated by numerous protein kinases such as glycogen synthase kinase-3β (GSK3β), cyclin dependent kinase 5 (CDK5), Protein kinase A (PKA), and mitogen-activated protein kinase (MAPK). Of particular interest is the relationship between PKA and MAPK with Cav-1. First, given that PKA is localized to caveolae and that the presence of the Cav-1 scaffolding domain is required for inhibition of PKA, perhaps loss of Cav-1 leads to increased activity of PKA thus leading to the abnormal phosphorylation of Tau (Razani and Lisanti 2001). Secondly, it is known that Cav-1

regulates the Ras-Raf-MEK-ERK pathway. An important kinase in this pathway is MAPK which could be abnormally active as a result of Cav-1 loss. Given our observation that there is increased pTau expression in diabetic animals, it would be of great interest to determine if increased kinase activity directly related to Cav-1 expression is responsible. Experiments designed to measure kinase activity as a direct result of Cav-1 loss would be informative.

It has been well established that impaired neurogenesis occurs early on in the development of AD, and that the hyperphosphorylation of tau contributes to impaired neurogenesis (Demars et al. 2010). Given that we observe an increase in AD pathology in T2D mice, and that neurogenesis plays an important role in hippocampal dependent learning and memory, we investigated if there were any observable deficits in hippocampal neurogenesis that could explain the apparent cognitive deficits seen in the models of T2D. Our observation that the neuroblast population is affected in the diabetic model is significant since this may indicate a loss of mature neurons that can be incorporated into the hippocampal network, thus having a negative impact on cognition. To more directly address if this is the case, follow up studies should investigate if there is a decrease in the number of mature neurons in the GCL of the hippocampus. Furthermore, functional and morphological studies of these neurons (for instance, measurements of dendritic branching and spine density) can be performed to determine if there are any deficits as a result of compromised neurogenesis. In addition, rescue of Cav-1 in neural progenitor cells specifically in mouse models of T2D may inform us of the role of Cav-1 in impaired neurogenesis in T2D and whether this restoration has therapeutic value on brain function. On the other hand, future experiments rescuing

endothelial Cav-1 may inform us of the effect on the vascular niche of neural progenitor cells and its implications for brain function in T2D.

We have also observed that there are significant deficits the ability of NPCs from db/db and Cav-1KO to form neurospheres. However, rescue of Cav-1 alone was not sufficient to restore the proliferative capacity *in vitro*. These results led us to believe that there are other mechanisms at work that are independent of Cav-1 which influence neurogenesis. Given our observations that there are significant deficits in insulin receptor expression in NPCs, one possible explanation is that there are changes in insulin receptor signaling which has been shown to be important for neural stem cell homeostasis and proliferation and is also compromised in T2D (Ziegler, Levison, and Wood 2015, Erickson et al. 2008).

4.2 Endothelial Cav-1 expression is critical for insulin transport across the blood-brain barrier.

Caveolae, and Cav-1, are abundant in endothelial cells and is home to several important receptors, which have a wide range of functions. One of these receptors is insulin receptor 1α which functions to bind and respond to insulin, a critical regulator of cell proliferation, differentiation and growth (Lee and Pilch 1994). We observe that depletion of Cav-1 downregulates IR1α and compromises insulin transport, however it is yet to be determined whether insulin is actively transported via receptor-ligand interactions into the brain. Future experiments are warranted to address the link between reduced insulin transport and decreased expression of IR in endothelial cells in the BBB. Nevertheless, we show that less insulin reaches the brain in T2D. We provide preliminary evidence that this negatively affects neurogenesis, but we cannot exclude the possibility

that the effect on neurogenesis is indirect. For example, it has been shown by other groups that Aβ levels can be enhanced by reduced insulin signaling via alterations in GSK3β activity which also affects tau phosphorylation (DaRocha-Souto et al. 2012, Cholerton, Baker, and Craft 2013). Given our observation that reconstitution of Cav-1 specifically in endothelial cells increases insulin transport across the BBB, it is possible that increasing expression of Cav-1 in endothelial cells of diabetic mice may be able to restore insulin transport. Furthermore, coupled with our previous observation that there are deficits in insulin receptor expression in NPCs leads us to conclude that there are significant deficits in the insulin signaling pathway which could negatively affect neuronal function and possibly enhance Aβ production. Future experiments should aim to uncover the link between Cav-1 and GSK3β activity, insulin transport, and neuronal function.

4.3 Regulation of neurogenesis by Cav-1

Next, we determined that endogenous neural Cav-1 plays a critical role in neurogenesis. Given that even very low concentrations of insulin are enough to stimulate proliferation in NPCs, perhaps this observation could be explained by the fact that enough insulin is still able to cross the BBB via caveolae-independent mechanisms (Erickson et al. 2008). We also observed that there were deficits in neurogenesis in the Cav-1KO and Cav-1RC mice. Taken together with our earlier results that loss of Cav-1 in NPCs results in loss of insulin receptor 1α, it follows that even if insulin were to make it across the BBB there needs to be adequate expression of its receptor for it to have an effect.

In support of earlier findings, we found that loss of Cav-1 compromised proliferative capacity of neurospheres. EGFR is known to stimulate cell growth, proliferation, and differentiation. Importantly, it has also been found to be associated with lipid rafts and its

localization to this region of the membrane is vital for its ability to bind its specific ligands (Freeman et al. 2007). Given that it's localization is critical for proper activation of its signaling cascade, and that we observe it to be mislocalized in the Cav-1KO condition, we hypothesize that loss of Cav-1 MLRs results in some kind of compensatory mechanism where more EGFR is produced but cannot be properly maintained in its correct location in the membrane. Clathrin-mediated endocytosis is a critical pathway for EGFR internalization resulting in attenuation of its signaling (Sigismund et al 2008). If there is loss of EGFR localization to the MLR, it may be possible that it is now mainly localized to clathrin-coated pits, thus resulting in increased internalization and loss of signaling.

We were then interested to see if there were any changes in negative regulators of neurogenesis. One candidate is the bone morphogenetic protein receptors (BMPR). Their signaling mediates is known to mediate neural progenitor cell proliferation (Brooker et al. 2017). Activation of these receptors leads to the phosphorylation of SMAD1/5/8 which then translocates to the nucleus to induce changes in gene expression. Excitingly, we found mRNA levels of BMPR1α and BMPR2 to be significantly increased in Cav-1KO neurospheres compared to WT. In support of this, pSMAD1/5/8, a downstream target of BMP signaling, is also increased. Target genes of BMPR include GATA3 which is critical for normal development of the nervous system, as well as Id1 and Id3 which are both important for cell growth, senescence, and differentiation. Early differentiation of NPCs can result in cell death and/or compromised neuronal morphology. Thus, proper control of these genes is critical for the proper maintenance of the neurogenic niche.

Additionally, it has been shown that BMP signaling increases substantially during aging in both the mouse and human hippocampus, and that inhibition of BMP signaling can enhance neurogenesis (Meyers et al. 2016). To fully determine if Cav-1 mediates neurogenesis via upregulation of BMP signaling we utilized noggin, a BMP inhibitor. Our observation that noggin treatment restores the number of cells in the Cav-1KO condition back to levels comparable with control indicates that loss of Cav-1 expression in neural progenitor cells enhances with BMP signaling thereby inhibiting neurogenesis, and potentially leading to compromised hippocampal plasticity. Further experiments to determine if there is a correlation between Cav-1 depletion and increased BMP signaling would be important to address if there is a causal relationship.

In summary, this work unravels a novel molecular mechanism underlying the development of cognitive deficits and AD-associated neuropathology in T2D. We have shown that as a result of chronic inflammation, Cav-1 is depleted in several cell types within the brain. This in turn leads to increased amyloidosis, decreased insulin transport, as well as alterations in critical regulators of neurogenesis. Because we have been able to identify a point of convergence between T2D and AD, it is possible that this offers a valuable target for future therapies aiming to improve cognitive deficits and potentially attenuate the development of neuropathology in T2D patients.

5. CONCLUSIONS AND FUTURE DIRECTIONS

The elucidation of the molecular mechanism linking T2D and AD is necessary for both understanding how these diseases are interlinked, as well as for the potential development of treatment strategies for those who have T2D to help prevent the progression to AD. This study shows that depletion of Cav-1 in T2D compromises insulin access to the brain regions critical for learning and memory and alters APP and tau metabolism leading to the development of sporadic Alzheimer's disease.

Previous studies have shown that T2D induces a chronic inflammatory state and that there are several cerebrovascular complications. Taken together with these previous findings, the work here shows, for the first time, that this chronic inflammatory state induces depletion of Cav-1 in endothelial cells, neurons, and neural progenitor cells in the hippocampus. This in turn leads to increases in amyloidosis and tau pathology, compromised vasculature, decreased insulin transport, and alterations of critical regulators of neurogenesis. Collectively, these events cause neuronal dysfunction and compromised neurogenesis leading to learning and memory impairments and AD neuropathology.

There are several follow up studies to be done in order to prove unequivocally that rescue of Cav-1 in the vasculature and in neural stem cells can restore the pathologies observed in T2D-induced AD. To this end, three novel mouse models would be essential to this understanding. The first animal model would be to cross the AD mouse (APPswe/PS1ΔE9) with the global Cav-1KO to determine if Cav-1 loss accelerates and/or enhances AD *in vivo*. The second animal model would aim to generally increase expression of Cav-1 in T2D mice to determine if this could ameliorate the development of

T2D and AD-associated neuropathologies. Lastly, creation of a T2D model in which Cav-1 has been reconstituted in endothelial cells would be informative to observe if this could rescue cerebrovascular abnormalities that are known to occur in T2D as well as restore insulin transport into the brains of these mice.

Next, we would like to determine if loss of Cav-1 specifically from NSCs contributes to cognitive decline and compromised neurogenesis. To this end, there are two major experiments that could be done. First, would be to generate a novel mouse model by crossing the Nestin-Cre and Cav-1 floxed lines to specifically remove Cav-1 from NSCs. This would provide crucial information about how Cav-1 influences the neurogenic niche. The second major experiment would be the targeted overexpression of Cav-1 in neural stem cells. This would require the generation of a virus in which drives Cav-1 expression under the nestin promoter so as to target NSCs specifically.

In summary, the goal of this thesis was to unravel the role of Cav-1 in T2D induced AD. The data presented here offers novel information concerning the link between these two diseases. Coupled with the experiments described above, our data would provide clear evidence of the importance of Cav-1 in the development of T2D-induced AD and potentially importantly offer novel targets to stop the progression of these diseases.

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APPENDIX 1

Supplementary Figures

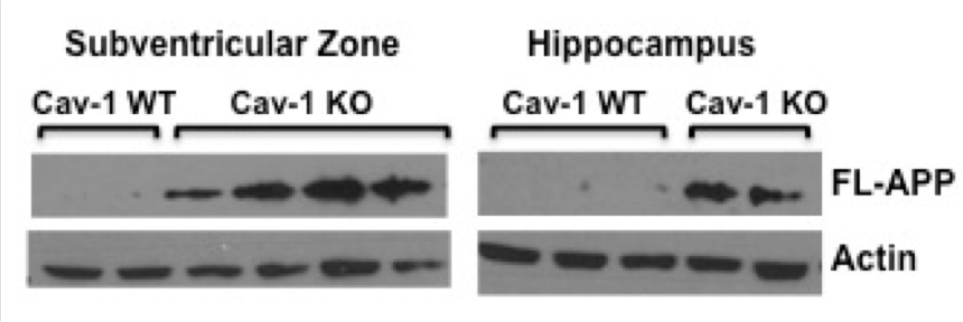

Figure S1. Loss of Cav-1 induces increases in FL-APP in the subventricular zone and hippocampus of aged mice. Comparison of levels of FL-APP in lysate prepared from the subventricular zone (SVZ) and hippocampus (HPC) of WT and Cav-1KO mice at various ages (SVZ: WT Lane 1 and 2 – 6 months, Cav-1KO Lane 3 – 7 months, Lane 4 – 12 months, Lanes 5 and 6 – 20 months; HPC: WT Lane 1 and 2 – 6 months, Lane 3 and 4 – 12 months).

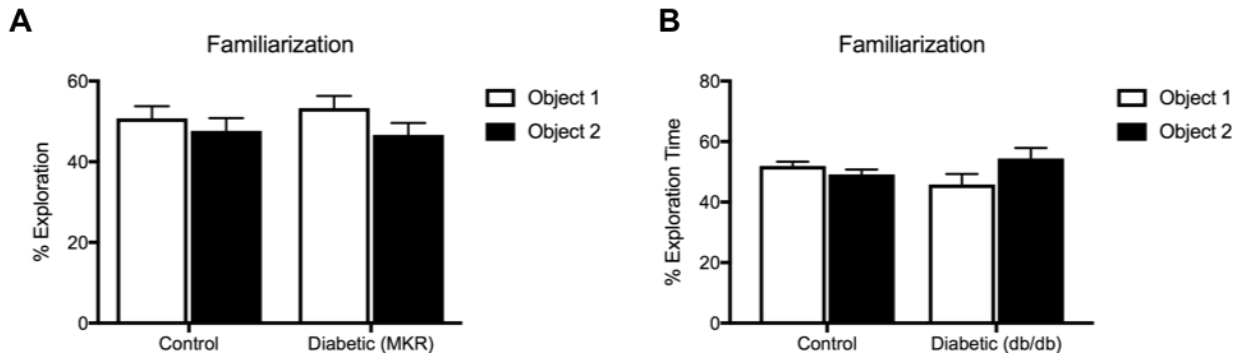

Figure S2. Familiarization of MKR and db/db mice in the novel object recognition test. Familiarization days 1 and 2 with identical objects in the novel object recognition test. On testing days 3 and 4, objects were placed on the non-preferred side of the arena.

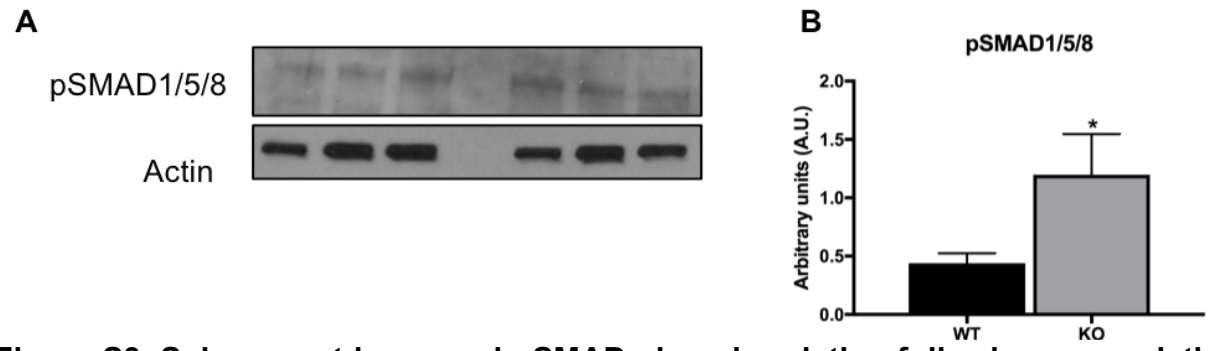

Figure S3. Subsequent increase in SMAD phosphorylation following upregulation of BMPR in Cav-1KO. A. Western blot analysis of pSMAD1/5/8 shows a significant increase in Cav-1KO neurospheres compared to WT control. **B.** Quantification of panel A. (N = 3; Unpaired t-test, *P<0.05)

APPENDIX 2

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APPENDIX 3

UIC

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Permission Clarification

2 messages

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Please advise if there is anything else that is required.

Thank you,
Jacqueline Bonds

--
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Curriculum Vitae

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EDUCATION

University of Illinois at Chicago, Chicago, IL **August 2011 – Present**
- Predoctoral Fellow, Graduate Program in Neuroscience

University of San Diego, San Diego, CA **August 2005 – May 2009**
- Bachelor of Arts in Biochemistry, Minor in English

RESEARCH EXPERIENCE

Laboratory of Dr. Orly Lazarov, University of Illinois at Chicago **June 2012 – Present**
Graduate Student, Department of Anatomy and Cell Biology
- Multi-photon Microscopy
- Middle cerebral artery occlusion in mice
- Neural progenitor cell and Primary endothelial cell isolation from mice
    - Culturing and Confocal imaging
- Training of medical and undergraduate students in laboratory techniques
- Co-advisor: Drs. Dale Pelligrino and Richard Minshall

Research Rotations, University of Illinois at Chicago **October 2011 – May 2012**
Graduate Student, Department of Biological Sciences
- Brain microvascular endothelial cell isolation and culture
- Neuromuscular motor junction isolation and immunofluorescence staining using *drosophila melanogaster*

Veterans Administration Medical Center, UCSD **January 2007 – August 2011**
Laboratory Technician, Department of Anesthesiology
- Lab manager - biochemistry component of the neuroscience and cardiac research group
- Controlled cortical impact model for traumatic brain injury in mice
- Stereotaxic intracerebral viral vector delivery
- Isolation and culture of mouse primary neurons and adult rat cardiac myocytes
- Isolation of membrane lipid rafts, synaptosomes, and mitochondria

Molecular and Genomic Imaging Center, Washington University in Saint Louis **Summer 2007 and 2008**
Summer Research Intern, Department of Genetics
- Performed DNA extractions from dried blood spots collected from Guthrie cards for pooled genomic analysis for the study and quantification of single nucleotide polymorphisms
- Shadowed in Hematology and Oncology Clinic at the Children's Hospital of Saint Louis

FUNDED AWARDS

Department of Anatomy and Cell Biology NIH Training Grant **August 2017 – August 2018**
Predoctoral Fellow (T32 1T32AG057468)
- Orly Lazarov, PhD – University of Illinois at Chicago

Midwestern Affiliate 2014 Predoctoral Fellowship **July 2014 – 2016**
- American Heart Association (14-PRE-20460380)

Abraham Lincoln Graduate Fellowship **August 2011 – 2016**
- University of Illinois at Chicago

Chancellor's Graduate Research Fellowship Award **January 2014-2015**
- University of Illinois at Chicago

Department of Physiology and Biophysics NIH Training Grant **August 2013-August 2014**
Predoctoral Fellow (5T32 HL 07692-24)
- John Solaro, PhD - University of Illinois at Chicago

Chancellor's Committee on the Status of Women Professional **January 2013**
Development Award
- University of Illinois at Chicago

AWARDS

Dr. Harry and Mildred Monsen Award for Excellence
December 2014
- University of Illinois at Chicago College of Medicine
  Department of Anatomy and Cell Biology

Neuroscience Day Travel Award for Outstanding Poster Presentation
September 2013, 2014
- Graduate Program in Neuroscience, University of Illinois at Chicago

PUBLICATIONS

1. **Bonds JA**, Haus JM, Bonini M, Minshall RD, Lazarov O. Inflammation induced caveolin-1 loss in diabetic mouse models leads to compromised neurogenesis and Alzheimer's pathology. *In Prep*.

2. **Bonds JA**, Minshall RM, Lazarov O. Role of caveolin-1 in the regulation of the neurogenic niche. *In Prep*.

3. **Bonds JA**, Hart PC, Minshall RD, Lazarov O, Haus JM, Bonini M. "Type 2 Diabetes Mellitus as a Risk Factor for Alzheimer's Disease." *Genes, Environment and Alzheimer's Disease*. By Orly Lazarov and Giuseppina Tesco. London, UK: Elsevier, Academic, 2016. 387-413. Print.

4. **Bonds JA**, Kuttner-Hirshler Y, Bartolotti N, Tobin MK, Pizzi M, Marr R, Lazarov. Presenilin-1 Dependent Neurogenesis Regulates Hippocampal Learning and Memory. PLoS ONE. 2015; 10(6): e0131266. doi:10.1371/journal.pone.0131266

5. Tobin MK, **Bonds JA**, Minshall RD, Pelligrino DA, Lazarov O. Neurogenesis and inflammation following ischemic stroke: what is known and where we go from here. J Cereb Blood Flow Metab. 2014 Oct;34(10):1573-84.

6. Niesman IR, Schilling JM, Shapiro LA, Kellerhals SE, **Bonds JA**, Kleschevnikov A, Cui W, Voong A, Krajewski S, Ali SS, Roth DM, Patel HH, Patel PM, Head BP. Traumatic brain injury enhances neuroinflammation and lesion volume in caveolin deficient mice. J Neuroinflammation. 2014 Mar 3; 11(1):39.

7. Fridolfsson HN, Kawaraguchi Y, Ali SS, Panneerselvam M, Niesman IR, Finley JC, Kellerhals SE, Migita MY, Okada H, Moreno AL, Jennings M, Kidd MW, **Bonds JA**, Balijepalli RC, Ross RS, Patel PM, Miyanohara A, Chen Q, Lesnefsky EJ, Head BP, Roth DM, Insel PA, Patel HH. Mitochondria-localized caveolin in adaptation to cellular stress and injury. FASEB. 2012 Nov; 26(11):4637-49. PMID: 22859372

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9. Head BP, Peart JN, Panneerselvam M, Yokoyama T, Pearn ML, Niesman IR, **Bonds JA**, Schilling JM, Miyanohara A, Headrick J, Ali SS, Roth DM, Patel PM, Patel HH. Loss of caveolin-1 accelerates neurodegeneration and aging. PLoS One. 2010 Dec 23; 5(12): e15697. PMID: 21203469

10. Panneerselvam M, Tsutsumi YM, **Bonds JA**, Horikawa Y, Saldana M, Dalton ND, Head BP, Patel PM, Roth DM, Patel HH. Dark chocolate receptors: epicatechin-induced cardiac protection is dependent on {delta} opioid receptor stimulation. Am J Physiol Heart Circ Physiol. 2010 Sep 10. PMID: 20833967

11. Druley TE, Vallania FL, Wegner DJ, Varley KE, Knowles OL, **Bonds JA**, Robison SW, Doniger SW, Hamvas A, Cole FS, Fay JC, Mitra RD. Quantification of rare allelic variants from pooled genomic DNA. Nat Methods. 2009 Apr 6 (4):263-5. PMID: 19252504

RESEARCH PRESENTATIONS

1. **JA Bonds**, A Bheri, Z Chen, L Tai, MG Bonini, J Haus, RD Minshall, and O Lazarov. Chronic inflammation insuced depletion of caveolin-1 in type-2 diabetes mediates the development of Alzheimer's disease neuropathology. Poster, Translational Sciences. Washington, D.C. 2018

2. **JA Bonds**, A Bheri, MG Bonini, RD Minshall, and O Lazarov. Role of caveolin-1 in adult neurogenesis and cognition: Implications for type 2 diabetes-induced Alzheimer's disease. Poster, Brain Research Foundation. Chicago, IL 2018

3. **JA Bonds**, A Bheri, RD Minshall, and O Lazarov. Alterations in neurogenesis following caveolin-1 deletion in the adult mouse brain. Poster, UIC Neuroscience Day. Chicago, IL 2017.

4. **JA Bonds**, A Bheri, RD Minshall, and O Lazarov. Alterations in neurogenesis following caveolin-1 deletion in the adult mouse brain. Poster, UIC College of Medicine Research Day. Chicago, IL 2017.

5. **JA Bonds**, Z Chen, MG Bonini, J Haus, RD Minshall, and O Lazarov. Chronic inflammation-induced depletion of brain endothelial cell caveolin-1 in Type 2 Diabetes and its role in the development of Alzheimer's disease. Poster, Society for Redox Biology and Medicine. Chicago, IL. 2016

6. **JA Bonds**, RD Minshall, and O Lazarov. Loss of endothelial caveolin-1 results in compromised neurogenesis within the hippocampus of the adult murine brain. Nanosymposia, Society for Neuroscience. San Diego, CA 2016.

7. **JA Bonds**, RD Minshall, and O Lazarov. Endothelial caveolin-1 regulates the neurogenic niche. Nanosymposia, Society for Neuroscience, Chicago, IL 2015.

8. **JA Bonds**, Y Kuttner, N Bartolotti, M Pizzi, A Gadadhar, R Marr, and O Lazarov. Presenilin-1 dependent neurogenesis regulates hippocampal learning and memory. Nanosymposia, Society for Neuroscience, Washington, D.C. 2014.

9. **JA Bonds**, Y Kuttner, N Bartolotti, M Pizzi, A Gadadhar, R Marr, and O Lazarov. Down-regulation of presenilin-1 in neural progenitor cells impairs learning and memory. Poster, Society for Neuroscience, San Diego, CA 2013.

10. **JA Bonds**, RD Minshall, and O Lazarov. Caveolin-1 regulates the vascular neurogenic niche response to stroke. Poster, UIC Neuroscience Day. Chicago, IL 2013.

11. **JA Bonds**, HH Patel, DM Roth, JN Peart, PM Patel, and BP Head. Age related decrease in post-synaptic signaling components and caveolin-1 in membrane/lipid rafts and synaptosomal membrane fractions in the CNS. Poster, Experimental Biology. Anaheim, CA 2010

12. **JA Bonds**, Y HU, IR Niesman, HH Patel, S Krajewski, PM Patel, and BP Head. Traumatic brain injury leads to decreased expression of synaptic pro-survival signaling components in membrane lipid rafts. Poster, Society for Neuroscience, San Diego, 2010.

TEACHING EXPERIENCE

Instructor – Neuroscience and the Mind (Science 3519) **August 2014 – Present**
School of the Art Institute of Chicago, Chicago, IL
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Invited Instructor – Neuroscience and the Mind (Science 3519) **April 2014**
School of the Art Institute of Chicago, Chicago IL
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Invited Instructor – Neurobiology Seminar (BioS 386) **March – April 2014**
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- Department of Biological Sciences

Invited Speaker – Brain Power: From Perception to Consciousness **March – April 2013**
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- UIC Urban Health and Diversity Programs

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